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Binding of a hemoregulatory tetrapeptide by a bis-guanidinium crown ether

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ABSTRACT

A synthetic receptor for the molecular recognition of a tetrapeptide in aqueous buffer was obtained by combining a luminescent crown ether with two pyrrole-guanidinium moieties. The compound interacts with ammonium carboxylates of complementary geometry and binds the hemoregulatory peptide Ac-Ser-Asp-Lys-Pro with $K=7\times10^3\ M^{-1}$ at physiological pH. Shorter fragments and other tetrapeptides show no or significant reduced affinity. The binding of the target peptide to the functionalized crown ether is signalled by an increase of its emission intensity.

1. Introduction

The naturally occurring hemoregulatory peptide Ac-Ser-Asp-Lys-Pro (**1**) shows *anti*-inflammatory and antifibrotic properties, thus decreasing hypertension-induced target organ damage.[1] It is known to inhibit the proliferation of hematopoietic stem cells[2] and has recently been reported to inhibit cardiac fibrosis.[3] It has been shown to reduce the damage to specific compartments in the bone marrow resulting from treatment with chemotherapeutic agents, ionizing radiations, hyperthermy, or phototherapy.[4] The peptide acts as a specific inhibitor for the N-terminal site of the angiotensin I-converting enzymes (ACE). The effect is characterized by the conversion activity of ACE.[5] Fluorescent substrates like coumaryl-acetyl-Ser-Asp-Lys-Pro were used for the determination of the kinetic constants and to develop a sensitive assay for ACE activity in human plasma.[6]

We report a synthetic receptor capable of indicating the presence of the peptide by change of its luminescence. The emission increase of the host depends on the concentration of the target peptide, which allows the determination of its concentration without further functionalisation with a fluorophore or radiolabel.

The structure of the peptide Ac-Ser-Asp-Lys-Pro (**1**) contains two carboxylate groups and the amino acid lysine bearing an ammonium ion side chain. Recently, we presented different receptors for zwitterionic amino acids, recognizing combinations of ammonium and carboxylate ions, based on luminescent crown ethers with guanidinium ion binding sites.[7] Here we extended this modular approach to a tridentate receptor **2** with complementary binding sites for the target peptide **1**.

The crown ether moiety[8,9] of compound **2** is a luminescent ammonium ion indicator based on photoinduced electron transfer (PET),[10] while the guanidinium ion group strongly interacts with anions through charge pairing and hydrogen bonding.[11] It is used as recognition motif for carboxylates[12] and its interactions are well-studied.[13] A pyrrole substituent improves the binding strength of the recognition motif further.[14] Several interesting examples employing this carboxylate binding site have been published by the Schmuck group, recently.[15] Supramolecular studies in water[16] are challenging, because solvent hydrogen bonds disturb the binding process and diminish or even prevent molecular recognition. Multiple intermolecular interactions can stabilize host/guest aggregates. The spacer units between the binding sites of compound **2** were therefore designed to complement the hydrogen-bonding pattern of the target peptide zwitterion (Fig. 1).

2. Results and discussion

2.1. Synthesis

The synthesis of the substructures of receptor **2**, the crown ether amino acid ester **6**[9] and pyrrole substituted isothiourea **4**[7] were published previously. 3,5-Bis-aminomethyl-benzoic acid methyl ester (**3**)[17] was reacted with methyl-isothiourea **4** yielding the protected pyrrole-guanidine **5a** (Scheme 1). The ester group was hydrolyzed giving **5b**, which was coupled to the crown ether amino acid ester **6a** using standard peptide coupling methods to yield compound **7**. Compound **6b** was prepared for comparison by acylation under standard conditions (Scheme 2). The guanidilation reaction can also be achieved by mercury(II) catalysis in DMF in 6 h.[18] The EDC promoted reaction is slower, but the use of toxic metal ions is avoided, the overall yield is higher and less side products are formed.[19] A small amount of methanol has to be added

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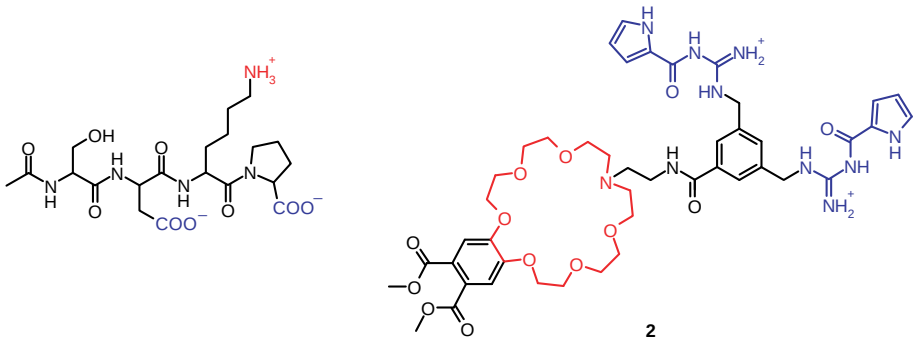
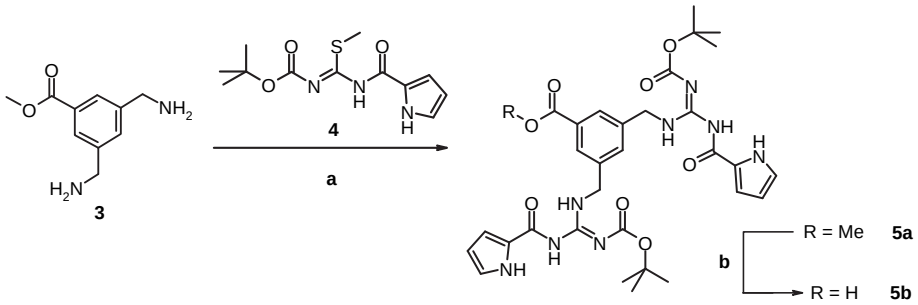
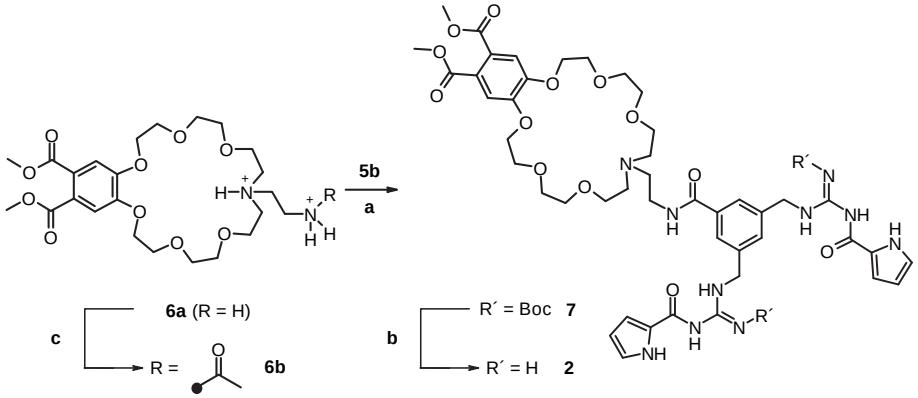


