Flexible but with a Defined Turn—Influence of the Template on the Binding Properties of Two-Armed Receptors

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Abstract: Combinatorial binding studies revealed that the di(*trans*-4-aminoproline)diketopiperazine is an ideal template for two-armed receptors with highly selective binding properties towards peptides. It is not only superior to structurally very different diamines but also to the diastereomeric di(*cis*-4-aminoproline)diketopiperazine. These empiric results are rationalized by the analysis of the conformation of the diastereomeric diketopiperazines in the solid state, by X-ray crystal structure analysis, as well as by NMR studies in solution: to observe highly selective binding, the template needs to be not only conformationally rigid but it must have a specific turn geometry. The combination of combinatorial binding studies, X-ray crystal structure analysis, and NMR spectroscopy gave insight into why the *trans*,*trans*-diketopiperazine is a superior template compared to other diamines. Additionally, the results provide a guide for the rational design of two-armed receptors with good binding properties towards peptidic guests.

Keywords: combinatorial chemistry · conformation analysis · diketopiperazine · molecular recognition · proline · receptors

Introduction

The binding specificities of molecular hosts and receptors are often altered significantly by seemingly small structural modifications.[1–6] Thus, understanding the correlation between structure and binding specificity is an important challenge for rationalizing molecular recognition phenomena. This task is particularly challenging for receptors that consist of a template and two peptidic or sulfonopeptidic recognition elements.[3–6] In spite of their structural flexibility many of such two-armed receptors bind peptidic guests with moderate to excellent selectivities and affinities. The lack of apparent preorganization within the receptor structure, combined with the many degrees of freedom of even simple di- and tripeptides renders the principles that govern their intermolecular interactions difficult to understand. However, such an understanding is crucial for the rational design of new receptors. In this paper we aim to understand the principles that determine the binding properties of two-armed diketopiperazine receptors, which were recently reported.[6] These receptors consist of a central diketopiperazine derived from 4-aminoproline and two peptidic side chains as receptor arms (Figure 1).[6]

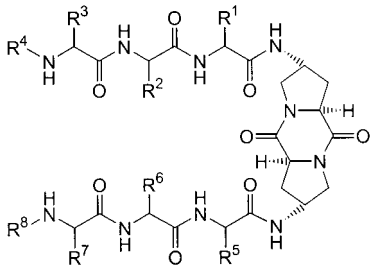

Figure 1. Two-armed diketopiperazine receptors.

Diketopiperazine receptors are highly selective binders for certain peptides as revealed by combinatorial screenings of several receptor prototypes against a tripeptide library. Within the receptor structure, the receptor arms function as the selectivity-determining modules, since small structural modifications in the arms lead to significantly altered binding specificities.[6]

The central diketopiperazine template must provide a structural basis by directing the arms into positions that allow for intermolecular binding. To understand the crucial structural prerequisites that are necessary for the observed highly

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selective binding of diketopiperazine receptors we evaluated the central diketopiperazine as a template of two-armed receptors. By a combination of combinatorial binding studies, crystal structure analysis as well as NMR studies we provide here a rationale why the di(*trans*-4-aminoproline)diketopiperazine[7] is superior to other diamines as a template of two-armed receptors.

Results and Discussion

Combinatorial binding studies: Combinatorial binding experiments allow for the simultaneous analysis of the binding properties of many thousands of peptides towards a given receptor, or vice versa. This technique was therefore used as a tool to elucidate the empiric effects of exchanging the *trans*,*trans*-diaminodiketopiperazine against other diamines on the binding properties of two-armed receptors with peptidic arms.

To examine the influence of a rather small structural difference in the template on the binding properties of two-armed diketopiperazine receptors, we prepared two-armed molecules based on the di(*cis*-4-aminoproline)diketopiperazine, the diastereomer of the originally employed *trans*,*trans*-diketopiperazine (Figure 2).[7]

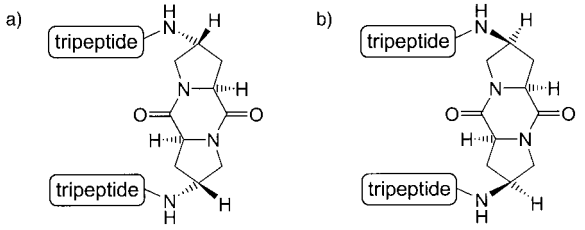

Figure 2. Two-armed receptors based on a) the di(*trans*-4-aminoproline)- and b) the di(*cis*-4-aminoproline)diketopiperazine.[7]

Abstract in German: *Kombinatorische Bindingungsstudien zeigten, dass bis(trans-4-aminoprolin)diamindiketopiperazin ein ideales Templat für zweiarmige Rezeptoren mit hochselektiven Bindungseigenschaften gegenüber Peptiden ist. Es ist nicht nur im Vergleich zu strukturell sehr unterschiedlichen Diaminen sondern auch gegenüber dem diastereomeren bis-(cis-4-aminoprolin)diketopiperazin das bessere Templat. Diese empirischen Resultate konnten durch die Aufklärung der Konformationen der beiden diastereomeren Diprolindiketopiperazine im Festkörper mittels Kristallstrukturanalyse als auch durch NMR-Studien in Lösung verstanden werden: Um eine hochselektive Wechselwirkung erzielen zu können, muss das Templat nicht nur konformationell rigide sein, sondern auch einen bestimmten "Knick" aufweisen. Die Kombination aus kombinatorischen Bindungsstudien, Kristallstrukturanalyse und NMR-Spektroskopie zeigte einerseits, dass das trans,-trans-diaminodiketopiperazin ein besseres Template im Vergleich zu anderen Diaminen ist. Andererseits geben die Ergebnisse eine Anleitung für das rationale Design von zweiarmigen Rezeptoren mit guten Bindungseigenschaften gegenüber peptidischen Gastmolekülen.*

cis,*cis*-Diketopiperazine receptors were prepared by a similar synthetic route as described for the *trans*,*trans*-diketopiperazine receptors.[6a] To allow for a direct analysis of the effect of the diastereomeric templates on the binding properties of two-armed receptors, the same tripeptides were used as receptor arms for prototypes of the *cis*,*cis*-diketopiperazine receptors as had been previously employed for the *trans*,*trans*-diketopiperazine receptors.[6]

