Potent and Prolonged Acting Cyclic Lactam Analogues of α-Melanotropin: Design Based on Molecular Dynamics

Fahad Al-Obeidi,[†] Ana M. de L. Castrucci,[‡,§] Mac E. Hadley,[‡] and Victor J. Hruby*,[†]

Departments of Chemistry and Anatomy, University of Arizona, Tucson, Arizona 85721. *Received February 13, 1989*

Utilizing results from previous structure–activity relationships and theoretical studies of α-melanotropin (α-MSH, Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2) and its related superpotent analogues, Ac-[Nle^4,D-Phe^7]-α-MSH and Ac-[Cys^4,Cys^{10}]-α-MSH, we have designed a new class of α-MSH_{4-13} and α-MSH_{4-10} cyclic lactam fragment analogues of α-melanotropin. The cyclic peptides have the following general structures: Ac-[Nle^4,Xxx^5,D-Phe^7,Yyy^{10},Gly^{11}]-α-MSH_{4-13}-NH_2 and Ac-[Nle^4,Xxx^5,D-Phe^7,Yyy^{10}]-α-MSH_{4-10}-NH_2, where Xxx = Glu or Asp and Yyy = Lys, Orn, Dab, or Dpr. Formation of the lactam bridge between the side-chain groups Xxx and Yyy was performed either in solution or on a solid-phase support. Seven cyclic peptides were prepared and bioassayed for their melanotropic potency by using standard frog (*Rana pipiens*) and lizard (*Anolis carolinensis*) skin bioassays. Relative to α-MSH (relative potency = 1), the potencies of the cyclic peptides in the lizard skin bioassay were as follows: α-MSH (1); Ac-[Nle^4,Glu^5,D-Phe^7,Lys^{10},Gly^{11}]-α-MSH_{4-13}-NH_2 (6); Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10},Gly^{11}]-α-MSH_{4-13}-NH_2 (100); Ac-[Nle^4,Glu^5,D-Phe^7,Lys^{10}]-α-MSH_{4-10}-NH_2 (9); Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10}]-α-MSH_{4-10}-NH_2 (90); Ac-[Nle^4,Asp^5,D-Phe^7,Orn^{10}]-α-MSH_{4-10}-NH_2 (20); Ac-[Nle^4,Asp^5,D-Phe^7,Dab^{10}]-α-MSH_{4-10}-NH_2 (5); Ac-[Nle^4,Asp^5,D-Phe^7,Dpr^{10}]-α-MSH_{4-10}-NH_2 (5). Similar results were obtained in the frog skin bioassay, but the analogues were much less potent. Cyclic melanotropins with 23-membered rings exhibited 100-fold higher melanotropic potency than α-MSH with selectivity for the lizard melanocyte receptors over the frog melanocyte receptors. Increasing or decreasing the ring size of these cyclic melanotropins from 23 diminishes the biological potency of the resulting cyclic peptide. The 23- and 24-membered ring analogues showed prolonged (residual) biological activities in both biological assays, but the smaller ring systems (20, 21, 22) did not. These results provide new insights into the structural and conformational requirements of α-MSH and its analogues at two different types of pigment cell (melanocyte) receptors.

α-Melanocyte-stimulating hormone (α-MSH, α-melanotropin), which is derived from the precursor protein proopiomelanocortin, is a tridecapeptide (Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2)[1] secreted from the pars intermedia of the pituitary gland.[2,3] Although α-MSH exhibits diverse central and peripheral biological effects, the hormone is particularly known for its classical role in regulating pigmentation of the skin of many vertebrates.[3,4] Like most linear peptide hormones, α-MSH has a short duration of action due to its rapid enzymatic degradation by proteolytic enzymes.[5,6] One of our goals has been to design α-MSH analogues with high potency and prolonged duration of action. Several years ago, on the basis of chemical and biological considerations, we prepared an analogue, [Nle^4,D-Phe^7]-α-MSH,[7] which exhibited superpotency and a prolonged duration of action in several bioassays.[4,7-9] On the basis of these results and conformational considerations we utilized the concept of pseudoisosteric cyclization by replacing Met^4 and Gly^{10} by a Cys^4,Cys^{10} disulfide bridge, to give a cyclic disulfide containing analogue, Ac-[Cys^4,Cys^{10}]-α-MSH, which was found to possess superpotent bioactivity in the frog skin bioassay, but which lacked significant prolonged activity.[10,11]

In the present investigation, we have designed a new class of cyclic α-MSH analogues based on an examination of the molecular dynamics of α-MSH with the objective of creating superpotent cyclic analogues of α-MSH that also exhibit prolonged biological activity. We have synthesized α-MSH analogues of the general formulas Ac-[Nle^4,Xxx^5,D-Phe^7,Yyy^{10}]-α-MSH_{4-10}-NH_2 and Ac-[Nle^4,Xxx^5,D-Phe^7,Yyy^{10},Gly^{11}]-α-MSH_{4-13}-NH_2 in which the acidic amino acids (Asp or Glu) and the basic amino acids (Lys, Orn, Dab, or Dpr) were substituted at positions

* To whom reprint requests should be addressed.
[†] Department of Chemistry.
[‡] Department of Anatomy.
[§] Permanent address: Departamento de Fisiologia, Universidade de Sao Paulo, CP 11176 Sao Paulo, Brazil.

(1) Abbreviations used in this paper for amino acids, protecting groups, and peptides follow the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature and Symbols as described in *Eur. J. Biochem.* **1972**, *27*, 201, and *J. Biol. Chem.* **1975**, *250*, 3215; all optically active amino acids are of L variety unless otherwise started. Other abbreviations: α-MSH, α-melanotropin; Nle, norleucine; Dab, 2,4-diaminobutyric acid; Dpr, 2,3-diaminopropionic acid; DCM, dichloromethane; DMF, *N,N*-dimethylformamide; DEAE, *N,N*-diethylaminoethyl; DPPA, diphenyl phosphorazidate; DIC, diisopropyl carbodiimide; HOBt, *N*-hydroxybenzotriazole; BOP, (benzotriazolyloxy)tris(dimethylamino)phosphonium hexafluorophosphate; Bom, benzyloxymethyl; TLC, thin-layer chromatography; DMAP, 4-(*N,N*-dimethylamino)pyridine; UV, ultraviolet; Fmo, fluorenylmethyloxy; RP-HPLC, reverse-phase high-pressure liquid chromatography; EDAC·MeI, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide methyl iodide; DIEA, diisopropylethylamine.