Figure 1. The hemoregulatory peptide **1** and synthetic receptor **2** with complementary ammonium and carboxylate ion binding sites.



Scheme 1. Synthesis of the protected pyrrole-guanidine **5b**; conditions: (a) DCM, MeOH, NEt_3, EDC·HCl, rt, then 40 °C over night, 77%; (b) NaOH, MeOH, H_2O, rt, 20 h, quant.



Scheme 2. Peptide coupling of compound **5b** with **6a**; conditions: (a) EDC·HCl, HOBt, $CHCl_3$, DMF, N_2, rt, then 40 °C, over night, 75%; (b) DCM, HCl in Et_2O, 5 h, rt, quant.; (c) DCM, NEt_3, Ac_2O, 3 h, rt, quant.

to ensure a homogeneous reaction mixture.[20] The subsequent deprotection step gives quantitative yields of **2**. Peptides for the determination of binding selectivity were prepared in solution and by standard solid phase methods.

2.2. Physical properties

Compound **2** shows absorption maxima in methanol at 205 nm, with a shoulder at 220 nm, and at 290 nm, corresponding to the crown ether and pyrrole moieties, respectively. Upon excitation at 310 nm, the system emits at 390 nm. The quantum yield is φ=0.1.[21] (Fig. 2)

The compounds emission intensity is sensitive to pH: protonation of the crown ether nitrogen atom interrupts PET fluorescence quenching.[22] Solutions of the compound **2** in methanol were mixed with aqueous buffer at defined pH values and the emission of the crown ether was recorded (Fig. 3). At pH values smaller than

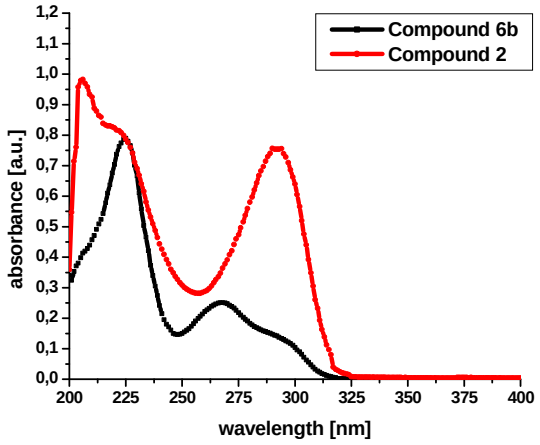


Figure 2. Absorption spectra of compounds **2** and **6b** (c=3.4×10^{-5} and 3.2×10^{-5} mol/L).

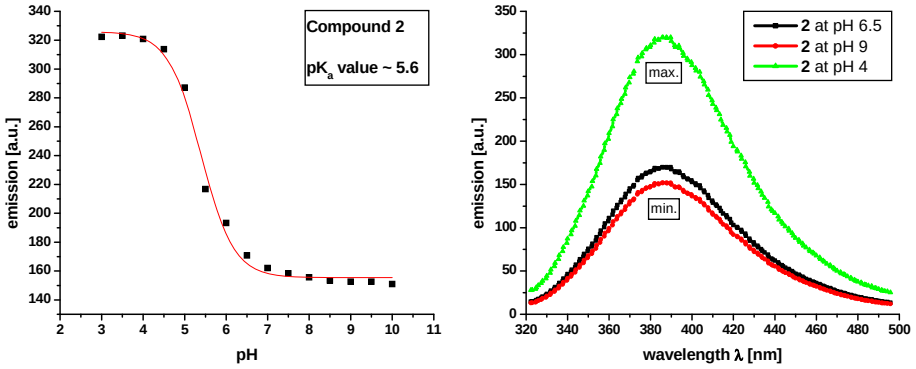


Figure 3. Emission intensity of compound **2** in methanol/aqueous buffer 4:1 at different pH values of the buffer; λ_{ex}=300 nm, λ_{em}=390 nm; (c=3.0×10^{-5} mol/L).

6 an emission intensity increase is observed by protonation of the aza-crown nitrogen atom.

