Compounds That Activate the Mouse Melanocortin-1 Receptor Identified by Screening a Small Molecule Library Based upon the β-Turn

Carrie Haskell-Luevano,[‡,▽] Åsa Rosenquist,[§] Andrew Souers,[§] Kathy C. Khong,[‡] Jonathon A. Ellman,*[,§] and Roger D. Cone*[,‡]

Vollum Institute, Oregon Health Sciences University, Portland, Oregon 97201, and Department of Chemistry, University of California at Berkeley, Berkeley, California 94720

Received April 20, 1999

A library of 951 compounds based upon the β-turn motif were examined for their ability to stimulate the melanocortin-1 receptor. From this screening process, we have identified two compounds possessing low micromolar agonist activity at the mMC1R. The compound **EL1** with racemic Nal(2′) in the $i + 1$ position, D-Pro in the $i + 2$ position, and Trp in the $i + 3$ position possesses an EC_{50} of 42.5 ± 6.9 μM. Compound **EL2** with Trp in the $i + 1$ position, D-Lys in the $i + 2$ position, and Phe in the $i + 3$ position possesses an EC_{50} value of 63.4 ± 26.9 μM. The results of the library screening process are consistent with a hypothesis dating back to the 1980s proposing that a β-turn conformation involving the melanocortin "Phe-Arg-Trp" core amino acids provides the key recognition element. Additionally, these compounds represent the first nonpeptidic heterocyclic molecules reported to date that are able to activate the MC1R, a melanocyte receptor involved in skin pigmentation and animal coat coloration.

Introduction

The melanocortin peptides include α-, β-, γ-melanocyte stimulating hormones (MSH) and adrenocorticotropin (ACTH). These hormones are derived by posttranslational processing of the pro-opiomelanocortin (POMC) preprohormone and all possess a central His-Phe-Arg-Trp sequence (Table 1). This His-Phe-Arg-Trp consensus sequence is also referred to as the "message" sequence for melanocortin hormones due to its conservation in these peptides. This family of peptides mediates a number of diverse physiological responses, including skin pigmentation and animal coat coloration, energy homeostasis, and adrenocortical steroidogenesis.[1−5] These hormones and neuropeptides are known to mediate their responses via interactions with the G-protein coupled melanocortin receptors and stimulate the adenylyl cyclase second messenger signal transduction cascade.

Five melanocortin receptor subtypes have been identified to date in humans and a variety of animal species.[6−11] The MC1R subtype is involved in skin pigmentation[12] and animal coat coloration.[13] The MC2R responds only to ACTH and is involved in adrenocortical steroidogenesis.[6] The MC3R is present in a variety of tissues including the brain, gut, and testes,[8,11] but its physiological role remains to be determined. The MC4R participates in energy homeostasis.[4,5] Finally, the MC5R has been demonstrated to be highly expressed in exocrine glands and plays a role in these glandular functions.[14]

Structure−activity studies on the melanocortin peptides dates back to the 1960s.[15,16] A linear tridecapeptide known as NDP-MSH, Ac-Ser-Tyr-Ser-Nle[4]-Glu-His-D-Phe[7]-Arg-Trp-Gly-Lys-Pro-Val-NH_2,[17] was initially developed for investigations of the melanocortin system. Subsequently, a cyclic peptide referred to as MTII, Ac-Nle[4]-c[Asp-His-D-Phe[7]-Arg-Trp-Lys]-NH_2, was reported.[18,19] The substructural and biological information gained from these peptides resulted in the generation of the hypothesis that the bioactive conformation of the MSH peptides involves a β-turn.[1] Subsequent SAR studies established that the core "His-Phe-Arg-Trp" tetrapeptide and "Phe-Arg-Trp" tripeptide were active in generating a physiological response in the frog and lizard skin bioassays.[20−22] Further studies examining stereochemically modified tetra- and tripeptides established that Ac-His-D-Phe-Arg-Trp-NH_2 bound and activated the human melanocortin receptor subtypes hMC1R and hMC4R at sub micromolar concentrations.[23] Additionally, tripeptides Ac-D-Phe-Arg-Trp-NH_2 and Ac-D-Phe-Arg-D-Trp-NH_2 were determined to bind and stimulate the hMC4R subtype only at 10 μM concentrations.[23] These data, in conjunction with the involvement of the MC4R subtype in energy homeostasis, prompted this study which involves the screening of a library of small molecules based upon the β-turn. The general synthesis sequence as reported previously[24−26] is shown in Scheme 1, and the building blocks used to prepare the library are shown in Figure 1.

Results

The 951 compounds derived from a small molecule library consisting of 5544 compounds were evaluated for agonist activity at the melanocortin-1 receptor subtype (data for all the compounds provided in Supporting Information). The 951-compound subset of the small molecule library was selected based upon peptides containing the putative melanocortin pharmacophore side chain moieties identified from previous studies to be important for molecular recognition and receptor stimulation.[22,23] These compounds were generated using

* To whom reprint requests should be addressed.
‡ Oregon Health Sciences University.
§ University of California at Berkeley.
▽ Present address: Department of Medicinal Chemistry, University of Florida, P.O. Box 100485, Gainesville, FL 32610-0485. E-mail: Carrie@cop.ufl.edu.

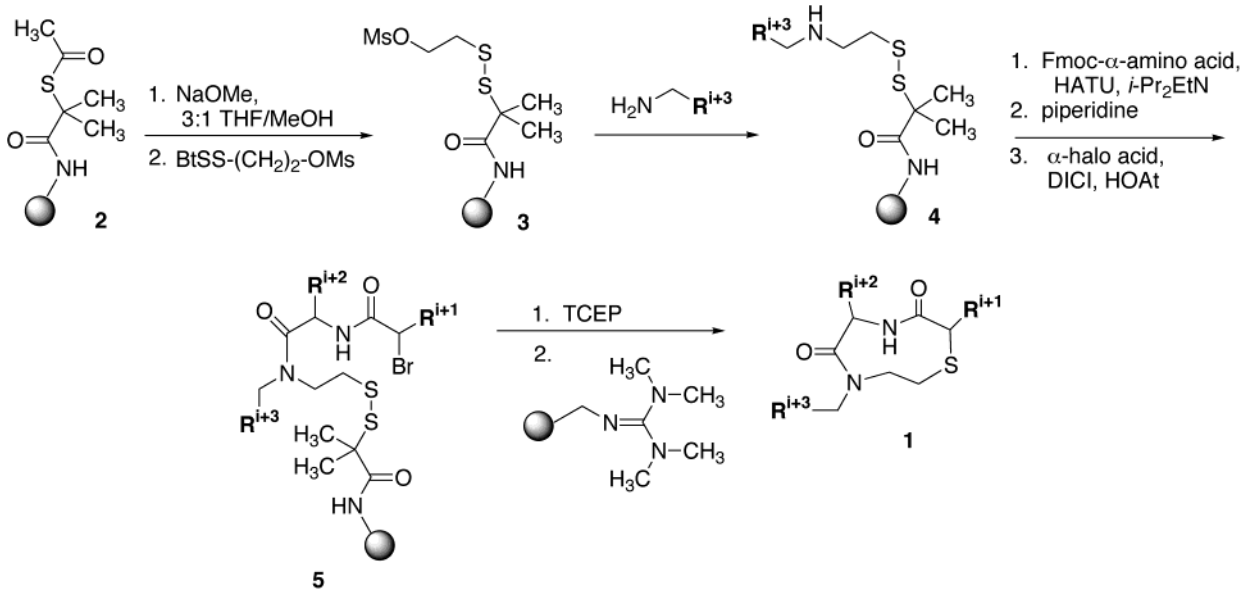
10.1021/jm990190s CCC: $18.00
Published on Web 09/23/1999

Table 1. Primary Sequence of POMC-Derived Melanocortin Peptides with the Core "His-Phe-Arg-Trp" Sequence Emphasized[a]

name	sequence
ACTH (1-39)	NH_2-SYSME**HFRW**GKPVGKKRRPVKVYPNGAEDESAEAFPLEF-OH
α-MSH	Ac-SYSME**HFRW**GKPV-NH_2
β-MSH	NH_2-AEKKDEGPYRME**HFRW**GSPPKE-OH
γ-MSH	NH_2-YVMG**HFRW**DRF-OH

[a] Sequences of human MCs are provided.

