Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry Letters 13 (2003) 1409–1413

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Peptoid Mimics of Agouti Related Protein

Darren A. Thompson,[a,*] Biao-Xin Chai,[b] Hilary L. E. Rood,[a] Michael A. Siani,[c] Nicholai R. Douglas,[a] Ira Gantz[b] and Glenn L. Millhauser[a]

[a]*Department of Chemistry and Biochemistry, University of California, Santa Cruz, CA 95064, USA*
[b]*Department of Surgery, University of Michigan Medical Center, 6504 MSRB I 1150 W. Medical Center Drive, Ann Arbor, MI 48109-0682, USA*
[c]*Department of Bioinformatics Affymetrix - Berkeley, 6550 Vallejo Street, Emeryville, CA 94608, USA*

Received 6 January 2003; revised 6 January 2003; accepted 11 February 2003

Abstract—The Agouti Related Protein (AGRP) is an endogenous antagonist of melanocortin-3 and -4 receptors, each of which plays a key role in body weight homeostasis. We designed a peptoid trimer based on AGRP 111–113 in which a single chiral atom is used to partially restrain the backbone structure. Peptoid **5** displaced both radiolabeled Nle4-α-MSH ($IC_{50} = 3.1$ μM) and AGRP (86–132) ($IC_{50} = 1.9$ μM) from the human melanocortin-4 receptor and functioned as an antagonist of α-MSH stimulated cAMP generation, thus providing an important lead in the development of AGRP mimetics.

The agouti related protein (AGRP), 112 amino acids, is a secreted signaling molecule produced in the hypothalamic arcuate nucleus.[1] AGRP stimulates feeding behavior in mammals and functions, in part, by blocking α-melanocyte stimulating hormone (α-MSH) activity at the hypothalamic melanocortin-3 and melanocortin-4 receptors (MC3R and MC4R).[2] Targeted disruption of MC4R in the mouse hypothalamus leads to obesity and transgenic mice over expressing AGRP are also obese.[1,3] Ablation of the MC3R coding sequence from the mouse genome results in metabolic malfunctions suggesting that this receptor operates in concert with MC4R to control energy balance.[4] Intracerebroventricular (ICV) injection of melanocortin receptor agonists reduces food intake, while injection of antagonists such as AGRP increases food consumption in lean mice.[5,6] Several studies implicate MC4R mutations in human morbid obesity.[7]

The C-terminal domain of AGRP, residues 87–132, forms the principal MCR binding domain. Alanine scanning of the homologous agouti protein reveals contiguous residues Arg-Phe-Phe, within this domain, as crucial for high affinity binding.[8] In AGRP, binding is eliminated by replacement of amino acids 111–114 (RFFN) with alanine; AGRP (87–132, R111A) fails to antagonize NDP-MSH (a potent α-MSH analogue) stimulated cAMP production at MC4R.[9,10] Within the melanocortin receptor, a number of anionic residues significantly modulate AGRP's affinity. A pair of aspartate residues separated by a helical turn on predicted transmembrane helix 3 are implicated in the hMC4R/AGRP interaction and a glutamate on helix 2 in mMC4R also contributes to AGRP affinity.[11,12] It is thought that these anionic residues help anchor AGRP through interaction with R111. Together, these findings implicate the sequence 111–113—the RFF triplet—as an active MC4R pharmacophore in AGRP.

AGRP's C-terminal domain contains an inhibitor cystine knot (ICK) motif (residues 87–120) that is responsible for high affinity MCR antagonism.[13] Within this ICK motif the RFF triplet is presented in the turn region of a β-hairpin formed by residues 107–120. Analysis of the AGRP 87–132 NMR structure shows that RFF triplet is fully solvent exposed and projects out of the protein with similar side chain orientations.[14] The backbone torsion angles for Phe 113 lie in the α_R region of Ramachandran space, while those of Phe 112 are extended. A cyclic peptide corresponding to AGRP (110–117), cyclo[CRFFNAFC], antagonizes MC4R, albeit with 1–2 logs less potency than that of AGRP(87–132) or AGRP(87–120).[9] Interestingly, the linear analogue SRFFNAFS has no detectable MC4R affinity. These finding suggest that activity of the RFF triplet requires that its backbone take on a turn configuration.

