Sequence-Selective Peptide Binding with a Peptido-A,B-*trans*-steroidal Receptor Selected from an Encoded Combinatorial Receptor Library

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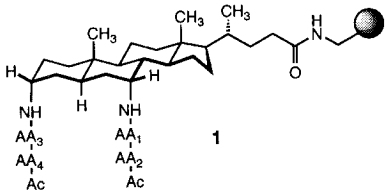
Researchers from this laboratory recently described the first preparation of a combinatorial library of synthetic receptors and screening for members that bound a certain pentapeptide (Leu enkephalin).[1] In that work, 10^4 different receptors were built from an A,B-*cis*-cholic acid core with two variable tripeptidic arms extending down from the concave, α-face of the steroidal nucleus at C3 and C7. Using a solid phase color assay to screen for binding, only a small fraction of the receptor library (∼100 of the 10 000 different receptors) was found to tightly bind the chosen substrate, N-acylated ^{5}Leu enkephalin (RCO-Tyr-Gly-Gly-Phe-Leu, concentration ∼100 μM in $CHCl_3$). In this paper, we describe a related receptor library (**1**) built around an A,B-*trans*-steroidal core that is axially 3,7-disubstituted with conformationally less flexible dipeptidic arms. As we will show, a much smaller fraction of this library (∼10 out of 10 000 different receptors) binds N-acylated ^{5}Leu enkephalin methyl ester and every one of these has the same AA1−AA3: (L)Asn, (D)Asn, and (D)Phe, respectively. By resynthesizing one of these enkephalin-binding receptors, we find that it binds ^{5}Leu enkephalin derivatives with binding energies that diminish by 1−3 kcal/mol when many single-residue modifications to the Leu enkephalin substrate are made.



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Peptidosteroid **1** was designed to be less flexible than our previously described peptidosteroids that were built around an A,B-*cis*-steroidal diol core. Thus **1** has an increased spacing between the two convergent, substrate-binding variable chains and amino substitution that allows direct attachment of peptidic substituents to C3 and C7, avoiding the flexible glycine spacers previously employed at these sites. The new 3α,7α-diamino A,B-*trans*-steroidal core was prepared from chenodeoxycholic acid as described in the supporting information and attached to aminomethyl polystyrene synthesis beads. We then used encoded split synthesis[2] as described previously to add the following 10 amino acids at each of the four variable (AA_n) positions of **1**: (D and L) Ala, Phe, Pro, Ser(*O*-tBu), and Asn(*N*-trityl).[1] This combinatorial synthesis yielded a bead library containing 10^4 variants of **1** such that each synthesis bead carried one variant and a unique encoding array of 16 haloaromatic tags.

We then screened our side-chain-protected receptor library for peptide binding using a red dye labeled methyl ester of the pentapeptide ^{5}Leu enkephalin (**P1**):

P1: dye-$CO(CH_2)_2CO$-(L)Tyr-Gly-Gly-(L)Phe-(L)Leu-OMe

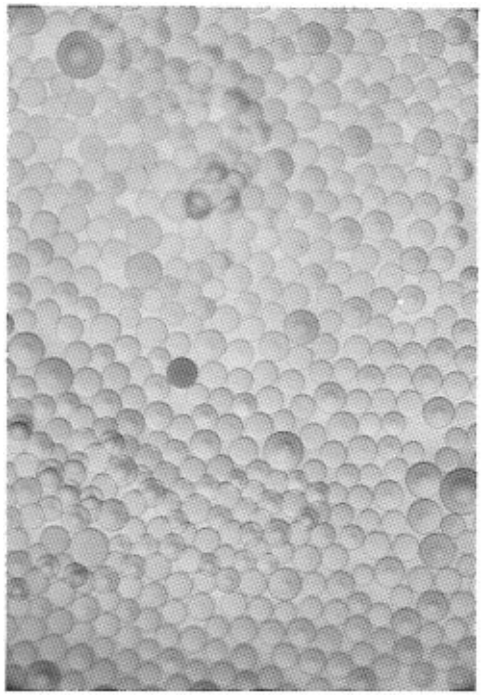

Figure 1. Bead library **1** upon equilibration with ^{5}Leu enkephalin derivative **P1** in $CHCl_3$.