Scheme 1. Synthetic Route for the Preparation of Heterocyclic β-Turn Mimetics **1**



parallel synthesis techniques,[24−26] and screened in parallel at concentrations of 10 μM using a 96-well colorimetric bioassay.[27] Figures 2 and 3 summarize the bioassay data from the two 96-well plates containing the lead compounds (10 μM), both of which possessed reproducible agonist activity at the mMC1R. The plate possessing the Phe side chain in the $i + 3$ position (Figure 3) contained compounds that had activities above the basal activity of the system. Figure 4 illustrates the three compounds that demonstrated the greatest reproducible agonist activity at the mouse MC1R subtype at concentrations of 10 μM.

Two compounds were then individually resynthesized, purified, and characterized (see Experimental Section). These two compounds, **EL1** (rac-Nal(2′) $i + 1$, DPro $i + 2$, Trp $i + 3$) and **EL2** (Trp $i + 1$, DLys $i + 2$, Phe $i + 3$) were tested in triplicate and in at least two independent experiments (10^{-4} M to 10^{-10} M) at the mMC1R, mMC3R, mMC4R, and mMC5R subtypes (Figure 5). **EL1** possessed an EC_{50} value of 42.5 ± 6.9 μM, and **EL2** possessed an EC_{50} value of 63.4 ± 26.9 μM at the mMC1R subtype. As illustrated in Figure 5, these two compounds did not generate an agonist response at the mMC3R and mMC4R subtypes. Interestingly, **EL1** possessed some agonist activity at the mMC5R.

Discussion

Rational design approaches toward the generation of small molecules that mimic peptide−receptor interactions have been pursued for decades. The use of peptide hormones as therapeutic agents has several shortcomings including poor enzymatic stability, bioavailability, and receptor specificity. Classic approaches in pursuit of generating small molecule agonists and antagonists include determining the minimally active peptide fragment, incorporating conformational restraints (global and local), and inverting amino acid chirality. For our screening efforts we selected a biased small molecule library based upon the β-turn as a result of prior classical studies of melanocortins.[20−23] First, these studies implicated the Phe-Arg-Trp side chains as important for melanocortin agonist activity. Substitution of the melanocortin Phe[7] with Gly or Ala in the 6−9 and 7−9 tetrapeptides (α-MSH numbering nomenclature) resulted in peptides that were inactive.[22] Substitution of the melanocortin Arg[8] by Lys, Gln, Ala, and Gly resulted in tetrapeptides possessing 2−65 μM EC_{50} values,[22] whereas substitution of Trp[9] by Gly or Ala resulted in inactive tri- and tetrapeptides (unpublished results, C. Haskell-Luevano, M. E. Hadley, and V. J. Hruby). Second, based upon biological characterization of NDP-MSH,[17] the design and evaluation of c[Cys[4], Cys[10]]-α-MSH,[28] and extensive model building,[1,18] Sawyer, Hadley, and Hruby proposed that the bioactive conformation of α-MSH involves a β-turn structure involving the Glu-His-Phe-Arg-Trp region. This hypothesis was further supported by the enhanced potency observed for the cyclic lactam analogue MTII.[18,19] Conformational analysis of MTII, α-MSH, and NDP-MSH by nuclear magnetic resonance (NMR) techniques provided further evidence that a β-turn is the bioactive conformation of melanocortin peptides.[29,30]

We have screened a library of compounds based upon the β-turn motif, with the library members biased to include side chains present in the putative β-turn region of α-MSH, against the melanocortin-1 receptor. These studies have led to the identification of the first heterocyclic small molecules that are able to stimulate the

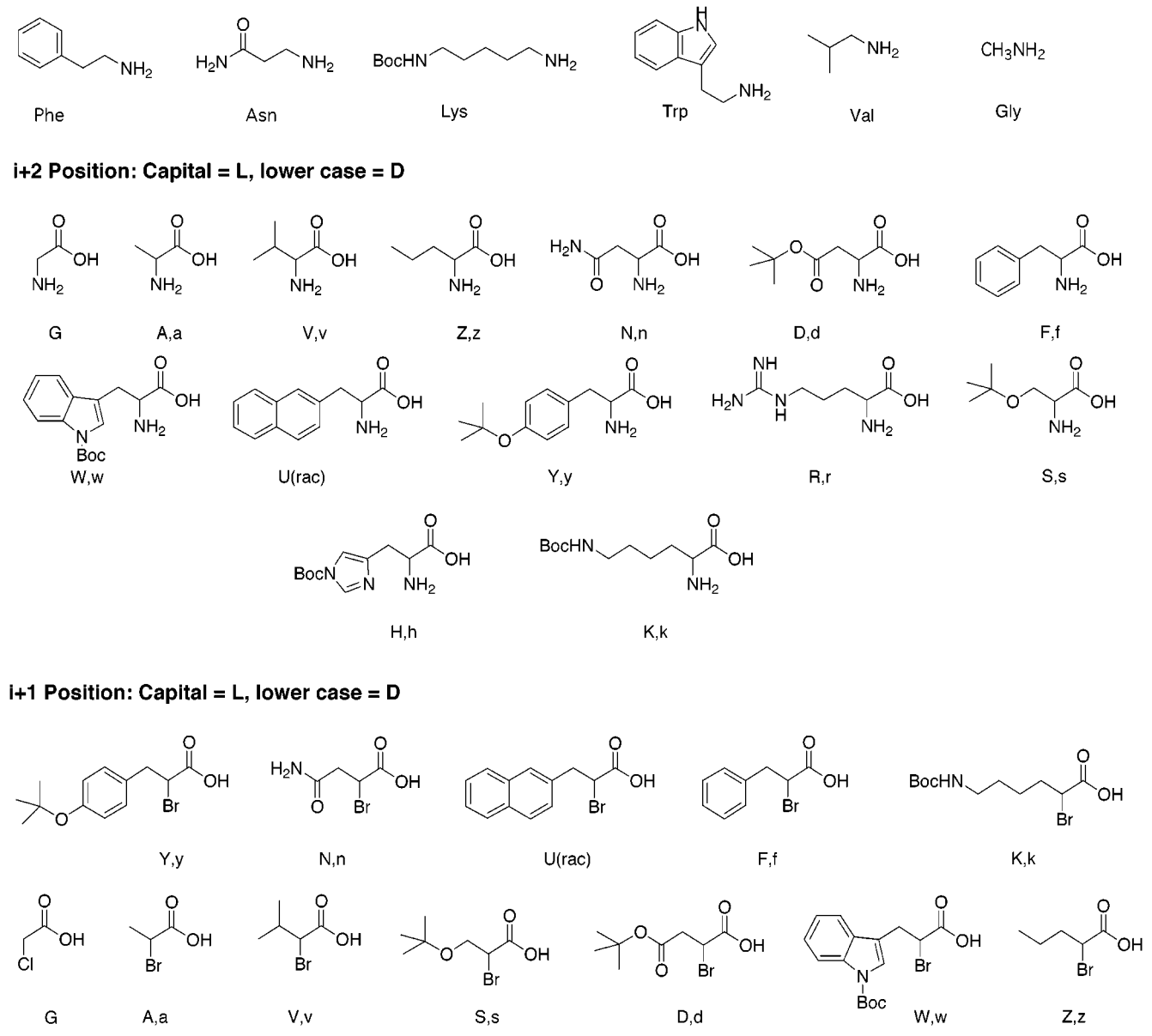


Figure 1. Side chain building blocks used in the generation of the β-turn library examined at the melanocortin receptor subtypes.

