Characterizations of the Unusual Dissociation Properties of Melanotropin Peptides from the Melanocortin Receptor, hMC1R

Carrie Haskell-Luevano,[†] Hiroto Miwa,[‡] Chris Dickinson,[§] Mac E. Hadley,[||] Victor J. Hruby,*[,†] Tadataka Yamada,[‡,⊥] and Ira Gantz[▽]

Departments of Chemistry and Anatomy, University of Arizona, Tucson, Arizona 85721, and Departments of Internal Medicine, Pediatrics, Physiology, and Surgery, University of Michigan Medical Center, Ann Arbor, Michigan

Received June 5, 1995[⊗]

Variation in the degree of prolonged (residual) biological activity of the melanotropin peptides α-MSH (α-melanocyte-stimulating hormone, Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2) and the superpotent analogues [Nle[4],D-Phe[7]]α-MSH (MT-I) and Ac-[Nle[4],Asp[5],D-Phe[7],Lys[10]]α-MSH(4−10)-NH_2 (MT-II) has stimulated considerable interest regarding this biological phenomena. We have examined the differences in their relative dissociation rates from the melanocortin receptor, hMC1R, to try and correlate peptide dissociation rates with the observations of prolonged biological activity. Interestingly, these studies revealed that α-MSH remained 25% bound, MT-I 65% bound, and MT-II 86% bound 6 h after the ligand had been removed from the assay medium. The relative dissociation rate of MT-II was 4 times slower than that for α-MSH and 2 times slower than that for MT-I, which was 2 times slower than that for α-MSH. These data suggest that slow dissociation kinetics (hours) may contribute to the prolonged biological activities observed for both MT-I and MT-II peptides *in vitro* and *in vivo*. The prolonged binding, biological activities, and enzymatic stability of MT-I and MT-II make them putative candidates for clinical uses such as external scintigraphy for the localization of tumors (i.e., melanoma).

Introduction

α-Melanotropin (α-melanocyte-stimulating hormone, α-MSH) is a linear tridecapeptide consisting of the amino acid sequence Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2. This melanocortin hormone is derived from the preprohormone, proopiomelanocortin (POMC), and secreted from the pars intermedia of the pituitary gland of most vertebrates.[1,2] This peptide hormone is thought to possess a variety of central and peripheral biological activities, but its most recognized role is regulating skin pigmentation.[3−7] The peripheral melanocortin receptor, designated MC1, has been isolated from human melanoma[8,9] and is the G-protein-coupled receptor believed to be involved in regulating skin pigmentation.

Utilizing the classical frog (*Rana pipiens*) and lizard (*Anolis carolinensis*) skin bioassays,[10,11] extensive structure−function studies of a diverse number of melanotropin analogues have been examined to identify compounds possessing increased biological potencies and prolonged residual activity, as compared to the native hormone α-MSH. Substitution of a Nle (side chain = CH_2-CH_2-CH_2-CH_3) for Met[4] resulted in peptides possessing more potent biological activity. Upon inversion of configuration of L-Phe[7] to D-Phe[7], to give [Nle[4],D-Phe[7]]α-MSH (hereafter referred to as MT-I, Figure 1), a substantial increase in potency[12] as well as prolonged (or residual) biological activity resulted.[3,13]

The design of the cyclic lactam peptide Ac-Nle[4]-Asp[5]-His[6]-D-Phe[7]-Arg[8]-Trp[9]-Lys[10]-NH_2 (hereafter referred to as MT-II, Figure 2), based on conformational considerations and molecular dynamic simulations, resulted in potencies ca. 10 times greater than that of α-MSH,[12] in addition to possessing prolonged (residual) biological activity.[14,15] This study was undertaken to examine the relative dissociation rates of these melanocortin peptides from the human peripheral melanocortin MC1 receptor to examine ligand−receptor kinetics as a possible mechanism for residual or prolonged biological activity.