(2) O'Donohue, T. L.; Dorsa, D. M. *Peptides* **1982**, *3*, 353.
(3) Hadley, M. E. *Endocrinology*, 2nd ed.; Prentice-Hall Inc.: Englewood Cliffs, NJ, 1988; p 160.
(4) Hruby, V. J.; Wilkes, B. C.; Cody, W. L.; Sawyer, T. K.; Hadley, M. E. *Pept. Protein Rev.*, **1984**, *3*, 1, for a review.
(5) Akiyama, K.; Yamamura, H. I.; Wilkes, B. C.; Cody, W. L.; Hruby, V. J.; Castrucci, A. M. L.; Hadley, M. E. *Peptides* **1984**, *5*, 1191.
(6) Castrucci, A. M. L.; Hadley, M. E.; Sawyer, T. K.; Hruby, V. J. *Comp. Biochem. Physiol.* **1984**, *78*, 519.
(7) Sawyer, T. K.; Sanfilippo, P. J.; Hruby, V. J.; Engel, M. H.; Heward, C. B.; Burnett, J. B.; Hadley, M. E. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 5754.
(8) Marwan, M. M.; Abdel-Malek, Z. A.; Kreutzfeld, K. L.; Hadley, M. E.; Wilkes, B. C.; Hruby, V. J.; Castrucci, A. M. L. *Mol. Cell. Endocrinol.* **1985**, *41*, 171.
(9) Hadley, M. E.; Anderson, B.; Heward, C. B.; Sawyer, T. K.; Hruby, V. J. *Science* **1981**, *213*, 1025.
(10) Sawyer, T. K.; Hruby, V. J.; Darman, P. S.; Hadley, M. E. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 1751.
(11) Knittel, J. J.; Sawyer, T. K.; Hruby, V. J.; Hadley, M. E. *J. Med. Chem.* **1983**, *26*, 125.

0022-2623/89/1832-2555$01.50/0 © 1989 American Chemical Society

Table I. Structure and Relative Potencies of Cyclic Melanotropin Analogues

peptide	structure	relative potencies (prolongation)[a]	
		frog skin	lizard skin
I	α-MSH	1.0 (–)	1.0 (–)
II	Ac-[Nle^4,$\overline{Glu^5,D\text{-}Phe^7,Lys^{10}}$,$Gly^{11}$]-α-$MSH_{4-13}$-$NH_2$	1.0 (+)	6.0 (+)
III	Ac-[Nle^4,$\overline{Asp^5,D\text{-}Phe^7,Lys^{10}}$,$Gly^{11}$]-α-$MSH_{4-13}$-$NH_2$	1.0 (+)	100.0 (+)
IV	Ac-[Nle^4,$\overline{Glu^5,D\text{-}Phe^7,Lys^{10}}$]-α-$MSH_{4-10}$-$NH_2$	0.5 (+)	9.0 (+)
V	Ac-[Nle^4,$\overline{Asp^5,D\text{-}Phe^7,Lys^{10}}$]-α-$MSH_{4-10}$-$NH_2$	0.8 (+)	90.0 (+)
VI	Ac-[Nle^4,$\overline{Asp^5,D\text{-}Phe^7,Orn^{10}}$]-α-$MSH_{4-10}$-$NH_2$	1.0 (–)	20.0 (–)
VI	Ac-[Nle^4,$\overline{Asp^5,D\text{-}Phe^7,Dab^{10}}$]-α-$MSH_{4-10}$-$NH_2$	1.0 (–)	5.0 (–)
VIII	Ac-[Nle^4,$\overline{Asp^5,D\text{-}Phe^7,Dpr^{10}}$]-α-$MSH_{4-10}$-$NH_2$	0.01 (–)	5.0 (–)

[a] Potencies relative to α-MSH (defined as 1.0). Prolongation was determined by adding α-MSH to one group of frog (*R. pipiens*) or lizard (*A. carolinenses*) skins and the peptide analogue to a second group of skins. The final concentration of each peptide in the medium was the one that elicited maximal darkening response. After 60 min the skins (five per group) were rinsed and transferred to Ringer's solution in the absence of melanotropins. Thus, (+) and (–) indicate whether the biological effect continued after the exogenous peptide analogue had been removed from the assay medium and the skins rinsed several times.

Xxx and Yyy, respectively, and a lactam bridge is formed between the residues in the 5- and 10-positions (see Table I for structures). In previous papers we reported the structure–function analysis of a series of related linear peptides[12] and have provided a short report on the design of the most potent cyclic analogue.[13] Here we present a detailed structure–function analysis of this new class of cyclic α-melanotropin analogues.

Results and Discussion

The syntheses of the precursor peptides for the cyclic lactam analogues of Ac-α-MSH_{4-13}-NH_2 and Ac-α-MSH_{4-10}-NH_2 discussed herein were accomplished by solid-phase synthetic methods, and the final products were purified by procedures similar to those previously reported for other α-melanotropins,[12,14,15] as well as by reverse-phase high-pressure liquid chromatography (RP-HPLC). The details of the synthetic methods are given in the Experimental Section, and the analytical data for the final cyclic products are given in Table II.