mouse melanocortin-1 receptor subtype. Furthermore, these findings identify a combinatorial chemistry scaffold upon which further modifications can be examined to increase ligand affinities to the mMC1R.

Because the identified compounds, **EL1** and **EL2**, are agonists at the mMC1R, it is possible that a similar mode of receptor interactions and activation are common to these molecules and the pharmacophore elements of the melanocortin peptides. Previously reported homology molecular modeling of the MC1R[31] suggested specific receptor side chain residue interactions with the ligand DPhe-Arg-Trp. As shown in Figure 6, the model proposes two hydrophobic pockets and an electrostatic pocket for accommodating binding of the peptidic ligand. Genetics and in vitro mutagenesis of the MC1R have clearly implicated the receptor residues Glu92, Asp115, and Asp119 (mouse numbering nomenclature) in transmembrane (TM) domains 2 and 3 as being particularly important for endogenous melanocortin ligand binding and receptor activation.[13,32] These studies and functional activities of α-MSH analogues with Arg^8 replacements clearly implicate the importance of Arg^8 in melanocortin recognition and receptor activation. Interestingly, although **EL1** lacks any potential electrostatic/hydrophilic functionality that corresponds to the Arg^8 side chain, this molecule possesses low micromolar EC_{50} values at the mMC1R. These finding are consistent with previous reports on activation of frog skin MC1R with a series of tri- and tetrapeptides, Ac-DPhe-Xaa-DTrp-NH_2 and Ac-His-DPhe-Xaa-DTrp-NH_2, where Xaa is Lys, Gln, Ala, or Gly. The tetrapeptides possess low micromolar EC_{50} values, and the tripeptides possess EC_{50} values in the range of 100 μM. These studies suggest that spatial orientation of the hydrophobic Phe and Trp side chains play a critical role in receptor activation. Figure 7 illustrates putative **EL1** interactions with the mMC1R side chains based upon the assumption that the ligand receptor interactions mimic the melanocortin peptide−receptor hydrophobic interactions. Notably, although **EL2** possesses a DLys side chain at the $i + 2$ position

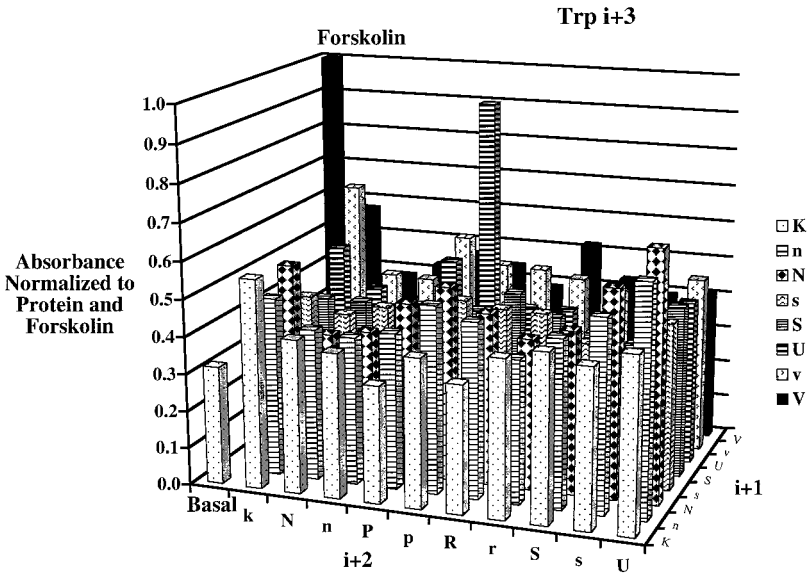


Figure 2. Summary of the β-turn library plate containing the **EL1** compound evaluated at concentrations of 10 μM in the β-galactosidase bioassay at the mMC1R.

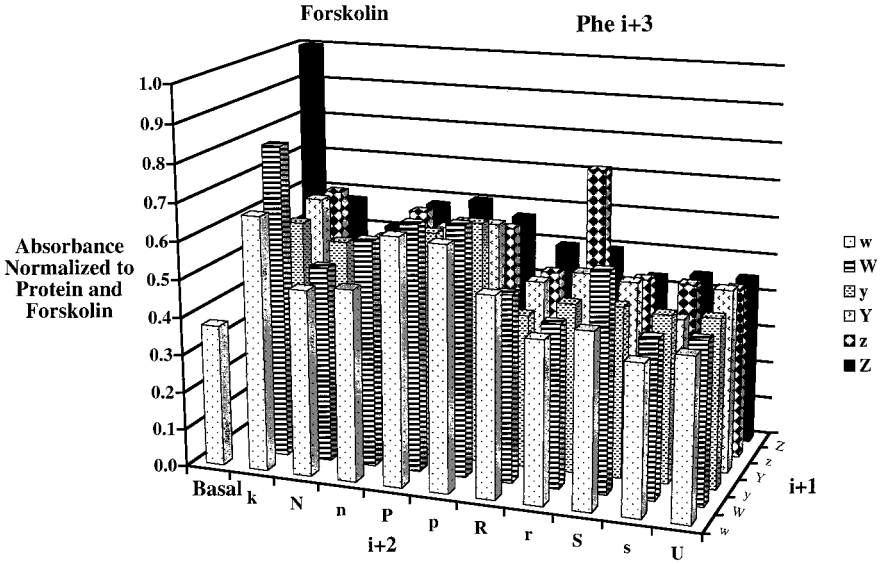


Figure 3. Summary of the β-turn library plate containing the **EL2** compound evaluated at concentrations of 10 μM in the β-galactosidase bioassay at the mMC1R.

that could substitute for Arg^8-mMC1R interactions, slightly poorer agonist activity is observed (Figure 5). In addition, the library members that incorporated D or L Arg in place of Pro in **EL1** and D or L Arg in place of Lys in **EL2** were not as potent as either **EL1** or **EL2** (Figures 3 and 4). Detailed SAR of **EL1** and **EL2** along with mutagenesis studies of mMC1R will be required in order to evaluate the validity of the proposed binding model.

