Novel 3D Pharmacophore of α-MSH/γ-MSH Hybrids Leads to Selective Human MC1R and MC3R Analogues†

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To further evaluate elements that could contribute to the 3D topographical structure of γ-MSH, we have systematically designed a group of linear γ-MSH analogues and evaluated their biological activities: without a N-terminal acetyl, with and without a C-terminal amide, with Nle^3, with L- or D-Phe^6 or D-Nal(2′)^6, and with D-Trp^8 or D-Nal(2′)^8. It was found that changing the C-terminal acid in γ-MSH to an amide and replacing Met with Nle leads to increased binding affinities at all four subtypes of melanocortin receptors (10−100 fold). Substitution of Trp^8 with D-Nal(2′)^8 and Phe^6 with D-Phe^6 in γ-MSH-NH_2 forms a selective antagonist for the hMC3R, whereas, substitution of Phe^6 with D-Nal(2′)^6 and replacing Trp^8 with D-Trp^8 at γ-MSH-NH_2 yields a selective partial agonist for the hMC1R. Finally, substitution of His^5 with Pro^5 and Trp^8 with D-Nal(2′)^8 in γ-MSH-NH_2 leads to a highly potent and selective agonist for the hMC1R. Molecular modeling showed that, at the C-terminal of Nle^3-γ-MSH-NH_2, there is a reverse-turn-like structure, suggesting that there might be a secondary binding site involved in ligand−receptor interaction for γ-MSH analogues that may explain the enhanced binding affinities of the Nle^3-γ-MSH-NH_2 analogues. Our results indicate that increasing the hydrophobicity and replacing Phe^6 and Trp^8 with bulkier aromatic amino acid residues is very important for selectivity of α-MSH/γ-MSH hybrids for hMCRs.

Introduction

α-Melanocyte-stimulating hormone (α-melanotropin, α-MSH, Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2) and γ-melanocyte-stimulating hormone (γ-melanotropin, γ-MSH, H-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH) belong to the family of melanotropin peptides derived by post-translational processing of the pro-opiomelanocortin (POMC) gene. It has been discovered that the melanocortin receptors (hMCR) and their ligands control a surprisingly large number of multifaceted biological actions including skin pigmentation,[1] erectile function,[2−5] blood pressure and heart rate,[6] control of feeding behavior,[7−10] and many others.[11−15] To date, five melanocortin receptor subtypes with different patterns of tissue expression in the brain and in the periphery have been cloned and characterized.[11,16−17] The hMC1R is mainly expressed in melanocytes and leukocytes and has been implicated to play a role in skin pigmentation, inflammatory responses, and control of the immune system.[13−15] The hMC2R is expressed in the adrenal cortex and mediates glucocorticoneogenesis,[12] the hMC3R is mainly distributed in central nervous system,[17] and the hMC4R is expressed in virtually all brain regions, including the cortex thalamus, hypothalamus, brainstem, and spinal cord.[11,17] The distribution of the hMC4R in the central nervous system is both different and wider than that of the hMC3R.[17] Both the hMC3R and the hMC4R have been implicated to play complimentary roles in weight control.[10,18,19] Finally, the hMC5R, found in a variety of peripheral tissues, plays a role in regulating exocrine gland function.[20] Recent studies have shown[7,21,22] that highly selective and potent agonists at the hMC4R might find therapeutic applications for treating obesity. The hMC3R also is physiologically involved in the control of energy partitioning.[22] Therefore, selective ligands at this receptor in conjunction with a hMC4R agonist might provide a novel approach in the treatment of obesity and related disorders. Thus, investigating the interactions of melanocortin receptors with designed ligands will help develop rational drug design for these receptors. The availability of cloned melanocortin receptor cell lines allowed us to use high throughput screening and thus determine receptor selectivity for ligands.

While most of the research on the melanotropins has concentrated on α-MSH, the most potent of the three melanotropins in nature, little is known of γ-MSH in terms of its structure−activity relationships (SAR). The initial SAR work from our group was an L-alanine scan,[23] a D-amino acid scan,[24] and a D-Nal(2′) scan.[25] These studies led to a few selective analogues for the human melanocortin receptors (hMCRs). As an extensive study, to understand the importance of each residue in its sequence and their topographical requirements for biological activity, we have further systematically investigated modified structures of γ-MSH (analogue **11**), NDP-α-MSH (analogue **15**),[26] Ac-NDP-γ-MSH-NH_2 (analogue **13**), and the C-terminal amide of γ-MSH (γ-MSH-NH_2, analogue **1**). As shown earlier, all melanotropins are characterized by the core or central tetrapeptide sequence, His-Phe-Arg-Trp, flanked by N- and C-terminal residues. This core sequence is the minimal fragment essential for biological activity and is believed to form the pharmacophore.[27−33] The bioactive confor-

† Dedicated to the memory and legacy of Dr. Paul A. J. Janssen, who was an inspiration for his multidisciplinary approach to drug discovery.

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Table 1. Sequence of α-MSH/γ-MSH Hybrid Peptides

no.	peptide sequence
1	H-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-NH_2
2	H-Tyr-Val-Nle-Gly-Pro-Phe-Arg-D-Nal(2′)-Asp-Arg-Phe-Gly-NH_2
3	H-Tyr-Val-Nle-Gly-Pro-D-Nal(2′)-Arg-D-Nal-Asp-Arg-Phe-Gly-NH_2
4	H-Tyr-Val-Nle-Gly-His-D-Phe-Arg-D-Nal(2′)-Asp-Arg-Phe-Gly-NH_2
5	H-Tyr-Val-Nle-Gly-His-D-Phe-Arg-D-Trp-Asp-Arg-Phe-Gly-NH_2
6	H-Tyr-Val-Nle-Gly-His-D-Nal(2′)-Arg-D-Trp-Asp-Arg-Phe-Gly-NH_2
7	H-Tyr-Val-Nle-Gly-His-D-Nal(2′)-Arg-D-Phe-Asp-Arg-Phe-Gly-NH_2
8	H-Tyr-Val-Nle-Gly-D-Nal(2′)-Phe-Arg-Trp-Asp-Arg-Phe-Gly-NH_2
9, Nle^3 γ-MSH	H-Tyr-Val-Nle-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH
10, Nle^3 γ-MSH-NH_2	H-Tyr-Val-Nle-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-NH_2
11, γ-MSH	H-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH
12, D-Trp^8-γ-MSH	H-Tyr-Val-Met-Gly-His-Phe-Arg-D-Trp-Asp-Arg-Phe-Gly-OH
13, Ac-NDP-γ-MSH-NH_2	Ac-Tyr-Val-Nle-Gly-His-D-Phe-Arg-Trp-Asp-Arg-Phe-Gly-NH_2
14, α-MSH	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2
15, NDP-α-MSH	Ac-Ser-Tyr-Ser-Nle-Glu-His-D-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2