The formation of the cyclic lactam bridges between the side-chain groups of the amino acid residues in positions 5 and 10 of the α-melanotropin analogues II–VIII (Table I) was accomplished either on the solid-phase support or in solution as outlined in Scheme I for the cyclic peptide V. In solid-phase cyclization the substitution level of the first amino acid residue (the C-terminal residue) was 0.30–0.35 mmol/g of resin, which was found to give the best results in terms of yield and purity of the final products. The synthesis of the cyclic peptides on the solid-phase resin (method A in Scheme I, see Experimental Section for details) was found to provide two major advantages: (a) easier workup and (b) higher overall yields of the cyclic peptide in comparison to those obtained by solution methods (method B in Scheme I, see Experimental Section for details). The solid-phase cyclization procedure was used to prepare cyclic peptides III, V, and VII (Table I). All analogues were prepared by the solution-phase cyclization (method B in Scheme I). The yield of purified V was 55–60% following method A and 30–40% following

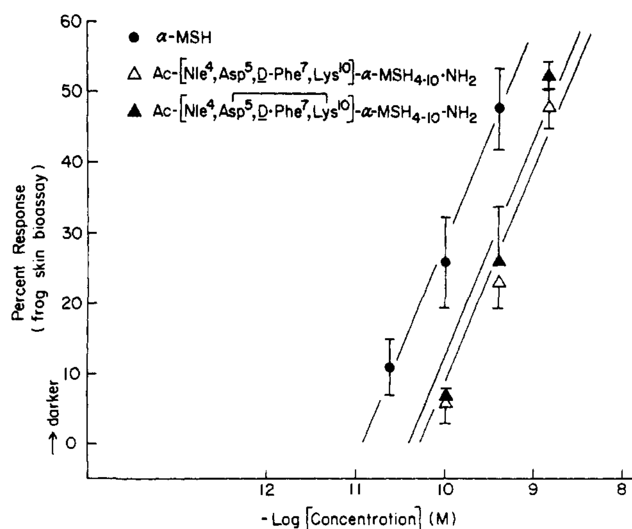


Figure 1. Comparison of biological potencies of the cyclic and linear analogues Ac-[Nle^4,$\overline{Asp^5,D\text{-}Phe^7,Lys^{10}}$]-α-$MSH_{4-10}$-$NH_2$ and Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10}]-α-MSH_{4-10}-NH_2 in the frog skin bioassay. Each value represents the mean (±SE) darkening response of skins (N = 6 or more in all experiments) to the melanotropins at the concentrations noted.

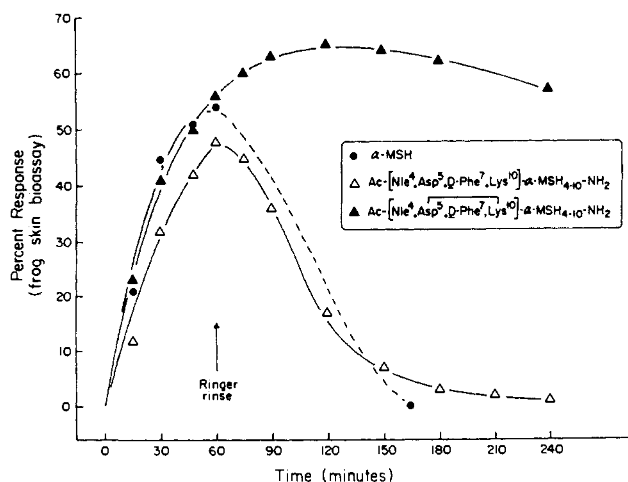


Figure 2. Demonstration of prolongation effect of the cyclic analogue Ac-[Nle^4,$\overline{Asp^5,D\text{-}Phe^7,Lys^{10}}$]-α-$MSH_{4-10}$-$NH_2$ in the frog skin bioassay system, and the reversal of the corresponding linear analogue Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10}]-α-MSH_{4-10}-NH_2 in the same assay.

(12) Al-Obeidi, F.; Hruby, V. J.; Castrucci, A. M. L.; Hadley, M. E. *J. Med. Chem.* **1989**, *32*, 174.
(13) Al-Obeidi, F.; Hruby, V. J.; Pettitt, M.; Hadley, M. E. *J. Am. Chem. Soc.* **1989**, *111*, 3413.
(14) Cody, M. L.; Wilkes, B. C.; Muska, B. J.; Hruby, V. J.; Castrucci, A. M. L.; Hadley, M. E. *J. Med. Chem.* **1984**, *27*, 1186.
(15) Lebl, M.; Cody, W. L.; Wilkes, B. C.; Hruby, V. J.; Castrucci, A. M. L.; Hadley, M. E. *Int. J. Pept. Protein Res.* **1984**, *24*, 472.

Table II. Characteristics of Cyclic Melanotropin Analogues

peptide	$[\alpha]^{22}_{589}$ in 10% HOAc	TLC R_f[a] in various systems			HPLC K'[b]	FAB-MS (M + H) found (calcd)
		A	B	C		
II	−57.8 (*c* 0.045)	0.76	0.79	0.89	4.00	1291.0 (1290.7)
III	−53.3 (*c* 0.04)	0.74	0.76	0.86	3.92	1277.0 (1277.4)
IV	−13.3 (*c* 0.07)	0.89		0.65	5.87	1040.0 (1041.1)
V	−54.2 (*c* 0.07)	0.65	0.73	0.80	6.02	1025.0 (1026.1)
VI	−37.1 (*c* 0.07)	0.66	0.71	0.79	5.78	1011.0 (1012.1)
VII	−54.5 (*c* 0.01)	0.77	0.80	0.80	4.33	997.0 (998.08)
VIII	−33.3 (*c* 0.045)	0.77	0.78	0.87	3.45	983.0 (984.05)

peptide	amino acid analyses													
	Nle	Glu	Asp	His	Phe	Arg	Trp	Lys	Gly	Pro	Val	Orn	Dab	Dpr
II	0.95	1.00		0.96	1.00	0.98	0.90	0.98	1.02	0.90	1.02			
III	0.99		0.96	0.98	1.01	0.98	0.91	0.96	1.01	0.89	1.01			
IV	1.00	0.97		0.90	1.10	0.96	1.10	0.90						
V	1.10		1.10	0.90	0.97	1.00	0.90	0.93						
VI	1.10		1.10	0.99	1.00	1.10	0.90					0.90		
VII	1.10		1.10	0.91	1.10	0.96	1.10						0.91	
VIII	1.10		1.00	1.10	0.90	0.94	0.95							0.91

[a] R_f values on thin-layer chromatograms of silica gel were observed in the following solvent systems: (A) 1-butanol/HOAc/H_2O (4:1:5, upper phase only); (B) 1-butanol/HOAc/pyridine/H_2O (15:3:10:12); (C) 1-butanol/pyridine/HOAc/H_2O (5:5:1:4). [b] HPLC K' = [(retention time − solvent front)/solvent front] − solvent system was 20% acetonitrile in 0.1% trifluoroacetic acid: isocratic for 5 min, then gradient to 30% acetonitrile over 10 min; isocratic for 5 min, then gradient 6–85% acetonitrile over 2 min. Then, 5 min to go to 20% acetonitrile and an additional 5 min for equilibration.

