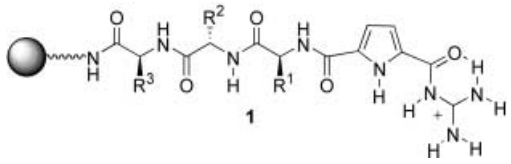
Peptide Binding by One-Armed Receptors in Water: Screening of a Combinatorial Library for the Binding of Val-Val-Ile-Ala



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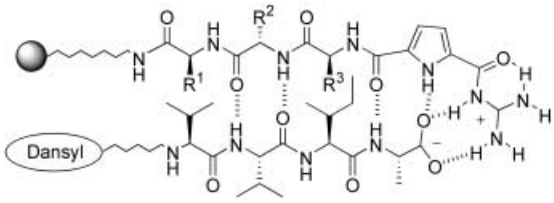
The development of artificial receptors for the molecular recognition of peptides[1] in aqueous solvents is of great importance for increasing our knowledge of peptide–peptide interactions and for the design of biosensors, the targeting of cellular processes, or the discovery of new therapeutics. However, there are only very few known receptors that allow the complexation of a peptidic substrate in water.[2] One reason for this is that the strength of H-bonds, often successfully used for molecular recognition in organic solvents, decreases rapidly with increasing polarity of the solvent.[3] Fortunately, peptides offer the advantage that various other noncovalent interactions can also be exploited for substrate binding. In addition to H-bonds to the amide backbone, further interactions to polar or nonpolar groups in the amino acid side chains can be used, thereby not only increasing the binding affinity, but also rendering the recognition process selective, since these interactions depend on the specific peptide sequence. In water, the combination of electrostatic interactions (H-bonds, ion pairs) with hydrophobic ones is of considerable interest.[4] Our work is currently focused on the design of artificial receptors that allow the complexation of a hydrophobic tetrapeptide, Ac-Val-Val-Ile-Ala-OH, in water. This tetrapeptide represents the C-terminal sequence of the amyloid-β-peptide responsible for the formation of protein plaques within the brains of patients suffering from Alzheimer's disease.[5] This specific sequence is thought to promote the formation of self-aggregated β-sheets stabilized through a combination of H-bonds and hydrophobic interactions.[6] In accordance with this, the tetrapeptide substrate **5** used in this study forms insoluble, fibril-like aggregates upon prolonged standing in solution, despite its hydrophilic triethylene glycol chain, underlining its high tendency to self-aggregate. This validates the use of this Val-Val-Ile-Ala tetrapeptide as a suitable model with which to study the self-aggregation of the amyloid-β-peptide. An artificial receptor that effectively binds to this tetrapeptide should therefore allow us to learn more about the molecular basis of the self-aggregation of the amyloid peptide and hence the origin of one of today's most devastating diseases.

To identify suitable receptors for this substrate, we decided to use a combinatorial approach,[2c,d, 7] screening a solid-phase-bound library of structurally related receptors **1** for their binding properties against a fluorophore-labeled derivative **5** of the tetrapeptide. The general structure of our receptor library is as follows: a linear tripeptide unit was attached to a solid support through its C terminus. The N terminus was further modified with a cationic guanidiniocarbonyl pyrrole group (=Gua), which is one the most efficient binding motifs so far known for the complexation of carboxylates in polar solvents.[8] This cationic guanidinium group would be expected to form an ion pair with the negatively charged C-terminal carboxylate of the tetrapeptide.[9] The peptidic nature of the receptor allows the formation of a hydrogen-bonded antiparallel β-sheet with the backbone of the substrate. In addition to these multiple electrostatic



interactions, hydrophobic contacts between the amino acid side chains both in the substrate and the receptor should further stabilize the complex, especially in water, not only through direct hydrophobic interactions between the residues, but also by burying the H-bonds within a less-polar microenvironment. We wish to report here the synthesis of such a combinatorial receptor library of 512 members and the successful evaluation of their binding properties in aqueous buffer solution by use of both a quantitative on-bead fluorescence binding assay and UV titration in solution.[10]

The library was synthesized on amino-TentaGel as the solid support according to a standard Fmoc protocol by the split-mix approach[11] in combination with IRORI-radio frequency tagging technology.[12] The same eight different amino acids—Lys(Boc), Tyr(*t*Bu), Ser(*t*Bu), Glu(OBzl), Phe, Val, Leu, and Trp—were used in each of the three coupling steps. These amino acids were selectively chosen from the proteinogenic amino acids to provide a wide range of structurally varying hydrophobic or steric interactions.[13]

In order to assess the binding properties of these 512 receptors in aqueous solvents, a fluorescent and water-soluble derivative **5** of the tetrapeptide Val-Val-Ile-Ala was synthesized on a solid support, again by a standard Fmoc protocol. The free amino group of the obtained tetrapeptide was then treated with a) succinic anhydride (**2**), b) the diamine **3**, and finally c) dansyl chloride **4** (Scheme 1). Product **5** was cleaved off the resin with TFA/CH_2Cl_2 (1:1) to give analytically pure **5** in 69% yield.

