Selective Binding of Two-Armed Diketopiperazine Receptors to Side-Chain-Protected Peptides

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Abstract: The binding properties of two-armed receptors based on a diketopiperazine template toward side-chain-protected peptides have been elucidated. Selective binding of these diketopiperazine receptors is not limited to side-chain-free peptides, but combinatorial on-bead assays show that also side-chain-protected peptides are recognized with high specificity. Furthermore, the screening of several dye-marked diketopiperazine receptors against an encoded side-chain-protected tripeptide library demonstrated not only their high binding specificities but also revealed that small structural changes suffice to alter the binding preferences significantly.

Studying the selective recognition of peptides by synthetic receptors is not only of importance as model studies for understanding noncovalent interactions but also for the development of diagnostic sensors and therapeutics.[1] Furthermore, synthetic receptors can be immobilized on a solid phase and used as chiral stationary phases for the separation of peptides.[2] In this respect, the discrimination between enantiomeric as well as diastereomeric peptides is of particular interest. Here, we report on the selective binding of various two-armed diketopiperazine receptors to side-chain-protected tripeptides.

We have recently developed the class of diketopiperazine receptors that consist of a rigid, structure-directing diketopiperazine template and tripeptides as "receptor arms" (Figure 1).[3]

The hydroxyproline derived diketopiperazine template was designed to provide conformational rigidity, while the peptidic receptor arms should allow for the formation of noncovalent interactions to peptidic substrates. These two-armed receptors[4] can be assembled by standard peptide chemistry and their structure can be varied easily. The receptor prototypes **1**−**5** (Figure 2) have already been found to bind with exceptionally high sequence selectivity to certain tripeptides within a combinatorial library.[3]

We have now turned our attention to the binding properties of receptors **1**−**5** toward side-chain-protected

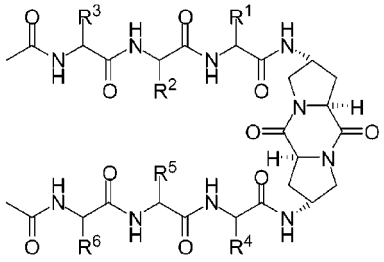

Figure 1. General structure of diketopiperazine receptors.

peptides. Thus, the dye-marked receptors **1**−**5** were screened against a tripeptide library with acid-sensitive protecting groups still attached to the side-chain functional groups of the tripeptides.

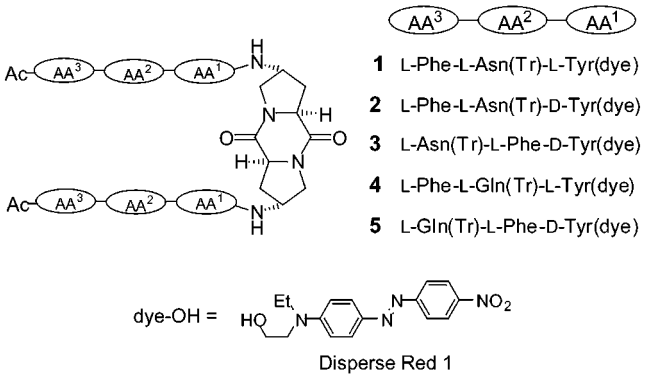

Figure 2. Dye-marked diketopiperazine receptors **1**−**5**.

The library with the general structure Ac-AA3-AA2-AA1-NH$(CH_2)_5$CONH-PS had been generated on polystyrene resin following the protocol for encoded[5] split-and-mix synthesis.[6] We used the standard FMOC procedure for peptide couplings;[7] thus, N-α-FMOC-protected amino acids with acid-sensitive protecting groups on the side-chain functional groups were employed in the synthesis. The side-chain functional groups of the amino acids aspartic acid, glutamic acid, serine, threonine, and lysine were protected with *tert*-butyl (*t*-Bu) groups, trityl (Tr) groups served for asparagine, glutamine, and histidine, and the 2,2,5,7,8-pentamethylchroman-6-sulfonyl (PMC) group for arginine. Since 29

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Table 1. Binding Specificities of Receptors 1−5 for Tripeptides within the Side-Chain-Protected Library Ac-AA3-AA2-AA1-NH(CH$_2$)$_5$CONH-PS