Scheme I

Method A: Cyclization on the Solid-Phase Resin

Boc-Lys(Fmoc)-pMBHA-resin

1. Boc-Arg(Tos)
2. Boc-D-Phe
3. Boc-His(Bom)
4. Boc-Asp(Fmo)
5. piperdine DMF (1:1)

Boc-Asp(OH)-His(Bom)-D-Phe-Arg(Tos)-Trp(For)-Lys(NH_2)-pMBHA-resin

6. Bop reagent + Et_3N in DMF (cyclization)

Boc-Asp-His(Bom)-D-Phe-Arg(Tos)-Trp(For)-Lys-pMbHA

7. Boc-Nle (solid–phase methods)
8. Ac_2O pyridine
9. HF cleavage and purification

Ac-Nle-Asp-His-D-Phe-Arg-Trp-Lys-NH_2

Method B: Cyclization in Solution

Boc-Lys (2-ClZ)-pMBHA

1. Boc-Arg(Tos)
2. Boc-D-Phe
3. Boc-His(Bom)
4. Boc-Asp(Bzl)
5. Boc-Nle
6. Ac_2O/pyridine
7. HF cleavage and purification

Ac-[Nle[4], Asp[5], D-Phe[7], Lys[10]]-a-MSH_{4-10}-NH_2•3HF

8. HCl, DIEA-cellulose

Ac-[Nle[4],Asp[5], D-Phe[10]]-α-MSH_{4-10}-NH_2•3HCl

9. DPPA + K_2HPO_4 in DMF (cyclization)

method B. The yields for the other cyclic peptides listed in Table I were similar according to the methods outlined in Scheme I (see the Experimental Section). The in vitro biological potencies and prolonged (residual) activities of the peptides were determined in both the frog (*Rana pipiens*)[7,14,15] and the lizard (*Anolis carolinensis*) skin bioassays[14] over the entire dose–response range of the peptides. The results of the latter studies are given in Figures 1 and 2, and the relative potencies from both studies are summarized in Table I.

On the basis of earlier structure–biological activity studies on α-melanotropin it was suggested by ourselves and others[4] that a salt bridge may exist between the Glu[5] (−COOH) and Lys[11] (ε-NH_2) side-chain groups of α-melanotropin in its "bioactive" conformation. However, efforts to confirm this hypothesis using theoretical calculations[16] or with synthetic analogues[4] were inconclusive. Recently, however, we have performed molecular dynamic simulations in conjunction with molecular mechanics calculations on α-melanotropin and the superpotent and prolonged acting analogue [Nle[4],D-Phe[7]]-α-MSH to further examine this question.[13,17] On the basis of these investigations we suggested that if the Lys[11] side chain group were transposed to the prochiral *S* hydrogen at the α-carbon Gly[10], an intramolecular interaction between the Glu[5] γ-CO_2H and the resultant Lys[10] ε-NH_2 side-chain functionalities might be highly probable and energetically favorable. To test this hypothesis, we synthesized a number of α-MSH, α-MSH_{4-13}, and α-MSH_{4-10} analogues in which position 5 (Glu[5]) contained either a Glu or an Asp residue, position 10 (Gly[10]) was replaced with Lys or other dibasic amino acid residues (Orn, Dab, Dpr), and position 11 (Lys[11]), if present, was replaced by Gly. As previously reported,[12] these analogues generally were found to be equipotent with or even more potent than the corresponding hormone or hormone fragment analogue.[12]

(16) Nikiforovich, G. V.; Shenderovich, M. E.; Chipens, G. I. *FEBS Lett.* 1981, *126*, 180.

(17) Hruby, V. J.; Kazmierski, W.; Pettitt, M.; Al-Obeidi, F. *Molecular Biology of Brain and Endocrine Peptidergic Systems*; Chretien, M., McKerns, K. W., Eds.; Plenum Publishing Corp.: New York, 1988; pp 13–27.

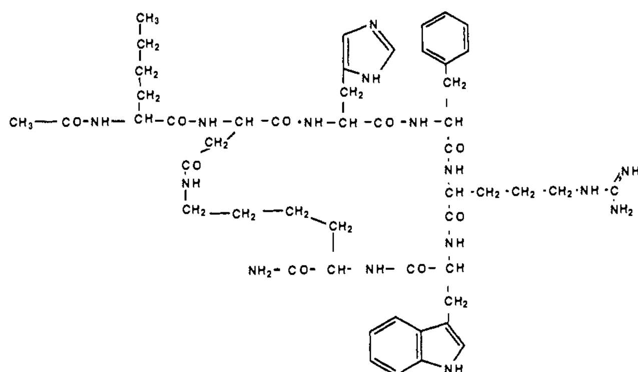


Figure 3. Structure of superpotent and prolonged acting cyclic lactam analogue Ac-[Nle[4],Asp[5],D-Phe[7],Lys[10]]-α-MSH_{4-10}-NH_2 (V).

We have now carried this examination further by preparing the corresponding cyclic lactam analogues of these linear 4–13 and 4–10 fragment analogues (Table I) to obtain ring structures that varied in size from 24 membered (II and IV, Table I) to 20 membered (VIII, Table I).