Conclusions

By screening a small molecule library based upon the β-turn, we have identified, to our knowledge, the first reported non-peptide heterocyclic compounds which activate the mouse melanocortin-1 receptor subtype. These results support the hypothesis put forth a decade ago that the melanocortin peptide possesses a β-turn conformation involving the melanocortin Phe-Arg-Trp core amino acids as a key recognition element. The compounds reported herein are the first leads for future generations of non-peptide heterocyclic agonists at the MC1R. (During the preparation of this manuscript, reports of MC1R peptoids and oligomeric α-MSH analogues were reported.[33,34])

Experimental Section

General Methods and Reagents. All materials, unless otherwise noted, were obtained from commercial suppliers and used without further purification. When used as a reaction solvent CH_2Cl_2 was distilled from CaH_2; THF and dioxane were distilled under N_2 from sodium benzophenone ketyl, all immediately prior to use. Deoxygenation of solvents and reaction mixtures was achieved by N_2 or Ar bubbling for 15−20 min. *N*-Fmoc-protected amino acids and side-chain-protected amino acids were purchased from Calbiochem-Novabiochem Corp. (San Diego, CA) or Bachem Bioscience Inc. (King of Prussia, PA). α-Bromo acids were prepared from the corresponding side-chain-protected amino acids in one step in optically enriched form according to the previously reported procedure.[35] Chromatography was carried out using Merck 60 230−240 mesh silica gel. Compounds were visualized either by ultraviolet light, by cobalt nitrate/ammonium molybdate staining,

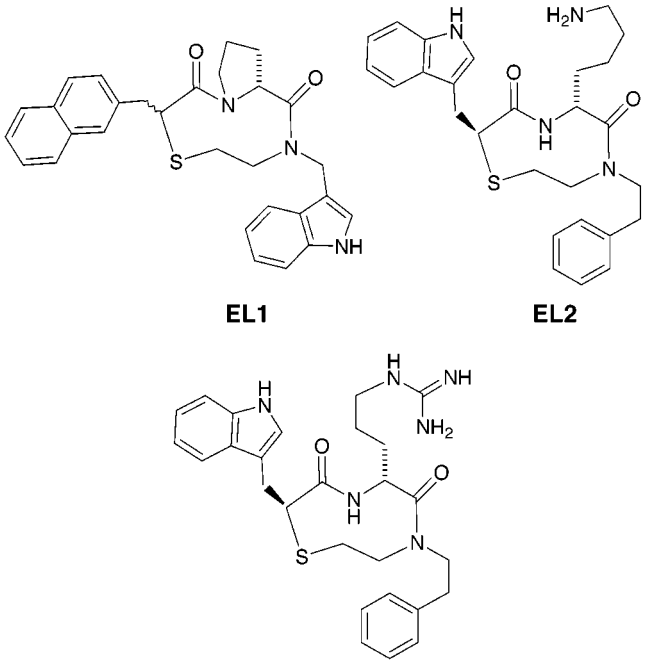


Figure 4. Structures of **EL1**, **EL2**, and the third compound which were identified as possessing agonist activity at the mMC1R.

or by a 0.3% ninhydrin solution in butanol/3% acetic acid. Melting points were determined in open Pyrex capillaries and are uncorrected. NMR spectra were recorded on Bruker AMX 300 (300 MHz), AMX 400 (400 MHz), AM 400 (400 MHz), or AM 500 (500 MHz) machines. Proton-decoupled ^{13}C spectra were obtained at 101 or 126 MHz with the same instruments with a line broadening of 1.5 Hz. Samples for NMR analysis which contained rotamers were heated in order to cause coalescence of the different rotameric peaks. NMR chemical shifts are expressed in ppm downfield, relative to internal solvent peaks. Coupling constants, *J*, are listed in hertz and chemical shifts δ are reported in ppm. MALDI TOF mass spectrometry was performed on a VoyagerTM BioSpectrometry workstation from PerSeptive Biosystems (South San Francisco, CA). Elemental analyses were performed by M-H-W Laboratories (Phoenix, AZ) or by the Microanalytical Laboratory at the University of California at Berkeley.

Synthesis of Amine (*i* + 3 Side Chain) Building Blocks.[36] **1-*N*-*tert*-Butoxycarbonyl-1,5-diaminopentane.** Di-*tert*-butyl dicarbonate (16 g, 73 mmol) in 300 mL of 9:1 dioxane/water was added to a solution of 1,5-diaminopentane (15 g, 147 mmol) in 450 mL of 9:1 dioxane/water over a period of 4 h. The solution was stirred at room temperature overnight and concentrated, and the residue was taken up in 300 mL of water. The precipitated *N*,*N*′-di-Boc-1,5-diaminopentane was removed by filtration through a fritted glass funnel, and the filtrate was extracted with CH_2Cl_2 (4 × 250 mL). The combined organic extracts were concentrated, and the crude product was chromatographed over silica gel (80:20:4 CH_2Cl_2/methanol/40% aqueous NH_4OH) and dried to provide 9.3 g (46 mmol, 63%) of a dense liquid which appears as a solid: boiling point 97−98° at 2×10^{-1} Torr;[37] ^1H NMR (400 MHz, $CDCl_3$) δ 1.28 (m, 2H), 1.36 (s, 9H), 1.37−1.46 (m, 4H), 2.65 (t, 2H, $J = 7.0$), 2.81 (br s, 2H), 2.98−3.08 (m, 2H), 4.78 (br s, 1H); ^{13}C NMR (81 MHz, $CDCl_3$) δ 23.9, 28.3, 29.7, 32.3, 40.3, 41.4, 78.8, 155.9.

Library Synthesis. Coupling of the SAMI Linker to Support. Aminomethylated resin beads (85 g, 100−200 mesh, purchased form California Bachem, Lot # ZL725, loading level 0.6 mequiv/g) were slowly stirred in a solution of *S*-acetyl 2-mercapto-2-methyl propionic acid (22.7 g, 140 mmol), PyBOP (73 g, 140 mmol), HOBt-H_2O (21.4 g, 140 mmol), and *i*-Pr_2-EtN (48.8 mL, 280 mL) in 650 mL of DMF for 12 h. The resin was then isolated by filtration and rinsed with DMF (8×), CH_2Cl_2 (4×), methanol (2×), CH_2Cl_2 (3×), and methanol (2×). The resin was dried in vacuo. Complete disappearance of support-bound amine was confirmed by a negative bromophenol blue test.

Disulfide Formation for Backbone Coupling. Thioester resin **2** (49 g) was solvated in 500 mL of THF under an inert atmosphere. Sodium (3.2 g, 140 mmol) was dissolved in 170 mL of methanol and slowly added to the solvated resin with mechanical stirring. The reaction slurry was stirred for 1 h, quenched by addition of 20 mL (350 mmol) of acetic acid. After being stirred for 5 min, the resin was filtered and rinsed with 2.5% acetic acid in THF, methanol, THF, methanol, THF, methanol (2×), and dry CH_2Cl_2 (2×). 2-Benzothiazolyl 2-methanesulfonoxyethyl disulfide (20.5 g, 63.7 mmol) dissolved in 300 mL of dry CH_2Cl_2 was added to the deprotected resin. The mixture was mechanically stirred under an inert atmosphere. After 18 h, Ellman's free thiol test showed that no free thiol remained on the resin. The resin was isolated by filtration and rinsed with CH_2Cl_2 (2×) and MeOH (3×) and dried in vacuo overnight to give **3**.

Amine Displacements (*i* + 3 Side Chain). Each support-bound secondary amine **4** was prepared by treating 4 g of resin **3** in a 50 mL round bottom flask with a 0.75 M solution of the corresponding primary amine in 24 mL of NMP for 18 h at 50−55 °C. The resin was isolated by filtration, rinsed with DMF (3×) and (CH_2Cl_2 (2×), MeOH (2×)) (2×), and dried in vacuo.