To probe the entire receptor library for its binding properties, in a first experiment, aliquots of the 512 resin-bound receptors **1** were pooled, and the combined mixture was incubated with a

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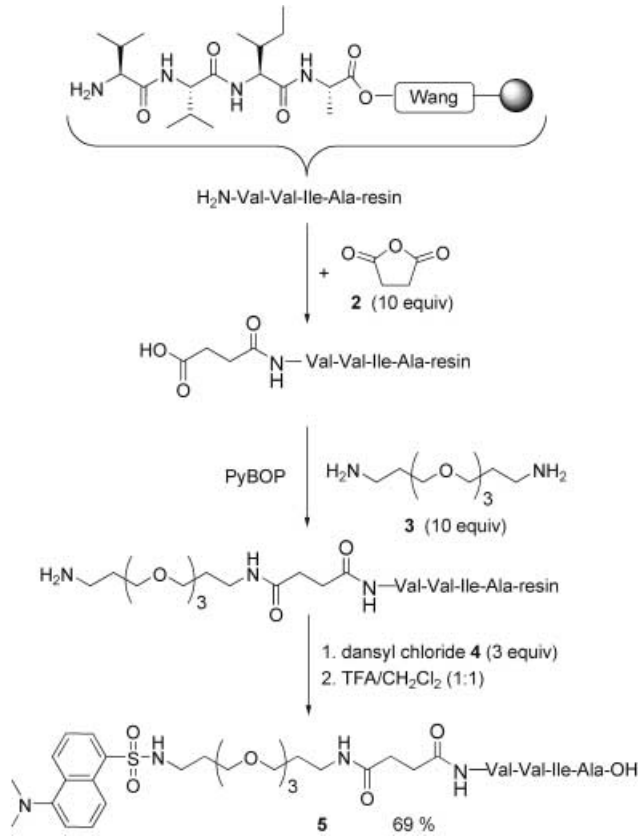

Scheme 1. *Synthesis of substrate* ***5****.*

2.5 μM solution of the tetrapeptide substrate **5** in 5 μM bis-tris buffer (pH 6) in water. After the supernatant solution had been washed off, the beads were screened under UV light. Selective binding of the tetrapeptide substrate **5** by some—but not all—of the 512 receptors **1** could indeed be observed, as indicated by the strong fluorescence activity of individual beads (Figure 1). Only those beads to which the attached receptor is able to bind the peptide even in water can show the characteristic fluorescence of the dansyl group. All the other receptors, which do not bind the peptide under these experimental conditions, remain dark.

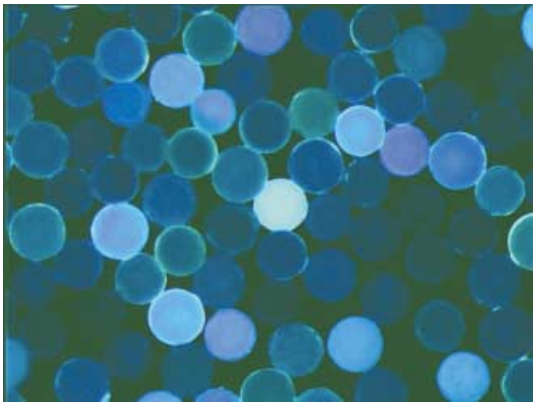

Figure 1. *On-bead binding assay in water (5 μM bis-tris buffer pH 6). The strong fluorescence activity of some beads indicates selective binding of the substrate* ***5*** *(2.5 μM) to some of the cationic receptors* ***1*** *within the library.*

Control experiments showed that the observed fluorescence activity is indeed due to selective complexation by the receptor of the peptide part of **5** and not of, for example, the dansylated spacer. Furthermore, the uncharged methyl ester of the peptide requires much higher concentrations to show only a weak and rather unselective binding to the receptor library;[10] this suggests that side-chain interactions alone are not strong enough to form a stable complex. Hence, the binding of the tetrapeptide by the receptors requires both electrostatic and hydrophobic interactions, neither of these interactions alone being sufficiently strong to ensure complex formation.

In a second experiment, we performed a quantitative on-bead binding assay with all 512 receptors.[14, 15] Table 1 summarizes the binding constants for the 10 best and the 10 worst receptors as identified in this assay. These data show that the binding, even in water, is still very efficient, with association constants of $4 \times 10^3\,M^{-1}$ for the best receptors. However, the association constants are about 1–2 orders of magnitude smaller than those seen in organic solvents such as methanol.[10] Furthermore, the receptors are remarkably different in their binding properties towards this specific substrate despite their similar general structure. The binding constants vary from $20\,M^{-1}$ for the worst up to $4200\,M^{-1}$ for the best, hence differing by a factor of more than 200! Within such a rather small library, with only a limited structural diversity, this represents a surprisingly high selectivity.

Table 1. *Selected association constants (M^{-1}) for the complexation of* ***5*** *by receptors of type* ***1*** *(Gua-AA¹-AA²-AA³-resin) in water.*

AA^1	AA^2	AA^3	K_{ass} [M^{-1}]
Lys(Boc)	Ser(O*t*Bu)	Phe	4200
Tyr(O*t*Bu)	Leu	Ser(O*t*Bu)	4000
Lys(Boc)	Lys(Boc)	Glu(OBz)	3000
Lys(Boc)	Phe	Val	2900
Lys(Boc)	Tyr(O*t*Bu)	Val	2600
Lys(Boc)	Phe	Phe	2500
Lys(Boc)	Lys(Boc)	Phe	2400
Lys(Boc)	Lys(Boc)	Val	2200
Lys(Boc)	Lys(Boc)	Leu	2000
Lys(Boc)	Ser(O*t*Bu)	Val	1900
Glu(OBz)	Glu(OBz)	Phe	120
Glu(OBz)	Glu(OBz)	Glu(OBz)	120
Ser(O*t*Bu)	Glu(OBz)	Tyr(O*t*Bu)	110
Val	Ser(O*t*Bu)	Glu(OBz)	100
Ser(O*t*Bu)	Glu(OBz)	Leu	90
Glu(OBz)	Trp	Glu(OBz)	85
Tyr(O*t*Bu)	Tyr(O*t*Bu)	Glu(OBz)	80
Glu(OBz)	Trp	Lys(Boc)	70
Ser(O*t*Bu)	Glu(OBz)	Lys(Boc)	25
Ser(O*t*Bu)	Glu(OBz)	Trp	20

Binding constants were determined on-bead in 10 μM bis-tris buffer (pH 6.1) at 20 °C; error limits are estimated to be ±25 %.