The melanocyte-stimulating activity of the cyclic decapeptide Ac-[Nle[4],Glu[5],D-Phe[7],Lys[10],Gly[11]]-α-MSH_{4-13}-NH_2 (II) was 6-fold greater than that of α-MSH in the in vitro lizard skin assay and was equipotent to that of α-MSH in the in vitro frog skin assay (Table I). In addition, II exhibited very prolonged biological activity in both of the bioassays.

This very promising result led us to examine the synthesis and bioactivities of the corresponding cyclic heptapeptides of the general structure Ac-[Nle[4],Xxx[5],D-Phe[7],Yyy[10]]-α-MSH_{4-10}-NH_2. These truncated analogues were prepared to investigate the contribution of the 11–13 residues on the potency and prolonged biological activity of this new cyclic series, as well as the importance of the ring size and the side-chain substitutions on the biological activity in this cyclic lactam series (Table I). Ac-[Nle[4],Glu[5],D-Phe[7],Lys[10]]-α-MSH_{4-10}-NH_2 (IV) (24-membered ring) was found to have 0.5 and 9 times the potency of α-MSH in the frog and lizard skin bioassays, respectively, and the duration of action of peptide IV was prolonged in both assays (Table I). It should be pointed out that the corresponding linear analogue was not prolonged acting in either bioassay. It thus appears that residues 11–13 of the modified melanotropins (which now have the sequence Gly-Pro-Val-NH_2 in the 4–13 analogues) have very little effect on the potency and prolonged activity of these cyclic compounds. Since in previous melanotropin analogues the C-terminal tripeptide residues (Lys-Pro-Val-NH_2) generally had potentiating effects, the substitution of Gly at position 11 for Lys may be at least in part responsible for this difference in potency. The structural and other features responsible for prolonged activity are complex[4,18] and are outside the scope of this study.

Interestingly, it was found that the 23-membered ring analogues, Ac-[Nle[4],Asp[5],D-Phe[7],Lys[10]]-α-MSH_{4-10}-NH_2 (V) (Figure 3) and Ac-[Nle[4],Asp[5],D-Phe[7],Lys[10],Gly[11]]-α-MSH_{4-13}-NH_2 (III), possessed the highest potencies among the cyclic heptapeptides synthesized in the present work. They were equipotent to α-MSH in the frog skin assay and 90–100 times more potent than α-MSH in the lizard skin bioassay. It should be noted that whereas α-MSH has 13 amino acid residues, V has only 7 amino acid residues and yet is 90–100 times more potent than the native hormone. The time course study demonstrated that peptide V was not only a superpotent analogue but also a prolonged acting one. The melanocyte-stimulating effect was observed for periods lasting from 6 to 48 h after removal of the exogenous cyclic peptide from the assay medium even at a minimal effective dose level. In contrast, α-melanotropin (Figure 2) and the linear analogue of V do not have prolonged actiity when tested at similar doses (Table I and Figure 2).

In evaluating the effect of ring size on the potencies of the cyclic lactam heptapeptides, it was found that potency and prolongation decreased with reduction of the ring size from a 23- (V) to a 20- (VIII) membered ring compound (Table I). In general, the resultant cyclic peptides showed a gradual reduction of potency from 0.50 to 0.01 the potency of α-MSH in the frog skin assay, and from 90-fold to 5-fold greater potency than that of α-MSH in the lizard skin assay when the ring size was reduced from 23 to 20 atoms (Table I). A correlation between ring size and biological potency in the frog skin bioassay system was found in cyclic Cys[4],Cys[10] disulfide containing melanotropin analogues.[14,15] However, it is interesting to emphasize that whereas the Cys[4],Cys[10] cyclic analogues of α-MSH generally were found to be superpotent only in the frog skin bioassay systems, the new cyclic lactam fragment analogues involving residues 5 and 10 of α-melanotropin reported here are superpotent only in the lizard skin bioassay system. Thus the receptor selectivities for the new cyclic lactam analogues are the opposite of those found for the cyclic disulfide containing analogues reported earlier. In fact, the cyclic lactam melanotropin analogues reported here represent the first class of cyclic melanotropins with superpotency in the lizard skin assay. Further studies of the conformational and dynamic properties of these two classes of cyclic conformationally constrained melanotropins should provide new insights into the differential structural and conformational requirements of various melanotropin receptors. In addition, the successful use of molecular dynamics simulations and molecular mechanics calculations in the design of the cyclic lactam analogues reported here provides incentive for the further development of these methods in peptide ligand design.

Experimental Section

Materials. *p*-Methylbenzhydrylamine (pMBHA) resin (0.6 mmol/g)[7] was used as polymer support. N^{α}-*tert*-Butyloxycarbonyl (N^{α}-Boc) protected amino acids were purchased from Bachem Inc., Torrance, CA, or were prepared by standard literature methods.[19] The reactive side chains of the amino acids were protected as follows: Lys, with 2-chlorobenzyloxycarbonyl (2-ClZ) or fluorenylmethoxycarbonyl (Fmoc); Orn, with benzyloxycarbonyl (Z); Asp and Glu, with benzyl (Bzl) or fluorenylmethyl (see below) esters (Fmo); His, with benzyloxymethyl (Bom);[20] Arg, with tosyl (Tos); Trp, with formyl (For); Dab and Dpr, with benzyloxycarbonyl (Z) or fluorenylmethoxycarbonyl (Fmoc) (see below), which were prepared as previously reported.[21,22] All reagents and solvents were ACS grade or better and were used without further purification.

Synthesis of N^{α}-Boc-Asp-β-fluorenylmethyl Ester [N^{α}-Boc-Asp(β-Fmo)]. This compound was prepared from Z-Asp

(18) Hruby, V. J.; Cody, W. L.; Wilkes, B. C.; Hadley, M. E. *Peptides 1984*; Ragnarsson, U., Ed.; Almquist & Wiksell International: Stockholm, 1984; pp 505-508.