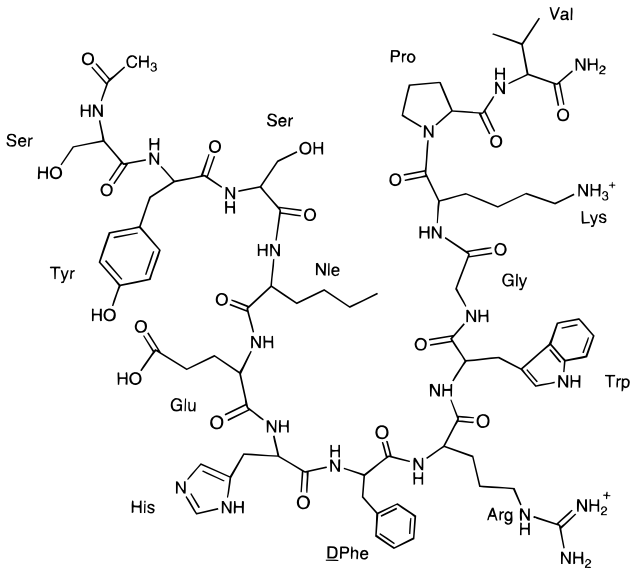

Figure 1. Structure of the peptide Ac-[Nle[4],D-Phe[7]]α-MSH-NH_2, also referred to as NDP-MSH and MT-I. This peptide differs in primary sequence from the native hormone α-MSH by Nle replacing Met at position 4 and inversion of chirality of the α-carbon of phenylalanine at position 7 to D-Phe.

* To whom reprint correspondences should be addressed.
[†] Department of Chemistry.
[‡] Department of Internal Medicine.
[§] Department of Pediatrics.
[||] Department of Anatomy.
[⊥] Department of Physiology.
[▽] Department of Surgery.
[⊗] Abstract published in *Advance ACS Abstracts,* December 15, 1995.

0022-2623/96/1839-0432$12.00/0

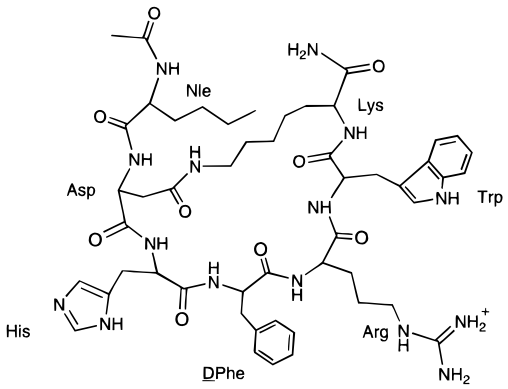

Figure 2. Structure of the superpotent monocyclic peptide Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH(4−10)-NH_2, referred to as MT-II.

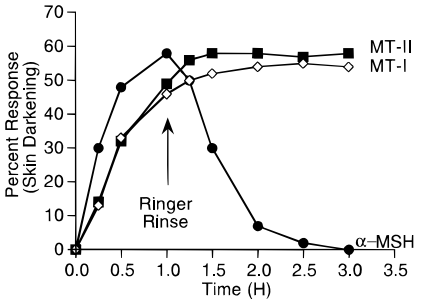

Figure 3. Prolonged activity of α-MSH, MT-I, and MT-II in the frog skin bioassay.[16] The experiment is conducted by incubating the peptide in the assay medium for 1 h and measuring the light reflectance of the assay skins. After 1 h, the peptide is removed from the assay medium, and the skins are washed with medium lacking the melanotropin and monitored over time for changes in skin darkening.