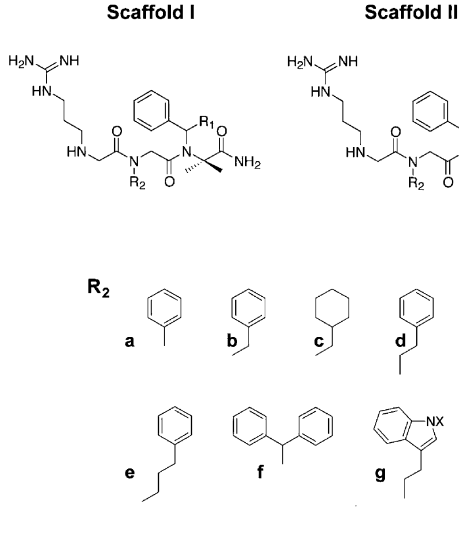
*Corresponding author. Tel.: +1-831-459-3390; fax: +1-831-459-2935; e-mail: thompson@chemistry.ucsc.edu

0960-894X/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0960-894X(03)00164-1

Successful molecular mimicry depends upon constraining the mimic's structure enough to allow conformational adaptation to the target's molecular shape. *N*-substituted glycines (peptoids) offer a unique backbone, which can be used in molecular design. The peptoid backbone and side chain rotamers are partially constrained by the steric bulk of the *N*-alkyl substituent.[15] Peptoid chemical diversity is virtually unlimited and easily exploited by combinatorial solid phase synthesis.[16] The peptoid amide bond, unlike the L-peptide amide bond, is resistant to protease cleavage.[17] Peptoids make fewer intramolecular H-bonds than the corresponding peptide, possibly contributing to enhanced lipophilicity.[18]

The progressive decrease in size, from a 12 kDa protein to a 5 kDa C-terminal domain to a 4 kDa ICK motif to a 1 kDa cyclic peptide, while still maintaining MC4R functionality, motivates the search for an even smaller AGRP-like ligand. In this light, several non-peptide ligands have been developed. An agonist based on NDP-MSH is active in a mouse MC1R assay at 42.5 μM and ligands, characterized by binding studies, have been identified in a large peptoid combinatorial library and in peptidic *N*-alkylamino acid derivatives.[19–21]

As noted, AGRP residues 111–113 lie at the beginning of a hairpin turn. In solution, the critical residues of NDP-MSH, a high affinity MC4R agonist, also form a β-turn.[22] It is therefore reasonable to design a scaffold with a structural turn restraint in order to properly mimic the RFF triplet. Scaffolds I and II (Fig. 1) were designed to incorporate such a restraint.[23] In scaffold I, the two backbone methyl groups of an Aib residue at the i+2 backbone position sterically restrain the local conformation. Indeed, conformational sampling using distance geometry techniques followed by MM2* force field calculations and minimization indicated the significant reduction in degrees of backbone rotational freedom in **1** resulting in high population of a turn conformations.[24] Compound **1** did demonstrate weak displacement of the radiolabeled agonist ^{125}I-[Nle4]-α-MSH at MC4R (data not shown). However, synthetic difficulties with scaffold I impeded construction of multiple compounds; consequently, this design was not considered further.



Compound #	R_1	R_2	Scaffold #
1	H	d	I
2	(R)-methyl	b	II
3	H	b	II
4	(S)-methyl	b	II
5	(R)-methyl	f	II
library	(R)-methyl	a-g	II

Figure 1. Two-dimensional drawings of compounds used in this study.

It has been established elsewhere that chiral *N*-substituted glycines form helical conformers in solution.[25] A methyl group at the R1 position confers a chiral center in the benzylamide and thus introduces helical backbone torsion angles. The specific *R* and *S* stereoisomers confer a left hand helical pitch with a positive circular dichroism (CD) signal or right hand helical pitch with a negative CD signal, respectively.[26]

Scaffold II tripeptoids, were synthesized individually as *R* and *S* isomers corresponding to compounds **2** and **4**, respectively. Compound **3**, lacking a chiral center, was also prepared. CD spectra for these three constructs are shown in Figure 2a. Spectra for **2** and **4** are characteristic of peptoid helices and differ by a change of sign consistent with molecules of opposite chirality.[27] Compound **3** lacks a stereo center and, as expected, is not CD active.