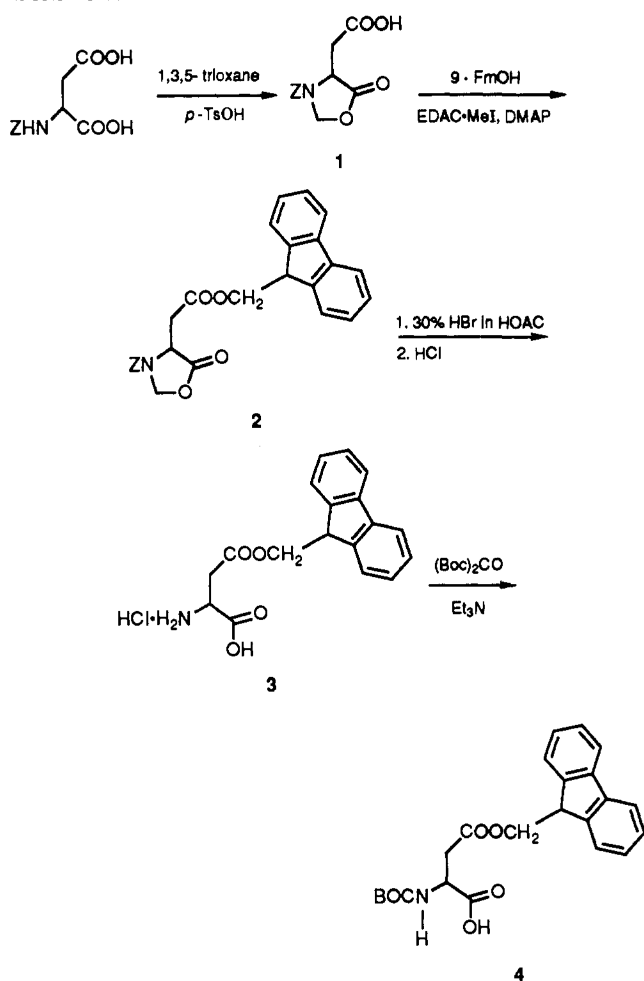
(19) Stewart, J. M.; Young, J. D. *Solid Phase Peptide Synthesis*, 2nd ed.; Pierce Chemical Co.: Rockford, IL, 1984.

(20) Pipkorn, R.; Ekberg, B. *Int. J. Pept. Protein Res.* **1986**, *27*, 583.

(21) Waki, M.; Kitajima, Y.; Izumiya, N. *Synthesis* **1981**, 266.

(22) Loudon, G. M.; Radhakrishna, A. S.; Almon, M. R.; Blodgett, J. K.; Boutin, H. R. *J. Org. Chem.* **1984**, *49*, 4272.

Scheme II



(Bachem) according to Scheme II. Z-Asp was converted to (*S*)-3-(benzyloxycarbonyl)-5-oxo-4-oxazolidineacetic acid (1, Scheme II) according to the method of Itah.[23] Then 1 (7.16 g, 28.96 mmol) and 9-fluorenylmethanol (4.92 g, 25.62 mmol) were mixed together in 50 mL of degassed DMF at 0 °C in the presence of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide methyl iodide (EDAC·MeI) (8.58 g, 30.0 mmol) and 4-(*N*,*N*-dimethylamino)-pyridine (DMAP) (0.06 g). The reaction mixture was stirred at 0–5 °C for 6 h, and at room temperature overnight, with addition of 2.5 g of EDAC·MeI and 3 g of 1 5 h after starting the reaction. The progress of the reaction was monitored by TLC in $CHCl_3$/MeOH/AcOH (94:5:1) (M) and visualized by ninhydrin and UV. The reaction mixture was concentrated in vacuo, and the residue was redissolved in ethyl acetate (150 mL). This solution was washed with water (3 × 50 mL), 5% aqueous $NaHCO_3$ (3 × 50 mL), water (1 × 50 mL), 5% aqueous K_2HSO_4 (3 × 50 mL), and water (3 × 50 mL) and dried over $MgSO_4$. Filteration and concentration in vacuo gave a product that was crystallized from hexane to give a white solid (2 in Scheme II): 8.91 g (85%); mp 128–130 °C; IR (KBr) 3042 (C–H aromatic), 2972 (C–H aliphatic), 1785 (C=O of 5-oxazolidinone ring), 1731 (C=O of ester), 1688 (C=O of benzyloxycarbonyl) cm^{-1}; NMR (Me_2SO-d_6) δ 7.85 (d, 2 H), 7.61 (d, 2 H), 7.34 (m, 9 H), 5.12 (2s, 4 H), 4.52 (m, 2 H), 4.41 (m, 2 H), 4.21 (m, 1 H), 3.08 (m, 2 H); MS (EI) *m*/*z* (relative intensity) 458 (0.6), 457 (2.0), 179 (16), 178 (100), 92 (1), 91 (5.9); calcd. for $C_{27}H_{24}NO_6$ (mol wt 458.46); R_f (M) 0.88. Then 4.10 g of 2 (Scheme II) was ground to a fine powder and mixed with 30 mL of 30% HBr in acetic acid (Aldrich), the suspension was stirred at room temperature for 4 h, and the orange-colored solution was concentrated in vacuo to a viscous oil. This oil was mixed with diethyl ether (100 mL) and concentrated HCl (3.5 mL) and the solution rotary evaporated to dryness to give a red powder. This powder was suspended in ethyl acetate (100 mL), filtered, and washed with ethyl acette (100 mL) to give a white powder of 3 in Scheme II: 2.48 g (75%); mp 218–221 °C; R_f 0.28 ($CHCl_3$/MeOH/AcOH, 80:18:2) (N); NMR (Me_2SO-d_6) δ 7.86 (d, 2 H), 7.64 (d, 2 H), 7.35 (m, 4 H), 4.34 (d, 2 H), 4.25 (m, 2 H), 3.07 (d, 2 H); MS (EI) *m*/*z* (relative intensity) 311 (1.2), 310 (0.4), 180 (1.7), 179 (100), 178 (18.9), 91 (6.5); calcd for $C_{18}H_{17}NO_4$ (mol wt 311.32). Then 1.5 g of 2 (Scheme II) (4.3 mmol) was dissolved in 15 mL of degassed DMF and added dropwise through an additional funnel over 30 min to a stirred, cooled (ice bath) mixture of di-*tert*-butyl dicarbonate (Boc-anhydride) (1.38, 6.33 mmol) and triethylamine (2 mL, 14.3 mmol) in a 2:1 DCM/DMF mixture (30 mL). This reaction mixture was stirred at 0–5 °C for an additional 4 h, and the progress of the reaction was monitored by TLC and visualized by ninhydrin and UV. After 5 h, the reaction mixture was mixed with ethyl acetate (100 mL) and washed with water (3 × 30 mL), 5% aqueous $NaHCO_3$ (1 × 30 mL), water (1 × 30 mL), 5% aqueous K_2HSO_4, and water (2 × 30 mL), dried over $MgSO_4$, and concentrated in vacuo. Crystallization from 10% ethyl acetate in hexane gave a white solid (4): 1.35 g (71%); mp 132 °C; R_f (N) 0.76; NMR (Me_2SO-d_6) δ 7.86 (d, 2 H), 7.66 (m, 2 H), 7.35 (m, 4 H), 7.20 (d, 1 H), 4.27 (m, 4 H), 2.80 (m, 2 H), 1.36 (s, 9 H); $[\alpha]_{589}$ = +1.7 (*c* = 0.71, MeOH); MS (EI) *m*/*z* (relative intensity) 411 (0.1), 179 (17.8), 178 (100), 56 (8.7); calcd for $C_{23}H_{25}NO_6$ (mol wt 411.32).