2.3. Binding studies

Peptide binding to compound **2** was investigated in methanol/aqueous buffer mixtures adjusted to pH 6.3. This ensures a large gain in emission intensity upon ammonium ion binding to the crown ether, the aza-crown nitrogen atom quenching the emission is mainly deprotonated at this pH. The acyl guanidines are protonated under these conditions and interact with carboxylate ions. First, the interaction of **2** with ammonium and carboxylate ions was investigated separately: the addition of up to 1000 equiv guanidine hydrochloride or *tert*-butylammonium acetate in methanol/aqueous buffer 4:1 (10 mM HEPES adjusted to pH 6.3 with dilute hydrochloric acid) did not affect the emission at 390 nm. Butylammonium chloride addition

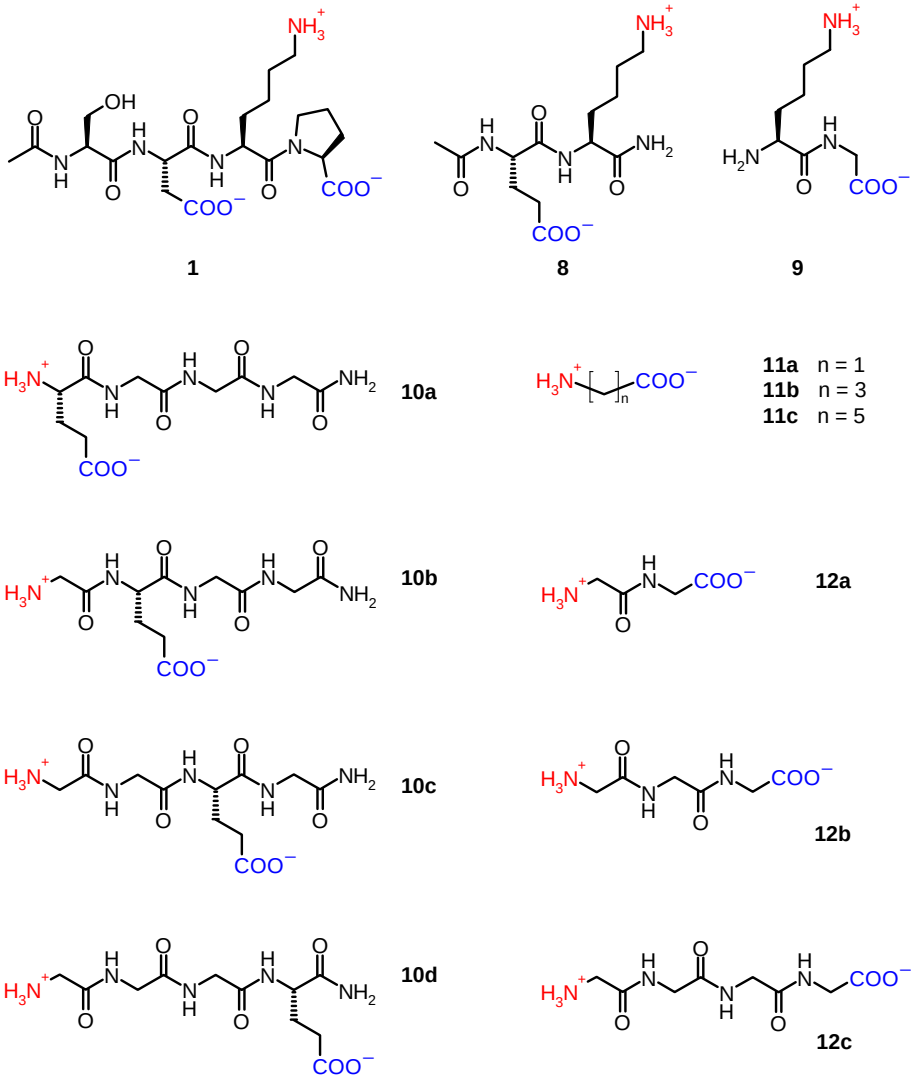


Figure 4. Peptides investigated for binding to compound **2** in methanol/aqueous buffer 4:1 solution.

increases the emission intensity only negligible ($F/F_0<1.05$).[23] The possible effect of ionic strength on the emission intensity was probed by addition of sodium perchlorate in large excess (1000 equiv), which induced no observable emission changes. Sodium acetate[24] was added to investigate the pyrrole guanidinium carboxylate binding. Analysis of changes in the UV/vis spectrum revealed a binding constant of $<10^3\ M^{-1}$ with 1:1 stoichiometry, but no changes of the emission intensity at 390 nm are observed.[25]

The binding of compound **2** to peptide **1** and related peptide structures was investigated by emission titration. Ac-Glu-Lys-amide (**8**),[26] Cbz-Lys-Gly and Lys-Gly (**9**) are partial structures of the target peptide. For comparison glycine (**11a**), γ-aminobutyric acid (**11b**), ε-aminohexanoic acid (**11c**), Gly-Gly-OH (**12a**), Gly-Gly-Gly-OH (**12b**), Gly-Gly-Gly-Gly-OH (**12c**) and the isomeric peptides Glu-Gly-Gly-Gly-NH_2 (**10a**), Gly-Glu-Gly-Gly-NH_2 (**10b**), Gly-Gly-Glu-Gly-NH_2 (**10c**) and Gly-Gly-Gly-Glu-NH_2 (**10d**) were used (Fig. 4). To quickly identify the strongest interactions, 200 and 500 equiv of the peptides were added to a solution of **2** (0.5 mL, 3×10^{-5} M) in methanol/buffer 4:1 (10 mM HEPES; pH 6.3). All peptides were soluble in the used concentration range.[27] Compound **2** shows a significant 1.7-fold fluorescence enhancement with **1** in 20% aqueous, buffered methanol (Fig. 5, left). Addition of glycine, γ-aminobutyric acid (GABA) and ε-aminohexanoic acid (AHX) gives no response, while $(Gly)_3$, $(Gly)_4$, (Cbz, H)-Lys-Gly, Gly-Glu-Gly-Gly-amide and Gly-Gly-Glu-Gly-amide induce a weak response of the emission intensity (F/F_0=1.1–1.3). In 50% aqueous buffer/methanol mixture the emission change upon addition of **1** is still evident (F/F_0=1.5), while all other peptides induce only small emission changes (F/F_0=<1.1; Fig. 5, right). In aqueous buffer without added methanol only the addition of **1** leads to detectable emission intensity changes ($F/F_0\approx1.3$) with the receptor **2**. Addition of the other peptides under these conditions caused no emission intensity change.