Results and Discussion

The relative potencies of these compounds in the frog[16] and lizard[14] skin bioassays, and on the human melanocortin receptor, hMC1R,[12] are summarized in Table 1. Figure 3 illustrates the prolonged biological activity observed for the peptides α-MSH, [Nle^4,D-Phe^7]α-MSH (MT-I), and Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH(4−10)-NH_2 (MT-II) in the frog skin bioassay, with similar results observed in the lizard skin bioassay.[1,14] Although the biological actions of α-MSH are rapidly reversed after transfer of the frog skins to a medium lacking α-melanotropin, the actions of MT-I and MT-II are prolonged almost indefinitely (for weeks in the case of MT-I). Since frog skin darkening results from receptor activation, signal transduction, and cyclic AMP production, it might be assumed that the prolonged actions of the MT-I and MT-II melanotropin analogues are due to irreversible receptor occupation and activation. This hypothesis is rather dramatically illustrated in Figure 4, using the lizard skin bioassay. Melanotropin receptor signal transduction (and skin darkening)

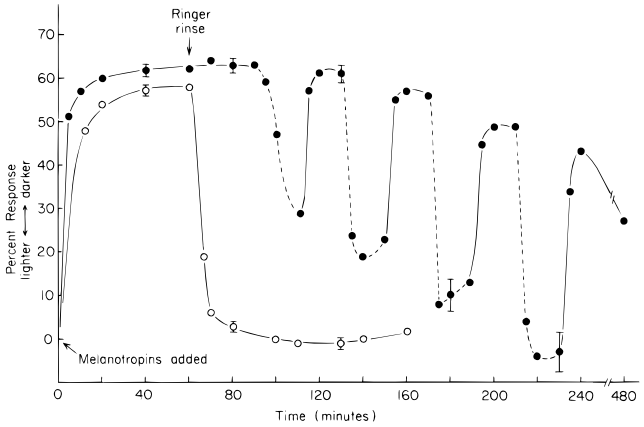

Figure 4. Illustration of the effect that Ca^{2+} plays on prolonged biological activity of (●) MT-I and the signal transduction mechanism(s) involving melanotropin peptides in general. Once Ca^{2+} (1 mM) is removed (− − −) from the assay medium, the signal transduction pathway is disrupted, as demonstrated by lightening of the assay skins. However, when Ca^{2+} is returned (—) to the assay medium, the signal transduction process is resumed, as demonstrated by darkening of the assay skins. α-MSH (○) is included to illustrate the lack of prolonged biological activity in this experiment. These studies support the hypothesis that MT-I is associated with the receptor over an extended period of time.

can be interrupted by intermittent removal of Ca^{2+} (1 mM) from the incubation medium (Figure 4). When Ca^{2+} is returned to the assay medium, the signal transduction process is resumed, as demonstrated by skin darkening. This supports our hypothesis that the ligand remains bound to the receptor in order for continued signal transduction. In the case of α-MSH, where no prolonged biological activity is observed, removal of Ca^{2+} and its return to the assay medium do not produce darkening of the skin. Although it is probable that MT-I is irreversibly bound to the melanocortin receptor, these physiological bioassays are limited in examining the dissociation kinetics of these peptides from the melanocortin receptor specifically.

The dissociation properties of α-MSH, MT-I, and MT-II from the human melanocortin receptor, hMC1R, are plotted (Figure 5) according to the first-order equation[17]

$$\ln [LR]/[LR]_e = -k_{-1}t \qquad (1)$$

where [LR] is the concentration of the ligand−receptor complex at time t and $[LR]_e$ is the concentration found at the 6 h time point. A linear plot of the peptide bound (percent of specific binding) versus time is obtained, and the apparent dissociation rate, k_{-1} was determined from the $t_{1/2}$ value, using eq 2.

$$k_{-1} = (\ln 1/2)/t_{1/2} \qquad (2)$$

The values obtained from this study (Figure 5) are summarized in Table 2. Because of very slow dissocia-

Table 1. Previously Reported Relative Potencies of These α-Melanotropin Peptides

peptide	structure	peptide activity (relative potency) frog	lizard	binding[c]	cAMP[c]
α-MSH	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	1.0	1.0	1.0	1.0
MT-I	Ac-Ser-Tyr-Ser-Nle-Glu-His-D-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	60.0[a]	5.0[a]	5.4	4.0
MT-II	Ac-Nle-Asp-His-D-Phe-Arg-Trp-Lys-NH_2	0.8[b]	10.0[b]	11.4	10.0