Table 2. Physicochemical Properties of the α-MSH/γ-MSH Hybrid Peptides

		m/*z* (M + 1)		HPLC[a]		TLC[b]	
no.	sequence	calcd	obsd	system 1	system 2	system 1	system 2
1	H-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-NH_2	1569.8	1569.9	14.6	19.5	0.54	0.06
2	H-Tyr-Val-Nle-Gly-Pro-Phe-Arg-D-Nal(2′)-Asp-Arg-Phe-Gly-NH_2	1522.8	1522.9	16.7	23.1	0.71	0.25
3	H-Tyr-Val-Nle-Gly-Pro-D-Nal(2′)-Arg-D-Nal(2′)-Asp-Arg-Phe-Gly-NH_2	1572.0	1572.8	17.5	23.9	0.77	0.36
4	H-Tyr-Val-Nle-Gly-His-D-Phe-Arg-D-Nal(2′)-Asp-Arg-Phe-Gly-NH_2	1562.8	1562.2	14.9	19.6	0.64	0.21
5	H-Tyr-Val-Nle-Gly-His-D-Phe-Arg-D-Trp-Asp-Arg-Phe-Gly-NH_2	1551.8	1551.9	14.1	18.0	0.57	0.16
6	H-Tyr-Val-Nle-Gly-His-D-Nal(2′)-Arg-D-Trp-Asp-Arg-Phe-Gly-NH_2	1601.8	1601.9	14.9	19.6	0.62	0.23
7	H-Tyr-Val-Nle-Gly-His-D-Nal(2′)-Arg-D-Phe-Asp-Arg-Phe-Gly-NH_2	1562.8	1562.8	14.8	19.6	0.64	0.21
8	H-Tyr-Val-Nle-Gly-D-Nal(2′)-Phe-Arg-Trp-Asp-Arg-Phe-Gly-NH_2	1611.8	1611.9	18.6	25.9	0.76	0.37
9	H-Tyr-Val-Nle-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH	1552.7	1552.9	15.1	20.4	0.53	0.15
10	H-Tyr-Val-Nle-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-NH_2	1551.8	1551.9	15.0	20.3	0.56	0.06
11	H-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH	1570.7	1570.9	14.9	19.8	0.49	0.06
13	Ac-Tyr-Val-Nle-Gly-His-D-Phe-Arg-Trp-Asp-Arg-Phe-Gly-NH_2	1593.8	1593.9	15.9	21.6	0.62	0.33

[a] HPLC column, YMC-Pack ODS-AM 150 × 4.6 mm, S-3 μm, 120A. HPLC system 1: solvent A, 0.1% TFA in water; solvent B, 0.08% TFA in acetonitrile; gradient, 2−80% B in A over 30 min, flow rate 0.8 mL/min. HPLC system 2: solvent A, 1% formic acid in water; solvent B, 1% formic acid in methanol; gradient, 2−80% B in A over 30 min, flow rate 0.8 mL/min. [b] TLC system 1, *n*-butanol/acetic acid/water/pyridine (4:1:2:1); TLC system 2, *n*-butanol/acetic acid/water (4:1:1).

Table 3. Binding Affinities and cAMP Activities of α-MSH/γ-MSH Hybrid Ligands at hMCRs[a]

	hMC1R			hMC3R			hMC4R			hMC5R		
no.	IC_{50}, nM	EC_{50}, nM	% max effect	IC_{50}, nM	EC_{50}, nM	% max effect	IC_{50}, nM	EC_{50}, nM	% max effect	IC_{50}, nM	EC_{50}, nM	% max effect
1	1.2 ± 0.2	70 ± 10	100	40 ± 10	520 ± 50	38	45 ± 5	65 ± 12	76	330 ± 57	390 ± 25	73
2	20 ± 2	55 ± 10	120	32 ± 3	13 ± 2	28	350 ± 50	2.6 ± 0.3	14	650 ± 98	250 ± 20	46
3	2 ± 0.2	2.0 ± 0.3	60	3.0 ± 0.3	11 ± 1	39	4.0 ± 0.6	3.3 ± 0.4	8	2.0 ± 0.2	NA	−
4	0.7 ± 0.1	2.0 ± 0.2	87	6.0 ± 0.5	NA	−	6.0 ± 1	36 ± 4	100	5.0 ± 1	200 ± 20	67
5	1 ± 0.1	8.0 ± 1	63	25 ± 0.3	140 ± 5	60	17 ± 2	29 ± 3	130	11 ± 1.7	410 ± 50	88
6	0.30 ± 0.02	3.0 ± 0.2	70	5.0 ± 0.6	300 ± 6	6	6.0 ± 1	630 ± 70	18	3.5 ± 0.5	NA	6
7	0.16 ± 0.01	1.5 ± 0.2	24	6.0 ± 0.7	951 ± 11	5	20 ± 2	2700 ± 300	16	3.3 ± 0.4	490 ± 50	−
8	−	−	−	3000 ± 400	>10000	−	1990 ± 200	> 10000	10	1000 ± 100	870 ± 90	19
9	60 ± 5	80 ± 7	100	62 ± 8	1000 ± 100	100	45 ± 5	70 ± 7	100	636 ± 100	82 ± 8	100
10	−	−	−	45 ± 7	1.6 ± 0.2	100	64 ± 7	34 ± 4	105	200 ± 23	99 ± 10	114
11	1000 ± 20	300 ± 30	100	71 ± 10	700 ± 89	100	760 ± 80	710 ± 70	100	2200 ± 200	550 ± 60	100
12	−	−	−	6.7 ± 1	0.33 ± 0.01	100	600 ± 70	100 ± 11	99	340 ± 40	82 ± 10	97
13	0.5 ± 0.01	1.5 ± 0.1	100	2. ± 0.02	2. ± 0.2	100	1.2 ± 0.2	1.4 ± 0.1	100	2.4 ± 0.3	1.9 ± 0.2	100
14	0.4 ± 0.01	0.7 ± 0.01	100	30 ± 3.9	6.7 ± 1	100	5 ± 1	2.1 ± 0.6	100	18 ± 2	8.1 ± 1.5	100
15	−	−	100	3.3 ± 0.3	0.8 ± 0.1	100	0.4 ± 0.02	0.2 ± 0.04	100	2.2 ± 0.5	1 ± 0.3	100

[a] IC_{50} = concentration of peptide at 50% specific binding ($N = 4$). EC_{50} = effective concentration of peptide that was able to generate 50% maximal intracellular cAMP accumulation ($N = 4$). The peptides were tested at a range of concentration from 10^{-10} to 10^{-5} M. NA, no activity.

mation of α-MSH was suggested to exist as a reverse-turn-type conformation by this group.[26,34]

Results and Discussion

In our efforts to produce highly receptor selective agonists and antagonists for the hMC1R, hMC3R, hMC4R, and hMC5R, we have designed and synthesized (see Experimental Section) a series of hybrid α-/γ-MSH analogues (Table 1). The purified peptides were evaluated by MS, HPLC, and TLC in three different solvent systems (Table 2). Their binding affinities and biological activities were determined at the hMC1R, hMC3R, hMC4R, and hMC5R (Table 3).