A closer examination of the data in Table 1 reveals that there are obviously certain structural features that can be assigned to good and bad binding. Most striking is perhaps the fact that nearly all of the receptors with high affinity have a Lys(Boc) in

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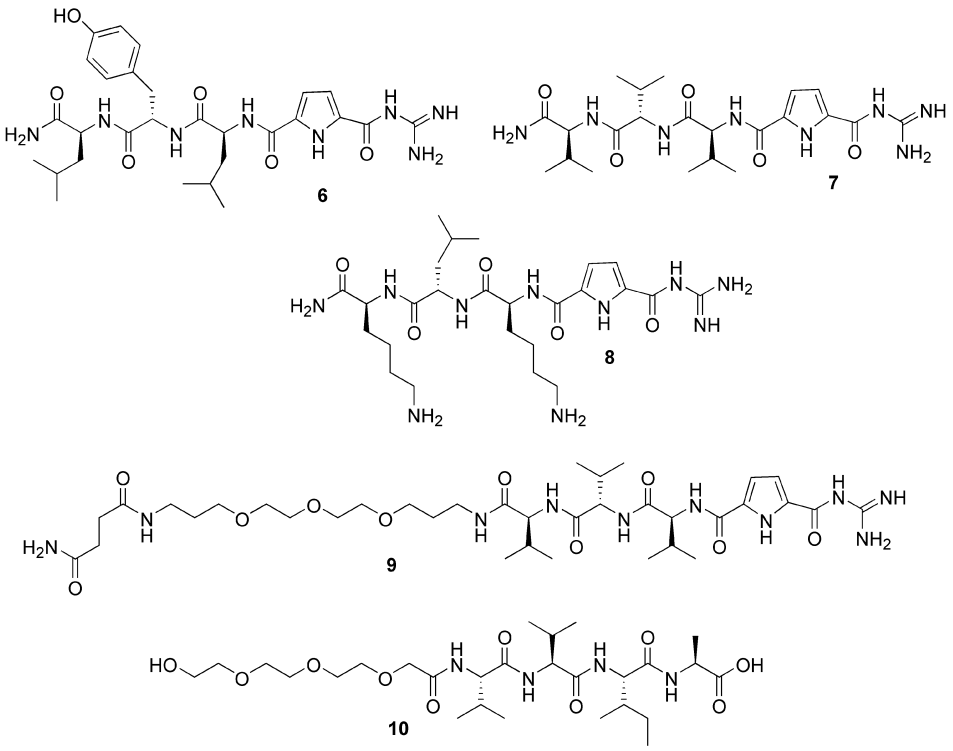
position-1 next to the guanidinium cation (9 out of 10). The presence, however, of a Glu(OBz)—regardless of its position in the receptor—seems to be incompatible with complex formation; all of the receptors with low affinity contain this amino acid. Interestingly, these findings are quite different from our previous results obtained for peptide binding by this receptor class in organic solvents.[10] In methanol, for example, amino acids such as Val or Phe were much better than Lys(Boc), with the two most efficient receptors being Val-Val-Val-Gua and Phe-Val-Val-Gua, with association constants of 9000 M^{-1} relative to a formate counter-ion. In water, though, their association constants are only of the order of 1400 M^{-1} and therefore only moderately high within the series of receptors studied here. This underlines the important influence of the solvent on supramolecular complex formation, both in terms of binding affinity and selectivity. Similarly to the situation in methanol, however, the presence of an apolar amino acid such as Val, Phe, or Leu in the third position of the receptor also seems to be preferable in water (8 out of 10); this probably reflects the importance of hydrophobic interactions to the N-terminal valine side chain of the substrate, which is opposite this position in the β-sheet-like complex. This is in good agreement with model studies that suggested that hydrophobic interactions with Val39, which corresponds to the N-terminal valine of our model tetrapeptide, are especially important for the Aβ self-aggregation.[16]

In order to check whether our one-armed receptors of type **1** were also able to bind the substrate **5** in free solution, and not only when attached to the solid support, we resynthesized a selection of receptors—Gua-Leu-Tyr-Leu-NH_2 (**6**), Gua-Val-Val-Val-NH_2 (**7**), and Gua-Lys-Leu-Lys (**8**)—by a standard Fmoc protocol. Because of solubility problems with the hydrophobic peptide **7**, this receptor was also synthesized with a C-terminal triethylene glycol chain to improve its solubility in aqueous solution, to provide Gua-Val-Val-Val-TEG (**9**).

The association constants for these receptors were determined in a UV titration by addition of aliquots of the substrate **10** to a solution of the receptors in water (bis-tris buffer, pH 6.1, [receptor] $= 5 \times 10^{-5}$ M^{-1}).[17] Changes in the UV spectrum of the receptor were recorded and used to determine the binding constants, as the absorbance of the pyrrole moiety at $\lambda = 290$ nm decreases upon complex formation.[18] Receptors **6** and **9** gave binding constants of 1770 M^{-1} and 2660 M^{-1}, in reasonably good agreement with the data obtained on the solid support (1800 M^{-1} and 1400 M^{-1}, respectively).[19] In order to check whether more polar receptors were more advantageous than the rather hydrophobic ones tested so far within the library, we also studied the tris-cationic receptor Gua-Lys-Leu-Lys (**9**)—obtained by deprotection of one of the former library members—under the same conditions. In the on-bead assay the association constant increased from $K_{ass} \approx 1300$ M^{-1} for the *t*Boc-protected receptor to $K_{ass} = 8800$ M^{-1}, which again was confirmed by UV titration in free solution, which provided $K_{ass} = 6025$ M^{-1}.