[a] Relative potencies reported from Sawyer et al.[1] [b] Relative potencies reported from Al-Obeidi et al.[14] [c] Relative potencies were calculated based on the IC_{50} and EC_{50} values relative to α-MSH.[12]

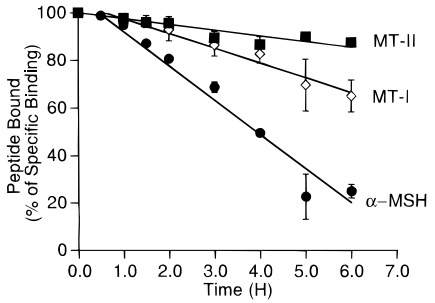

Figure 5. Percentage of α-MSH, MT-I, and MT-II remaining bound to the melanocortin receptor, hMC1R, over time. The peptide is incubated for a minimum of 1 h and removed from the assay medium, the cells are washed, and radioligand is added to the medium and monitored for a duration of 6 h to determine dissociation properties of these peptides on the human MC1 receptor believed to be involved in skin pigmentation ($n = 4$).

Table 2. Observed Dissociation Characteristics of Melanotropin Peptides on hMC1 Receptor over a Time Period of 6 H

peptide	extrapolated[a] $t_{1/2}$ value (h)	apparent[b] k_{-1} (h^{-1})	relative[c] dissociation rate
α-MSH	4.00	0.17	1.0
MT-I	8.50	0.08	0.50
MT-II	19.5	0.04	0.24

[a] Determined by extrapolation of the slope until 50% of the peptide was bound relative to the specific binding, Figure 5. [b] Dissociation rate calculated from the first-order equation (ln 1/2)/$t_{1/2} = k_{-1}$, using the $t_{1/2}$ value listed. [c] The relative rates were determined by setting α-MSH equal to 1, and dividing the apparent k_{-1} by the α-MSH value.

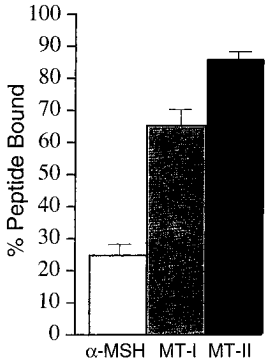

Figure 6. Percentage of α-MSH, MT-I, and MT-II remaining bound to the hMC1 receptor after being removed from the assay medium for 6 h.

tion rates, the $t_{1/2}$ values for MT-I and MT-II were determined by extrapolation using the slopes obtained from Figure 5.

Examination of the residual binding of the peptides 6 h after they were removed from the assay medium revealed that α-MSH was only 25% bound, whereas MT-I was ca. 65% bound, and MT-II was observed to be a remarkable 86% bound (Figure 6) at that time. These results compare with previously reported results in the frog skin bioassay (Figure 3), where MT-I and MT-II possess superprolonged residual activity. α-MSH, however, was previously observed to possess little or no residual activity. However, the interaction of α-MSH with hMC1R demonstrates some prolonged activity (Table 2, Figures 5 and 6). This may be accounted for evolutionarily when considering that chameleons, and frogs to a lesser extent, are able to rapidly induce pigment dispersion (skin darkening), while humans require an extended time period to induce melanosome