Coupling of Fmoc Amino Acids (*i* + 2 Side Chain) and Fmoc Cleavage. Each secondary amine resin **4** was weighed (mass depended on R^{i+3}, 62.5 mmol/cartridge) into 28 12 mL filter cartridges with 70 mm hydrophobic polyethylene frits {Applied Separations (Allentown, PA), cat.# 2427)} for coupling of the 28 Fmoc-amino acids. Solutions of each Fmoc amino acid (0.2 M) with HATU (0.2 M) and *i*-Pr_2EtN (0.4 M) in DMF were prepared, and 2 mL of the coupling solution containing the appropriate Fmoc amino acid was added to each filter cartridge. HOAt (0.2 M) was added to coupling solutions of Fmoc-Arg and Fmoc-D-Arg to protect the guanidine side chain as the HOAt salt.[38] The filter cartridges were stoppered and shaken for 12 h. After complete consumption of starting material as confirmed by negative bromophenol blue test, the resin was rinsed with DMF (2×), CH_2Cl_2 (2×), and MeOH (2×) and allowed to air-dry. Each resin batch was then treated with 2 mL of DMF containing 20% (v/v) piperidine for 30 min with shaking. The resin was drained, rinsed with DMF (2×), CH_2Cl_2 (2×), and MeOH (2×), and dried in vacuo.

Coupling of α-Bromo Acids (*i* + 1 Side Chain). Filter cartridges (152) of deprotected resin (62.5 mmol) were equilibrated in 5 mL of a 17:13 comixture of 1,2-dichloroethane/DMF for at least 10 min. Resin from each filter cartridge was then transferred as an isopycnic slurry to each of 22 respective wells of a high-throughput solid-phase synthesis block (∼200 mL of slurry/well, 2.6 mmol/well). Each block was then fitted into a deep-well (2 mL) microtiter plate (Beckmann, cat.# 267006) with one glass bead present in each well. The coupling solution corresponding to one of the 22 α-bromo acids (0.1 M), DICI (0.1 M), and HOAt (0.1 M) in DMF was added to each well (300 mL/well). After 12 h, the solvent was drained, and the resin in each high-throughput synthesis block was rinsed with DMF (2×), CH_2Cl_2 (2×), and 1:1 CH_2Cl_2/MeOH (2×) to provide support-bound **5**.

Cleavage and Cyclization. Each high-throughput synthesis apparatus was fitted into a microtiter plate and transferred into a glovebag under a N_2 atmosphere, and TCEP (6 mM) in ∼1 mL of oxygen-free 19:1 DMF/water was added to each well. The plates were transferred into a glovebag under a N_2 atmosphere within an oven maintained at 50 °C. After 24 h, the cleaved resin in each synthesis block was rinsed once with 200 mL of DMF, and the synthesis blocks were removed from the microtiter plates to provide the acyclic β-turn mimetics in 1.2 mL of DMF.

Guanidine resin (50−60 mg, 108−120 mmol, 100−200 mesh) was then added to each well of each microtiter plate. The microtiter plates were transferred to a glovebag under a N_2 atmosphere and allowed to remain for at least 24 h at room

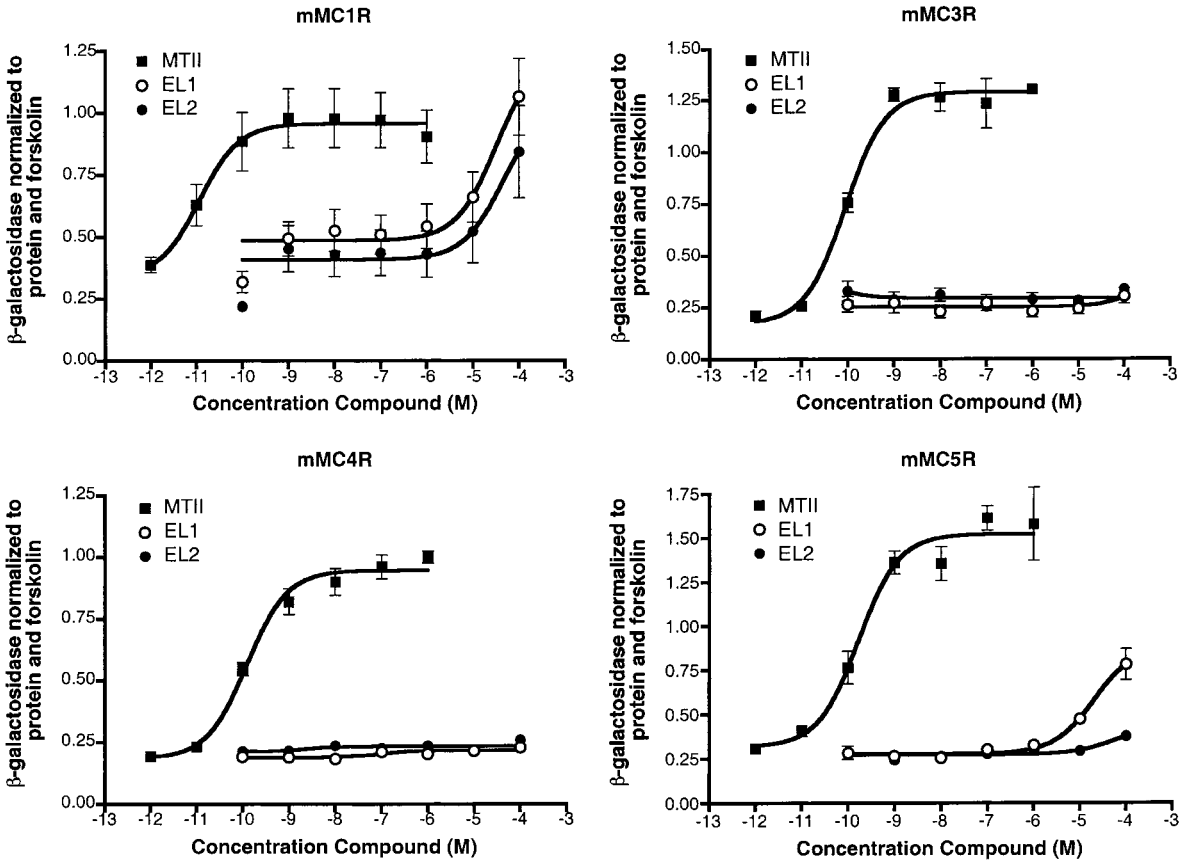


Figure 5. Agonist pharmacological analysis of purified **EL1** and **EL2** compound activity at the melanocortin receptor subtypes.