Of course, the on-bead data are not exactly the same as the solution data, since the solid support does influence the binding event to some extent.[20] For the calculation of the binding constant, for example, it is assumed in the on-bead assay that all of the immobilized receptors can participate in intermolecular association with the peptide substrate. Especially for hydrophobic receptors, such as Gua-Val-Val-Val (**7**), and for high resin-loadings, aggregation of the receptor within the resin might occur, affecting the measured binding constant. Furthermore, the polarity of the resin and its swelling properties can also influence complex formation, especially in water. However, the above comparison of selected binding data for our receptors both on the solid support and in free solution, together with other studies,[15, 20] shows that at least the general trends that one observes in on-bead binding assays will be the same as those in free solution. The determination of binding data in an on-bead assay is therefore not only a fast and convenient but also a reliable way to identify receptors with high affinity and selectivity within a combinatorial library and to arrive at relative conclusions relating to intermolecular complex formation.

To obtain an idea of why the presence of a Lys(Boc) group next to the guanidinium cation is favorable for binding, we turned to molecular dynamics calculations (Macromodel V8.0, Amber* force field, GB/SA water solvation treatment, 100 ps simulation time, 1.5 fs time steps at 300 K).[21] These calculations suggest the formation of



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a hydrogen-bonded β-sheet between the tetrapeptide Val-Val-Ile-Ala and our receptors, with additional hydrophobic interactions between the side chains and an ion pair between the carboxylate and the guanidinium cation (Figure 2).

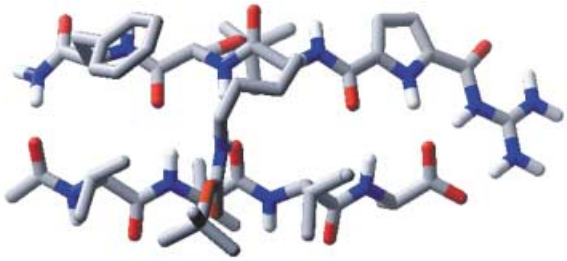

Figure 2. *Calculated structure for the complex formed between receptor Gua-Lys(Boc)-Ser(OtBu)-Phe (above) and Ac-Val-Val-Ile-Ala (below).*

The formation of such a β-sheet-like complex is also supported experimentally. ESI-MS experiments showed strong signals for the formation of a discrete 1:1 complex between receptor **7** and the peptide substrate N-Ac-Val-Val-Ile-Ala in solution. Interestingly, the intensity of the 1:1 complex relative to the free cationic receptor increases on going from less polar organic solvents (DMSO/CH_2Cl_2) to aqueous solution (DMSO/water), again this suggests that hydrophobic interactions, which only occur in water and not in dichloromethane, play a significant role in stabilizing the complex. According to NMR studies in DMSO,[22] both the free receptor **7** and the free peptide substrate adopt an extended zigzag conformation as required for the formation of a β-sheet. All coupling constants for the amide NH groups along the backbones are around 8 Hz. Complex formation therefore does not require any extensive conformational changes. Upon addition of the substrate to a solution of the receptor in DMSO, complexation-induced shift changes in the NMR spectrum can be observed for the amide NH groups; this indicates their involvement in hydrogen bonds.[25] As would be anticipated for complexation of a carboxylate, the signal for the guanidinium NH_2 protons is split into two signals.[8b] Furthermore, a weak intermolecular NOE can be observed between the alanine methyl group of the substrate and the pyrrole CH of the receptor. All these spectroscopic data are in full agreement with the proposed β-sheet-like structure suggested by the modeling and depicted in Figure 2.

On the basis of such a β-sheet complex structure, the high preference for Lys(Boc) in position-1 next to the guanidinium cation might be due to favorable hydrophobic interactions between the bulky *t*Boc group and the first (Val) and third (Ile) amino acids of the tetrapeptide. According to the MD simulations, the *t*Boc group is positioned between these two side chains of the substrate, thereby closing the hydrophobic gap in between. This minimizes the solvent-accessible surface (Figure 3) and therefore not only stabilizes the complex but also provides a less-polar microenvironment for the H-bonds between the amide backbones of substrate and receptor, as they are buried within the hydrophobic interior of the complex.

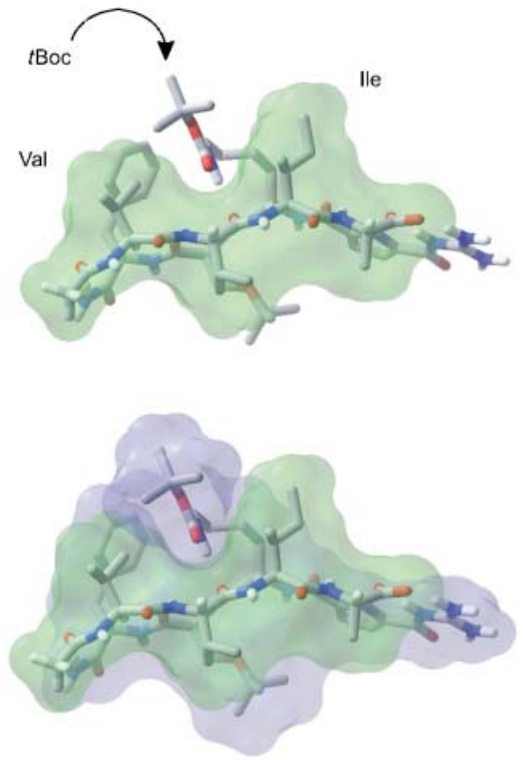

Figure 3. *Solvent-accessible surfaces of substrate (green) and receptor (blue). The tBoc group of the lysine side chain (see arrow) fits perfectly into the hydrophobic gap between the first (Val) and third (Ile) side chains of the substrate (side view of the complex). Nonpolar hydrogen atoms are omitted for clarity.*

The higher affinity of the tris-cationic receptor Gua-Lys-Leu-Lys (**9**) for the tetrapeptide is probably the result of the additional positive charges on the lysine side chains, which favor the complexation of the negatively charged tetrapeptide. According to molecular modeling studies, Lys1 next to the guanidinium moiety enters into an additional electrostatic interaction with the negatively charged carboxylate (Figure 4).