For the peptides showing a significant emission enhancement the binding affinities stoichiometries were determined by emission titration of solutions of **2** ($c=3\times10^{-5}$ M) in 4:1 methanol/aqueous buffer-mixture (10 mM HEPES, pH 6.3). For comparison, compound **6b** was titrated with peptide **1** under identical conditions. The interaction of Ac-Ser-Asp-Lys-Pro (**1**) with **2** was additionally investigated in 1:1 methanol/aqueous buffer. Emission titration data were analyzed using the Hill equation.[28] Figure 6 shows the emission titration data and Job's plot analyses of compound **2** titrated with Ac-Ser-Asp-Lys-Pro (**1**) in methanol/aqueous buffer 4:1 (left) and 1:1 (right). Table 1 summarizes the results of all titrations with receptor **2**. The investigated peptides Lys-Gly (**8**), Gly-Glu-Gly-Gly-NH_2 (**10b**), Gly-Gly-Glu-Gly-NH_2 (**10c**), $(Gly)_3$ (**12b**) and $(Gly)_4$ (**12c**), bind to **2** with affinities in the range of 220–930 M^{-1}, which is significantly weaker than the binding of **2** to **1**.

The enhanced binding affinity of **2** for peptide **1** of log K=3.8 can be rationalized by the additional electrostatic interactions due to the carboxylate groups in **1**. The dipeptide guest Lys-Gly (**8**), which reflects a fragment of the structure of **1**, is missing this additional carboxylate/guanidinium interaction and therefore shows an affinity to **2** of log $K<3$. All other zwitterion guests, which can undergo ditopic binding, show even weaker binding to **2**. It was not possible to derive a binding constant for **1** versus **6b**. Even if a large excess of the guest (>2000 equiv) is added, no saturation of titration curve is reached.[29] This illustrates the importance of the guanidinium/carboxylate interaction for the formation of stable aggregates.

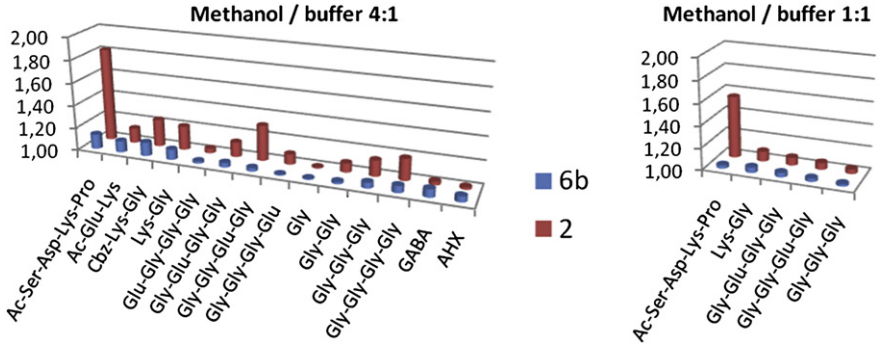


Figure 5. Comparison of the emission response of compound **2** ($c=3\times10^{-5}$ M) and of crown ether **6b** ($c=3\times10^{-5}$ M) upon addition of 200 equiv of different peptides (c=0.006 M) in MeOH/buffer (10 mM HEPES) 4:1 (left) and 1:1 (right) at pH 6.3 adjusted with Et_4NOH and HCl.

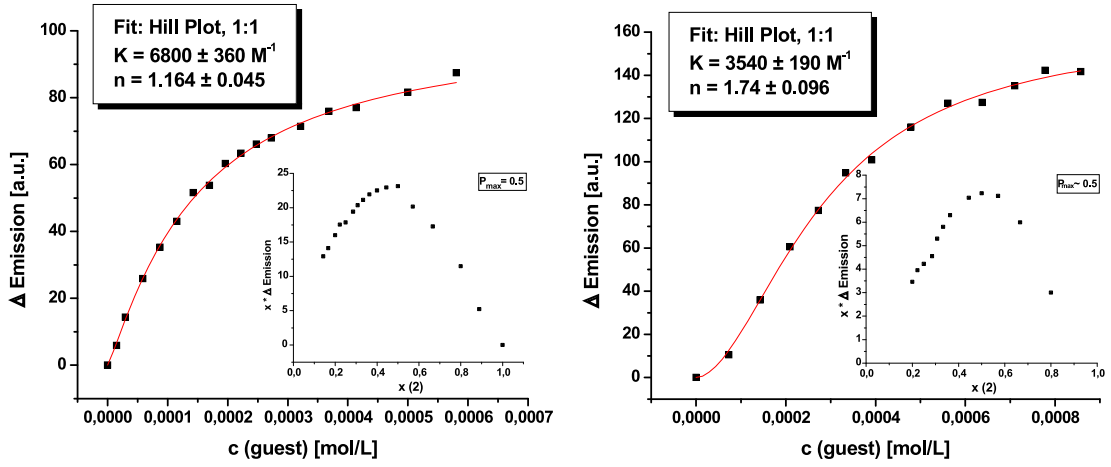


Figure 6. Titration of compound **2** with the tetrapeptide **1** in methanol/aqueous buffer 4:1 (left) and 1:1 (right) at pH 6.3; Small inserts: Job plots.

Table 1
Absolute and relative binding constants of compound **2** to different peptides **1** methanol/aqueous buffer 4:1 at pH 6.3

Peptide	Binding constant K [M^{-1}]	Relative binding strengths K/K (**1**)	Fluorescence enhancement F/F_0
Ac-Ser-Asp-Lys-Pro (**1**)	6800±360 (3540±190)[a]	1	1.62 (1.69)[a]
Lys-Gly (**8**)	930±50	0.137	1.41
Gly-Glu-Gly-Gly-NH_2 (**10b**)	220±30	0.032	1.32
Gly-Gly-Glu-Gly-NH_2 (**10c**)	800±60	0.117	1.34
$(Gly)_3$ (**12b**)	510±60	0.075	1.21
$(Gly)_4$ (**12c**)	390±40	0.057	1.17

[a] Values for 1:1 methanol/aqueous buffer.