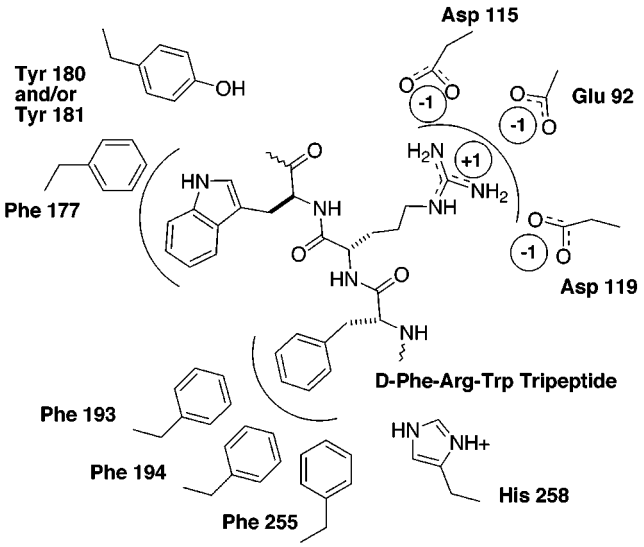


Figure 6. Illustration of the putative ligand DPhe-Arg-Trp amino acids interactions with the mouse MC1R.

temperature. After the cyclization step, each microtiter plate containing solutions of the cyclic β-turn mimetics and the guanidine resin was positioned atop another microtiter plate so that the corresponding wells of the lower plate were directly beneath those of the upper plate. Syringe 18-gauge holes were then punched into each well of the upper plate using a microtiter-based punch apparatus. Filtration to remove the guanidine resin was accomplished by draining the solution into the new microtiter plate. To expedite the draining process the plates were centrifuged. The wells of the upper plate containing the guanidine resin were rinsed with 400 mL of DMF and then with 200 mL of DMF. Centrifugation was performed after each rinse. After filtration and rinsing, the solvent containing the β-turn mimetics was removed in vacuo on a microtiter plate concentrator. Methanol was added to each well followed by reconcentration as before to provide a white residue for most of the β-turn mimetics **1**.

Library Characterization. The presence of compounds at their specified locations within the library was confirmed by mass spectral (MALDI TOF) analysis. Next, 110 of the deprotected compounds containing the lysine side chain functionality at one or more positions were submitted for analysis. Of the compounds analyzed, 95% (104/110) displayed the desired mass spectral ions. ^{1}H NMR was used to confirm the structure, assess the purity, and determine the yield of a selected set of compounds from the library. Thus, 26 compounds were selected from the library to span a range of side chain functionality at all three positions and to provide easily interpretable spectra. An average of 1.4 ± 0.4 mmol of compound was obtained per well as determined by comparison of peak areas to that of an internal standard (*p*-xylene). The analytical results from the library characterization are given in the Supporting Information.

Compounds **EL1** and **EL2** (Figure 4), which showed interesting activity in the preliminary screenings were prepared on a 20 mg scale on solid support following the previously described conditions in 65 and 74% total yield, respectively.

EL1: ^{1}H NMR (MeOH-d_4, 300 MHz) δ 1.10−1.28 (m, 2H), 1.33−1.69 (m, 3H), 1.77−1.90 (m, 1H), 2.63−2.89 (m, 6H), 3.07−3.37 (m, 5H), 3.62−3.79 (m, 2H), 4.62−4.74 (m, 1H), 6.94−7.07 (m, 3H), 7.12−7.32 (m, 5H), 7.53 (d, 1H, J = 7.87), 8.75 (d, 1H, J = 9.56). HRMS (FAB) *m/z* calcd for $C_{27}H_{35}N_4O_2S$ (MH^+), 479.2481; found, 479.2482.

EL2: ^{1}H NMR (DMSO-d_6, 300 MHz) δ 2.42−2.95 (m, 4H), 2.97−3.45 (m, 2H), 3.48−3.90 (m, 4H), 4.54−4.67 (m, 1H), 4.81−5.18 (m, 2H), 6.12 (d, 0.5H, J = 7.13), 6.36 (t, 0.5H, J = 7.21), 7.68−7.90 (m, 3H), 8.04−8.69 (m, 8H), 10.52 (b, 0.5H), 10.84 (b, 0.5H). HRMS (FAB) *m/z* calcd for $C_{30}H_{32}N_3O_2S$ (MH^+), 498.2215; found, 498.2219.

Cell Culture and Transfection. Briefly, HEK-293 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum and seeded 1 day prior to transfection at

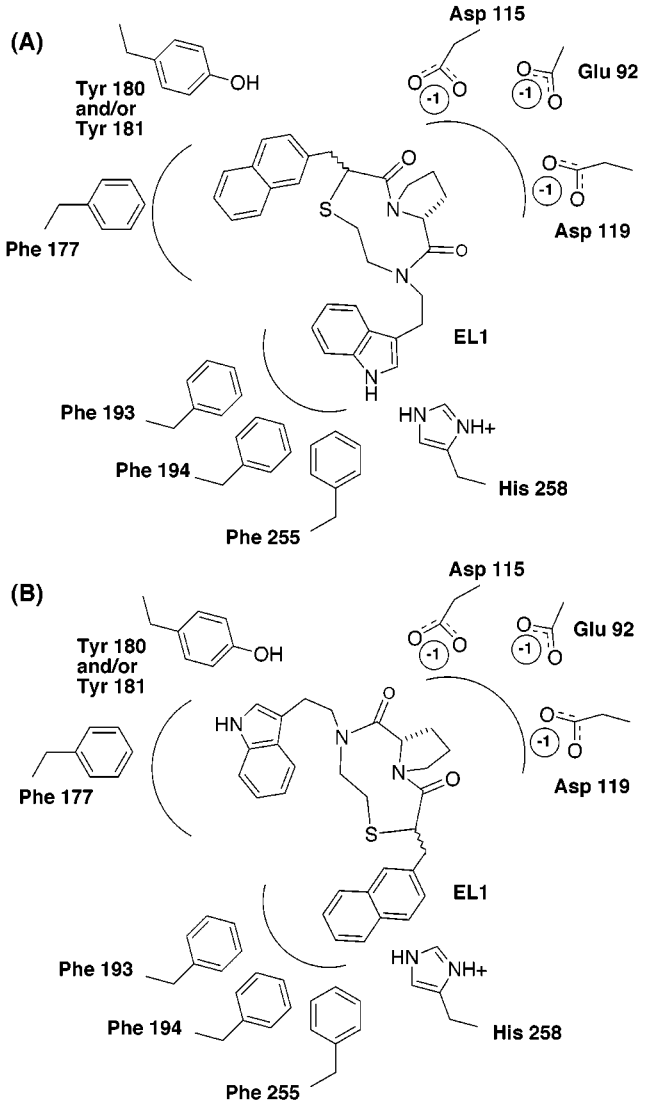


Figure 7. Putative **EL1** ligand−receptor interactions which may mimic the DPhe-Arg-Trp receptor interactions at the mMC1R resulting in agonist activity.

1 to 2 × 10^6 cell/100 mm dish. Melanocortin receptor cDNA subcloned into the $pCDNA_3$ expression vector (Invitrogen) was transfected (20 μg) using the calcium phosphate method. Stable receptor populations were generated using G418 selection (1 g/mL) for subsequent bioassay analysis.[27]

***β*-Galactosidase Bioassay.** Cells stably expressing wild-type receptors were transfected with 4 μg of CRE/β-galactosidase reporter gene as previously described.[27] Briefly, 5 000 to 15 000 post-transfection cells were plated into 96-well Primeria plates (Falcon) and incubated overnight. At 48 h post-transfection, the cells were stimulated with compound or peptide at concentrations indicated, or forskolin (10^{-4} M), in assay medium (DMEM containing 0.1 mg/mL BSA and 0.1 mM isobutylmethylxanthine) for 6 h. The assay media was aspirated, and 50 μL of lysis buffer (250 mM Tris-HCl pH = 8.0 and 0.1% Triton X-100) was added. The plates were stored at −80° overnight. The plates containing the cells lysates were thawed the following day. Aliquots of 10 μL were taken from each well and transferred to another 96-well plate for relative protein determination. The 40 μL of phosphate-buffered saline with 0.5% BSA was added to each well. Subsequently, 150 μL of substrate buffer (60 mM sodium phosphate, 1 mM $MgCL_2$, 10 mM KCl, 5 mM β-mercaptoethanol, 200 mg of ONPG) was added to each well, and the plates were incubated at 37°. The sample absorbance, OD_{405}, was measured using a 96-well plate reader (Molecular Devices). The relative protein was determined by adding 200 μL of 1:5 dilution Bio Rad G250 protein dye:water to the 10 μL cell lysate sample taken previously, and the OD_{595} was measured on a 96-well plate reader (Molecular Devices). Data points were normalized both to the relative protein content and nonreceptor dependent forskolin stimulation. Data analysis and EC_{50} values were determined using nonlinear regression analysis with the PRISM program (v2.0, GraphPad Inc.).