In vitro biological activities (Table 3) showed that for γ-MSH (analogue **11**) the IC_{50} values at the hMC3R, hMC4R, and hMC5R are 71, 760, and 2200 nM, respectively, and for γ-MSH-NH_2 (analogue **1**), the IC_{50} values at the hMC3R, hMC4R, and hMC5R are 40, 45, 330 nM, respectively. Clearly a 10-fold increase in binding affinity was observed for the hMC4R as well as the

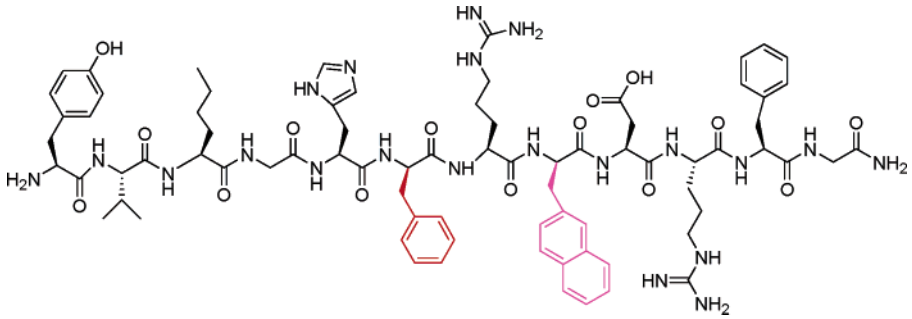

Figure 1. Primary structure of peptide **4**, a selective antagonist for the hMC3R.

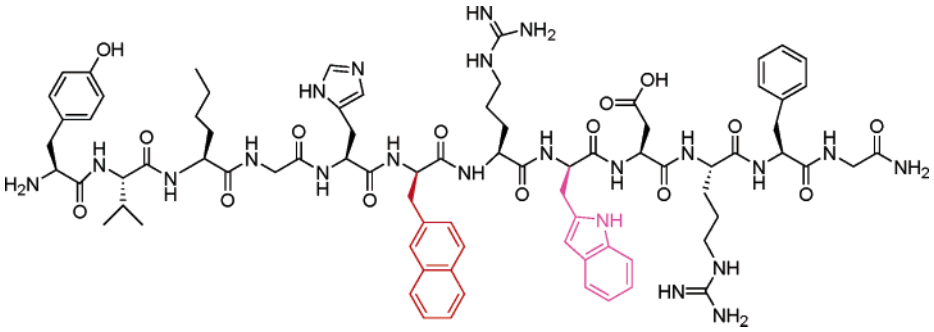

Figure 2. Primary structure of peptide **6**, a selective agonist for the hMC1R.

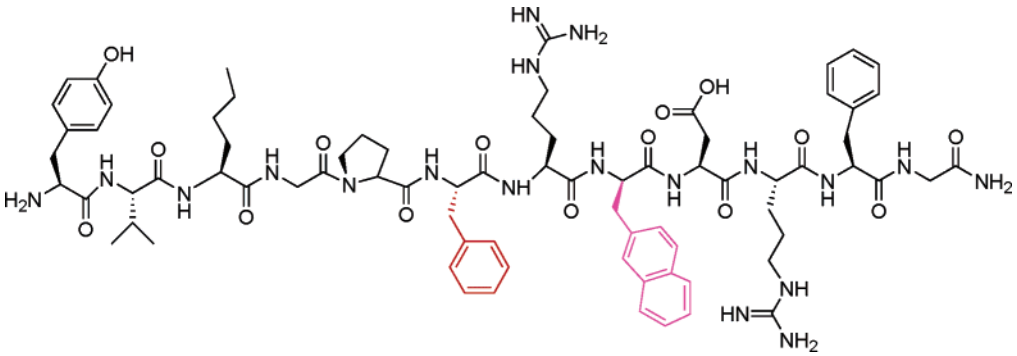

Figure 3. Primary structure of peptide **2**, a selective agonist for the hMC1R.

hMC5R. Similar biological activity comparisons were made for γ-MSH vs Nle^3-γ-MSH (analogue **9**) and Nle^3-γ-MSH-NH$_2$ (analogue **10**) (Table 3). These studies suggest that the C-terminal amide plays a critical role in increasing the ligand−receptor interactions at hMC4R and hMC5R. One possible reason could be that the C-terminal amide aids the peptide in anchoring to the cell membrane because of the net change in the dipole moment. The functional assays showed that Nle^3-γ-MSH increased cyclic AMP activities by 250-fold for the hMC3R and 2−3-fold for the hMC4R, when compared to γ-MSH-NH$_2$. The same observations were made for Nle^3-γ-MSH-NH$_2$ vs γ-MSH-NH$_2$. These data suggest that the Nle^3 residue increases selectivity for melanocortin receptors, especially for the hMC3R. It was generally observed previously (unpublished results from our laboratories) that N-terminal acetyl has a small influence on binding and functional activity for each subtype of human melanocortin receptors and no significant influence on selectivity. In addition, when comparing Ac-NDP-γ-MSH-NH$_2$ with NDP-α-MSH, we found that both Ac-NDP-γ-MSH-NH$_2$ and NDP-α-MSH show the highest binding affinities and cAMP activities among the compounds under study.