The binding properties of the two-armed molecules **1**–**5** (Figure 3) were examined towards a resin-bound tripeptide library with the general structure Ac-AA3-AA2-AA1-$NH(CH_2)_5CONH$-PS (PS = polystyrene resin); the same library that had been used for the analysis of the binding properties of the *trans*,*trans*-diketopiperazine receptors. The

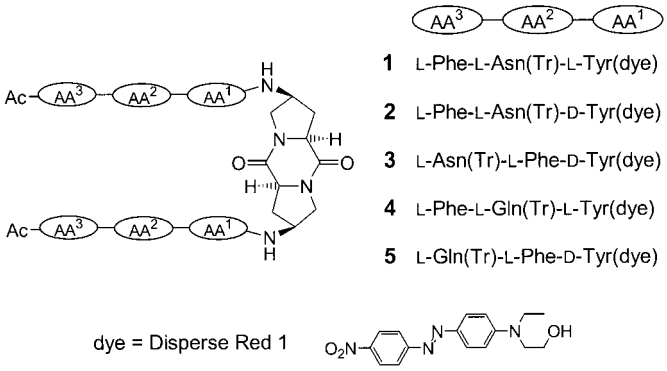

Figure 3. Two-armed molecules **1**–**5** based on the *cis*,*cis*-diketopiperazine.[8]

library had been prepared following the protocol for encoded[9] split-and-mix synthesis[10] and contained a maximum of $29^3 = 24389$ different tripeptides, since 29 different D- and L-amino acids were employed in each position.[11] Each combinatorial binding assay was carried out with an amount of the library that corresponded to at least five theoretical copies in order to ensure representative screening results.[12]

When dilute solutions ($\approx 30\,\mu M$) of receptors **1** and **4** in chloroform were equilibrated with the library, only one bead out of approximately 1500 picked up the red color of the receptors. This indicates a highly selective binding to certain tripeptides within the library. Isolation of these colored beads and analysis of the encoding tag molecules by gas chromatography using electron capture detection[9] revealed the peptide binding specificities of receptors **1** and **4** (Table 1).[13]

Both **1** and **4** select preferentially peptides with a D-His followed by two hydrophobic D-amino acids (D-Hph). These binding specificities are similar to the ones previously observed for the diastereomeric receptors based on the *trans*,*trans*-diketopiperazine.[6a, 14] Besides, both receptors, which differ only in a single methylene group from each other exhibit further distinct binding specificities. Receptor **1** selects for peptides that contain a L-Asn in the middle or a D-Asn at the N-terminal position along with two hydrophobic amino acids. Receptor **4** shows a further preference for peptides with a D-Gln at the N-terminal position followed by a pair of hydrophobic D-amino acids. These selectivities are

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Table 1. Binding specificities of the two-armed molecules **1**–**5** based on the *cis*,*cis*-diketopiperazine for tripeptides within the library Ac-AA3-AA2-AA1-NH$(CH_2)_5$CONH-PS.

	AA3	AA2	AA1	Freq. found [%][a]	Freq. expec. [%][a]
1	D-Hph[b]	D-Hph	D-His	59	0.10
	D-Ala	L-Asn	D/L-Hph	26	0.04
	D-Asn	D-Hph	D-Val/Gly	9	0.04
2			No binding		
3			No binding		
4	D-Val	D-Hph	D-His	56	0.02
	D-Phe/D-Ala	D-Phe/D-Ala	D-His	19	0.02
	D-Gln	D-Val/D-Phe	D-Val/D-Leu	19	0.02
5			No binding		

[a] 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. [b] Hph = hydrophobic amino acid can be either Gly, Ala, Val, Leu, or Phe.

not exhibited by the diastereomeric receptors based on the *trans*,*trans*-diketopiperazine.

In contrast to the highly selective binding properties observed for receptors **1** and **4**, no intermolecular association was observed when solutions of the potential receptors **2**, **3**, and **5** were mixed with the tripeptide library up to concentrations of 500 μM. Thus, the two-armed molecules **2**, **3**, and **5** do not interact with any of the approximately 24000 tripeptides present in the library. This is remarkable, since the diastereomeric molecules based on the *trans*,*trans*-diketopiperazine are highly selective receptors with distinct binding preferences.[6]

The observed binding properties demonstrate that the template plays the most crucial role in allowing or preventing an intermolecular interaction of two-armed receptors towards peptides. This result is supported by combinatorial binding studies with two-armed molecules based on the diamines **6**–**9** (Figure 4). None of these two-armed molecules interact with any of the ≈24000 peptides within the tripeptide library.

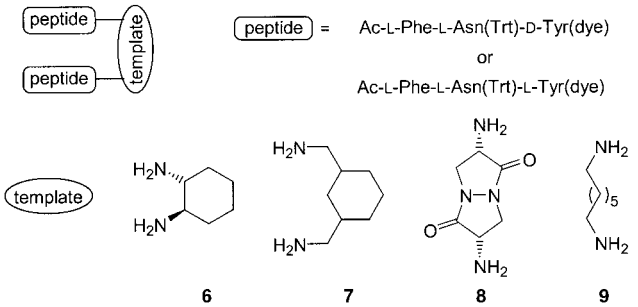

Figure 4. Diamines used as templates for two-armed molecules.[15]

A closer look at the structures of two-armed molecules **1**–**5** reveals that those with an initial L-Tyr are highly selective receptors, while those with an initial D-Tyr do not associate with any peptide. Thus, the combinatorial binding studies revealed that the template influences the binding properties of two-armed receptors the most and that the second largest effect is due to the initial amino acid, the structural element closest to the template.

Conformational analysis: To understand the different binding properties of two-armed receptors based on the *trans*,*trans*- and the *cis*,*cis*-diketopiperazine, we analyzed the conformation of substituted diproline diketopiperazines in the solid state by X-ray single-crystal structure analysis and by NMR studies in solution. In particular, we studied the conformation of the acetamides **10** and **11** as well as the azides **12** and **13** (Figure 5). These minimal fragments of the receptors were used since it was not possible to obtain crystals of the two-armed receptors, suitable for X-ray structure analysis, and the signals in the NMR spectra of the receptors largely overlap.

Figure 5. The acetamides **10** and **11**, and the azides **12** and **13**.