Conformational minimizations were performed on compound **2**. As with **1**, the resulting low energy structures

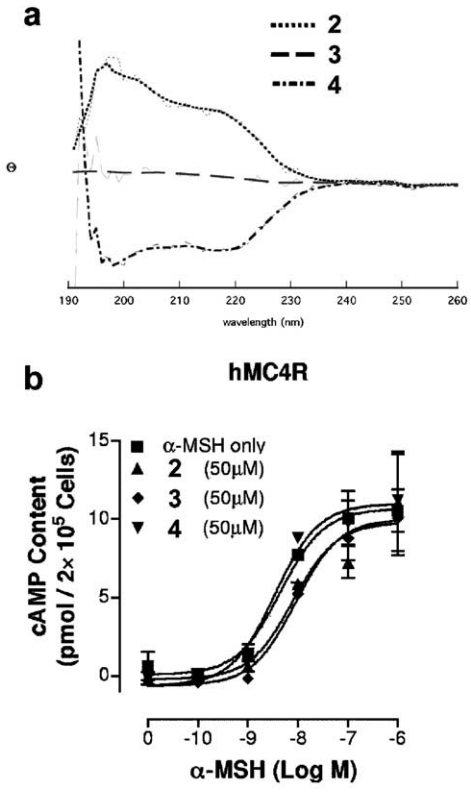


Figure 2. (a) Far-UV CD (25 °C 10 mM phosphate pH 7) of 200 μM **2**, **3**, and **4**. (b) Dose response curves for α-MSH stimulated cAMP production at hMC4R in the absence (■) and presence of 50 μM **2** (▲), **3** (◆), and **4** (▼).

displayed significant population of turn conformations for **2**.

Compounds **2**, **3** and **4** were tested for their ability to inhibit α-MSH stimulated cAMP production in hMC4R-transfected cells. The rightward shift in cAMP activity shown in Figure 2b demonstrates that **2** and **3** have weak antagonist properties. In contrast, **4** has no effect on activity of hMC4R at 50 μM.

The partial success with compound **2** motivated the synthesis of a library in which the i (3-Guanidinopropylamine) and i + 2 (R-α-Methylbenzylamide) side chain and backbone were kept constant and the i + 1 side chain (R2) was varied. All library members were synthesized in one pot in anticipation that ultimately one may be interested in the simultaneous preparation of a large library consisting of thousands of compounds.[28]

The specific choices for the R2 side chains, based on variations of phenylalanine or tryptophan, are shown in Figure 1. Unpurified compounds, direct from TFA cleavage, gave a 13.3 μM IC_{50} (Table 1).[29] RP-HPLC purified library had IC_{50} of 6.6 μM for [Nle4]-α-MSH and also displaced radiolabeled AGRP (86–132) with an IC_{50} of 27 μM. RP-HPLC fractions from the collection were lyophilized separately then recombined for activity studies. Table 1 shows pooled fractions A-D displaced ^{125}I-[Nle4]-α-MSH poorly while pooled fractions E - H exhibit significant activity with an IC_{50} of 9 μM. Individual cAMP activity assays demonstrated fractions G and H inhibited hMC4R α-MSH cAMP production at 10 μM, with fraction H having greater inhibition than fraction G.[29]

ESI-MS of fraction H showed a major component of 558 g/mol consistent with the MH+ of compound **5** (Fig. 1). This molecule was synthesized separately, product confirmed by ESI-MS, and purified by HPLC. Although the peptoid showed no significant hMC4R cAMP inhibition at 100 nM, there is significant inhibition at 1.0 μM (Fig. 3). In addition, **5** is an improved antagonist over fraction H at 10 μM. Partial agonist activity is seen with 10 μM peptoid at hMC1R. At high dosage **5** is also a partial agonist at hMC4R (data not shown). No significant antagonist activity is detected for **5** at hMC1R or hMC3R at 10 μM concentration (Fig. 3).

Dose displacement curves indicated remarkably similar IC_{50} values for **5** when assayed against all three radiolabeled ligands at MC4R. Fifty percent displacement by **5** at hMC4R was at 3.1 μM for ^{125}I-[Nle4]-α-MSH, 2.1 μM for ^{125}I-NDP-MSH, and 1.9 μM for ^{125}I-AGRP (86–132).

Table 2 contains IC_{50}'s for **5** at hMC4R, as well as hMC1R and MC3R. **5** gives a 9.1 μM IC_{50} for ^{125}I-[Nle4]-α-MSH displacement and 25 μM IC_{50} for displacement of ^{125}I-hAgouti (ASIP (90–132, L89Y)) at the human MC1R. Compound **5** is less potent at hMC3R as evidenced by a 48 μM IC_{50} for radiolabeled [Nle4]-α-MSH, and $IC_{50} > 50$ μM for AGRP (86–132).