In this group of hybrid melanotropin peptides, analogue **4** (Figure 1) was found to be a hMC3R selective antagonist (IC_{50} = 6 nM). In this analogue the sterically bulky D-Nal(2′)^8 was substituted for D-Trp^8, which converts a hMC3R selective agonist[24] to a hMC3R selective antagonist. Interestingly, analogue **6** (Figure 2) is a selective hMC1R partial agonist (EC_{50} = 3 nM). It appears that increasing the hydrophobicity by replacing Phe^6 and Trp^8 with D-Nal(2′)^6 and D-Trp^8 (analogue **6**), respectively, in the parent analogue **10** (Nle^3-γ-MSH-NH$_2$) leads to a very selective partial agonist with hMC1R selectivity (maximum cAMP activity at hMC1R is 70% vs 6%, 18%, and 6% at the hMC3R, hMC4R, and hMC5R, respectively). Interestingly, analogue **2** (Figure 3) is a potent full agonist at the hMC1R [maximum cAMP activity 120% at the hMC1R compared with 100% for MT-II and 28%, 14%, and 46% (partial agonist activities) at the hMC3R, hMC4R, and hMC5R, respectively]. In this case, Trp^8 was substituted with D-Nal(2′)^8 and His^5 with Pro^5 in γ-MSH-NH$_2$ (analogue **1**). Thus, increasing the hydrophobic environment leads to a more potent hMC1R agonist (analogue **2**) versus analogue **6**, which is a less potent partial agonist at the hMC1R. Modeling-guided synthesis of Ac-NDP-γ-MSH-NH$_2$ pro-

Table 4. Backbone Torsion Angles (deg) for the Low-Energy Conformations for MT-II, SHU-9119, PG-901, and α-MSH/γ-MSH Hybrid Peptides Based on LLMOD/OPLS-AA Calculations

	Nle^3		Asp/Gly^4		His/Pro^5		D-Phe/D-Nal^6		Arg^7		Trp^8		Lys/Asp^9	
code	Φ	Ψ	Φ	Ψ	Φ	Ψ	Φ	Ψ	Φ	Ψ	Φ	Ψ	Φ	Ψ
MT-II	−94	63	54	66	−90	145	70	35	−77	154	−136	77	−120	49
SHU-9119	−145	178	−80	97	−123	148	70	61	−80	56	−93	83	−154	169
PG-901	−143	176	−73	141	−72	−10	93	37	−87	179	−75	128	−136	49
α-MSH	−84	148	−66	−51	−90	72	−65	109	48	67	−151	161	−94	70
NDP-α-MSH	−90	115	−135	167	73	−52	113	−56	−82	80	−71	150	179	53
γ-MSH	−79	158	−94	58	−158	157	−147	145	−140	137	−111	144	−146	164
Ac-γ-MSH-NH_2	−61	121	129	−55	−83	−46	−83	157	−115	103	−82	91	−85	71
Nle^3-γ-MSH-NH_2	−135	170	−178	180	−148	172	−82	150	−145	142	−118	146	−143	156
Ac-NDP-γ-MSH-NH_2	−86	65	143	−170	−85	156	136	−164	−108	132	−88	85	−85	72
4	−93	161	−84	52	−171	−167	73	−156	−92	−53	65	39	−107	−77
6	−131	166	−176	−175	−79	138	52	−107	−130	37	112	−44	−118	175

Table 5. Side Chain Torsion Angles (deg) of Low-Energy Conformations for MT-II, SHU-9119, PG-901, and the α-MSH/γ-MSH Hybrid Peptides Based on LLMOD/OPLS-AA Calculations

	His/Pro^5			D-Phe/D-Nal^6			Arg^7			Trp^8	
code	χ^1	$\chi^{2.3.1}$	$\chi^{2.3.2}$	χ^1	$\chi^{2.3.1}$	$\chi^{2.3.2}$	χ^1	χ^2	χ^1	$\chi^{2.3.1}$	$\chi^{2.3.2}$
MT-II	−67	178	−2	61	−104	75	54	170	−173	78	−103
SHU-9119	−170	−94	84	−58	−101	75	−62	176	−172	79	−102
PG-901	29	−32	–	58	−132	48	59	158	−175	91	−89
α-MSH	26	−179	4	−166	−80	102	−69	−171	−172	49	−129
NDP-α-MSH	−166	−135	47	63	−95	85	−174	177	−172	87	−92
γ-MSH	176	69	−111	179	−103	75	177	175	−178	94	−85
Ac-γ-MSH-NH_2	−162	−167	16	−172	−119	63	−178	180	−179	77	−105
Nle^3-γ-MSH-NH_2	62	86	−94	−179	−105	73	−177	180	−177	92	−88
Ac-NDP-γ-MSH-NH_2	−165	−165	19	67	−104	76	179	177	−172	75	−107
4	−162	−112	68	61	−159	21	−162	177	54	16	−164
6	−176	73	−108	73	−94	88	−166	167	63	100	−81

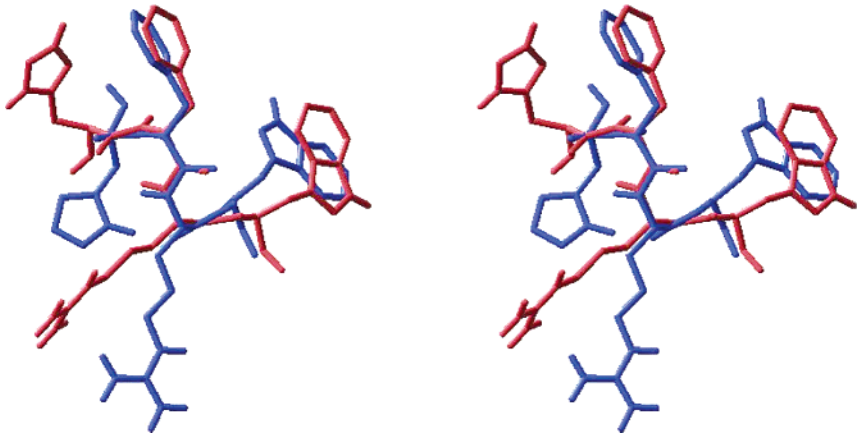

Figure 4. NMR structure of the MT-II core sequence His-D-Phe-Arg-Trp (red) and the LLMOD-derived conformation of the core sequence of NDP-α-MSH (blue) (His-D-Phe-Arg-Trp).

vided a highly potent agonist analogue **13**, equipotent to NDP-α-MSH (analogue **15**) at all four subtypes of the receptors (Table 3).