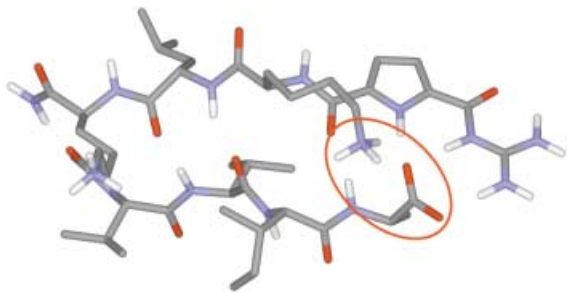

Figure 4. *Calculated structure for the complex between receptor Gua-Lys-Leu-Lys (**9**, above) and Ac-Val-Val-Ile-Ala (below). Nonpolar hydrogen atoms are omitted for clarity.*

In conclusion, one-armed cationic guanidiniocarbonyl pyrrole receptors efficiently bind the anionic tetrapeptide Val-Val-Ile-Ala with high affinity even in pure water, as shown by UV titration studies in free solution. Complex formation is based on a combination of electrostatic and hydrophobic interactions within a β-sheet-like structure. To identify high-affinity receptors

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within a combinatorial receptor library in a fast and convenient way, a quantitative fluorescence on-bead screening assay can be successfully used and provides relative data for binding affinities at the same time. Analysis of these data shows that, even within a small library of only 512 different members, the binding affinity can be tuned over two orders of magnitude by small changes within the receptor structure. Hence, small libraries designed for a specific case can be as efficient as large, random libraries in identifying suitable receptors for a specific target.

Experimental Section

Synthesis of tetrapeptide 5: The fluorophore-labeled tetrapeptide **5** was synthesized on Wang resin by a standard protocol: Wang resin (300 mg, 1.11 mmol g^{-1}, 0.33 mmol) was swollen in CH_2Cl_2/DMF (8:2) for 1.5 h, and the first amino acid was coupled to the resin by use of Fmoc-Ala (2.0 equiv), diisopropylcarbodiimide (DIC, 2.0 equiv), and dimethylaminopyridine (DMAP, 0.1 equiv) in CH_2Cl_2/MeOH (8:2), with a reaction time of 20 h. The coupling step was repeated twice. The Fmoc group was cleaved with piperidine in DMF (20%). The other three amino acids (Ile, Val, Val) were coupled by use of the following procedure: 2.5 equiv of amino acid and 2.5 equiv of (benzotriazol-1-yloxy)trispyrrolidinophosphonium hexafluorophosphate (PyBOP) in DMF containing 3% *N*-methyl morpholine (NMM; 10 mL) for 4 h. The free amino function of the tetrapeptide was then coupled with succinic anhydride (10.0 equiv) in CH_2Cl_2/DMF mixture (8:2) for 3 h. The resulting carboxylic acid was coupled with 1,13-diamino-4,7,10-trioxaundecane with PyBOP (10.0 equiv) in DMF (reaction time 3 h). The resulting amine was treated with dansyl chloride (3.0 equiv) in DMF for 20 h. Cleavage from the resin was performed with a CH_2Cl_2/TFA mixture (1:1) for 2 h. The solvent was evaporated, and the resulting red oil was treated with dry diethyl ether to obtain a light green solid that was analytically pure: ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 0.76-0.83$ (m, 18H; 6CH_3), 1.06 (m, 1H; CH_2), 1.23 (d, $^3J_{H,H} = 7.3$ Hz, 3H; CH_3), 1.41 (m, 1H; CH_2), 1.51 (q, $^3J_{H,H} = 6.6$ Hz, 2H; CH_2), 1.58 (q, $^3J_{H,H} = 6.8$ Hz, 2H; CH_2), 1.70 (m, 1H; CH_2), 1.94 (m, 1H; CH), 2.24–2.42 (m, 4H; CH_2), 2.83 (s, 6H; CH_3), 3.04 (m, 3H; CH), 3.20–3.23 (m, 4H; CH_2), 3.34–3.51 (m, 12H; CH_2), 4.12–4.20 (m, 4H; CH), 7.25 (d, $^3J_{H,H} = 7.6$ Hz, 1H; CH), 7.65 (d, $^3J_{H,H} = 9.0$ Hz, 1H; NH), 7.77 (t, $^3J_{H,H} = 5.6$ Hz, 1H; NH), 7.79 (d, $^3J_{H,H} = 7.1$ Hz, 1H; NH), 7.82 (t, $^3J_{H,H} = 5.8$ Hz, 1H; NH), 7.84 (d, $^3J_{H,H} = 6.8$ Hz, 1H; NH), 8.09 (d × d, $^3J_{H,H} = 7.1$ Hz, $^3J_{H,H} = 7.3$ Hz, 1H; CH), 8.11 (m, 1H; NH), 8.29 (d, $^3J_{H,H} = 8.6$ Hz, 1H; CH), 8.45 ppm (d, $^3J_{H,H} = 8.6$ Hz, 1H; CH); MS (ESI, DMSO/H_2O) *m/z*: 958 $[M+Na]^+$, 936 $[M+H]^+$, 758 $[M-C_9H_{17}N_2O_3+Na]^+$, 576 $[C_{26}H_{39}N_3O_7S+K]^+$; HR-MS (pos. ESI) $C_{45}H_{74}N_7O_{12}S^+$ calcd: 936.511; found: 936.511.