Acknowledgment. This work was supported by grants from the U.S. Public Health Service, RO1-AR42415 (R.D.C.), R01-53696 (J.E.), and DK09231 (C.H.L.). Carrie Haskell-Luevano is a recipient of a Burroughs Wellcome fund Career Award in the Biomedical Sciences. Åsa Rosenquist thanks the Knut and Alice Wallenberg Foundation for funding.

Supporting Information Available: Characterization of selected library members by low-resolution mass spectral (MALDI-TOF) analysis and ^{1}H NMR is available. Additionally, the data for plates indicating the exact side chain combinations (i + 1, i + 2, and i + 3) of the small molecules which were pharmacological evaluated at the mMC1R are shown. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Hruby, V. J.; Wilkes, B. C.; Cody, W. L.; Sawyer, T. K.; Hadley, M. E. Melanotropins: Structural, Conformational and Biological Considerations in the Development of Superpotent and Super-prolonged Analogues. *Pept. Protein Rev.* **1984**, *3*, 1−64.
(2) Eberle, A. N. *The Melanotropins: Chemistry, Physiology and Mechanisms of Action*; Karger: Basel, 1988.
(3) Cone, R. D.; Lu, D.; Kopula, S.; Vage, D. I.; Klungland, H.; Boston, B.; Chen, W.; Orth, D. N.; Pouton, C.; Kesterson, R. A. The Melanocortin Receptors: Agonists, Antagonists, and the Hormonal Control of Pigmentation. *Recent Prog. Horm. Res.* **1996**, *51*, 287−318.
(4) Fan, W.; Boston, B. A.; Kesterson, R. A.; Hruby, V. J.; Cone, R. D. Role of Melanocortinergic Neurons in Feeding and the *agouti* Obesity Syndrome. *Nature* **1997**, *385*, 165−168.
(5) Huszar, D.; Lynch, C. A.; Fairchild-Huntress, V.; Dunmore, J. H.; Smith, F. J.; Kesterson, R. A.; Boston, B. A.; Fang, Q.; Berkemeir, L. R.; Gu, W.; Cone, R. D.; Campfield, L. A.; Lee, F. Targeted Disruption of the Melanocortin-4 Receptor Results in Obesity in Mice. *Cell* **1997**, *88*, 131−141.
(6) Mountjoy, K. G.; Robbins, L. S.; Mortrud, M. T.; Cone, R. D. The Cloning of a Family of Genes that Encode the Melanocortin Receptors. *Science* **1992**, *257*, 1248−1251.
(7) Chhajlani, V.; Wikberg, J. E. S. Molecular Cloning and Expression of the Human Melanocyte Stimulating Hormone Receptor cDNA. *FEBS Lett.* **1992**, *309*, 417−420.
(8) Gantz, I.; Konda, Y.; Tashiro, T.; Shimoto, Y.; Miwa, H.; Munzert, G.; Watson, S. J.; DelValle, J.; Yamada, T. Molecular Cloning of a Novel Melanocortin Receptor. *J. Biol. Chem.* **1993**, *268*, 8246−8250.
(9) Gantz, I.; Miwa, H.; Konda, Y.; Shimoto, Y.; Tashiro, T.; Watson, S. J.; DelValle, J.; Yamada, T. Molecular Cloning, Expression, and Gene Localization of a Fourth Melanocortin Receptor. *J. Biol. Chem.* **1993**, *268*, 15174−15179.
(10) Gantz, I.; Shimoto, Y.; Konda, Y.; Miwa, H.; Dickinson, C. J.; Yamada, T. Molecular Cloning, Expression, and Characterization of a Fifth Melanocortin Receptor. *Biochem. Biophys. Res. Commun.* **1994**, *200*, 1214−1220.
(11) Roselli-Rehfuss, L.; Mountjoy, K. G.; Robbins, L. S.; Mortrud, M. T.; Low, M. J.; Tatro, J. B.; Entwistle, M. L.; Simerly, R. B.; Cone, R. D. Identification of a Receptor for g Melanotropin and Other Proopiomelanocortin Peptides in the Hypothalamus and Limbic System. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 8856−8860.
(12) Levine, N.; Sheftel, S. N.; Eytan, T.; Dorr, R. T.; Hadley, M. E.; Weinrach, J. C.; Ertl, G. A.; Toth, K.; McGee, D. L.; Hruby, V. J. Induction of Skin Tanning by Subcutaneous Administration of a Potent Synthetic Melanotropin. *J. Am. Med. Assoc.* **1991**, *266*, 2730−2736.
(13) Lu, D.; Väge, D. I.; Cone, R. D. A Ligand-Mimetic Model for Constitutive Activation of the Melanocortin-1 Receptor. *Mol. Endo.* **1998**, *12*, 592−604.
(14) Chen, W.; Kelly, M. A.; Opitz-Araya, X.; Thomas, R. E.; Low, M. J.; Cone, R. D. Exocrine Gland Dysfunction In MC5-R Deficient Mice: Evidence For Coordinated Regulation Of Exocrine Gland Functions By Melanocortin Peptides. *Cell* **1997**, *91*, 789−798.