In summary, we explored two strategies for incorporating a turn into a peptoid backbone. Of these, one scaffold where

Table 1. hMC4R IC_{50} (μM)

Cold ligand	^{125}I-Radiolabeled ligand		
	[Nle4]-α-MSH	NDP-MSH	AGRP (86–132)
1	>100	—	—
2	>100	—	>100
3	>100	—	>100
4	NA[a]	—	NA[a]
5	3.1 (0.9)	2.1 (0.4)	1.9 (0.2)
Crude lib.	13.3	—	—
Semi-pure	6.6	—	27
Fract. A-D	>100	—	—
Fract. E-H	9	—	—
[Nle4]-α-MSH	0.016 (0.007)	0.019[b]	0.015 (0.001)
AGRP	0.001 (0.0002)	0.011[b,c]	0.003 (0.0009)
NDP-MSH	0.001 (0.0003)	0.0013[b]	0.0038[b,d]
$_C$110-117	—	0.621[b]	—

All data points are mean values from at least 3 different experiments. The standard deviations are in parentheses.
[a]NA = no activity.
[b]From ref 2, 9, 13.
[c]AGRP 87–132 displace 125I-NDP-MSH.
[d]NDP-MSH displace 125I-AGRP 87–132.

Table 2. IC_{50} (μM) of **5** displacing agonist and antagonist ligands at human melanocortin receptors

	^{125}I-[Nle4] α-MSH	^{125}I-AGRP (86–132)	^{125}I ASIP Y [90–132]
hMC1R	9.1 (0.3)	—	25 (4)
hMC3R	48 (4)	>50	—
hMC4R	3.1 (0.9)	1.9 (0.2)	—

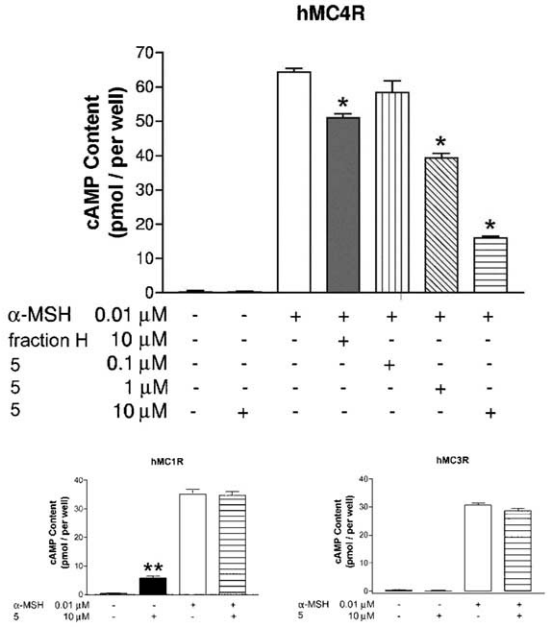


Figure 3. cAMP activity at the hMC1, 3, and 4R for 10 nM α-MSH alone, with various concentrations of **5**, and with 10 μM fraction H at hMC4R. * = significantly different from 10 nM α-MSH $p < 0.001$. ** = significantly different from control $p < 0.001$ (95% confidence interval).

a single chiral center was placed on a fixed side chain, was found to be synthetically convenient. CD experiments confirmed that backbone stereochemistry was indeed influenced by this single stereoactive center. A ligand displacement assay found that **2** was active whereas its enantiomer **4** was not. Interestingly, a non-chiral version, compound **3**, was also active according to this assay. Using **2** as a starting point, a restricted library was developed and lead to the identification of compound **5** as an MC4R antagonist with activity in the low micromolar range.

Several recent studies have focused on the development of MCR agonists. For example, Haskell-Luevano et al. recently used a heterocycle scaffold based on a β-turn motif to produce a 951 membered library.[19] Within this library, an MC1R agonist with an EC_{50} of 42 μM was identified. Using an *N*-alkylaminoacid design, Mutulis et al. identified several compounds with low μM binding activity; however, agonist versus antagonist function was not explored.[21] In light of these pioneering studies, rapid identification of an AGRP mimetic with low μM affinity is remarkable.

Molecules with AGRP-like function may play an important role in treating disorders related to negative energy balance. An AGRP polymorphism has been linked to anorexia nervosa and it has been suggested that MC4R targeted antagonists may serve as appropriate therapeutics.[30] Cachexia is the wasting condition often associated with cancer and AIDS. Recent work on mice, in an anorexic state induced by tumor growth, has demonstrated that administration of AGRP stimulated feeding and helped reduce loss of lean body mass.[31] The tunable molecular scaffold developed here is the first rationally designed functional mimic of AGRP 111–113 and may serve as an ideal starting point for the development of molecules for treating these and related conditions. The low molecular weight, protease resistance, enhanced lipophilicity, and synthetic accessibility of this template make it ideal for further development of AGRP analogues.