3. Conclusions

Compound **2** is a synthetic receptor for the hemoregulatory peptide **1** presumably binding the lysine ammonium side chain, the terminal and the aspartic acid side chain carboxylate of the peptide.[30] The binding of the peptide is signalled by an increase in emission intensity of the crown ether fluorophor, as the ammonium ion binding intercepts the photoinduced electron transfer (PET) quenching mechanism. The combination of the crown ether/ammonium ion interaction with the guanidinium/carboxylate ion interaction is essential for sensing the target peptides: The crown ether/ammonium ion interaction is too weak to be effective in the aqueous buffer, while the acyl-guanidinium/carboxylate binding itself will not affect the emission intensity. Small peptides, which were used for comparison, revealed that the additional carboxylate group in **1** and the ammonium/carboxylate distance complementary to **2** are responsible for the observed increased affinity. Figure 7 shows the proposed structure of the peptide–receptor aggregate. The results provide another example that the combination of several weak reversible interactions may lead to synthetic receptors with affinity and selectivity of biological targets. Although the binding and emission properties of compound **2** are not sufficient for analytical applications in complex matrices, its use as an indicator is already possible.

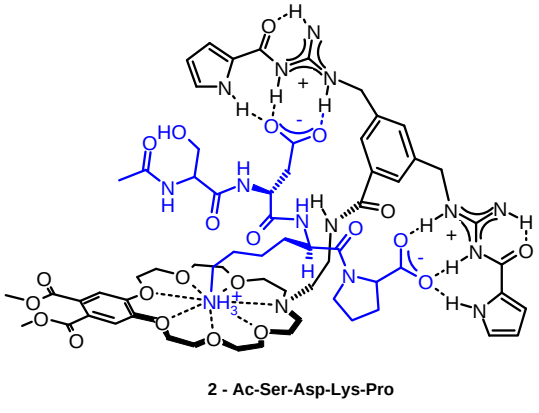


Figure 7. Proposed structure of the aggregate of **2** and the tetrapeptide **1**.

4. Experimental

4.1. Syntheses

Compounds **6**,[9] **4**[31] and **3**[17] were synthesized in solution according to published procedures. Ac-Ser-Asp-Lys-Pro (**1**) was purchased from GENSCRIPT in a purity >95% and used as is.

*4.1.1. Methyl 3,5-bis-(1-(tert-butyloxycarbonyl)-3-(pyrrol-2-carbonyl)-guanidino-methyl)benzoate (**5a**).* To a cooled solution (2 °C) of compound **4** (312 mg, 1.1 mmol) and triethylamine (0.66 mL, 500 mg, 5.0 mmol) in 3.0 ml of DCM and methanol (19:1), 3,5-bis-aminomethyl-benzoic acid methyl ester (**3**)[32] (105 mg, 0.5 mmol) and EDC·HCl (272 mg, 1.5 mmol) were added. The reaction was stirred for 3 h, allowed to warm up to room temperature, refluxed for another 2 h and cooled to room temperature. A second portion EDC·HCl (272 mg, 1.5 mmol) was added, the reaction mixture was stirred for 30 min at room temperature and then refluxed for 2 h. The reaction mixture was diluted with 30 mL of DCM and filtered; washed with 10% citric acid (10 mL), water (10 mL), dried over $MgSO_4$ and the solvent was evaporated. The residue was purified by column chromatography with ethyl acetate/ethanol, 6:1, to give the product as an orange solid (256 mg, 77%).

Mp: (uncorrected)=127–128 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ [ppm]=1.44–1.54 (m, 18H), 3.90 (s, 3H), 4.68–4.81 (m, 4H), 6.22 (m, 2H), 6.71–7.01 (m, 4H), 7.59–7.72 (m, 1H), 7.85–8.01 (m, 2H), 8.80 (m, 2H), 9.23 (m, 1H), 9.53 (br s, 1H), 9.76 (br s, 1H), 9.98 (br s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$):[33] δ [ppm]=28.1 and 28.3 (+, 6C), 44.5 (−, 2C), 52.3 (+, 1C), 79.8 and 83.3 (C_{quat}, 2C), 110.4 (+, 2C), 114.5 (+, 2C), 122.1 (+, 2C), 128.2 (+, 2C), 131.0 (C_{quat}, 2C), 131.6 (+, 1C), 139.1 (C_{quat}, 2C), 153.3 (C_{quat}, 2C), 156.0 and 156.3 (C_{quat}, 2C), 160.6 (C_{quat}, 1C), 164.0 (C_{quat}, 1C), 166.5 (C_{quat}, 1C), 171.1 (C_{quat}, 1C); MS (ESI-MS, CH_2Cl_2/MeOH+10 mmol NH_4OAc): m/z (%)=665.2 (90, MH^+), 333.0 (100, $(M+2H^+)^{2+}$); HRMS (FAB LSI-MS Glycerine): calcd for $C_{32}H_{41}N_8O_8^+$: 665.3047, found: 665.3033; MF: $C_{33}H_{43}N_8O_8$—FW: 679.76 g/mol.

*4.1.2. Sodium 3,5-bis-(1- (tert-butyloxycarbonyl)-3-(pyrrol-2-carbonyl)-guanidino-methyl)benzoate (**5b**).* Compound **5a** (166 mg, 0.25 mmol) in methanol (1.0 mL) was treated with 1 M aqueous sodium hydroxide solution (0.25 mL) by vigorous stirring at room temperature over night. Methanol was evaporated and the aqueous solution was lyophilised to give the product as a yellowish solid (164 mg, 98%).