Modeling studies of the new analogues were performed using Kolossvary's large scale low mode conformational search method (LLMOD)[35] and the OPLS-AA force field. The results of these modeling experiments were compared with the NMR conformation of MT-II[36,37] and are summarized in Tables 4 and 5. Earlier, our laboratories have reported the NMR-determined conformation[36,37] of the superpotent agonist MT-II,[38,39] which was found to fit the LLMOD/OPLS-AA-derived global minimum. A superimposition of the NMR structure[36] of MT-II with the LLMOD-derived global minimum of another potent agonist, NDP-α-MSH, also produces a satisfactory fit within the core sequence His-D-Phe-Arg-Trp (Figure 4). This observation correlates well with the reported biological activities of these nonselective superpotent agonists of hMCRs.[40] On the other hand, a structural comparison of NDP-α-MSH and the natural hormone α-MSH revealed strikingly different global minimum conformations, with the only good fit observed in the backbone of the Phe-Arg-Trp sequence (Figure 5). As is evident from Figure 5, the side chains of Phe^7/D-Phe^7 occupy different positions in χ-space [χ^1(Phe) = −166°, χ^1(D-Phe) = +63°]. We postulate that the gauche(+) conformation of the D-phenylalanine side chain is important for high potency of melanotropin peptides. This hypothesis also is supported by the χ^1 angle of D-Phe^6 (+66°) determined in the NMR structure of the superagonist MT-II (Table 5).[35]

α-MSH and its potent analogues (MT-II, SHU-9119) have been extensively studied by NMR and computa-

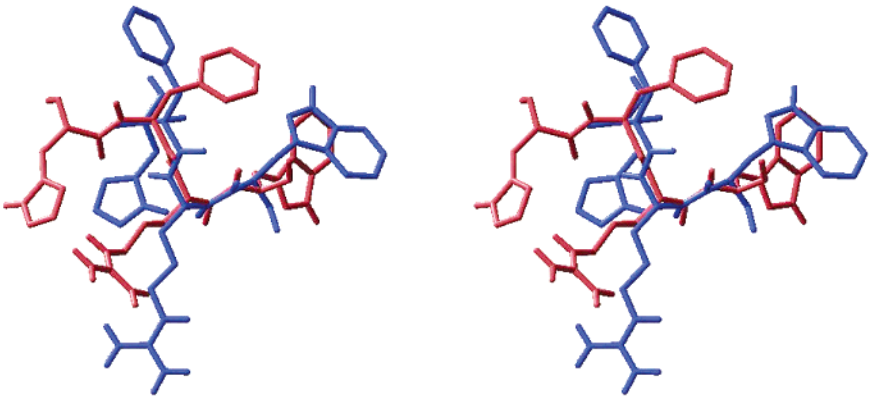

Figure 5. Superimposed LLMOD-derived conformation of the core sequence (His-D-Phe-Arg-Trp) of NDP-α-MSH (blue) and α-MSH (red) core sequence (His-Phe-Arg-Trp).

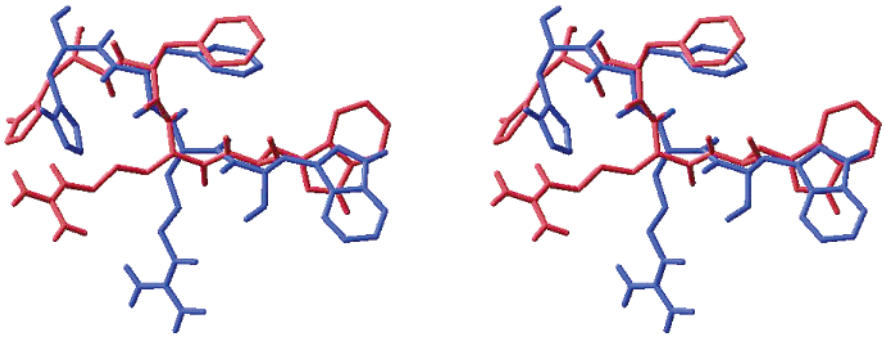

Figure 6. Superimposed LLMOD-derived conformation of the core sequence (His-Phe-Arg-Trp) of γ-MSH (blue) and α-MSH (red).

tional methods,[36,37,41−43] which prompted us to perform investigations of γ-MSH and its analogues. The starting point was to compare the structures of α-MSH and γ-MSH. γ-MSH itself was found to form an extended structure (not shown). Superimposition of α-MSH and γ-MSH revealed similar backbone and side chain conformations within the core sequence of His-Phe-Arg-Trp (Figure 6). It was interesting to note the differences in the binding affinities of α-MSH [IC_{50} = 0.4 nM (hMC1R), 30 nM (hMC3R), 5 nM (hMC4R), 18 nM (hMC5R)[33]] and γ-MSH [IC_{50} = 1000 nM (hMC1R), 71 nM (hMC3R), 760 nM (hMC4R), 2200 nM (hMC5R) (Table 3)] at the four receptors. These observed differences in binding affinities may be due at least in part to the C-terminal amide group of α-MSH. Similar observations were made for glucagon vs glucagon-amide, where it was stated that the C-terminal amide changes the dipole moment, resulting in a favorable anchoring of the peptide on the cell membrane.[44,45] Introduction of the terminal amide group into the γ-MSH sequence (analogue **1**) greatly increased the binding affinities at all but the hMC3R [IC_{50} = 1.2 nM (hMC1R), 40 nM (hMC3R), 45 nM (hMC4R), 330 nM (hMC5R) (Table 3)], which supports our hypothesis on the role of the C-terminal amide group in the increased binding affinities.

An intriguing observation was noted when the LLMOD-derived global minima of Nle^3-γ-MSH-NH_2 (analogue **10**) and γ-MSH (analogue **11**) were compared (Figure 7). It was observed that their conformations within the pharmacophore sequence His-Phe-Arg-Trp were nearly identical (not shown), though the binding affinities of these peptides differed by an order of magnitude (Table 3). A closer examination of the conformations of the C-terminal sequence Trp-Asp-Arg-Phe-Gly of these compounds showed identical backbone conformations but a significant difference in the side chain conformations of Phe^{11} (Figure 8). We suggest that this sequence creates a secondary binding site (ancillary site), which is responsible in part for the increased binding affinity. At the same time, the topography of the Trp-Asp-Arg-Phe-Gly-NH_2 sequence of Nle^3-γ-MSH-NH_2 was found to be similar to that of the core sequence (His-D-Phe-Arg-Trp) of the superpotent agonist MT-II (Figure 9).