Acknowledgements

Supported by NIDDK grants DK58606 & DK064265 (G.L.M.), DK54032 (I.G.), and by the University of Michigan Gastrointestinal Peptide Research Center (NIH Grant P30DK34933). The authors are grateful to Justin Bursch for valuable synthesis assistance and Dr. Ying-kui Yang for preliminary pharmacological experiments. D.T. also thanks Drs. Reyna Simon and Lynne Canne for helpful discussions.

References and Notes

1. Ollmann, M. M.; Wilson, B. D.; Yang, Y. K.; Kerns, J. A.; Chen, Y.; Gantz, I.; Barsh, G. S. *Science* **1997**, *278*, 135.
2. Yang, Y. K.; Thompson, D. A.; Dickinson, C. J.; Wilken, J.; Barsh, G. S.; Kent, S. B. H.; Gantz, I. *Mol. Endocrinol.* **1999**, *13*, 148.
3. Huszar, D.; Lynch, C. A.; Fairchild-Huntress, V.; Dunmore, J. H.; Fang, Q.; Berkemeier, L. R.; Gu, W.; Kesterson, R. A.; Boston, B. A.; Cone, R. D.; Smith, F. J.; Campfield, L. A.; Burn, P.; Lee, F. *Cell* **1997**, *88*, 131.
4. Butler, A. A.; Kesterson, R. A.; Khong, K.; Cullen, M. J.; Pelleymounter, M. A.; Dekoning, J.; Baetscher, M.; Cone, R. D. *Endocrinology* **2000**, *141*, 3518.
5. Fan, W.; Boston, B. A.; Kesterson, R. A.; Hruby, V. J.; Cone, R. D. *Nature* **1997**, *385*, 165.
6. Rossi, M.; Kim, M. S.; Morgan, D. G.; Small, C. J.; Edwards, C. M.; Sunter, D.; Abusnana, S.; Goldstone, A. P.; Russell, S. H.; Stanley, S. A.; Smith, D. M.; Yagaloff, K.; Ghatei, M. A.; Bloom, S. R. *Endocrinology* **1998**, *139*, 4428.
7. MacNeil, D. J.; Howard, A. D.; Guan, X.; Fong, T. M.; Nargund, R. P.; Bednarek, M. A.; Goulet, M. T.; Weinberg, D. H.; Strack, A. M.; Marsh, D. J.; Chen, H. Y.; Shen, C. P.; Chen, A. S.; Rosenblum, C. I.; MacNeil, T.; Tota, M.; MacIntyre, E. D.; Van der Ploeg, L. H. *Eur. J. Pharmacol.* **2002**, *440*, 141.
8. Kiefer, L. L.; Veal, J. M.; Mountjoy, K. G.; Wilkison, W. O. *Biochemistry* **1998**, *37*, 991.
9. Tota, M. R.; Smith, T. S.; Mao, C.; MacNeil, T.; Mosley, R. T.; Van der Ploeg, L. H.; Fong, T. M. *Biochemistry* **1999**, *38*, 897.
10. Bolin, K. A.; Anderson, D. J.; Trulson, J. A.; Thompson, D. A.; Wilken, J.; Kent, S. B. H.; Gantz, I.; Millhauser, G. L. *FEBS Lett.* **1999**, *451*, 125.
11. Yang, Y. K.; Fong, T. M.; Dickinson, C. J.; Mao, C.; Li, J. Y.; Tota, M. R.; Mosley, R.; Van Der Ploeg, L. H.; Gantz, I. *Biochemistry* **2000**, *39*, 14900.
12. Haskell-Luevano, C.; Cone, R. D.; Monck, E. K.; Wan, Y. P. *Biochemistry* **2001**, *40*, 6164.
13. Jackson, P. J.; McNulty, J. C.; Yang, Y. K.; Thompson, D. A.; Chai, B.; Gantz, I.; Barsh, G. S.; Millhauser, G. L. *Biochemistry* **2002**, *41*, 7565.
14. McNulty, J. C.; Thompson, D. A.; Bolin, K. A.; Wilken, J.; Barsh, G. S.; Millhauser, G. L. *Biochemistry* **2001**, *40*, 15520.
15. Mohle, K.; Hofmann, H. J. *J. Pept. Res.* **1998**, *51*, 19.
16. Figliozzi, G. M.; Goldsmith, R.; Ng, S. C.; Banville, S. C.; Zuckermann, R. N. *Meth. Enzymol.* **1996**, *267*, 437.
17. Miller, S. M.; Simon, R. J.; Ng, S.; Zuckermann, R. N.; Kerr, J. M.; Moos, W. H. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 2657.
18. Lipinski, C. A.; Lombard, F.; Dominy, B. W.; Feeney, P. *J. Adv. Drug Del. Rev.* **1997**, *23*, 3.
19. Haskell-Luevano, C.; Rosenquist, A.; Souers, A.; Khong, K. C.; Ellman, J. A.; Cone, R. D. *J. Med. Chem.* **1999**, *42*, 4380.
20. Heizmann, G.; Hildebrand, P.; Tanner, H.; Ketterer, S.; Pansky, A.; Froidevaux, S.; Beglinger, C.; Eberle, A. N. *J. Rec. Sig. Trans. Res.* **1999**, *19*, 449.
21. Mutulis, F.; Mutule, I.; Wikberg, J. E. S. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 1039.
22. Lee, J.-H.; Lim, S.-K.; Huh, S.-H.; Lee, D.; Lee, W. *Eur. J. Biochem.* **1998**, *257*, 31.
23. Compound **1** synthesis 0.5 g Fmoc-Rink amide MBHA resin Novabiochem (San Diego, CA) was deprotected with 2×5 min 20% piperidine/DMF. The free amine was acylated with 2 mmol Fmoc-Aib-OH and DIC in DMF for 30 min, deprotected as before, the resulting amine was alkylated with a 1:1:3 Benzylbromide:DIEA:DMF solution for 1 h. Acylation of secondary amines was accomplished by DIC and Bromoacetic acid for 1.5 h. A 20% solution of Phenethylamine in DMSO was added and left overnight (typical yield $<50\%$). The secondary amine acylated with bromoacetic acid/DIC for 30 min. Diaminopropane was added as a 2 M solution in DMSO for 1 h, followed by guanidinylation by 3,5-dimethylpyrazole carboxamidine nitrate (4 mmol 20% DIEA/DMF 50 °C 1.5 h) Aldrich (Milwaukee, WI). The reaction vessel was DMF washed after every synthetic step. Reactions were at