Mp: (uncorrected)=145–146 °C (decomp.); ^{1}H NMR (300 MHz, $CDCl_3$):[33] δ [ppm]=1.47–1.53 (s, 18H), 4.69–4.78 (m, 4H), 6.20–6.31 (m, 2H), 6.83–6.89 (m, 4H), 7.64 (m, 1H), 7.90–8.05 (m, 2H), 8.84 (m, 2H), 9.41 (m, 2H), 9.86 (br s, 1H), 12.43 (br s, 1H); MS (ESI-MS, CH_2Cl_2/MeOH+10 mmol NH_4OAc): m/z (%)=651.2 (94, MH^+), 326.0 (100, $(M+2H^+)^{2+}$); HRMS (FAB LSI-MS Glycerine): calcd for $C_{31}H_{39}N_8O_8^+$: 651.2891, found: 651.2897; MF: $C_{31}H_{37}N_8O_8$—FW: 672.68 g/mol.

*4.1.3. Dimethyl 14-[2-[3,5-bis-(1-(tert-butyloxycarbonyl)-3-(pyrrol-2-carbonyl)-guanidino-methyl)benzamido]-ethyl]-6,7,9,10,13,14,15,16,18,19,21,22-dodecahydro-12H-5,8,11,17,20,23-hexaoxa-14-aza-benzocycloheneicosene-2,3-dicarboxylate (**7**).* Compound **5b** (135 mg, 0.20 mmol) was dissolved in 1.0 ml of DMF and DCM (1:1) at 5 °C, triethylamine (0.06 mL, 0.05 g, 0.5 mmol), HOBt (29 mg, 0.22 mmol) and EDC hydrochloride (36 mg, 0.21 mmol) were added subsequently and the mixture was stirred for 30 min in the ice bath. The TFA salt of compound **6a** (163 mg, 0.22 mmol) dissolved in 1.0 mL of DCM containing 0.06 mL triethylamine (0.05 g, 0.5 mmol) was added slowly and the reaction mixture was stirred for 2 h at room temperature. Another portion of triethylamine (0.06 mL, 0.05 g, 0.5 mmol), HOBt (29 mg, 0.22 mmol) and EDC hydrochloride (36 mg, 0.21 mmol) was added, stirring was continued for 1 h, the mixture was warmed to 40 °C and stirred for additional 8 h under nitrogen. The solvents were removed and the residue was re-dissolved in 10 mL of ethyl acetate. The solution was filtered and the filter cake was washed with a small portion of ice cold ethyl acetate. The clear filtrate was washed with 5% aqueous ammonium chloride solution and with water (3×3 mL). The organic phase was dried over $MgSO_4$, evaporated to dryness and the

remaining solid was purified by column chromatography with ethyl acetate/ethanol 10:1 to yield 173 mg of a pale yellow glass (75%).

^{1}H NMR (600 MHz, $CDCl_3$): δ [ppm]=1.42–1.54 (m, 18H), 2.56 (br m, 4H), 3.49–3.68 (br m, 6H), 3.59 (m, 4H), 3.65–3.70 (m, 4H), 3.81 (m, 4H), 3.82–3.88 (m, 8H), 4.62–4.78 (m, 8H), 6.15–6.33 (m, 2H), 6.81–6.95 (m, 4H), 7.04 (m, 1H), 7.12 (m, 2H), 7.46 (m, 1H), 7.70 (m, 1H), 7.82 (br s, 1H); ^{13}C NMR (150 MHz, $CDCl_3$):[33] δ [ppm]=28.0 and 28.3 and 30.9 (+, 6C), 40.9 (–, 1C), 44.2 and 44.4 (–, 2C), 46.3 (–, 1C), 52.6 (+, 2C), 54.5 (–, 2C), 68.8 (–, 2C), 69.4 (–, 2C), 70.2 (–, 4C), 70.8 (–, 2C), 79.6 and 82.2 (C_{quat}, 2C), 110.3 and 110.8 (+, 2C), 113.1 and 113.6 (+, 2C), 114.3 (+, 2C), 121.9 (+, 2C), 124.2 (+, 1C), 125.3 (C_{quat}, 2C), 126.1 (+, 2C), 130.1 (C_{quat}, 2C), 138.9 (C_{quat}, 1C), 150.0 and 153.1 (C_{quat}, 2C), 155.7 and 155.8 (C_{quat}, 2C), 165.1 (C_{quat}, 1C), 167.8 (C_{quat}, 2C), 170.9 (C_{quat}, 2C); IR (KBr): ν (cm^{-1})=3300 (br m), 2948 (m), 2929 (m), 2876 (m), 2356 (w), 2247 (w), 1718 (m), 1607 (s), 1578 (s), 1546 (s), 1406 (s), 1349 (s), 1289 (s), 1256 (m), 1229 (m), 1187 (m), 1124 (s), 1027 (m), 980 (m), 910 (m), 843 (m), 780 (m), 729 (s), 646 (m); MS (ESI-MS, CH_2Cl_2/MeOH+10 mmol NH_4OAc): *m/z* (%)=1147.6 (21, MH^+), 574.2 (100, $(M+2H^+)^{2+}$), 383.2 (22, $(M+3H^+)^{3+}$); HRMS (FAB LSI-MS Glycerine): calcd for $C_{56}H_{75}N_{10}O_{17}^+$: 1147.5312, found: 1147.5307; UV (MeOH): λ (ε)=298 (18,900), 205 (30,500); MF: $C_{55}H_{74}N_{10}O_{17}$—FW: 1147.26 g/mol.