Crystal structure analysis: Within the crystal structures of **10** and **11** the conformation of the tricyclic skeletons differ only marginally. The central diketopiperazine moieties adopt boat conformations; the pyrrolidine rings possess envelope ($E_{C\beta}$) conformations that are slightly perturbed to twisted ($^{C\gamma}T_{C\beta}$)conformations[16](Figure 6). The major difference between the conformation of **10** and **11** is the orientation of the *N*-acetyl groups. In the crystal structure of the *trans*,*trans*-diketopiperazine **10**, they occupy the pseudo axial positions at the Cγ-atoms, in the *cis*,*cis*-diketopiperazine **11** the pseudo equatorial positions. As a result, the distances between the *N*-acetyl groups, as well as the angle formed by the side chains and the tricyclic sceleton are considerably different in the two diastereomers. The N atoms of the *N*-acetyl groups (N1 and N2) are at a distance of 7.8 Å apart in the *trans*,*trans*-diketopiperazine **10** and 8.7 Å apart in the *cis*,*cis*-diketopiperazine **11**. This difference of 1 Å apart is reflected in a significantly narrower angle formed by the *N*-acetyl groups and the diketopiperazine skeleton in the *trans*,*trans*-diketopiperazine **10** compared to the *cis*,*cis*-diketopiperazine **11**. The *trans*,*trans*-diketopiperazine **10** therefore adopts a turn conformation while the conformation of the *cis*,*cis*-diketopiperazine **11** resembles a rather linear structural element.

NMR spectroscopic studies: The asymmetric unit within the unit cell of both crystal structures consists of two molecules that are connected by two hydrogen bonds.[16] The observed conformations might therefore be stabilized and are not likely to reflect the conformation in solution. Thus we analyzed the conformations of the acetylated diketopiperazines **10** and **11** in chloroform solution by a combination of one- and two-dimensional NMR spectroscopy. The conformations of the azides **12** and **13** were also investigated as the proton signals of the *cis*,*cis*-diketopiperazine **11** did not allow for an unambig-

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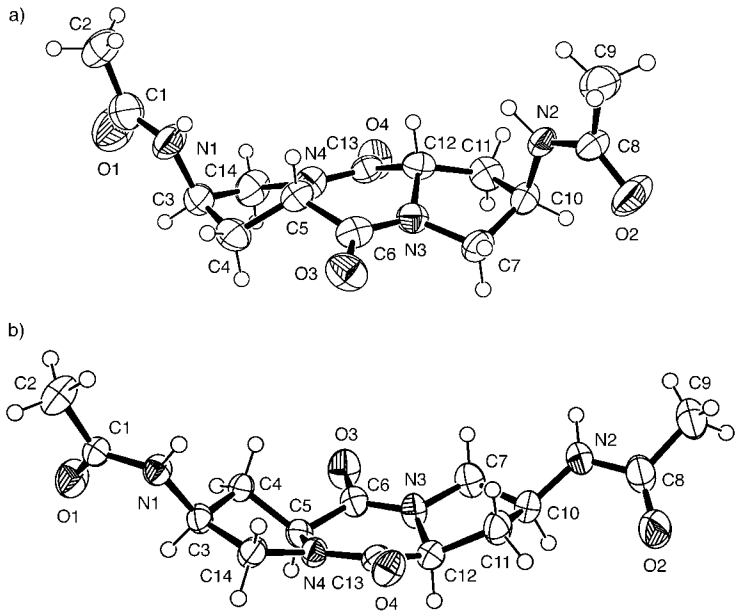


Figure 6. Crystal structures of the *trans,trans*-diketopiperazine **10** (a) and the *cis,cis*-diketopiperazine **11** (b).

uous analysis of the coupling constants of all protons due to overlapping signals. All NMR spectra show only one six-spin system for the pyrrolidine ring protons, indicating that on the average time scale of the NMR measurement, compounds **10–13** possess C_2- symmetry.

Judged by the Karplus curve,[17] the observed couplings between the pyrrolidine protons of the *trans,trans*-diketopiperazine **10** and **12** are in good agreement with the torsion angles found in the crystal structure of **10** (Table 2). The coupling constants of the acetamide **10** and the azide **12** differ by ≈ 1 Hz. This indicates that the azide is conformationally slightly more fixed and reveals that the overall conformation is not dramatically changed by the substituent at Cγ. Both compounds prefer a conformation with the substituents in the pseudo axial positions, the same turn like conformation as observed in the crystal structure of **10**.

Unfortunately, several coupling constants of the acetamide **11** could not be determined unambiguously due to overlapping signals. Since the coupling constants ^3J(Hγ,Hδ) and ^3J(Hγ,Hδ') are almost identical for **11** and the azide **13**, we assume that their conformations look alike (Table 3). In contrast to the good correlation of the conformations in solution and the solid state of the *trans,-trans*-diketopiperazines, the coupling constants observed for the *cis,cis*-diketopiperazines **11** and **13** do not match with the torsion angles found in the crystal structure of **11**. For example, the torsion angles of $\approx 150^\circ$ should be reflected by couplings of $^3J \approx 9-11.5$ Hz, and the torsion angles of $\approx 30^\circ$ by couplings of $^3J \approx 6.5-8.5$ Hz.[17] The observed coupling constants differ significantly from these expected values and are in fact not in agreement with any single conformation. Two main conformations are conceivable for **11** and **13**: a conformation with the substituents at Cγ in the pseudo equatorial positions as seen in the crystal structure and another one with pseudo-axial-positioned substituents and ring flipped pyrrolidine moieties. The latter would result in torsion angles that are considerably different from the values found in the crystal structure (Figure 7).

Table 2. ^{1}H-^{1}H coupling constants [Hz, ± 0.1 Hz] observed for **10** and **12** compared to the torsional angles [°] found in the crystal structure of **10**.

Torsion angle[a]	NMR, ^3J(H,H) **10**	NMR, ^3J(H,H) **12**	X-ray **10**[b]
Hα-Cα-Cβ-Hβ	7.7	6.6	34 ± 5
Hα-Cα-Cβ-Hβ'	9.6	10.4	156 ± 5
Hβ-Cβ-Cγ-Hγ'	2.2	1.4	79 ± 2
Hβ'-Cβ-Cγ-Hγ'	6.1	5.1	44 ± 2
Hγ'-Cγ-Cδ-Hδ	1.5	1.4	90 ± 2
Hγ'-Cγ-Cδ-Hδ'	6.1	5.1	32 ± 2
Hβ-Cβ-Cγ-Cδ-Hδ[c]	–	1.4 (^4J(H,H))[c]	–

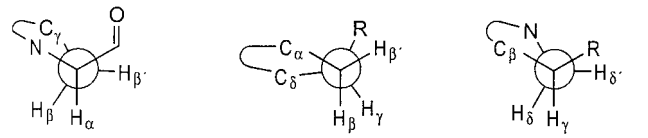
[a] Hα, Hβ, Hδ and Hγ', Hβ', Hδ', respectively, are on opposite faces of the pyrrolidine ring. [b] Average over all torsional angles within the pyrrolidine rings of the two molecules in the assymetric unit of the crystal structure.[15] [c] ^4J(H,H) coupling between Hβ and Hδ indicates their pseudo-equatorial positions at Cβ and Cδ.