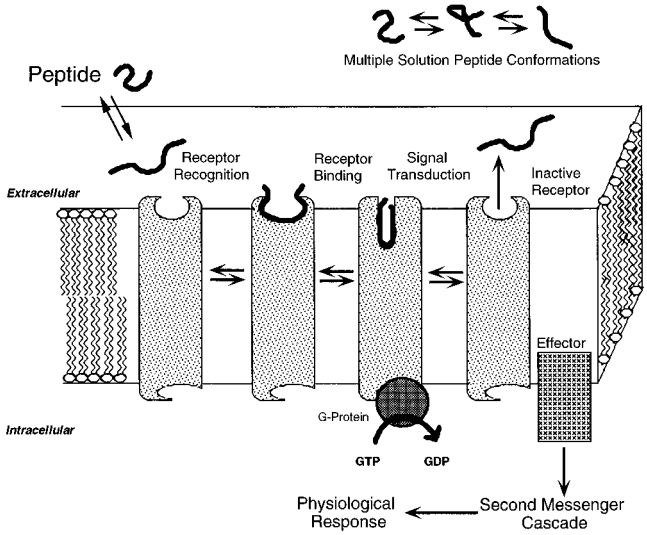

Figure 7. Illustration of the general mechanism by which peptide hormones interact with their receptors to transduce a signal into the intracellular region of cells. Several receptor states are illustrated, in addition to alternative conformational states of a linear peptide interacting with its representative receptor.

dispersion (typically UV induced), thus exemplifying species differences between humans, amphibians, and reptiles.

Figure 7 identifies the various receptor and peptide states that are believed to be occurring throughout the signal transduction pathway. Although a two-state model of receptor conformations can be proposed as the simplest case,[18] it is commonly believed that multiple receptor states exist and, in addition, most small peptide hormones and neurotransmitters possess a variety of conformations. The hypothesis of a "π state" has been previously proposed by our laboratories for the prolonged action of melanotropin peptides.[19,20] This hypothesis suggests that prolongation may result from peptide−receptor interactions. Since the dissociation constant is related to the ligand off rate, strong binding interactions, which can be attributed to highly potent analogues, might lead to slow off rates. This is the first study of dissociation kinetics of melanotropin peptides from the human melanocortin receptor, hMC1R. The results presented here demonstrate extremely slow dissociation rates (Table 2) for the superpotent melanotropin analogues and provide experimental evidence supporting the hypothesis of slow peptide−receptor dissociation rates. This study further provides evidence that prolonged biological activities can be incorporated into the design of peptide analogues which are desired to maintain a long duration of binding and biological action.

Conclusions

Prolonged, or residual, biological activity is a phenomenon that is desired for compounds considered for clinical applications. Peptides are notorious for their short *in vivo* and *in vitro* half-lives which may be attributed to multiple physiological degradation mechanisms. Peptides that are able to possess biological activity over extended periods of time (hours) are highly desirable and rarely reported in the literature. Multiple research endeavors are being pursued to design peptides maintaining prolonged action, in addition to isolating the mechanism(s) that sustain these physiological ac-

tivities. We report here peptides that exhibit prolonged activity (Figure 3), which correlates with their long dissociation rates from the cloned human receptor. Both MT-I and MT-II have been found to be stable to proteolytic enzymes such as α-chymotrypsin, trypsin, and pepsin.[21] Therefore, their residual activity may be related to improved circulating half-life, but in addition, the dissociation study reported here clearly indicates that the long dissociation half-life (hours) on the human receptor, hMC1R, also may be a contributing factor to the exceptional prolonged, or residual, biological activity that these compounds possess.

Experimental Section

Peptide Synthesis. Ac-Nle-Asp-His-D-Phe-Arg-Trp-Lys-NH_2 was synthesized by solid phase synthetic methods, as previously described.[14,22] The properties and purity of this peptide were assessed by FAB-MS, RP-HPLC, TLC in three solvent systems, optical rotation, and amino acid analysis and are in agreement with previously published values (data not shown). The other melanotropins, α-MSH and [Nle^4,D-Phe^7]α-MSH, were purchased from Peninsula Laboratories, Inc. (Belmont, CA).