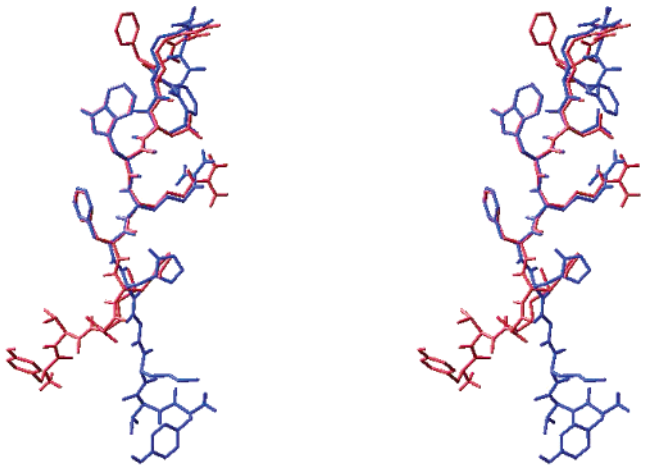

Figure 7. Superimposed LLMOD-derived conformation of γ-MSH (red) and Nle^3-γ-MSH-NH_2 (blue).

Comparing the LLMOD-derived core conformation of Ac-γ-MSH with that of γ-MSH (not shown), it is observed that both of them share nearly identical backbone conformations as well as local side chain conformations within the core sequence His-Phe-Arg-

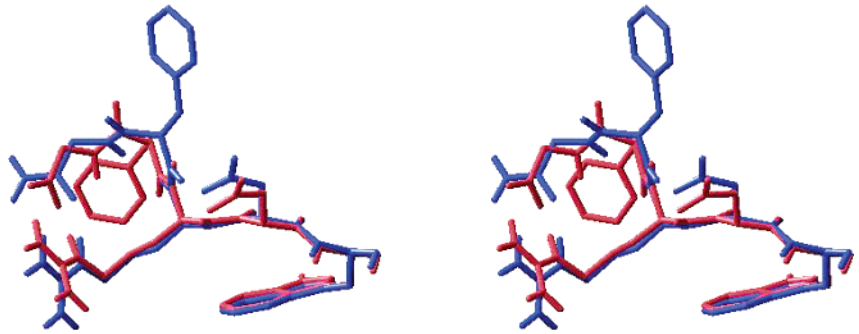

Figure 8. Superimposed LLMOD-derived conformation of the C-terminal sequences Trp-Asp-Arg-Phe-Gly of γ-MSH (red) and Nle^3-γ-MSH-NH$_2$ (blue).

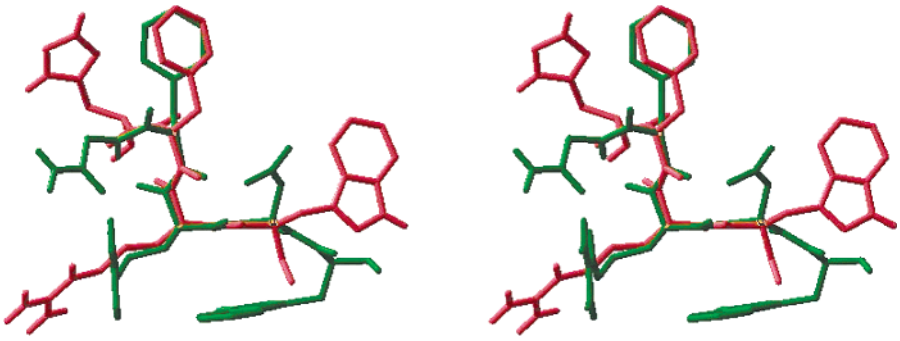

Figure 9. Superimposed NMR conformation of core sequence His-D-Phe-Arg-Trp (red) of MT-II and the LLMOD-derived conformational structure (green) of the sequence Trp-Asp-Arp-Phe-Gly of Nle^3-γ-MSH-NH$_2$. Please note that the backbone direction in the second fragment is reversed.

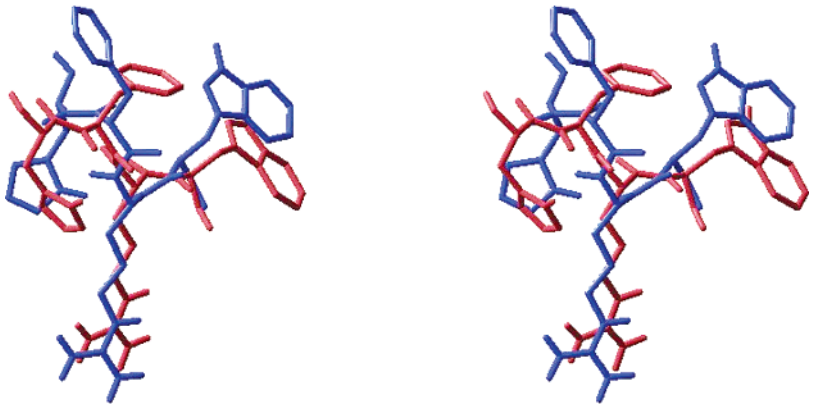

Figure 10. Superimposed LLMOD-derived core conformation of NDP-α-MSH (red) and γ-MSH (blue).

Trp. This observation suggests that the N-terminal acetyl has less influence on γ-MSH-like peptides.

It also was observed that, in the superimposition of the LLMOD-derived conformation of the NDP-α-MSH pharmacophore with that of γ-MSH (Figure 10), the backbones of the core sequence (His-Phe/D-Phe-Arg-Trp) fit well, but the χ-space of the D-Phe of NDP-α-MSH is entirely different from the Phe side chain of γ-MSH (Table 5). On the other hand, the superimposed LLMOD-derived conformation of the NDP-α-MSH and Ac-NDP-γ-MSH-NH$_2$ core sequences fit well (Figure 11). It was found that both of them share similar Φ, Ψ angles at D-Phe position, as well as the same χ angles for the D-Phe, Arg, and Trp positions (Tables 4 and 5). Thus the profiles of their 3-D topological structures are convergent.

The novel biological properties of analogues **4** and **6** led us to perform molecular modeling studies on them to obtain further structural insights. Their global minima were found to share similar backbone and side chain conformations within the core sequences (residues 5−8, Figure 12). The side chains of the residues 8 in peptides **4** [D-Nal(2′)] and **6** (D-Trp) favored the gauche(+) conformation ($\chi^1 = 54°$ and 63°, respectively), which was in contrast with the preferred trans conformation observed in Trp^8 side chain of the hMC3R agonists γ-MSH ($\chi^1 = -178°$) and Nle^3-γ-MSH-NH$_2$ ($\chi^1 = -177°$) (Table 5). We propose that the gauche(+) conformation of the side chain of the residue 8 may be important for selective hMC3R antagonist activity. Partial agonist activity was displayed at all the receptor subtypes in the case of analogue **7**, which had a D-Nal(2′) substitution for Phe^6 and a D-Phe for Trp^8. A drastic loss of the binding affinity followed the replacement of the His^5 residue with a D-Nal(2′) (**8**, Table 3), which was consistent with the earlier findings of Grieco et al.[31] that D-amino acids are not suitable for this position in α-MSH.