Table 1. Relative Binding of **1NnfS** to ^{5}Leu Enkephalin Analogs[a]

	R1	R2	R4	R5	rel binding[b] (kcal/mol)
P1	(L)Tyr	Gly	(L)Phe	(L)Leu-OMe	
P2	(L)Tyr	Gly	(L)Phe	(L)Met-OMe	0.0 ± 0.1
P3	(L)Tyr	Gly	(L)Phe	(L)Ala-OMe	0.1 ± 0.1
P4	(L)Tyr	Gly	(L)Phe	(D)Leu-OMe	1.7 ± 0.1
P5	(L)Tyr	Gly	(L)Phe-OMe	*c*	1.3 ± 0.1, 1.4 ± 0.2[d]
P6	(L)Tyr	Gly	(D)Phe-OMe	*c*	1.4 ± 0.1
P7	(L)Tyr	Gly	(L)Phe-NHMe	*c*	1.0 ± 0.1
P8	(L)Tyr	(D)Ala	(L)Phe	(L)Leu-OMe	1.0 ± 0.1, 1.0 ± 0.2[d]
P9	(L)Phe	Gly	(L)Phe	(L)Leu-OMe	3.5 ± 0.2
P10	(L)Tyr(*O*Me)	Gly	(L)Phe	(L)Leu-OMe	2.6 ± 0.1
P11	*c*	Gly	(L)Phe	(L)Leu-OMe	1.9 ± 0.1

[a] Sequence: dye-linker-R1-R2-R3-R4-R5; R3 = Gly. [b] Binding relative to **P1** (positive energies indicate weaker binding). [c] Residue deletions. [d] Energies independently measured as the difference in **P1**'s and **P5**'s or **P8**'s absolute binding energies (error limits correspond to 1 standard deviation).

The assay involved equilibrating ∼10 copies of library **1** (∼1 mg of beads) with ∼70 μM **P1** in chloroform for 48 h and selecting those beads that accumulated the red color of the dye.[3] Under these conditions, only ∼0.1% of the library beads took on a deep red color while the remainder of the beads remained virtually colorless (see Figure 1; compare with the range of color intensities shown in Figure 1 of ref 1 found with beads bearing the previous peptidosteroidal receptors). The presence of only deep red beads and colorless beads (but no pale red beads) indicates a large difference in binding constants for **P1** between those members of **1** that bind and those that do not.

Upon collecting 37 of these red beads and reading their tags with electron capture gas chromatography, we found only eight different receptor library members, each being replicated 2−12

(1) Boyce, R.; Li, G.; Nestler, H. P.; Suenaga, T.; Still, W. C. *J. Am. Chem. Soc.* **1994**, *116*, 7955.
(2) Ohlmeyer, M. H. J.; Swanson, R. N.; Dillard, L. W.; Reader, J. C.; Asouline, G.; Kobayashi, R.; Wigler, M.; Still, W. C. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 10922.
(3) Under the same conditions, no members of library **1** were observed to bind the dye (Disperse Red 1) to a significant extent.

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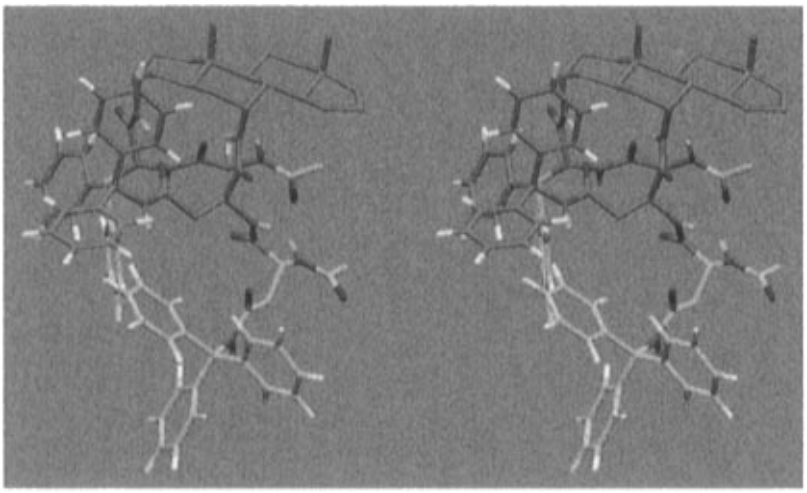

Figure 2. Low-energy conformation of receptor **1NnfA** from molecular mechanics. Conformationally homogeneous substructure in orange.

times. Remarkably, all 37 of the decoded beads had AA_1-AA_3 = (L)Asn(*N*-trityl), (D)Asn(*N*-trityl), and (D)Phe. AA_4 appeared random except for the absence of (D)Asn(*N*-trityl) and (D)Ser(*O*-tBu) at that position. Clearly, there is something special about peptidosteroids **1** having the AA_1-AA_3 appendages defined above (*i.e.*, **1NnfX**[4]).

To evaluate the selectivity of these enkephalin-binding peptidosteroids, we resynthesized one of them (**1NnfS**, AA_1-AA_4 = (L)Asn(*N*-trityl), (D)Asn(*N*-trityl), (D)Phe, (L)Ser(*O*-tBu)) on a gram scale and evaluated its binding to **P1** and certain analogs. Solid phase binding measurements in which the resynthesized peptidosteroid was attached to the solid support were carried out in two ways.[5] In the first, two different peptides were allowed to compete for a deficiency of **1NnfS** with HPLC being used to measure the quantities of the two peptides in the supernatant solution and on the beads after equilibration.[6] The results of these measurements are summarized in Table 1.