Frog and Lizard Skin Assays. The frog (*R. pipiens*) and lizard (*A. carolinensis*) skin bioassays were utilized to determine the relative potencies of the synthetic melanotropins.[10,11,14] The assays measure the amount of light reflected from the surface of the skins *in vitro*. In response to melanotropic peptides, melanosomes within integumental melanocytes migrate from a perinuclear position into the dendritic processes of the pigment cells. This centrifugal organellar dispersion results in a change in color (darkening) of the skins which is measured by a Photovolt reflectometer and expressed as the percent response compared to the initial (time zero) reflectance value. Subsequent removal of a melanotropin such as α-MSH usually results in a rapid perinuclear (centripetal) reaggregation of melanosomes within melanocytes leading to a lightening of the skin back to their original (base) value.

The experiments examining the effect of Ca^{2+} were performed on a group of lizard (*A. carolinensis*) skins. At time zero, MT-I (nM) was added to the skins ($n = 6$). At 60 min, the skins were transferred to Ringer solution in the absence of any melanotropins. At subsequent time periods the skins were alternatively transferred to Ringer solution in the absence (dahsed) and presence (solid line) of calcium ion (1 mM).

Dissociation Assays. L-Cells transfected with the human MC1 receptors were grown to confluence in 12-well (2.4 × 1.7 cm) tissue culture plates.[12] The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO) containing 4.5 g/100 mL glucose, 10% fetal calf serum, 100 units/mL penicillin and streptomycin, 1 mM sodium pyruvate, and 1 mg/mL geneticin. For the assays, the medium was removed and cells were washed twice with a freshly prepared binding buffer consisting of 97% minimum essential medium with Earle's salt (MEM; GIBCO), 2.5% HEPES (pH 7.4), 0.2% bovine serum albumin, 0.1% 1,10-phenanthrolone, 0.5 mg/L leupeptin, and 200 mg/L bacitracin. A 10^{-6} M concentration of the peptide being tested was added to the well, preincubated for at least 1 h, and removed from the assay medium at the designated time, and the cells were washed with binding buffer. Subsequently, a 100 μL solution of [^{125}I][Tyr^2,-Nle^4,D-Phe^7]α-MSH (100 000 cpm/well) was added to each well and incubated at 37 °C for up to 6 h. Upon completion of the incubation period, the medium was subsequently removed and each well was washed twice with binding buffer. The cells were lysed by the addition of 0.5 mL 0.1 M NaOH and 0.5 mL of 1% Triton X-100. The mixture was left to react for 5 min and the contents of each well transferred to labeled 16 × 150 mm glass tubes and quantified in a γ-counter. [^{125}I][Tyr^2,-Nle^4,D-Phe^7]α-MSH was prepared and purified by methods described previously.[23]

Acknowledgment. This work was supported by a grant from the U.S. Public Health Service, DK17420 and CA 27502 (V.J.H.). Dr. Gantz is a recipient of a Veterans Administration Research Associate Award, and Dr. Miwa is a recipient of a grant from Sasakawa Medical Research Foundation.