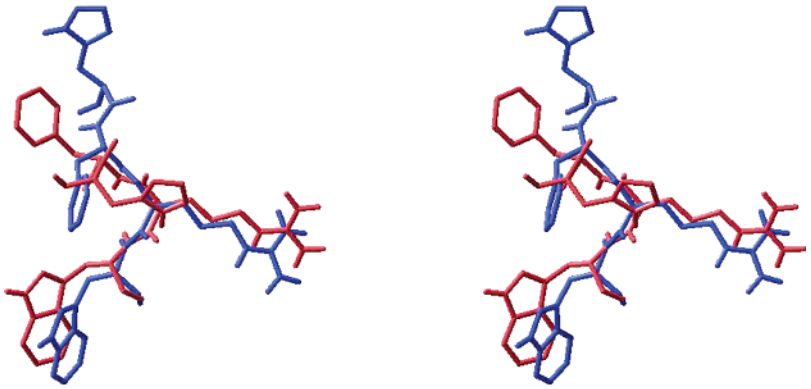

Figure 11. Superimposed LLMOD-derived conformation of the core sequence His-D-Phe-Arg-Trp of NDP-α-MSH (red) and Ac-NDP-γ-MSH-NH_2 (blue).

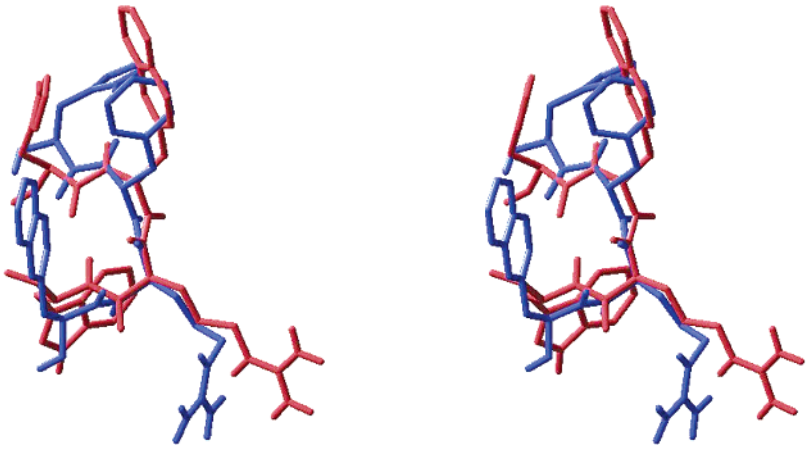

Figure 12. Superimposed LLMOD-derived core conformations of peptide **4** (red) and peptide **6** (blue).

Since NDP-α-MSH is a universal superagonist for all the subtypes of melanocortin receptors, we determined the LLMOD-derived conformation of Ac-NDP-γ-MSH-NH_2 and compared its conformation with the 3D pharmacophore conformation of NDP-α-MSH (and MT-II). Interestingly, we observed that the Φ, Ψ and χ^1, χ^2 angles in these two peptides were very close (for D-Phe^6, 113 vs 136 for Φ angle, −56 vs −164 for Ψ angle, 63 vs 67 for χ^1, and −95 vs −104 for χ^2). These results prompted us to synthesize Ac-NDP-γ-MSH-NH_2. This modeling-based, designed Ac-NDP-γ-MSH-NH_2 (analogue **13**) was found to be equipotent when compared with the universal agonist NDP-α-MSH (analogue **15**, Table 3) and it binds to melanocortin receptors with much stronger affinities than α-MSH and γ-MSH. Thus we have discovered a unique and superpotent analogue of γ-MSH in which the position of D-Phe^6 in χ space is critical for its enhanced biological activities, similar to that observed earlier in our laboratories.[26]

Our studies of α-MSH/γ-MSH hybrid structures demonstrate that the Trp^8 position (γ-MSH) is playing a critical role for selectivity at human melanocortin receptors. D-Trp^8-γ-MSH has previously been reported to be a potent and selective agonist for hMC3R, thus demonstrating the importance of the chirality of the residue 8 (Trp) for enhancing hMC3R potency in γ-MSH analogues.[24] In this study, we introduced D-Trp^8 and D-Nal(2′)^8 into the core sequence of Nle^3-γ-MSH-NH_2 analogues and obtained selective agonists for the hMC1R (peptides **2** and **6**) and a selective antagonist at the hMC3R (peptide **4**). Nle^3 was found to be important for increasing the binding affinity as well as the receptor selectivity. These studies also suggest that the Nle^3-γ-MSH chimeric analogues may have a secondary binding site, which is a novel discovery that can be further explored. Finally, the C-terminal amide substitution leads to increased binding as well as efficacy for all of these peptides compared to natural γ-MSH. Additionally, this latest study of α-MSH/γ-MSH hybrid revealed that conformational studies in χ-space[46] could be very valuable for the development of highly potent and receptor selective melanotropins.

Experimental Section

1. Abbreviations. Abbreviations used for amino acids and designation of peptides follow the rules of the IUPAC−IUB Commission of Biochemical Nomenclature in *J. Biol. Chem.* **1972**, *247*, 977−983. The following additional abbreviations are used: AAA, amino acid analysis; Boc, *tert*-butyloxycarbonyl; Fmoc, fluorenylmethoxycarbonyl; Fmo, fluorenylmethyl; Bzl, benzyl; *t*Bu, *tert*-butyl; CH_3CN, acetonitrile; DCM, dichloromethane; DIEA, diisopropylethylamine; DMF, *N,N*-dimethylformatmide; DIC, diisopropyl carbodiimide; HOBt, *N*-hydroxybenzotriazole; BOP reagent, (benzotriazolyloxy) tris(dimethylamino)phosphonium hexafluorophosphate; Nal(2′), 2′-naphthylalanine; TFA, trifluoroacetic acid; TIPS, triisopropylsilyl; SPPS, solid-phase peptide synthesis; RP-HPLC, reversed-phase high-performance liquid chromatography; hMC3R, human melanocortin-3 receptor; α-MSH, Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2; NDP-α-MSH, Ac-Ser-Tyr-Ser-Nle-Glu-His-D-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2; γ-MSH: H-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH.