*4.1.4. Dimethyl 14-[2-[3,5-bis-(1-(pyrrol-2-carbonyl)-guanidinomethyl)benzamido]-ethyl]-6,7,9,10,13,14,15,16,18,19,21,22-dodecahydro-12H-5,8,11,17,20,23-hexaoxa-14-aza-benzocycloheneicosene-2,3-dicarboxylate hydrochloride (**2**).* Compound **7** (115 mg, 0.10 mmol) was dissolved in 1.0 ml of dry DCM, 0.3 mL of HCl saturated diethylether was added and the mixture was stirred for 3 h at room temperature under moisture protection. The product was fully precipitated by slow addition of diethylether. The solvent was decanted off, the solid was suspended in a small volume of diethylether, allowed to precipitate completely and the ether was decanted off. This step was repeated once. After drying in vacuo the product was isolated as a fine yellow powder (96 mg, 91%).

^{1}H NMR (400 MHz, CD_3OD): δ [ppm]=3.48–3.59 (m, 6H), 3.65–3.71 (m, 8H), 3.75 (t, 2H, 6.4 Hz), 3.82 (s, 6H), 3.84 (m, 4H), 3.91 (m, 4H), 4.21 (m, 4H), 6.30 (m, 2H), 7.16 (m, 2H), 7.21 (s, 2H), 7.26 (m, 2H), 7.61 (s, 1H), 7.82 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ [ppm]= 45.6 (–, 2C), 51.7 (–, 1C), 53.2 (+, 2C), 55.3 (–, 2C), 64.1 (–, 1C), 70.2 (–, 4C), 70.8 (–, 2C), 71.4 (–, 2C), 71.7 (–, 2C), 111.8 (+, 2C), 114.3 (+, 2C), 115.9 (C_{quat}, 1C), 116.6 (+, 2C), 126.5 (C_{quat}, 2C), 127.0 (+, 2C), 130.9 (C_{quat}, 2C), 151.7 (C_{quat}, 2C), 161.7 (C_{quat}, 2C), 169.4 (C_{quat}, 2C), further signals were not detectable; IR (KBr): ν (cm^{-1})=3200 (br m), 2946 (m), 2883 (m), 2361 (m), 2347 (m), 1678 (s), 1632 (m), 1546 (m), 1434 (m), 1293 (s), 1186 (m), 1120 (s), 1051 (m), 974 (m), 948 (m), 887 (m), 746 (m), 667 (m); MS (ESI-MS, CH_2Cl_2/MeOH+10 mmol NH_4OAc): *m/z* (%)=947.7 (6, MH^+), 531.2 (40, $(M+2H^+)^{2+}$+TFA), 474.2 (100, $(M+2H^+)^{2+}$), 330.1 (98, $(M+3H^+)^{3+}$+MeCN), 316.4 (14, $(M+3H^+)^{3+}$+TFA); HRMS (FAB LSI-MS Glycerine): calcd for $C_{45}H_{59}N_{10}O_{13}^+$: 947.4263, found: 947.4241; UV (MeOH): λ (ε)=291 (22,300), 206 (29,400); MF: $C_{45}H_{61}N_{10}O_{13}Cl_3$—FW: 1056.40 g/mol.

4.2. Binding investigations

4.2.1. Screening of amino acid and peptide binding affinities. The screening was performed in UV star 96 well plates (400 μL volume per cell) in a 4:1 mixture of methanol and aqueous salt free HEPES buffer (10 mM) at pH 6.3. To a 3×10^{-5} M solution of the receptor compound 200 equiv of peptide or amino acid (1.5×10^{-2} M) were added (1:1 v/v). The fluorescence spectrum was recorded (λ_{ex}=310 nm); all measurements were repeated twice.

4.2.2. Binding affinity titrations. To a cuvette with 1.0 mL of the receptor (3×10^{-5} mol/L) in a 4:1 or a 1:1 mixture of methanol and aqueous HEPES buffer (10 mM, pH 6.3) small aliquots of the peptide solution, 5–40 μL for peptide **1** (1×10^{-3} mol/L) or 60–200 μL for G-E-G-G-NH_2, G-E-G-G-NH_2, K-G-OH, G-G-G-OH and G-G-G-G-OH (1×10^{-2} mol/L) were successively added. After each addition the solution was allowed to equilibrate for 5 min before the fluorescence intensity (λ_{ex}=310 nm) and the UV spectrum were recorded at 25 °C. The stoichiometry was determined by Job plot analysis.[34] Binding constants were determined from volume corrected emission intensities by non linear fitting. The pH of the buffer/methanol mixtures was determined before and after titrations. Measurements with deviations of more than 0.5 pH units were not used for binding constant determination.

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Supplementary data

Supplementary data associated with this article can be found in online version at 10.1016/j.tet.2010.06.024. These data include MOL files and InChIKeys of the most important compounds described in this article.