	AA3[a]	AA2[a]	AA1[a]	freq found[b] (%)	freq exp[b] (%)
1	D-Asn(Tr)	D-Ala	X	47	0.12
	D-Asn(Tr)	D-Val	X	35	0.12
2	X	L-Asn(Tr)	L-Ala	41	0.12
	X	L-Ser(*t*-Bu)/L-Asp(*t*-Bu)	L-Ala	17	0.24
	X	Gly	Gly	17	0.12
	L-Hph[c]	X	L-Pro/D-Pro	14	0.71
3	D-Val	D-Asn(Tr)	D-Phe	19	0.004
	D-Val	D-Asn(Tr)	D-X	37	0.06
	D-Ala/D-Val	L-Ala/L-Val	D-Gln(Tr)	37	0.02
4		no binding			
5		no binding			

[a] X = random amino acid. [b] The frequency found column lists the percentage of beads selected in the receptor binding assay for the indicated peptide sequence. The frequency expected column lists the expected frequency for the particular tripeptide sequence if the beads were picked randomly. The comparison between the percentage of "frequency found" and "frequency expected" is a measure for the selectivity level of the receptor. [c] Hph = hydrophobic amino acid can be either Ala, Val, or Leu.

different D- and L-amino acids[8] were employed in each position, the library contained maximally $29^3 = 24\,389$ different tripeptides.

Upon mixing of dilute solutions (40 mM) of receptors **1**−**3** in $CHCl_3$ with the library,[9] only a few beads (ca. one out of 1000−1500) picked up the color of the dye-marked receptors indicating selective binding to certain members within the library. In contrast, in the assays of receptors **4** and **5** none of the library beads picked up the red color of the receptors indicating no interaction with any of the tripeptides up to receptor concentrations of 500 mM. This result is most remarkable since receptors **4** and **5** differ from receptors **1** and **3**, respectively, only by a single additional methylene group, asparagine is exchanged by glutamine. Thus, subtle differences in the receptor structure suffice to alter the binding properties to a significant extent (Table 1).

The comparison of the assays of receptors **1**−**3** showed that the darkest colored beads with receptor **2** were significantly lighter than the darkest beads with receptors **1** and **3**, hinting at a somewhat weaker association between receptor **2** and its selected peptides.

Isolation of the colored beads of the assays of receptors **1**−**3** followed by analysis of the encoding tag molecules revealed the amino acid sequences of the selected peptides.[10]

Remarkably, all three structurally similar receptors select for different di- and tripeptides. Receptor **1** binds with high selectivity the dipeptidic motifs D-Asn(Tr)-D-Ala or D-Asn(Tr)-D-Val in positions AA3 and AA2 followed by a random amino acid in position AA1. In contrast, receptor **2**, differing from receptor **1** only in the configuration of the tyrosine, selects predominantly for tripeptides with the dipeptidic motif L-Asn(Tr)-L-Ala in positions AA2 and AA1. Thus, the diastereomeric receptors **1** and **2** select for the frame shifted enantiomers D-Asn(Tr)-D-Ala and L-Asn(Tr)-L-Ala, respectively. Furthermore, receptor **3** differing from **2** in the sequence of the last two amino acids in the receptor arms shows not only binding preferences for dipeptidic motifs but even for tripeptides such as Ac-D-Val-D-Asn(Tr)-D-Phe and Ac-D-Ala-L-Val-D-Gln(Tr). These results demonstrate that the binding preferences of diketopiperazine receptors are very responsive to small structural changes.

Table 2. Relative Binding Affinities of Receptors 1−3 toward Solid Supported Peptides; Peptide-NH(CH$_2$)$_5$CONH-PS

receptor	peptide	K_a (M^{-1})	ΔG (kcal mol^{-1})
1	Ac-D-Asn(Tr)-D-Ala-L-Val	260 ± 50	−3.3 ± 0.1
1	Ac-D-Asn(Tr)-D-Val-L-Lys(Boc)	220 ± 40	−3.2 ± 0.1
1	Ac-D-Asn(Tr)-D-Ala	35 ± 6	−2.1 ± 0.1
1	Ac-D-Phe-L-Asn(Tr)-L-Ala	≤15	≤−1.5
1	Ac-L-Asn(Tr)-L-Ala	≤15	≤−1.5
2	Ac-D-Phe-L-Asn(Tr)-L-Ala	≤15	≤−1.5
2	Ac-L-Asn(Tr)-L-Ala	≤15	≤−1.5
2	Ac-D-Asn(Tr)-D-Ala	≤15	≤−1.5
3	Ac-D-Val-D-Asn(Tr)-D-Phe	3900 ± 600	−4.9 ± 0.1
3	Ac-D-Ala-L-Val-D-Gln(Tr)	610 ± 100	−3.8 ± 0.1

As expected, these binding specificities toward side-chain-protected peptides are entirely different from the binding specificities observed toward side-chain free peptides.[3] For example, receptor **1** selects exclusively for tripeptides Ac-D-Hph-D-Hph-D-His (D-Hph = hydrophobic amino acid, either Ala, Val, Leu, or Phe) within side-chain-free peptides[3] and for the dipeptide motif D-Asn-(Tr)-D-Ala among side-chain-protected peptides. Receptor **3** selects the side-chain-protected peptide Ac-D-Ala-L-Val-D-Gln(Tr) but the side-chain-free peptide Ac-D-Hph-L-Hph-L-Ser/Thr.[3] Furthermore, while receptors **4** and **5** do not bind to any of the side-chain-protected peptides, they show distinct binding preferences among side-chain-free tripeptides.