35 °C unless otherwise noted. MH+ calculated: 510.7 (ave. isotopes) found: 511.3. Typical peptoid synthesis 0.3 g Fmoc-Rink amide MBHA resin was deprotected with 2×5 min 20% piperidine/DMF. Step I: the amine was acylated with excess Bromoacetic acid/DIC for 30 min. Step II: 2 M solutions of primary amines in DMSO were added for 1–2 h. Steps I and II repeated two additional times, followed by guanidinylation of 3-aminopropyl side chains as before. The reaction vessel was DMF washed after every synthetic step.

24. SMILES containing defined atoms, bonds, and stereochemistry were converted to 2000 random, low-energy 3-D conformations using distance geometry techniques by Rubicon (Daylight Chemical Information Systems, Irvine, CA), then subjected to 200 steps of conjugate gradient minimization using the MM2* force field (Macromodel/Batchmin, Columbia University, New York, NY).

25. Armand, P.; Kirshenbaum, K.; Falicov, A.; Dunbrack, R. L., Jr.; Dill, K. A.; Zuckermann, R. N.; Cohen, F. E. *Fold. Des*. **1997**, *2*, 369.

26. Armand P. *Dissertation*. 1998.

27. All compounds were purified using c18 RP-HPLC columns using water (0.1% w/v TFA)/ACN (0.1% w/v TFA) gradients. Analytical HPLC verified sample purity. Compound molecular weight was confirmed on a VG-Quattro II ESI-MS. Circular dichroism measurements were taken with an Aviv 60 DS spectrometer using 0.1 cm path length rectangular quartz cuvettes. Compounds were suspended in 0.010 M sodium phosphate pH 7 at 200–400 μM, filtered, and scanned from 300–191 nm at 25 °C. No significant changes in spectral shape were observed from 100–400 μM **2**.

28. Library synthesis resin was split at i+2 amine coupling and each amine was coupled separately. Recombined resin and proceeded as before.

29. For detailed cAMP and binding assays see ref 2.

30. Adan, R. A.; Vink, T. *Eur Neuropsychopharmacol*. **2001**, *11*, 483.

31. Marks, D. L.; Ling, N.; Cone, R. D. *Cancer Res*. **2001**, *61*, 1432.