Synthesis of N^α-Boc-Dpr-β-fluorenylmethoxycarbonyl [N^α-Boc-Dpr(β-Fmoc)]. N^α-(*tert*-Butyloxycarbonyl)asparagine (3.0 g, 12.91 mmol) was dissolved in 50 mL of ice bath cooled 1:1 mixture of water/DMF. Bis(trifluoroacetyl)iodobenzene (6.02 g, 14.0 mmoL) was added and the reaction mixture stirred at 0 °C for 30 min, followed by addition of pyridine (1.5 mL). The mixture was stirred at room temperature for 4 h, and the progress of the reaction was monitored by TLC (solvent system N), visualized by ninhydrin (R_f of product 0.29, R_f of reactant 0.36). Then the reaction mixture was concentrated in vacuo, and the residue was redissolved in a 1:1 mixture of acetone/water in presence of $NaHCO_3$ (4.21 g, 50.0 mmol) and cooled to 0 °C in an ice bath. To this reaction mixture at 0 °C was added 9-fluorenylmethyl succinimidyl carbonate (Fmoc-OSu) (4.70 g, 14.0 mmol), and the pH of the solution was kept at 8.5–8.8 by addition of 4 N NaOH aqueous solution. The reaction mixture was stirred at 0 °C for 5 h and then allowed to warm slowly to room temperature. Additional Fmoc-OSu (1.2 g) was added in addition to aliquots of 4 N NaOH to maintain the pH of the reaction at 8.5 for additional 1 h. The reaction mixture was concentrated to a small volume and the residue partitioned between ethyl acetate (150 mL) and ice-cooled water (50 mL). This stirred mixture was cooled in ice bath and acidified to pH 2.0 by cooled 2 N HCl. The organic layer was separated and washed further with 5% aqueous $NaHCO_3$ (1 × 50 mL), water (1 × 50 mL), and 5% aqueous K_2HSO_4 (2 × 50 mL), dried ($MgSO_4$), and concentrated in vacuo to give a viscous oil. The oil was dried in vacuo gave a white foamy solid: 3.23 g (66%); mp 62 °C; R_f 0.74 (solvent system N); NMR ($CDCl_3$) (1) δ 8.98 (br, 1 h, acid), 7.47 (m, 8 H, aromatic), 5.79 (br, d, 1 H, NH), 4.35 (m, 1 H), 4.31 (m, 2 H), 4.14 (m, 1 H), 3.62 (m, 2 H), 1.40 (S, 9 H); $[\alpha]^{24}_{589}$ = −5.0 (*c* = 0.3, MeOH); MS (EI) *m*/*z* (relative intensity) 426 (0.1), 238 (0.3), 196 (0.9), 179 (19), 178 (100), 101 (0.6), 57 (2.6); calcd for $C_{23}H_{26}N_2O_6$ (mol wt 426.46).

Peptide Synthesis. The peptides were synthesized by using a Vega 1000 peptide synthesizer or a manual synthesizer and standard solid-phase procedures.[14,19]

Method A (Cyclization on the Solid Support). Synthesis of Ac-Nle-Asp-His-D-Phe-Arg-Trp-Lys-NH_2 (V). The protected peptide resin to the title compound was prepared from 1.5 g of pMBHA resin (0.6 mmol of NH_2/g of resin) by first coupling N^α-Boc-Lys(N^ϵ-Fmoc) to the resin to a substitution level of 0.30 mmol/g of resin. The remaining amino groups were then blocked by acetylation using acetic anhydride. The remaining amino acid residues were then added to the growing peptide chain by stepwise addition of N^α-Boc-Trp(N^i-For), N^α-Boc-Arg(N^G-Tos), N^α-Boc-D-Phe, N^α-Boc-His(N^π-Bom), N^α-Boc-Asp(β-Fmo), and N^α-Boc-Nle by using standard solid-phase synthetic methodology. Each coupling reaction was achieved with a 3-fold excess of DIC and 2.4-fold excess of HOBt. After coupling the last amino acid, the Fmo and Fmoc protecting groups were removed by treating the

(23) Itah, M. *Chem. Pharm. Bull.* **1969**, *17*, 1679.

peptide resin with 50% piperidine in DMF for 1 h. The peptide resin was washed with DMF (3 × 40 mL), DCM (3 × 40 mL), 10% diisopropylethylamine (DIEA) (3 × 40 mL), and DCM (3 × 40 mL), suspended in 15 mL of DMF, and mixed with a 6-fold excess of BOP reagent[24,25] in the presence of an 8-fold excess of DIEA for 6 h. The coupling was repeated twice until the resin gave the negative ninyydrin test. After the cyclization, the N^{α}-Boc protecting group was removed and the amino group neutralized and acetylated with 25% of acetic anhydride in DCM for 0.5 h. The finished peptide resin weighed 1.86 g. Following cleavage of 0.5 g of the resin, workup, and purification as outlined below, V was obtained as white powder (87 mg, 58%). The analytical data for the compound are given in Table II, and the biological potencies are given in Table I.