(15) Koida, M.; Hano, K.; Iso, T. Evaluation of In Vitro Melanocyte-Darkening Activities of L-Histidyl-L-Phenylalanyl-L-Arginyl-L-Tryptophyl-Glycine and its Nine Stereoisomers in Rana Nigromaculata. *Jpn. J. Pharmacol.* **1966**, *16*, 243−249.
(16) Yajima, H.; Kubo, K. The Synthesis of D-Histidyl-L-Phenylalanyl-L-Arginyl-L-Tryptophylglycine and L-Histidyl-L-Phenylalanyl-L-Arginyl-D-Tryptophylglycine and Their Physiological Properties in Frog Melanocyte. *Biochim. Biophys. Acta* **1965**, *97*, 596−597.
(17) Sawyer, T. K.; Sanfillippo, P. J.; Hruby, V. J.; Engel, M. H.; Heward, C. B.; Burnett, J. B.; Hadley, M. E. 4-Norleucine, 7-D-Phenylalanine-α-Melanocyte-Stimulating Hormone: A Highly Potent α-Melanotropin with Ultra Long Biological Activity. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 5754−5758.
(18) Al-Obeidi, F.; Hadley, M. E.; Pettitt, B. M.; Hruby, V. J. Design of a New Class of Superpotent Cyclic α-Melanotropins Based on Quenched Dynamic Stimulations. *J. Am. Chem. Soc.* **1989**, *111*, 3413−3416.
(19) Al-Obeidi, F.; Castrucci, A. M.; Hadley, M. E.; Hruby, V. J. Potent and Prolonged Acting Cyclic Lactam Analogues of α-Melanotropin: Design Based on Molecular Dynamics. *J. Med. Chem.* **1989**, *32*, 2555−2561.
(20) Hruby, V. J.; Wilkes, B. C.; Hadley, M. E.; Al-Obeidi, F.; Sawyer, T. K.; Staples, D. J.; DeVaux, A.; Dym, O.; Castrucci, A. M.; Hintz, M. F.; Riehm, J. P.; Rao, K. R. a-Melanotropin: The Minimal Active Sequence in the Frog Skin Bioassay. *J. Med. Chem.* **1987**, *30*, 2126−2130.
(21) Castrucci, A. M. L.; Hadley, M. E.; Sawyer, T. K.; Wilkes, B. C.; Al-Obeidi, F.; Staples, D. J.; DeVaux, A. E.; Dym, O.; Hintz, M. F.; Riehm, J.; Rao, K. R.; Hruby, V. J. α-Melanotropin: The Minimal Active Sequence in the Lizard Skin Bioassay. *Gen. Comput. Endocrinol.* **1989**, *73*, 157−163.
(22) Haskell-Luevano, C.; Sawyer, T. K.; Hendrata, S.; North, C.; Panahinia, L.; Stum, M.; Staples, D. J.; Castrucci, A. M.; Hadley, M. E.; Hruby, V. J. Truncation Studies of a-Melanotropin Peptides Identifies Tripeptide Analogues Exhibiting Prolonged Agonist Bioactivity. *Peptides* **1996**, *17*, 995−1002.
(23) Haskell-Luevano, C.; Hendrata, S.; North, C.; Sawyer, T. K.; Hadley, M. E.; Hruby, V. J.; Dickinson, C.; Gantz, I. Discovery of Prototype Peptidomimetic Agonists at the Human Melanocortin Receptors MC1R and MC4R. *J. Med. Chem.* **1997**, *40*, 2133-2139.
(24) Virgilio, A. A.; Ellman, J. A. Simultaneous Solid-Phase Synthesis Of β-Turn Mimetics Incorporating Side-Chain Functionality. *J. Am. Chem. Soc.* **1994**, *116*, 11580−11581.
(25) Virgilio, A. A.; Schurer, S. C.; Ellman, J. A. Expedient Solid-Phase Synthesis Of Putative β-Turn Mimetics Incorporating the $i + 1$, $i + 2$, and $i + 3$ Side Chains. *Tetrahedron Lett.* **1996**, *37*, 6961−6964.
(26) Virgilio, A. A.; Bray, A. A.; Zhang, W.; Trinh, L.; Snyder, M.; Morrissey, M. M.; Ellman, J. A. Synthesis and Evaluation Of a Library Of Peptidomimetics Based Upon the β-Turn. *Tetrahedron* **1997**, *53*, 6635−6644.
(27) Chen, W.; Shields, T. S.; Stork, P. J. S.; Cone, R. D. A Colorimetric Assay for Measuring Activation of Gs- and Gq-Coupled Signaling Pathways. *Anal. Biochem.* **1995**, *226*, 349−354.
(28) Sawyer, T. K.; Hruby, V. J.; Darman, P. S.; Hadley, M. E. [half-Cys[4],half-Cys[10]]-α-Melanocyte-Stimulating Hormone: A Cyclic α-Melanotropin Exhibiting Superagonist Biological Activity. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 1751−1755.
(29) Al-Obeidi, F. A.; O'Connor, S.; Hruby, V. J.; Pettitt, M. NMR and Quenched Molecular Dynamics Studies of Superpotent Linear and Cyclic a-Melanotropins. *J. Pept. Res* **1998**, *51*, 420−431.
(30) Lee, J. H.; Lim, S. K.; Huh, S. H.; Lee, D.; Lee, W. Solution Structures of the Melanocyte-stimulating Hormones By Two-dimensional NMR Spectroscopy and Dynamical Simulated-annealing Calculations. *Eur. J. Biochem.* **1998**, *257*, 31−40.
(31) Haskell-Luevano, C.; Sawyer, T. K.; Trumpp-Kallmeyer, S.; Bikker, J.; Humblet, C.; Gantz, I.; Hruby, V. J. Three-Dimensional Molecular Models of the hMC1R Melanocortin Receptor: Complexes with Melanotropin Peptide Agonists. *Drug Des. Discovery* **1996**, *14*, 197−211.
(32) Yang, Y.-K.; Dickinson, C.; Haskell-Luevano, C.; Gantz, I. Molecular Basis for the Interaction of [Nle,[4]DPhe[7]] Melanocyte Stimulating Hormone with the Human Moleanocortin-1 Receptor (Melanocyte α-MSH Receptor). *J. Biol. Chem.* **1997**, *272*, 23000−23010.
(33) Brandenburger, Y.; Rose, K.; Bagutti, C.; Eberle, A. N. Synthesis and Receptor Binding Analysis of Thirteen Oligomeric α-MSH Analogues. *J. Recept. Signal Transduct. Res.* **1999**, *19*, 467−80.
(34) Heizmann, G.; Hildebrand, P.; Tanner, H.; Ketterer, S.; Pansky, A.; Froidevaux, S.; Beglinger, C.; Eberle, A. N. A Combinatorial Peptoid Library for the Identification of Novel MSH and GRP/bombesin Receptor Ligands. *J. Recept. Signal Transduct. Res.* **1999**, *19*, 449−66.
(35) Souers, A. J.; Schürer, S. C.; Kwack, H. H.; Virgilio, A. A.; Ellman, J. A. Preparation of Enantioenriched alpha-bromo Acids Incorporting Diverse Functionality. *Synthesis*, in press.
(36) Virgilio, A. A. The Design, Synthesis, and Evaluation of Libraries of β-Turn Mimetics. Ph.D. Thesis, University of California, Berkeley, 1997.
(37) Houssin, R.; Bernier, J. L.; Henichart, J. P. A Convenient and General Method for the Preparation of *tert*-Butoxycarbonylaminoalkanenitriles and Their Conversion to Mono-*tert*-butoxycarbonylalkanediamines. *Synthesis* **1988**, 259−261.
(38) Rzeszotarska, B.; Masiukiewicz, E. Arginine, Histidine and Tryptophan in Peptide Synthesis. The Guanidino Function of Arginine. *Org. Prep. Proc. Int.* **1988**, *20*, 429−464.
(39) Souers, A. J.; Virgilio, A. A.; Schurer, S. S.; Ellman, J. A.; Kogan, T. P.; West, H. E.; Ankener, W.; Vanderslice, P. Novel inhibitors of alpha 4 beta 1 integrin receptor interactions through library synthesis and screening. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 2297−302.
(40) Souers, A. J.; Virgilio, A. A.; Rosenquist, Å.; Fenuik, W.; Elman, J. A. Identification of a Potent Heterocyclic Ligand to Somatostatin Receptor Subtype 5 by the Synthesis and Screening of a β-turn Mimetic Libraties. *J. Am. Chem. Soc.* **1999**, *121*, 1817−1825.
(41) Fields, G.; Noble, R. L. Solid-Phase Peptide Synthesis Utilizing 9-Fluorenymethoxycarbonyl Amino Acids. *Int. J. Pept. Protein Res.* **1990**, *35*, 161−214.

JM990190S