These results indicate that **1NnfS** binding to **P1** is most sensitive to changes at **P1**'s N-terminal tyrosine (R1) and removal of that residue (**P11**) or modifications affecting phenolic hydroxyl (**P9**, **P10**) diminish binding by 2−3 kcal/mol. Alterations nearer **P1**'s C-terminal residues (R4, R5) are better tolerated, but significant changes (R5 deletion or chiral inversion) in this area still diminish binding significantly (**P4**−**P7**). More subtle C-terminal alterations (R5: (L)Leu → (L)Met, (L)-Ala) were not energetically distinguishable by **1NnfS.** Considering the insensitivity of binding to R5 side-chain structure, it is not surprising that virtually the same members of **1** (including **1NnfS**) were found when the corresponding [5]Met enkephalin substrate (**P2**) was used to screen the receptor library.

We also measured the absolute binding energy of **1NnfA** and **P1** in $CHCl_3$ by UV-monitored titration and found it to be -6.5 ± 0.1 kcal/mol.[7] Analogous binding energy measurements for **P5** and **P8** gave energies of -5.1 ± 0.1 and -5.5 ± 0.1 kcal/mol, respectively. These energies are consistent with the relative binding energies measured by the competition experiments described above.

While we know little about the structure of the **1NnfX·P1** complexes at this time, a conformational analysis of **1NnfA** alone provides a hint about how it might bind peptidic substrates. In particular, a 5000-step, SUMM conformational search[8] found substantial conformational homogeneity within the molecule. Thus all conformations of **1NnfA** within the lowest 2 kcal/mol shared a common three-dimensional substructure that included not only the steroidal nucleus but also chain residues AA1 and AA3. This conformationally fixed substructure is indicated in orange in the stereopair diagram of a representative low-energy conformation (Figure 2). The structure is stabilized by two strong interchain hydrogen bonds that create a compact, highly ordered core. It may be relevant that the majority of the low-energy conformations found by the conformational search contain only two strong intramolecular hydrogen bonds in spite of the fact that **1NnfA** contains eight secondary amides, the remaining unassociated hydrogen bond donors and acceptors being both conformationally restricted and available for hydrogen bonding to a peptidic substrate in an organic solvent.

While we can say little about the structural origin of **1NnfX**'s peptide-binding selectivity at this time, the receptor is clearly able to make some remarkable distinctions between closely related oligopeptides. These results not only support the proposal that receptor libraries can provide a rich source of highly selective receptor molecules but also provide an example of how decreased receptor flexibility can result in increased receptor selectivity. In the case at hand, this decreased flexibility follows primarily from deletion of two conformationally mobile glycines from our original peptidosteroid design.

Acknowledgment. Support by NSF CHE92 08254 is gratefully acknowledged.

Supporting Information Available: Synthetic scheme for preparation of the differentially protected steroidal core of **1** (1 page). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(4) Nomenclature defined by standard single letter code for amino acids (AA1−AA4).

(5) We were unable measure the binding of the methyl ester of **1NnfS** to **P1** in solution because we could find no variable (CD, UV, ^{1}H NMR) that could be monitored over a sufficiently large concentration range during a titration of **1NnfS** methyl ester with **P1**.

(6) $\Delta\Delta G_{\mathbf{P1}-\mathbf{Pn}} = -RT \ln([\mathbf{P1}]_{bound}/[\mathbf{Pn}]_{bound}/[\mathbf{P1}]_{free}/[\mathbf{Pn}]_{free})$ where $[\mathbf{P}]_{free}$ corresponds to the concentration of **P** in the supernatant solution over the beads at equilibrium.

(7) Binding constants were measured by additions of known quantities of **P1** (leading to $[\mathbf{P1}]_{total}$) to a constant quantity of **1NnfS** beads with monitoring of $[\mathbf{P1}]_{free}$ by UV after 2 h of equilibration. Because the concentration of bead-bound **1NnfS** is unknown, we collect [**P1**] data at two points (A, B) and solve the two binding equations simultaneously:

$$K = \frac{\{[\text{complex}]_B/[\mathbf{P1}]_{\text{free-B}} - [\text{complex}]_A/[\mathbf{P1}]_{\text{free-A}}\}}{\{[\text{complex}]_A - [\text{complex}]_B\}}$$

where $[\text{complex}] = [\mathbf{P1}]_{total} - [\mathbf{P1}]_{free}$. Data was collected at three such points, and *K*'s were calculated for each of the three pairs of points. The binding experiment was repeated. The resulting six *K*'s were averaged, and error limits were computed from the different *K*'s as 1 standard deviation. Related methodology: Smith, P. W.; Chang, G.; Still, W. C. *J. Org. Chem.* **1988**, *53*, 1587.

(8) Goodman, J. M.; Still, W. C. *J. Comput. Chem*. **1991**, *12*, 1110. The conformational search was carried out with MacroModel V5.0 utilizing the AMBER* force force field and the GB/SA continuum model for chloroform. For the unconserved residue AA_4, we used (L)Ala to simplify the search.