Table 3. ^{1}H-^{1}H coupling constants [Hz, ± 0.1Hz] observed for **11** and **13** compared to the torsional angles [°] found in the crystal structure of **11**.

Torsion angle[a]	NMR, ^3J(H,H) **11**	NMR, ^3J(H,H) **13**	X-ray **11**[b]	^3J(H,H) expec.[c]
Hα-Cα-Cβ-Hβ	nd[d]	8.8	30 ± 8	6.5–8.5
Hα-Cα-Cβ-Hβ'	nd[d]	5.7	152 ± 9	9.0–11.5
Hβ-Cβ-Cγ-Hγ	nd[d]	5.6	30 ± 8	6.5–8.5
Hβ'-Cβ-Cγ-Hγ	nd[d]	4.5	152 ± 9	9.0–11.5
Hγ-Cγ-Cδ-Hδ	5.9	5.6	17 ± 7	7.5–9.5
Hγ-Cγ-Cδ-Hδ'	3.7	3.8	139 ± 8	6.5–9.5
Hβ'-Cβ-Cγ-Cδ-Hδ'[e]	–	1.2 (^4J(H,H))[e]	–	–

[a] Hα, Hβ, Hδ, and Hγ, Hβ', Hδ', respectively, are on opposite faces of the pyrrolidine ring. [b] Average over all torsional angles within the pyrrolidine rings of the two molecules in the assymetric unit of the crystal structure.[16] [c] Vicinal coupling constants estimated for the conformation observed in the crystal structure of **11**.[17] [d] The coupling constants could not be determined unambiguously due to overlapping signals of Hα and Hγ as well as Hβ and Hβ'. [e] The $^4J_{(H,H)}$ coupling between Hβ' and Hδ' indicates their pseudo-equatorial positions at Cβ and Cδ.

The observed coupling constants of **11** and **13** are approximately an average of the ones expected for the conformation with pseudo equatorial substituents and the one with pseudo axial substituents. For example, the torsion angles of $\approx 150^\circ$ between Hα and Hβ' as well as between Hγ and Hβ' observed in the crystal structure should be reflected by coupling constants between 9–11.5 Hz. In the conformation with



$^3J_{(H\alpha,H\beta)} \approx 5.5\text{-}8.5$ Hz ($\approx 25\text{-}45^\circ$)
$^3J_{(H\alpha,H\beta')} \approx \leq 2$ Hz ($\approx 80\text{-}90^\circ$)

$^3J_{(H\beta,H\gamma)} \approx 4.0\text{-}7.0$ Hz ($\approx 35\text{-}55^\circ$)
$^3J_{(H\beta',H\gamma)} \approx \leq 2$ Hz ($\approx 80\text{-}90^\circ$)

$^3J_{(H\gamma,H\delta)} \approx 4.5\text{-}7.5$ Hz ($\approx 30\text{-}50^\circ$)
$^3J_{(H\gamma,H\delta')} \approx \leq 2$ Hz ($\approx 80\text{-}90^\circ$)

Figure 7. Newman projections of **11** (R = NHAc) and **13** (R = N_3) with pseudo-axially positioned substituents at Cγ and estimated vicinal coupling constants.[17, 18]

pseudo axial substituents, the torsion angles would be close to 90° and should result in couplings close to 0 Hz. The observed couplings of $^3J = 4.5$ Hz and $^3J = 5.7$ Hz are averages of the expected values. Analysis of the remaining values leads to the same conclusion. Thus, the coupling constants indicate that the conformation with pseudo axial as well as the one with pseudo-equatorial-positioned substituents are populated to more or less equal extents. This conformer equilibrium is further supported by NOE spectroscopic studies (Figure 8). Mutual NOEs are not only observed between Hβ' and Hδ' as would be expected for a conformation with pseudo equatorial substituents but also between Hα, Hβ and Hγ as expected for the conformation with pseudo axial substituents.

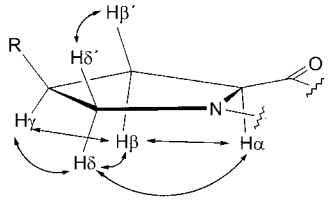


Figure 8. NOEs observed for **11** (R = NHAc) and **13** (R = N_3).

Thus, *cis*,*cis*-diketopiperazines are conformationally not well defined, but are rather flexible. In contrast, the diastereomeric *trans*,*trans*-diketopiperazines are highly preorganized molecules with a defined conformation.

Conclusion

The conformational analysis revealed significant differences between *cis*,*cis*- and *trans*,*trans*-diketopiperazines that are reflected in the different binding properties of two-armed receptors based on these templates. The highly preorganized, turn like *trans*,*trans*-diketopiperazine is superior to all other investigated diamines as a template for two-armed receptors with peptidic side chains. The rather linear and conformationally flexible *cis*,*cis*-diketopiperazine can only serve as a template with certain receptor arms (receptors **1** and **4**). Thus, rigidity paired with a turn-like conformation are necessary requirements of templates for two-armed receptors. Still, these requirements are not necessarily sufficient as revealed by the binding experiments with the two-armed molecules based on the diamines **6**–**9**. The diamines **6** and **8** are highly preorganized molecules with a defined turn. However, they are unsuitable as templates of two-armed receptors with peptidic receptor arms.[19] Our results demonstrate that a template with a defined narrow angle formed by the arms and the template, such that a distance of ≈8 Å exists between the starting points of the receptor arms, is ideal for this kind of two-armed receptor. Thus, for the rational design of two-armed receptors that bind tripeptides with high selectivity the template should not only be conformationally rigid and adopt a turn like conformation but the turn has to be defined.