References and notes

1. Sharma, U.; Rhaleb, N.-E.; Pokharel, S.; Harding, P.; Rasoul, S.; Peng, H.; Carretero, O. A. *Am. J. Physiol. Heart Circ. Physiol.* **2008**, *294*, H1226–H1232.
2. Lombard, M.-N.; Sotty, D.; Wdzieczak-Bakala, J.; Lenfant, M. *Cell Prolif.* **1989**, *23*, 99–103.
3. Pokharel, S.; Rasoul, S.; Roks, A.; van Leeuwen, R.; van Luyn, M.; Deelman, L. E.; Smits, J. F.; Carretero, O.; van Gilst, W. H.; Pinto, Y. M. *Hypertension* **2002**, *40*, 155–161.
4. Massé, A.; Ramirez, L. H.; Bindoula, G.; Grillon, C.; Wdzieczak-Bakala, J.; Raddassi, K.; Deschamps de Paillette, E.; Mencia-Huerta, J. M.; Koscielny, S.; Potier, P.; Sainteny, F.; Carde, P. *Blood* **1998**, *91*, 441–449.
5. Grillon, C.; Lenfant, M.; Wdzieczak-Bakala, J. *Growth Factors* **1993**, *9*, 133–138.
6. Cheviron, N.; Rousseau-Plasse, A.; Lenfant, M.; Adeline, M.-T.; Potier, P.; Thierry1, J. *Anal. Biochem.* **2000**, *280*, 58–64.
7. Späth, A.; König, B. *Tetrahedron* **2010**, *66*, 1859–1873.
8. Stadlbauer, S.; Riechers, A.; Späth, A.; König, B. *Chem.—Eur. J.* **2008**, *14*, 2536–2541; Kruppa, M.; Mandl, C. P.; Miltschitzky, S.; König, B. *J. Am. Chem. Soc.* **2005**, *127*, 3362–3365.
9. Mandl, C. . P.; König, B. *J. Org. Chem.* **2005**, *70*, 670–674.
10. de Silva, A. P.; Gunaratne, H. Q. N.; Gunnlaugsson, T.; Huxley, A. J. M.; McCoy, C. P.; Rademacher, J. T.; Rice, T. E. *Chem. Rev.* **1997**, *97*, 1515–1566; de Silva, A. P.; Fox, D. B.; Moody, T. S.; Weir, S. M. *Trends Biotechnol.* **2001**, *19*, 29–34.
11. Best, M. D.; Tobey, S. L.; Anslyn, E. V. *Coord. Chem. Rev.* **2003**, *240*, 3–15.
12. Fitzmaurice, R. J.; Kyne, G. M.; Douheret, D.; Kilburn, J. D. *J. Chem. Soc., Perkin Trans. 1* **2002**, 841–864; Brooks, S. J.; Edwards, P. R.; Gale, P. A.; Light, M. E. *New J. Chem.* **2006**, *30*, 65–70.
13. Czekalla, M.; Stephan, H.; Habermann, B.; Trepte, J.; Gloe, K.; Schmidtchen, F. P. *Thermochim. Acta* **1998**, *313*, 137–144.
14. Schmuck, C. *Coord. Chem. Rev.* **2006**, *250*, 3053–3067.
15. Schmuck, C. *Chem.—Eur. J.* **2000**, *6*, 709–718; Hernandez-Folgado, L.; Schmuck, C.; Tomic, S.; Piantanida, I. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 2977–2981 and literature therein.
16. Oshovsky, G. V.; Reinhoudt, D. N.; Verboom, W. *Angew. Chem., Int. Ed.* **2007**, *46*, 2366–2393.
17. Maeda, D. Y.; Mahajan, S. S.; Atkins, W. M.; Zebala, J. A. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 3780–3783; Ryu, E.-H.; Yan, J.; Zhong, Z.; Zhao, Y. *J. Org. Chem.* **2006**, *71*, 7205–7213.
18. Chand, P.; Elliot, A. J.; Montgomery, J. A. *J. Med. Chem.* **2001**, *44* (25), 4379–4392; Kent, D. R.; Cody, W. L.; Doherty, A. M. *Tetrahedron Lett.* **1996**, *37*, 8711–8714.
19. The cyclised by-product *1-oxo-1,2-dihydro-pyrrolo[1,2-c]imidazol-3-ylidene* is formed using Hg(II) as catalyst in DMF.
20. Heterogeneous reaction leads to increased amounts of the side product *1-oxo-1,2-dihydro-pyrrolo[1,2-c]imidazol-3-ylidene*.
21. All quantum yields were determined with quinine disulfate in 1 N H_2SO_4 as the reference compound (Φ=0.546).
22. The pK_a value for the crown ether nitrogen atom of the protected derivative **7** was determined to be 5.6 by titration with perchloric acid; the pK_a value of 5.8 for compound **6b** is comparable. The pK_a value of the pyrrole guanidinium groups is 6.5. See Supplementary data.
23. In pure methanol the crown ether binds this ammonium guest weakly (log *K* (**2**)=2.2 and log *K* (**6b**)=2.3).
24. Sodium is not bound by the crown ether (21-crown-7). Even if a 2000 fold excess is added in aqueous methanol no change in luminescence can be observed.

25. Schmuck, C. *Chem.—Eur. J.* **2006**, *6*, 709–718.
26. The amino acid Glu is used instead of Asp due to synthetic reasons. The structural difference should not influence the selectivity and binding strength to a large extent.
27. Fauchere, J. L.; Pliska, V. *Eur. J. Med. Chem.* **1983**, *18*, 369–375; Narita, M.; Chen, J. Y.; Sato, H.; Kim, Y. *Bull. Chem. Soc. Japan* **1985**, *58*, 2494–2501.
28. Connors, K. A. *Binding Constants*; Wiley: New York, NY, 1987.
29. The crown ether binding affinity for ammonium ions in aqueous solution is known to be small (log $K<2$) Rüdiger, V.; Schneider, H.-J.; Solov'ev, V. P.; Kazachenko, V. P.; Raevsky, O. A. *Eur. J. Org. Chem.* **1999**, *8*, 1847–1856.
30. Suhs, T.; König, B. *Mini-Rev. Org. Chem.* **2006**, *3*, 315–331.
31. Späth, A.; König, B. *Tetrahedron* **2009**, *65*, 690–695.
32. The free base was prepared from the hydrochloride by shaking its chloroform solution several times with an aqueous solution of potassium carbonate (2 M). The combined aqueous phases were re-extracted three times with chloroform.
33. The splitting of some signals in the spectrum indicates the presence of unequal populations of two conformers at room temperature. This observation caused by hindered rotation in the molecule is literature known: Dalton, D. R.; Ramey, K. E.; Gisler, H. J., Jr.; Lendvay, L. J.; Abraham, A. *J. Am. Chem. Soc.* **1969**, *91*, 6367–6370 and literature therein.
34. MacCarthy, P. *Anal. Chem.* **1978**, *50*, 2165; Schmuck, C.; Wich, P. *Angew. Chem., Int. Ed.* **2006**, *45*, 4277–4281.