To obtain a measure for the strength of the observed intermolecular interactions, we determined the binding affinities of receptors **1**−**3** toward peptides selected in the on-bead assays and several nonselected peptides by a solid-phase binding assay (Table 2).[3,4f]

Receptor **1** binds to tripeptides containing the dipeptide motifs Ac-D-Asn(Tr)-D-Ala or Ac-D-Asn(Tr)-D-Val with an affinity of $K_a = 260 \pm 50\ M^{-1}$ ($\Delta G = -3.3 \pm 0.1$ kcal/mol^{-1}). The binding affinity toward the dipeptide Ac-D-Asn(Tr)-D-Ala itself is 10-fold lower $K_a = 35 \pm 6\ M^{-1}$ ($\Delta G = -2.1 \pm 0.1$ kcal/mol^{-1}), indicating that the third amino acid is not selectively bound but nevertheless contributes to the intermolecular association, presumably due to hydrogen bonding toward the additional amide. Peptides containing the enantiomeric dipeptide Ac-L-Asn(Tr)-L-Ala are not bound with measurable affinity, demonstrating the ability of receptor **1** to discriminate between enantiomeric and diastereomeric peptides.

As already expected from the visual inspection of the combinatorial assay of receptor **2**, its binding affinity toward the preferred peptide Ac-D-Phe-L-Asn(Tr)-L-Ala is smaller than $K_a \leq 15\ M^{-1}$ ($\Delta G \leq -1.5$ kcal/mol^{-1}), the detection limit of the solid-phase binding assay. Thus, the color intensity observed in the combinatorial assay already allows for an estimate of the strength of the intermolecular interaction. Receptor **3**, which did not only select for a dipeptide motif but for tripeptides in the combinatorial screening, interacts with the selected

(8) AA1−AA3 = Gly, D- and L-Ala, Val, Leu, Phe, Pro, Ser(*t*-Bu), Thr(*t*-Bu), Asp(*t*-Bu), Glu(*t*-Bu), Asn(Tr), Gln(Tr), His(Tr), Lys(Boc), Arg(PMC).

(9) To ensure the presence of each library member 3−5 theoretical copies of the library were present in each assay: Burgess, K.; Liaw, A. I.; Wang, N. Y. *J. Med. Chem.* **1994**, *37*, 2985.

(10) A total of at least 20 beads were selected per receptor from at least three assays.

tripeptides considerably tighter than receptor **1** with its selected dipeptides. Receptor **3** binds to Ac-D-Val-D-Asn-(Tr)-D-Phe and Ac-D-Ala-L-Val-D-Gln(Tr) with association constants of $K_a = 3900 \pm 600\ M^{-1}$ ($\Delta G = -4.9 \pm 0.1$ kcal/mol^{-1}) and $K_a = 610 \pm 100\ M^{-1}$ ($\Delta G = -3.8 \pm 0.1$ kcal/mol^{-1}), respectively, which are 3- to 10-fold larger than the binding strength of receptor **1** to its preferred peptides. These results reflect that higher sequence specificity coincides with tighter intermolecular interactions.

Two-armed diketopiperazine receptors are versatile hosts for binding different peptidic substrates with high enantiomeric and diastereomeric specificity. We have demonstrated that binding is not limited to side-chain free peptides but that also side chain protected peptides, where hydrogen bonding presumably plays a less pronounced role, are recognized selectively. Diketopiperazine receptors not only bind peptides with high selectivity but small structural changes cause significant differences in their binding preferences to both side chain deprotected as well as side chain protected peptides. Thus, they could be used in any application which requires selective molecular recognition, for example the development of diagnostic sensors or the separation of peptidic substrates on chiral stationary phases.

Experimental Section

General Methods. Solid-phase syntheses were carried out on a manual Wrist Action Shaker with resins purchased from Calbiochem-Novabiochem-AG. ^{1}H and ^{13}C NMR spectra were recorded in 10% CD_3OD in $CDCl_3$ at 500 and 125.6 MHz, respectively, with chemical shifts reported in ppm relative to $CDCl_3$. The solid-phase binding assays were carried out with $CHCl_3$ freshly filtered through aluminum oxide.