Synthesis of receptors 6, 7, and 8: The synthesis of the receptors **6**, **7**, and **8** was performed on Rink amide resin by following a standard protocol. Rink amide resin (300 mg, 0.74 mmol g^{-1}, 0.22 mmol) was swollen in DMF for 1.5 h. The Fmoc protection group was removed by agitation with piperidine in DMF (20%) for 20 min. Coupling conditions for the amino acids: 2.5 equiv of Fmoc amino acid and 2.5 equiv PyBOP in DMF containing NMM 3% (10 mL). The mixture was shaken for 3.5 h to ensure quantitative coupling. The yield was monitored indirectly through the UV absorption of the Fmoc–piperidine adduct at 300 nm. The attachment of the 5-guanidiniocarbonylpyrrole-2-carboxylic acid was performed under related conditions: 2.5 equiv carboxylic acid, 2.5 equiv PyBOP, and DMF containing 5% NMM, with extended reaction times of 24 h. The last step was repeated to ensure quantitative coupling. The product was cleaved from the solid support by shaking the resin with CH_2Cl_2/TFA (5:95). The solvent was evaporated, and the remaining oil was treated with dry diethyl ether. To obtain the hydrochloride salt, the resulting white solid was dissolved in methanol (1 mL), acidified with hydrochloric acid (0.1 N, 4 mL), and lyophilized. To ensure quantitative hydrochlorination this was done twice.

*Gua-Leu-Tyr-Leu-NH$_2$ (**6**)*: ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 0.79-0.88$ (m, 12H; 4CH_3), 1.41–1.57 (m, 6H; 2CH_2, 2CH), 2.71 (m, 1H; CH_2), 2.89–2.94 (m, 1H; CH), 4.18 (m, 1H; CH), 4.37–4.48 (m, 2H; CH), 6.55 (d, 2H; $^3J_{H,H} = 8.5$ Hz; Ar-CH), 6.88 (m, 1H; py-CH), 6.97 (s, 1H; NH_2), 6.98 (d, $^3J_{H,H} = 8.5$ Hz, 2H; Ar-CH), 7.09 (s, 1H; NH_2), 7.49 (m, 1H; py-CH), 7.75 (d, $^3J_{H,H} = 8.6$ Hz, 1H; NH), 8.14 (d, $^3J_{H,H} = 8.1$ Hz, 1H; NH), 8.42 (s, 2H; gua-NH_2), 8.53 (d, $^3J_{H,H} = 7.8$ Hz, 1H; NH), 8.60 (s, 2H; gua-NH_2), 11.92 (s, 1H; gua-NH), 12.48 ppm (s, 1H; py-NH); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 21.7$ (2CH_2), 23.1, 23.2, 24.2, 24.4 (CH_3), 36.2, 40.7, 41.2 (CH_2), 51.1, 51.5, 54.4 (CH), 113.8, 115.8 (py-CH), 125.9, 132.5 (py-C_q), 115.0, 128.0 (Ar-CH) 130.2, 155.9 (Ar-C_q) 155.6 (gua-C_q), 159.0, 159.9, 170.8, 172.0, 174.0 (C_q) ppm; MS (ESI, DMSO/H_2O) *m/z*: 585 $[M]^+$, 568 $[M-NH_2]^+$, 455 $[M-C_6H_{13}N_2O]^+$, 292 $[M-C_{15}H_{22}N_3O_3]^+$, 179 $[C_7H_7N_4O_2]^+$; HR-MS (pos. ESI) $C_{28}H_{41}N_8O_6^+$ calcd: 585.3149; found: 585.315.

*Gua-Val-Val-Val-NH$_2$ (**7**)*: ^{1}H NMR (500 MHz, [D$_6$]DMSO): $\delta = 0.82-0.85$ (m, 18H; 6CH_3), 1.93–1.95 (m, 2H; 2CH), 2.05 (m, 1H; CH), 4.10 (m, 1H; CH), 4.17 (m, 1H; CH), 6.91 (m, 1H; py-CH), 7.02 (s, 1H; NH_2), 7.12 (m, 1H; py-CH), 7.30 (s, 1H; NH_2), 7.67 (d, $^3J_{H,H} = 8.8$ Hz, 1H; NH), 8.11 (d, $^3J_{H,H} = 8.7$ Hz, 1H; NH), 8.40 (s, 2H; gua-NH_2), 8.45 (d, $^3J_{H,H} = 8.5$ Hz, 1H; NH), 8.57 (s, 2H; gua-NH_2), 11.32 (s, 1H; gua-NH), 12.72 ppm (s, 1H; py-NH); ^{13}C NMR (125 MHz, [D$_6$]DMSO): $\delta = 17.9$, 18.4, 18.5, 19.2, 19.3, 19.3 (CH_3), 30.1, 30.4, 30.6, 57.4, 58.1, 57.9, (CH), 113.9, 115.0 (py-CH), 125.6, 132.4 (py-C_q), 155.1 (gua-C_q), 159.9, 170.7, 170.7, 170.9 ppm (C_q); MS (ESI, DMSO/H_2O) *m/z*: 493 $[M]^+$, 377 $[M-C_5H_{11}N_2O]^+$, 278 $[M-C_{10}H_{20}N_3O_2]^+$, 179 $[C_7H_7N_4O_2]^+$.

*Gua-Lys-Leu-Lys-NH$_2$ (**8**)*: ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 0.82$ (d, $^3J_{H,H} = 6.3$ Hz, 3H; CH_3), 0.87 (d, $^3J_{H,H} = 6.6$ Hz, 3H; CH_3), 1.25–1.77 (m, 15H; CH_2 + CH), 2.72–2.80 (m, 4H; CH_2), 4.13 (m, 1H; CH), 4.26 (m, 1H; CH), 4.43 (m, 1H; CH), 6.90 (m, 1H; py-CH), 7.03 (s, 1H; NH_2), 7.18 (m, 1H; py-CH), 7.29 (s, 1H; NH_2), 7.73 (d, 1H; NH), 7.75 (s, 6H; NH_3^+), 8.15 (d, $^3J_{H,H} = 8.1$ Hz, 1H; NH), 8.53 (s, 2H; gua-NH_2), 8.54 (d, $^3J_{H,H} = 7.6$ Hz, 1H; NH), 8.67 (s, 2H; gua-NH_2), 11.51 (s, 1H; gua-NH), 12.49 ppm (s, 1H; py-NH); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 21.6$ (2CH_3), 22.3 (CH), 22.7, 23.3, 24.4, 26.8, 31.5, 31.6, 38.8, 38.9 (CH_2), 51.5, 52.3, 52.9 (CH), 113.8, 115.3 (py-CH), 125.8, 132.5 (py-C_q), 155.4 (gua-C_q), 158.7, 160.1, 171.8, 171.9, 173.5 ppm (C_q); MS (ESI, DMSO/H_2O) *m/z*: 565 $[M+H]^+$, 283 $[M+2H]^{2+}$, 274 $[M-NH_2]^{2+}$, 189 $[M+3H]^{3+}$, 179 $[C_7H_7N_4O_2]^+$; HR-MS (pos. ESI) $C_{28}H_{41}N_8O_6^+$ calcd: 565.3574; found: 565.358.