Experimental Section

General aspects: Materials and reagents were of the highest commercially available grade and used without further purification. Reactions were monitored by thin-layer chromatography using Merck silica gel 60 F_{254} plates. Compounds were visualized by UV light, ceric ammonium molybdate (CAM) and ninhydrin. Flash chromatography was performed by using Merck silica gel 60, particle size 40–63 μm. Gel filtrations were performed on Sephadex LH20 resin purchased from Sigma. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DPX 500 spectrometer. Chemical shifts are reported in ppm with TMS as a reference. Infrared spectra were obtained on a Perkin–Elmer 1600 series; peaks are reported in cm^{-1}. Finnigan MAT LCQ and TSQ 700 instruments were used for electrospray ionization (ESI) mass spectrometry. HPLC analysis were carried out on a Nucleosil 100-5 (250 mm × 4.6 mm) from Macherey Nagel. The solid-phase binding assays were carried out with $CHCl_3$ freshly filtered through aluminumoxide.

Two-armed molecules **1**–**5** were assembled by standard peptide couplings by using *N*-α-Fmoc-protected amino acids and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) as a coupling reagent. For a detailed procedure for the preparation of compounds **1**–**5** [6a]

Receptor (1): ^{1}H NMR (500 MHz, 2% CD_3OD in $CDCl_3$, 25 °C): $\delta = 8.32$ (d, $J = 9.0$ Hz, 4H; dye), 7.99 (s, 2H; NH), 7.91 (d, $J = 9.0$ Hz, 4H; dye), 7.88 (d, $J = 9.3$ Hz, 4H; dye), 7.36 (d, $J = 6.7$ Hz, 2H; NH), 7.27–7.16 (m, 40H; trityl, Phe, NH), 7.09 (d, $J = 7.0$ Hz, 4H; Phe), 7.03 (d, $J = 8.6$ Hz, 4H; Tyr), 6.92 (d, $J = 6.5$ Hz, 2H; NH), 6.77 (d, $J = 9.3$ Hz, 4H; dye), 6.74 (d, $J = 8.6$ Hz, 4H; Tyr), 4.48 (m, 2H; Tyr-Hα), 4.36 (m, 6H; Pro-Hγ, Asn-Hα, Phe-Hα), 4.17 (ψt, $J = 8.0$ Hz, 2H; Pro-Hα), 4.03 (t, $J = 5.7$ Hz, 4H; OCH_2CH_2N), 3.74 (t, $J = 5.7$ Hz, 4H; OCH_2CH_2N), 3.61 (m, 2H; Pro-Hδ), 3.55 (q, $J = 7.1$ Hz, 4H; CH_2CH_3), 3.23 (m, 2H; Pro-Hδ'), 3.06 (dd, $J = 14.1$ Hz, 4.9 Hz, 2H; Tyr-Hβ), 2.90 (m, 4H; Tyr-Hβ', Phe-Hβ), 2.78 (dd, $J = 14.9$ Hz, 5.7 Hz, 2H; Asn-Hβ), 2.65 (dd, $J = 14.0$ Hz, 9.9 Hz, 2H; Phe-Hβ'), 2.56 (dd, $J = 14.9$ Hz, 6.9 Hz, 2H; Asn-Hβ'), 2.38 (m, 2H; Pro-Hβ), 2.15 (m, 2H; Pro-Hβ'), 1.78 (s, 6H; $COCH_3$), 1.25 ppm (t, $J = 7.1$ Hz, 6H; CH_2CH_3); ^{13}C NMR (125.6 MHz, 2% CD_3OD in $CDCl_3$, 25 °C): $\delta = 172.4$, 171.6, 171.4, 170.7, 170.2, 166.0, 157.3, 156.8, 151.3, 147.3, 144.1, 143.4, 136.3, 130.3, 129.4, 129.0, 128.7, 127.8, 127.0, 126.3, 124.7, 122.6, 114.4, 111.4, 70.6, 65.2, 58.9, 55.2, 54.9, 51.1, 49.8, 49.2, 47.5, 46.0, 37.4, 36.9, 35.8, 32.3, 22.5, 12.2 ppm; HRMS (ESI): *m*/*z*: calcd for $C_{128}H_{128}N_{20}O_{18}$ [M^{2+}+2H] 1117.4931; found: 1117.4933.

Two-armed molecule (2): ^{1}H NMR (500 MHz, 5% CD_3OD in $CDCl_3$, 25 °C): $\delta = 8.31$ (d, $J = 9.1$ Hz, 4H; dye), 7.90 (d, $J = 9.1$ Hz, 4H; dye), 7.85 (d, $J = 9.2$ Hz, 4H; dye), 7.26–7.12 (m, 36H; trityl, Phe-6H,), 7.08 (d, $J = 8.4$ Hz, 8H; Tyr, Phe), 6.78 (d, $J = 8.6$ Hz, 4H; Tyr), 6.73 (d, $J = 9.2$ Hz, 4H; dye), 4.37 (m, 2H; Tyr-Hα), 4.27 (m, 4H; Asn-Hα, Phe-Hα), 4.07 (m, $J = 6.0$ Hz, 8H; OCH_2CH_2N, Pro-HαPro-Hγ), 3.73 (t, $J = 6.0$ Hz, 4H; OCH_2CH_2N), 3.52 (q, $J = 7.1$ Hz, 4H; CH_2CH_3), 3.36 (br d), $J = 9.7$ Hz, 2H; Pro-Hδ), 3.18 (dd, $J = 13.6$, 6.7 Hz, 2H; Asn-Hβ), 3.10 (dd, $J = 14.3$, 4.2 Hz, 4H; Phe-Hβ, Tyr-Hβ), 2.92 (dd, $J = 13.6$, 7.8 Hz, 2H; Asn-Hβ'), 2.75 (dd, $J = 14.3$, 9.7 Hz, 2H; Phe-Hβ', Pro-Hδ'), 2.64 (dd, $J = 14.9$, 5.9 Hz, 2H; Tyr-Hβ'), 2.27 (m, 4H; Pro-Hβ, Pro-Hβ'), 1.60 (s, 6H; $COCH_3$), 1.21 ppm(t, $J = 7.1$ Hz, 6H; CH_2CH_3); ^{13}C NMR (125.6 MHz, 5% CD_3OD in $CDCl_3$, 25 °C): $\delta = 172.1$, 171.3, 171.1, 170.9, 170.7, 165.0, 157.1, 156.8, 151.3, 147.3, 144.2, 143.7, 136.0, 130.6, 129.8, 128.9, 128.9, 128.8, 127.9, 127.3, 127.0, 126.3, 124.7, 122.6, 114.4, 111.4, 70.8, 65.1, 58.2, 55.9, 50.9, 50.6, 49.8, 47.5, 46.0, 36.7,

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35.4, 32.4, 22.9, 12.3 ppm; HRMS (ESI): *m/z*: calcd for $C_{128}H_{128}N_{20}O_{18}$ $[M^{2+}+2H]$ 1117.4931; foun: 1117.4937.