General Procedure for the Synthesis of Peptides on the Solid Support Exemplified by the Synthesis of Ac-D-Asn-(Tr)-D-Ala-L-Val-NH(CH$_2$)$_5$CONH-resin. Amino methyl polystyrene resin (200 mg, 0.19 mmol, 100−200 mesh, loading 0.97 mmol/g) was placed in a 20 mL Merrifield vessel and suspended in CH_2Cl_2 (5 mL). *N*-ϵ-FMOC-aminocaproic acid (210 mg, 0.58 mmol) along with 1-hydroxybenzotriazole (80 mg, 0.58 mmol) dissolved in DMF (0.2 mL) were added, and the mixture was shaken for 5 min before diisopropylcarbodiimide (90 μL, 0.58 mmol) was added. The mixture was agitated for 2 h and then washed three times with DMF and three times with CH_2Cl_2 for 3 min each.

For FMOC-deprotection, the resin was washed three times with DMF and then agitated with 10 mL of a mixture of DMF/piperidine 4:1 for 3 min and then 10 min. After the resin was washed five times with DMF and five times with CH_2Cl_2, it was ready for the next coupling. Both the amino acid coupling and the FMOC-deprotection were closely monitored by the Kaiser test for free amines. Consecutive coupling and deprotection cycles employing *N*-Fmoc-D-Phe (225 mg, 0.58 mmol), *N*-α-Fmoc-*N*-β-trityl-D-Asn (350 mg, 0.58 mmol) and *N*-Fmoc-D-Val (200 mg, 0.58 mmol) yielded H-D-Val-D-Asn(Tr)-D-Phe-NH(CH$_2$)$_5$-CONH-resin. For the acetylation, the resin was shaken in a mixture of CH_2Cl_2 (5 mL), NEt_3 (0.15 mL, 0.97 mmol), and Ac_2O (0.1 mL, 0.97 mmol) for 1 h. Washing with CH_2Cl_2 (5 times) yielded Ac-D-Val-D-Asn(Tr)-D-Phe-NH(CH$_2$)$_5$CONH-resin.

To ascertain the identity of the immobilized peptide, a sample was prepared in parallel on 2-chlorotrityl chloride-resin that allows for the cleavage of the side-chain-protected peptide off the solid support by treatment with $CH_3CO_2H/CF_3CH_2OH/CH_2Cl_2$ 1:1:8 for 30 min.[11]

Ac-D-Val-D-Asn(Tr)-D-Phe-OH (6): ^{1}H NMR (10% CD_3OD in $CDCl_3$) δ 0.78 (d, $J = 6.9$ Hz, 3H), 0.84 (d, $J = 6.8$ Hz, 3H), 1.91 (s, 3H), 1.93 (m, 1H), 2.68 (dd, $J = 15.6$, 5.7 Hz, 1H), 2.88 (dd, $J = 15.6$, 5.6 Hz, 1H), 2.97 (dd, $J = 13.9$, 6.8 Hz, 1H), 3.08 (dd, $J = 13.6$, 5.7 Hz, 1H), 4.18 (d, $J = 6.2$ Hz, 1H), 4.62 (dd, $J = 6.8$, 5.7 Hz, 1H), 4.69 (ψt, $J = 5.6$ Hz, 1H), 7.08−7.28 (m, 20H); ^{13}C NMR (10% CD_3OD in $CDCl_3$) δ 17.4, 19.0, 22.4, 30.7, 37.0, 37.8, 49.5, 53.8, 58.4, 70.5, 126.8, 127.7, 127.7, 128.3, 128.6, 129.2, 136.0, 144.1, 170.3, 170.7, 171.4, 171.7, 172.7; ESI-MS *m*/*z* calcd for $C_{39}H_{42}N_4O_6$ $[M + Na]^+$ 685, found 685.

General Procedure for the Determination of Binding Constants on the Solid Support by UV. An accurately measured amount (ca. 5 mg) of resin-bound peptide was placed in a 1 mL UV cuvette. The UV cuvette was then charged with a solution of the receptor in $CHCl_3$ (freshly filtered through Al_2O_3) sealed with a Teflon stopper and slightly agitated for 72 h. After this period of time, the UV absorbance did not change anymore. The beads were allowed to float to the top of the $CHCl_3$ solution before determining the receptor concentration at equilibrium by UV. For the calculation of the binding constant it was assumed that all peptides on the resin are able to participate in the binding process.

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Supporting Information Available: Tripeptide sequences found in the assays of receptors **1**−**3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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