Synthesis of receptor 9: Rink amide resin (300 mg, 0.74 mmol g^{-1}, 0.22 mmol) was swollen in DMF for 1.5 h. The Fmoc protection group was removed by agitation with piperidine in DMF (20%) for 20 min. The resin was treated with succinic anhydride (10 equiv) and DMAP (1 equiv) in DMF/CH_2Cl_2 (8:2) for 4 h. The free carboxylic acid was coupled with 1,13-diamino-4,7,10-trioxaundecane (10 equiv) by use of PyBOP (2.5 equiv) in DMF for 3 h. The peptide was synthesized with the resulting free amine. Coupling conditions for the amino acids: 2.5 equiv of Fmoc amino acid, 2.5 equiv PyBOP, DMF containing NMM 3% (10 mL). The mixture was shaken for 3.5 h to ensure quantitative coupling. The attachment of the 5-guanidiniocarbonylpyrrole-2-carboxylic acid was performed under related conditions: 2.5 equiv carboxylic acid, 2.5 equiv PyBOP, and DMF containing 5% NMM, with extended reaction times of 24 h. The last step was repeated to ensure quantitative coupling. The product was cleaved from the solid support by shaking the resin with a CH_2Cl_2/TFA mixture (5:95). The solvent was evaporated, and the remaining oil

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was treated with dry diethyl ether. To obtain the hydrochloride salt, the white solid was dissolved in methanol (1 mL), acidified with hydrochloric acid (0.1 N, 4 mL), and lyophilized: ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 0.81 - 0.88$ (m, 18 H; 6 CH$_3$), 1.59 (m, 4 H; 2 CH), 1.85 – 2.09 (m, 3 H; CH), 2.25 (s, 4 H; CH$_2$), 3.05 (m, 4 H; CH$_2$), 3.35 – 3.45 (m, 12 H; CH$_2$), 4.07 (m, 1 H; CH), 4.18 (m, 1 H; CH), 4.46 (m, 1 H; CH), 6.83 (s, 1 H; NH$_2$), 6.90 (m, 1 H; py-CH), 7.24 (s, 1 H; NH$_2$), 7.36 (m, 1 H; py-CH), 7.69 (d, $^3J_{H,H} = 8.8$ Hz, 1 H; NH), 7.40 (t, $^3J_{H,H} = 5.5$ Hz, 1 H; NH), 7.87 (t, $^3J_{H,H} = 5.5$ Hz, 1 H; NH), 8.06 (t, $^3J_{H,H} = 8.8$ Hz, 1 H; NH), 8.44 (t, $^3J_{H,H} = 8.3$ Hz, 1 H; NH), 8.36 – 8.50 (br s, 4 H; gua-NH$_2$), 11.65 (s, 1 H; gua-NH), 12.70 ppm (s, 1 H; py-NH); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 18.4$, 18.5, 18.6, 19.3, 19.3, 19.4 (CH$_3$), 29.4, 29.5, 30.3, 30.4, 30.6, 30.7, 30.8 (3 CH, 2 CH$_2$, 2 N–CH$_2$) 35.8, 35.9 (CH$_2$) 58.0, 58.1, 58.3 (CH), 68.1, 68.2, 69.7, 69.7, 69.9, 69.9 (O–CH$_2$), 114.1, 115.6 (py-CH), 125.7, 132.6 (py-C$_q$), 155.5 (gua-C$_q$), 158.5, 159.9, 170.7, 170.8, 171.0, 171.4, 173.6 ppm (C$_q$).

Synthesis of tetrapeptide 10: The tetrapeptide **10** was synthesized on Wang resin by a standard protocol: Wang resin (300 mg, 1.11 mmol g^{-1}, 0.33 mmol) was swollen in CH$_2$Cl$_2$/DMF (8:2) for 1.5 h. The first amino acid was coupled to the resin with Fmoc-Ala (2.0 equiv), DIC (2.0 equiv), and DMAP (0.1 equiv) in CH$_2$Cl$_2$/MeOH (8:2); reaction time 20 h. The procedure was repeated, after which the Fmoc group was cleaved with piperidine in DMF (20 %). The next three amino acids (Ile, Val, Val) were coupled by the following procedure: 2.5 equiv of amino acid and 2.5 equiv of PyBOP in DMF containing 3 % NMM (10 mL) for 4 h. The free amino function of the tetrapeptide was then coupled with triethyleneglycol carboxylic acid by use of PyBOP in DMF (2.5 equiv in DMF for 3 h). Cleavage from the resin was performed with CH$_2$Cl$_2$/TFA (1:1) for 2 h. The solvent was evaporated, and the resulting brown oil was treated with dry diethyl ether to obtain a white solid, which was analytically pure: ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 0.76 - 0.83$ (m, 18 H; 6 CH$_3$), 1.07 (m, 1 H; CH$_2$), 1.23 (d, $^3J_{H,H} = 7.3$ Hz, 3 H; CH$_3$), 1.42 (m, 1 H; CH$_2$), 1.69 (m, 1 H; CH), 1.94 (m, 2 H; CH), 3.46 – 3.59 (m, 12 H; CH$_2$), 3.92 (s, 2 H; CH$_2$), 4.12 – 4.18 (m, 4 H; CH), 7.44 (d, $^3J_{H,H} = 9.1$ Hz, 1 H; NH), 7.72 (d, $^3J_{H,H} = 8.8$ Hz, 1 H; NH), 8.02 (d, $^3J_{H,H} = 8.8$ Hz, 1 H; NH), 8.13 ppm (d, $^3J_{H,H} = 7.1$ Hz, 1 H; NH); ^{13}C NMR (75 MHz, [D$_6$]DMSO): $\delta = 11.0$, 15.3, 17.2, 18.0, 18.0, 18.5, 19.3 (CH$_3$), 24.3 (CH$_2$), 30.4, 31.1, 36.8, 47.4, 56.5, 57.7, 58.1 (CH), 67.6, 67,7, 67,8, 68.4, 69.7, 69.9, 72.5 (O–CH$_2$), 168.9, 170.4, 170.6, 170.7, 173.9 ppm (C$_q$); MS (ESI, DMSO/H$_2$O) *m/z*: 613 [M+Na]$^+$, 502 [$M - C_3H_6NO_2$]$^+$, 389 [$C_{18}H_{33}N_2O_7$]$^+$, 290 [$C_{13}H_{24}NO_6$]$^+$, 203 [$C_8H_{15}O_5 - Na$]$^+$; HR-MS (pos. ESI) $C_{28}H_{41}N_8O_6Na^+$ calcd: 613.3424; found: 613.342.