Two-armed molecule (3): ^{1}H NMR (500 MHz, 5% CD_3OD in $CDCl_3$, 25 °C): $\delta = 8.31$ (d, $J = 9.1$ Hz, 4H; dye), 7.91 (d, $J = 9.1$ Hz, 4H; dye), 7.88 (d, $J = 9.2$ Hz, 4H; dye), 7.23–7.15 (m, 36H; trityl, Phe-6H), 7.05 (d, $J =$ 6.9 Hz, 4H; Phe), 6.78 (ψd, $J = 9.1$ Hz, 8H; dye, Tyr), 6.69 (d, $J = 8.8$ Hz, 4H; Tyr), 4.50 (ψt, $J = 5.9$, 2H; Asn-Hα 4.39 (ψt, $J = 7.3$ Hz, 2H; Pro-Hγ), 4.32 (t, $J = 6.6$ Hz, 2H; Tyr-Hα), 4.13 (t, $J = 8.4$ Hz, 2H; Pro-Hα), 4.07 (t, $J = 6.0$ Hz, 4H; OCH_2CH_2N), 4.06 (m, 2H; Phe-Hα), 3.78 (t, $J = 6.0$ Hz, 4H; OCH_2CH_2N), 3.61 (dd, $J = 12.0$, 7.6 Hz, 2H; Pro-Hδ), 3.56 (q, $J =$ 7.0 Hz, 4H; CH_2CH_3), 3.30 (dd, $J = 12.0$, 7.6 Hz, 2H; Pro-Hδ'), 2.95 (dd, $J =$ 13.4, 6.7 Hz, 2H; Phe-Hβ), 2.80 (dd, $J = 13.4$, 8.0 Hz, 2H; Phe-Hβ'), 2.74 (m, 8H; Tyr-Hβ Tyr-Hβ', Asn-HβAsn-Hβ'), 2.42 (m, 2H; Pro-Hβ), 2.27 (m, 2H; Pro-Hβ'), 1.77 (s, 6H; $COCH_3$), 1.25 ppm(t, $J = 7.0$ Hz, 6H; CH_2CH_3); ^{13}C NMR (125.6 MHz, 5% CD_3OD in $CDCl_3$, 25 °C): $\delta = 171.8$, 171.4, 171.1, 171.0, 170.3, 165.8, 157.3, 156.7, 151.2, 147.3, 144.1, 143.6, 136.6, 130.2, 129.3, 129.0, 128.6, 128.5, 127.7, 126.9, 126.2, 124.6, 122.5, 114.2, 111.3, 70.5, 65.2, 58.6, 55.9, 54.5, 53.4, 50.2, 49.9, 49.7, 47.0, 46.0, 37.5, 36.3, 35.3, 32.6, 22.5, 12.1 ppm; HRMS (ESI): *m/z*: calcd for $C_{128}H_{128}N_{20}O_{18}$ $[M^{2+}+2H]$ 1117.4931; found:1117.4933.

Receptor (4): ^{1}H NMR (500 MHz, $CDCl_3$, 43 °C): $\delta = 8.53$ (brs), 2H, NH), 8.30 (d, $J = 9.1$ Hz, 4H; dye), 7.88 (d, $J = 9.1$ Hz, 4H; dye), 7.84 (d, $J =$ 9.2 Hz, 4H; dye), 7.27 - 7.09 (m, 48H; trityl, Phe, Tyr-4H, NH-4H), 6.98 (brs), 2H, NH), 6.73 (ψd, $J = 9.2$ Hz, 8H; Tyr, dye), 5.73 (brs), 2H, NH), 4.56 (m, 2H; Tyr-Hα), 4.41 (m, 2H; Pro-Hγ), 4.32 (m, 2H; Phe-Hα), 4.13 (m, 2H; Pro-Hα), 4.03 (m, 6H; OCH_2CH_2N, Gln-Hα), 3.73 (t, $J = 5.8$ Hz, 4H; OCH_2CH_2N), 3.71 (m, 2H; Pro-Hδ), 3.52 (q, $J = 7.0$ Hz, 4H; CH_2CH_3), 3.35 (dd, $J = 11.3$, 7.4 Hz, 2H; Pro-Hδ'), 3.25 (dd, $J = 14.1$, 3.7 Hz, 2H; Tyr-Hβ), 3.05 (dd, $J = 14.1$, 4.5 Hz, 2H; Phe-Hβ), 2.83 (dd, $J =$ 14.1, 10.1 Hz, 2H; Tyr-Hβ'), 2.67 (dd, $J = 14.0$, 9.9 Hz, 2H; Phe-Hβ'), 2.42 (m, 2H; Pro-Hβ), 2.34 (m, 2H; Pro-Hβ'), 2.08 (m, 4H; Gln-Hγ, Gln-Hγ'), 1.87 (m, 2H; Gln-Hβ), 1.75 (m, 2H; Gln-Hβ'), 1.62 (s, 6H; $COCH_3$), 1.22 ppm (t, $J = 7.1$ Hz, 6H; CH_2CH_3); ^{13}C NMR (125.6 MHz, $CDCl_3$, 25 °C): $\delta = 173.3$, 172.9, 172.0, 171.3, 170.9, 165.8, 157.2, 156.8, 151.3, 147.4, 144.3, 143.7, 136.3, 130.4, 129.0, 128.8, 128.7, 128.0, 127.3, 127.1, 126.3, 124.7, 122.6, 114.3, 111.4, 70.8, 65.4, 58.9, 56.0, 54.8, 49.8, 49.4, 47.3, 46.1, 37.0, 36.1, 32.9, 25.8, 23.0, 12.3 ppm; HRMS (ESI): *m/z*: calcd for $C_{130}H_{132}N_{20}O_{18}$ $[M^{2+}+2H]$ 1131.5087; found:1131.5069.