References

(1) 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.
(2) Hadley, M. E. *Endocrinology*, 3rd ed.; Prentice Hall: Englewood Cliffs, NJ, 1992; pp 179−207.
(3) Lerner, A. B.; McGuire, J. S. Effect of Alpha- and Beta-Melanocyte Stimulating Hormones on the Skin Colour of Man. *Nature* **1961**, *189*, 176−179.
(4) Lerner, A. B.; McGuire, J. S. Melanocyte-Stimulating Hormone and Adrenocorticotrophic Hormone. Their Relation to Pigmentation. *N. Engl. J. Med.* **1964**, *270*, 539−546.
(5) Eberle, A. N. *The Melanotropins: Chemistry, Physiology and Mechanisms of Action*; Karger: Basel, 1988.
(6) Hadley, M. E. *The Melanotropic Peptides: Source, Synthesis, Chemistry, Secretion and Metabolism*; CRC Press: Boca Raton, FL, 1989; Vols. I−III.
(7) Vaudry, H.; Eberle, A. N. *The Melanotropic Peptides*; Annals of the New York Academy of Sciences: New York, 1993; Vol. 680.
(8) Chhajlani, V.; Wikberg, J. E. S. Molecular Cloning and Expression of the Human Melanocyte Stimulating Hormone Receptor cDNA. *FEBS Lett.* **1992**, *309*, 417−420.
(9) 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.
(10) Shizume, K.; Lerner, A. B.; Fitzpatrick, T. B. In Vitro Bioassay for the Melanocyte Stimulating Hormone. *Endocrinology* **1954**, *54*, 533−560.
(11) Wright, R. M.; Lerner, A. B. On the Movement of Pigment Granules in Frog Melanocytes. *Endocrinology* **1960**, *66*, 599−609.
(12) Haskell-Luevano, C.; Miwa, H.; Dickinson, C.; Hruby, V. J.; Yamada, T.; Gantz, I. Binding and cAMP Studies of Melanotropin Peptides with the Cloned Human Peripheral Melanocortin Receptor, hMC1R. *Biochem. Biophys. Res. Commun.* **1994**, *204*, 1137−1142.
(13) Hadley, M. E.; Anderson, B.; Heward, C. B.; Sawyer, T. K.; Hruby, V. J. Calcium-Dependent Proloned Effects on Melanophores of [4-Norleucine, 7-D-Phenylalanine]-α-Melanotropin. *Science* **1981**, *213*, 1025−1027.
(14) 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.
(15) 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 Simulations. *J. Am. Chem. Soc.* **1989**, *111*, 3413−3416.
(16) Sawyer, T. K.; Hruby, V. J.; Wilkes, B. C.; Draelos, M. T.; Hadley, M. E. Comparative Biological Activities of Highly Potent Active-Site Analogues of α-Melanotropin. *J. Med. Chem.* **1982**, *25*, 1022−1027.
(17) Fields, J. Z.; Roeske, W. R.; Morkin, E.; Yamamura, H. I. Cardiac Muscarinic Cholinergic Receptors. *J. Biol. Chem.* **1978**, *253*, 3251−3258.
(18) Bond, R. A.; Leff, P.; Johnson, T. D.; Milano, C. A.; Rockman, H. A.; McMinn, T. R.; Apparsundaram, S.; Hyek, M. F.; Kenakin, T. P.; Allen, L. F.; Lefkowitz, R. J. Physiological Effects of Inverse Agonists in Transgenic Mice with Myocardial Overexpression of the β_2-Adrenoceptor. *Nature* **1995**, *374*, 272−275.
(19) Hruby, V. J.; Cody, W. L.; Wilkes, B. C.; Hadley, M. E. Design and Synthesis of Peptide Hormones with Prolonged Activity at Specific Receptors. In *Peptides 1984*; Ragnarsson, U., Ed.; Almqvist & Wiksell: Stockholm, Sweden, 1984; pp 505−508.
(20) Hruby, V. J.; Hadley, M. E. Binding and Information Transfer in Conformationally Restricted Peptides. In *Design and Synthesis of Organic Molecules Based on Molecular Recognition*; van Binst, G., Ed.; Springer-Verlag: New York, 1986; pp 269−289.
(21) Hadley, M. E.; Marwan, M. M.; Al-Obeidi, F.; Hruby, V. J.; de Lauro Castrucci, A. M. Linear and Cyclic α-Melanotropin [4−10] Fragment Analogues That Exhibit Superpotency and Residual Activity. *Pigm. Cell Res.* **1989**, *2*, 478−484.
(22) Hruby, V. J.; Al-Obeidi, F.; Sanderson, D. G.; Smith, D. D. Synthesis of Cyclic peptides by Solid Phase Methods. In *Innovations and Perspectives in Solid Phase Synthesis*; Epton, R. Ed.; SPCC (U.K.) Ltd.: Oxford, England, 1989; pp 197−203.
(23) Tatro, J. B.; Reichlin, S. Specific Receptors for α-Melanocyte-Stimulating Hormone are Widely Distributed in Tissues of Rodents. *Endocrinology* **1987**, *121*, 1900−1907.

JM950407S