2. Peptide Design and Synthesis. *N*-Fmoc-amino acids were obtained from Bachem, NovaBiochem, and Advanced

ChemTech. The side chain protecting groups were Boc and *t*Bu [Fmoc-Asp(*t*Bu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Arg(Boc)$_2$-OH, Fmoc-His(Boc)-OH, and Fmoc-Tyr(*t*Bu)-OH]. Fmoc-Rink amide resin was purchased from Polymer Laboratories. Organic solvents and reagents were purchased from Aldrich and used without further purification. All peptides were synthesized by the N-Fmoc solid-phase peptide strategy using DIC and HOBt as the coupling reagents.[47−50]

Rink amide resin (100 mg, 0.065 mmol/g) was placed into the 5 mL polypropylene syringe with the frit on the bottom and swollen in DCM (2 mL) for 30 min and in DMF (2 mL) for 30 min. The Fmoc protecting group on the Rink linker was removed by 50% piperidine in DMF. After 20 min the solution of piperidine was removed and the resin was washed with DMF (2 mL, 10 times). *N*-Fmoc amino acid (3 equiv, 0.195 mmol) and HOBt (3 equiv, 0.195 mmol) were dissolved in 700 L of DMF, and then DIC (3 equiv, 0.195 mmol) was added. The coupling mixture was transferred into the syringe with the resin and shaken for 1−3 h. Coupling completion was monitored with a ninhydrin test. The coupling mixture was removed and the resin was washed with DMF (2 mL, five times). *N*-Fmoc groups were removed with 50% piperidine in DMF over 20 min. Each coupling and deprotection step was repeated until a linear peptide was assembled. The final wash of the resin was done with DMF (2 mL, five times) and DCM (2 mL, five times). The product was cleaved from the resin with a mixture of 95% TFA, 2.5% TIPS, and 2.5% water during 1.5 h. Side chain protecting groups were removed during the cleavage step as well. The cleaved mixture was evaporated on a rotary evaporator, and the crude peptide was dissolved in acetic acid and purified by HPLC.

HPLC Purification. The peptide was lyophilized and purified by preparative RP-HPLC on a C_{18} bonded silica column (Column YMC-Pack ODS-AM 150 × 4.6 mm, S-3 μm, 120A) eluted with a linear gradient of acetonitrile (gradient, 2−80% B in A over 30 min, flow rate 0.8 mL/min. System 1: solvent A, 0.1% TFA in water; solvent B, 0.08% TFA in acetonitrile. System 2: solvent A, 1% formic acid in water; solvent B, 1% formic acid in methanole) and aqueous 0.1% TFA (v/ v).

3. Biological Activity Assays. Receptor Binding Assay. Competition binding experiments were performed on whole cells. Transfected HEK293 cell line with hMCRs cells[29,40,51] were seeded on 96 well plates, 48 h before assay, 100 000 cells/well. For the assay, the medium was removed and cells were washed twice with a freshly prepared binding buffer containing 100% minimum essential medium with Earle's salt (MEM, GIBCO), 25 mM HEPES (pH 7.4), 0.2% bovine serum albumin, 1 mM 1,10-phenanthrolone, 0.5 mg/L leupeptin, and 200 mg/L bacitracin. Cells were then incubated with different concentrations of unlabeled peptide and ^{125}I-labeled [Nle^4,D-Phe^7]-α-MSH (PerkinElmer Life Science, 100 000 cpm/well, 0.1386 nM) for 40 min at 37 °C. The medium was subsequently removed and each well was washed twice with the assay buffer. The cells are lysed by the addition of 250 μL of 0.1 NaOH and 250 μL of 1% Triton X-100. The lysed cell was transferred to the 12 × 75 mm glass tubes and the radioactivity was measured by Wallac 1470 WIZARD Gamma Counter. Data were analyzed using Graphpad Prism 3.1 (Graphpad Software, San Diego, CA).

Adenylate Cyclase Assay. HEK 293 cells transfected with human melanocortin receptors[40] were grown to confluence in MEM medium (GIBCO) containing 10% fatal bovine serum, 100 units/mL penicillin and streptomycin, and 1 mM sodium pyruvate. The cells were seeded on 96 well plates 48 h before assay, (100 000 cells/well). For the assay, the medium was removed and cells were rinsed with 1 mL of MEM buffer (GIBCO) or with Earle's balanced salt solution (EBSS, GIBCO). An aliquot (0.4 mL) of the Earle's balanced salt solution was placed in each well along with isobutylmethylxanthine (IBMX; 5 μL; 0.5 mM) for 1 min at 37 °C. Varying concentrations of melanotropins (0.1 mL) were added and the cells incubated for 3 min at 37 °C. The reaction was stopped by aspirating the buffer and adding ice cold Tris/EDTA buffer to each well (0.15 mL). The 96 well plates were covered and placed on ice. After dislodging the cells with the help of trypsin, the cells were transferred to polypropylene microcentrifuge tubes, capped, and place in a boiling water bath for 15 min. The cell lysate was then centrifuged for 2 min (6500 rpm), and 50 μL of the supernatant was aliquoted into a clean Eppendorf tube. cAMP content was measured by competitive binding assay according to the assay kit instructions (TRK 432, Amersham Corp.).

Data Analysis. IC_{50} and EC_{50} values represent the mean of duplicate experiments performed in triplicate. IC_{50} and EC_{50} estimates and their associated standard errors were determined by fitting the data using a nonlinear least-squares analysis, with the help of Graphpad Prism 3.1 (Graphpad Software, San Diego, CA).

4. Computational Procedures. Molecular modeling experiments employed MacroModel 8.1 equipped with Maestro 5.0 graphical interface installed on a Linux RedHat 9.0 system. Peptide structures were built into extended structures with standard bond lengths and angles, and they were minimized using the OPLS-AA force field and the Polak-Ribier conjugate gradient (PRCG). Optimizations were converged to a gradient rmsd less that 0.05 kJ/Å mol or continued until a limit of 50 000 iterations was reached. Aqueous solution conditions were simulated using the continuum dielectric water solvent model (GB/SA) as implemented in MacroModel. Extended cutoff distances were defined at 8 Å for van der Waals, 20 Å for electrostatics, and 4 Å for H-bonds.

Conformational profiles of the cyclic peptides were investigated by Macromodel's LLMOD procedure of Kolossváry[35] using the energy minimization parameters as described above. A total of 25 000 search steps were performed and the conformations with energy difference of 50 kJ/mol from the global minimum were saved. Interatomic dihedral angles were measured for each peptide analogue using the standard Maestro measurement tool, and they are described in Tables 4 and 5. The superimpositions of peptide structures were performed using the α-carbons of the core sequence His-D-Phe-Arg-Trp unless otherwise specified.

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