Two-armed molecule (5): ^{1}H NMR (500 MHz, 5% CD_3OD in $CDCl_3$, 25 °C): $\delta = 8.31$ (d, $J = 9.1$ Hz, 4H; dye), 7.91 (d, $J = 9.1$ Hz, 4H; dye), 7.88 (d, $J = 9.2$ Hz, 4H; dye), 7.29–7.15 (m, 36H; trityl, Phe-6H), 7.05 (d, $J =$ 7.1 Hz, 4H; Phe), 6.77 (d, $J = 9.2$ Hz, 4H; dye), 6.76 (d, $J = 8.7$ Hz, 4H; Tyr), 6.71 (d, $J = 8.8$ Hz, 4H; Tyr), 4.36 (m, 2H, Tyr-Hα), 4.32 (m, 2H, Pro-Hγ), 4.19 (ψt, $J = 8.4$ Hz, 2H; Pro-Hα), 4.09 (m, $J = 5.8$ Hz, 8H; Phe-Hα, OCH_2CH_2N, Gln-Hα), 3.79 (t, $J =$ 5.8 Hz, 4H; OCH_2CH_2N), 3.61 (m, 2H; Pro-Hδ), 3.56 (q, $J = 7.1$ Hz, 4H; CH_2CH_3), 3.47 (m, 2H, Pro-Hδ'), 2.97–2.90 (m, 4H, Phe-HβTyr-Hβ), 2.79 (dd, $J = 13.8$, 8.2 Hz, 2H; Phe-Hβ'), 2.70 (dd, $J = 13.8$, 5.6 Hz, 2H; Tyr-Hβ'), 2.48 (m, 2H; Pro-Hβ), 2.20 (m, 6H; Pro-Hβ', Gln-Hγ, Gln-Hγ'), 1.86 (s, 6H; $COCH_3$), 1.80 (m, 4H, Gln-Hβ, Gln-Hβ'), 1.25 ppm (t, $J =$ 7.1 Hz, 6H; CH_2CH_3); ^{13}C NMR (125.6 MHz, 5% CD_3OD in $CDCl_3$, 25 °C): $\delta = 172.7$, 172.2, 171.7, 171.6, 171.1, 165.7, 157.3, 156.7, 151.3, 147.3, 144.3, 143.6, 136.8, 130.2, 129.0, 129.0, 128.6, 128.5, 127.8, 126.9, 126.8, 126.2, 124.6, 122.5, 114.4, 111.3, 70.5, 65.2, 58.6, 55.7, 54.4, 52.9, 49.5, 49.3, 47.1, 46.0, 36.2, 35.3, 32.8, 32.3, 26.7, 22.5, 12.2; ppm HRMS (ESI): *m/z*: calcd for $C_{130}H_{132}N_{20}O_{18}$ $[M^{2+}+2H]$ 1131.5087; found: 1131.5101.

Cyclo[(4*R*)-4-(*N*-acetyl)amino-L-proline]$_2$ (10): Cyclo[(4*R*)-(*N*-*tert*-butoxycarbonyl)amino-L proline]$_2$[6a] (44 mg, 104 mmol) was dissolved in 4M HCl in dioxane (1 mL) and allowed to stir at room temperature for 15 min. After removal of all volatiles at reduced pressure, the solid residue was triturated with Et_2O (3×2 mL) to yield the diammonium salt as a white solid which was isolated by decantation followed by removal of all residual volatiles in vacuo. Acetic anhydride (29 μL, 311 mmol) was added to the solution of the diammonium salt in CH_2Cl_2 (0.5 mL) and NEt_3 (43 μL, 311 mmol), and the mixture was allowed to stir for 1 h at room temperature. After removal of all volatiles at reduced pressure, flash chromatography on silica gel (gradient of CH_2Cl_2/MeOH from 100:0 to 100:6) afforded the diketopiperazine **10** (17 mg, 52%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$, 25 °C): $\delta = 6.43$ (d, $J = 6.0$ Hz, 2H; NH), 4.47 (m, 2H; Hγ'), 4.45 (dd, $J = 9.3$, 7.6 Hz, 2H; Hα), 3.84 (dd, $J = 12.8$, 6.1 Hz, 2H; Hδ'), 3.48 (dd, $J = 12.8$, 1.6 Hz, 2H; Hδ), 2.41 (ddd, $J = 13.6$, 9.6, 6.1 Hz, 2H; Hβ'), 2.34 (ddd, $J = 13.6$, 7.7, 2.2 Hz, 2H;Hβ), 2.00 ppm (s, 6H; CH_3); ^{13}C NMR (100.5 MHz, 5% CD_3OD in $CDCl_3$, 25 °C): $\delta = 170.8$, 166.3, 58.9, 51.3, 47.7, 33.7, 22.7 ppm; IR (KBr): $\tilde{\nu} = 3465$, 3288, 3071, 1662, 1650, 1547, 1446 cm^{-1}; FAB-MS (NBA): *m/z* (%): 309 (100) $[M^+ + H]$; elemental analysis calcd (%) for $C_{14}H_{20}N_4O_4 \cdot H_2O$ (326.4): C 51.52, H 6.79, N 17.17; found: C 51.80, H 7.10, N 17.00.

Cyclo[(4*S*)-4-(*N*-acetyl)amino-L-proline]$_2$ (11): Compound **11** was prepared analogously to **10**. ^{1}H NMR (500 MHz, $CDCl_3$, 25 °C): $\delta = 6.08$ (d, $J = 6.1$ Hz, 2H; NH), 4.33 (ψt, $J = 6.8$ Hz, 2H; Hα), 4.41 (ψtq, $J = 5.9$ Hz, 3.8 Hz, 2H; Hγ), 3.78 (dd, $J = 12.3$, 3.7 Hz, 2H; Hδ'), 3.57 (dd, $J = 12.3$, 5.9 Hz, 2H; Hδ), 2.52–2.49 (m, 4H; Hβ', Hβ), 1.91 ppm (s, 6H; CH_3); ^{13}C NMR (100.5 MHz, $CDCl_3$, 25 °C): $\delta = 170.2$, 167.1, 59.2, 51.4, 48.3, 32.2, 23.1 ppm; IR (KBr): $\tilde{\nu} = 3446$, 3292, 3071, 1661, 1549, 1438; FAB-MS (NBA): *m/z* (%): 309 (100) $[M^+ + H]$; elemental analysis calcd (%) for $C_{14}H_{20}N_4O_4 \cdot H_2O$ (326.4): C 51.52, H 6.79, N 17.17; found: C 51.26, H 6.76, N 16.84.