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Keywords: combinatorial chemistry · guanidinium cations · molecular recognition · peptides · receptors

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[19] Another receptor, Gua-Glu(OBnz)-Trp-Glu(OBnz)-NH_2, which is one of the weak binding receptors according to the on-bead assay ($K_{ass} = 90\,M^{-1}$), did not give any reasonable data. The binding constant was too low to be measured accurately in free solution by UV titration, which is again in good agreement with the rather poor binding affinity of this specific receptor observed in the on-bead assay.
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Synthetic Peptides Derived from the Angiostatin K4 Domain Inhibit Endothelial Cell Migration

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Angiogenesis, the process by which new capillaries are formed from preexisting blood vessels, represents a vital function for the growth of normal tissues during embryogenesis as well as for the pathological growth of tumors. Experimental evidence has shown that both primary and metastatic tumors need to recruit angiogenic vessels for their growth, and form their own circulatory system by up-regulating angiogenic stimulators and down-regulating angiogenesis inhibitors.[1] Blocking of positive regulators or utilization of negative regulators to suppress angiogenesis results in a delay or regression of induced tumors. In particular, negative regulators of angiogenesis, such as angiostatin, endostatin, and antagonists for integrin $\alpha v\beta 3$, displayed profound antitumor activities in vivo.[2]

Angiostatin is a 38 kDa internal fragment of plasminogen, produced by proteolytic cleavage, and consists of four triply-disulfide-bridged kringle structures. In mice, angiostatin inhibits primary tumor growth as well as angiogenesis-dependent growth of metastases. In vitro, angiostatin inhibits endothelial cell migration and reduces endothelial cell growth in proliferation assays. Furthermore, it has been shown that angiostatin induces endothelial cell-specific apoptosis in vitro.[3]

Despite intense research efforts, the exact mechanisms by which angiostatin down-regulates neovascularization remain to be determined. Little is also known about the nature of receptors mediating inhibitory effects on endothelial cells, although several receptors for angiostatin have been identified. ATP synthase has been described as a high-affinity angiostatin-binding protein, and antibodies to ATP synthase have been reported to inhibit the antiproliferative activity of angiostatin on endothelial cells. Plasminogen itself has been shown to bind to other cell-surface proteins such as annexin II and alpha enolase.[4] Recently, angiomotin has been identified in a yeast two-hybrid screen as a functional angiostatin-binding protein that mediates the inhibition of endothelial cell motility and tube formation in vitro.[5]

A fundamental step for deciphering the biology of angiostatin is to understand the role of the kringle functional domains in the structure/function of angiostatin. Each kringle is composed of approximately 80 amino acids in a double-loop conformation, held together by three disulfide bonds. Angiostatin was originally described as consisting of kringles 1 – 4, although the −COOH terminus was identified later on by Soff and co-workers, who described an angiostatin consisting of kringles 1 – 4, plus approximately 85 % of plasminogen kringle 5.[6] Early attempts to generate recombinant angiostatin indicated the importance of the disulfide-bonded kringle in the inhibition of angiogenesis. Several other findings suggested that the kringle structure itself confers a large part, if not all, of the antiangiogenic activity.[7] While sharing structural similarity and sequence homology, kringle domains appear to have distinct inhibitory profiles on angiogenesis-associated endothelial cell activities.[8] Specifically, the in vitro activity of angiostatin in endothelial-cell proliferation assays resides in kringles 1 – 3, with kringle 1 being the most potent inhibitor, while kringle 4 is relatively ineffective. In contrast, K4 is the smaller fragment with the most potent antimigratory activity. Finally, Cao and co-workers demonstrated that K5 of plasminogen presents the most potent activity against endothelial-cell proliferation.[9] The reasons why some of the kringle domains are significantly more potent than others and the mechanisms explaining the dissociation of antiproliferative and antimigratory activity attributed to K4, compared with other kringle domains, are still unknown. However, comparisons of different angiostatin-related proteins require careful scrutiny since differences in activity between different kringle domains, or different angiostatin isoforms with varying kringle compositions, may reflect artifacts of the recombinant technology, and not necessarily an inherent difference in the kringle composition.

To investigate the different contribution of K4 regions to the antimigratory activity, three linear peptides that mapped the entire K4 sequence were designed (Figure 1), synthesized, and tested for biological activity, the quantification of the biological activity being regarded as the first step in the process of identification of the minimum active sequence in the design of peptides of reduced length. All linear peptides present N- and C-terminal Cys residues protected by Acm or *t*Buthio groups. In

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