Cyclo[(4*R*)-4-azido-L-proline]$_2$ (12): For the preparation see [6a] ^{1}H NMR (500 MHz, $CDCl_3$, 25 °C): $\delta = 4.45$ (dd, $J = 10.3$, 6.6 Hz, 2H; Hα), 4.34 (tt, $J = 5.1$, 1.4 Hz, 2H; Hγ'), 3.69 (dd, $J = 13.0$, 5.1 Hz, 2H; Hδ'), 3.62 (dt, $J =$ 13.0, 1.3 Hz, 2H; Hδ), 2.43 (ddt, $J = 13.9$, 6.6, 1.4 Hz, 2H; Hβ), 2.29 (ddd, $J = 13.9$, 10.5, 5.1 Hz, 2H;Hβ'); ^{13}C NMR (100.5 MHz, $CDCl_3$, 25 °C): $\delta =$ 166.5, 58.8, 58.7, 50.7, 33.9 ppm; IR (KBr): $\tilde{\nu} = 2948$, 2124, 1661, 1437,

Table 4. Crystallographic data, structure solution and refinement of **10** and **11**.

	10	**11**
formula	$C_{28}H_{40}N_8O_8 \cdot 2(C_{14}H_{20}N_4O_4)$	$C_{28}H_{44}N_8O_{10} \cdot 2(C_{14}H_{20}N_4O_4 \times H_2O)$
M_r	616.68	652.71
crystal system	monoclinic	monoclinic
space group	$P2_1$	$P2_1$
a [Å]	9.400(3)	6.9113(4)
b [Å]	17.189(4)	17.8909(12)
c [Å]	10.156(2)	13.2950(11)
α [Å]	90	90
β[Å]	111.15(97)	104.632(5)
γ[Å]	90	90
V [Å^3]	1530.5	1590.6
Z	2	2
F(000) [e$^-$]	656	696
ρ [gcm^{-3}]	1.338	1.363
μ [mm^{-1}]	0.100	0.105
crystal size [mm]	$0.04 \times 0.12 \times 0.17$	$0.10 \times 0.40 \times 0.44$
T [K]	293	293
radiation	$Mo_{K\alpha}$ ($\lambda = 0.71073$)	$Mo_{K\alpha}$ ($\lambda = 0.71073$)
Θ_{max} [°]	23.87	27.52
measured reflections	8435	10179
independent reflections	4407	6105
reflections in refinement	3308	3902
variables	414	446
final *R*	0.0675	0.0532
final *Rw*	0.0548	0.0506
weighting scheme	Chebychev polynomial with 4 parameters[22b]	Chebychev polynomial with 5 parameters[22b]
last max/min in difference map	1.03/ – 0.40	0.41/ – 0.36

1274 cm^{-1}; FAB-MS (NBA): *m/z* (%): 277 (100) [M^++H]; elemental analysis calcd (%) for $C_{10}H_{12}N_8O_2$ (276.3): C 43.48, H 4.38, O 11.58; found: C 43.26, H 4.50, O 11.64.

Cyclo[(4*S*)-4-azido-L-proline]$_2$ (13):Compound **13** was prepared analogously to **12**. ^{1}H NMR (500 MHz, $CDCl_3$, 25 °C): $\delta = 4.27$ (dd, $J = 8.8$, 5.7 Hz, 2H; Hα), 4.19 (ψqd, $J = 5.7$, 4 Hz, 2H; Hγ), 3.81 (ddd, $J = 12.4$, 3.8, 1.2 Hz, 2H; Hδ'), 3.55 (dd, $J = 12.4$, 5.6 Hz, 2H; Hδ), 2.69 (dddd, $J = 13.8$, 5.7, 4.9, 1.4 Hz, 2H; Hβ'), 2.50 ppm(ddd, $J = 13.8$, 8.8, 5.7 Hz, 2H;Hβ); ^{13}C NMR (75.5 MHz, $CDCl_3$, 25 °C): $\delta = 165.5$, 58.2, 58.0, 50.3, 32.4 ppm; IR (film): $\tilde{\nu} = 2953$, 2103, 1680, 1422, 1269cm^{-1}; FAB-MS (NBA): *m/z* (%): 277 (100) [M^++H]; elemental analysis calcd (%) for $C_{10}H_{12}N_8O_2$ (276.3): C 43.48, H 4.38, N 40.56; found: C 43.33, H 4.54, N 40.26.

Crystal structure determination of 10 and 11: Crystals were obtained by slow vapour diffusion of Et_2O into a solution of **10** and **11** in EtOAc and a trace of CH_2Cl_2, respectively. Crystals of the compounds under investigation were stuck with glue on glass fiber and mounted on a Nonius KappaCCD. Data collection was carried out using the Nonius collect suite.[20] The structures were solved by using direct methods with the program SIR92.[21] Least square refinement was carried out using the program CRYSTALS.[22] Plots were produced using ORTEP3 for Windows.[23] Experimental details of the structure determinations of **10** and **11** are compiled in Table 4.
CCDC-190722 (**10**) and CCDC-190723 (**11**) contain the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB21EZ, UK; fax: (+44) 1223-336-033; or e-mail: deposit@ccdc.cam.ac.uk).

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[11] AA1 – AA3 = Gly, D-Ala, L-Ala, D-Val, L-Val, D-Leu, L-leu, D-Phe, L-Phe, D-Pro, L-Pro, D-Ser, L-Ser, D-Thr, L-Thr, D-Asp, L-Asp, D-Glu, L-Glu, D-Asn, L-Asn, D-Gln, L-Gln, D-His, L-His, D-Lys, L-Lys, D-Arg, L-Arg.
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[14] The binding affinities of **1** and **4** towards Ac-D-Val-D-Val-D-His-NH$(CH_2)_5$CONH-PS are $K_a = 1680 \pm 310$ M^{-1} ($\Delta G = -4.4 \pm 0.1$ kcal mol^{-1}) and $K_a = 1420 \pm 200$ M^{-1} ($\Delta G = -4.3 \pm 0.1$ kcal mol^{-1}) as determined by solid-phase binding assays[6a] and are thus similar to the binding affinity of the diastereomeric *trans,trans*-diketopiperazine receptor of **1**.[6a]
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