Method B (Cyclization in Solution). Synthesis of Ac-Nle-Asp-His-D-Phe-Arg-Trp-Lys-NH_2 (V). The protected linear peptide Ac-Nle-Asp-His-D-Phe-Arg-Trp-Lys-NH_2 was synthesized as previously reported.[12] A 40-mg sample of HPLC pure linear peptide, Ac-Nle-Asp-His-D-Phe-Arg-Trp-Lys-NH_2, was converted to the hydrochloride salt by passing the peptide solution through DEAE-cellulose in its HCl form, with 5% hydrochloric acid solution as the eluent solvent,[26] followed by lyophilization. The dried peptide salt was dissolved in anhydrous DMF (10 mL) and cyclized by using DPPA (4 equiv) in the presence of anhydrous K_2HPO_4 (4 equiv).[27] The reaction mixture was stirred at 0 °C for 6 h and overnight at room temperature; the completion of the reaction was monitored by analytical HPLC (see Table II). After completion of cyclization (ninyhdrin negative[28]), the reaction mixture was quenched by addition of 30% acetic acid (5 mL). The organic solvent was removed by rotary evaporation under vacuum at 40 °C and the crude cyclic peptide applied to a Sephadex G-15 column and eluted by 15% acetic acid. The eluted peptide was monitored at 280 nm. A 23-mg sample of the gel-filtered cyclic peptide was isolated and purified by the reverse-phase semipreparative HPLC (RP-HPLC) (see below). The major peak was collected and lyophilized to give 12.5 mg (30%) of the title peptide. The analytical data for the purified compound are given in Table II, and the biological activities are given in Table I.

General Procedure for Cleavage, Removal of Protecting Groups, and Purification. The peptide resin was cleaved in anhydrous HF in the presence of anisole (~10%) and 1,2-dithioethane (~0.8%)[29] at 0 °C for 45 min in the usual manner.[14,15,19] The crude peptide powder was purified by cation-exchange chromatography on a CMC column with discontinuous NH_4OAc buffer solution[4,7] followed by gel filtration through Sephadex G-15 using 15% aqueous acetic acid as eluting solvent. The final purification was effected by preparative RP-HPLC on a C18 bonded silica column (Vydac 218TP1010, 1.0 × 25 cm), eluted with a linear acetonitrile gradient (20–40%) with a constant concentration of trifluoroacetic acid (0.1% v/v). The linear gradient was generated with a Spectra-Physics SP 8800 ternary pump system. The separation was monitored at 280 nm with an ABI Spectra-Flow 757 absorbance detector, and the peaks were integrated with a Spectra-Physics SP 4270 integrator. The purity of the finished peptide was checked by TLC in at least three solvents and analytical RP-HPLC at 280 and 223 nm. The structures of the pure peptides were confirmed by fast atom bombardment (FAB) mass spectrometry, amino acid analysis (Table II), and 250-MHz 1D and 2D proton nuclear magnetic resonance (NMR) spectroscopy.

Ac-[Nle[4],Glu[5],D-Phe[7],Lys[10],Gly[11]]-α-MSH_{4-13}-NH_2 (II). This peptide was prepared by solution synthesis of the linear precursor peptide[12] followed by cyclization in solution (Scheme I, method B). Starting with 18.3 mg of hydrochloride salt of linear precursor of II which was cyclized and worked up as for V (vide supra), the title peptide II was obtained as a white powder (8.2 mg, 45%). The biological activities and the analytical data are given in Tables I and II, respectively.

Ac-[Nle[4],Asp[5],D-Phe[7],Lys[10],Gly[11]]-α-MSH_{4-13}-NH_2 (III). This peptide was synthesized by both method A and method B (Scheme I). A 42-mg sample of the HPLC pure linear precursor peptide[12] of III was cyclized in solution to give 18.6 mg of the title peptide. Alternatively, cleavage of 0.53 g of the precursor title peptide resin Ac-Nle-Asp-His(N^{π}-Bom)-D-Phe-Arg(N^G-Tos)-Trp(N^i-For)-Lys-Gly-Pro-Val-pMBHA-resin, 0.3 mmol/g substitution) gave 133 mg (83%) of crude peptide. A 25-mg sample of the crude peptide was HPLC purified to give 14.5 mg (58% yield) of the title peptide as a white powder. The biological activities and the analytical data are given in Tables I and II, respectively.

Ac-[Nle[4],Glu[5],D-Phe[7],Lys[10]]-α-MSH_{4-10}-NH_2 (IV). This peptide was synthesized according to method B. Starting with 25.2 mg of hydrochloride salt of the linear precursor peptide Ac-Nle-Glu-His-D-Phe-Arg-Trp-Lys-NH_2,[12] 10.6 mg (42% yield) of HPLC-pure cyclic peptide was obtained. The biological activities and the analytical data are given in Tables I and II, respectively.

Ac-[Nle[4],Asp[5],D-Phe[7],Orn[10]]-α-MSH_{4-10}-NH_2 (VI). This peptide was prepared by cyclization in solution (Scheme I, method B). A 24-mg sample of linear precursor peptide Ac-Nle-Asp-His-D-Phe-Arg-Trp-Orn-NH_2[12] gave 10.2 mg (43% yield) of cyclic peptide VI. The biological activities and the analytical data are given in Tables I and II, respectively.

Ac-[Nle[4],Asp[5],D-Phe[7],Dab[10]]-α-MSH_{4-10}-NH_2 (VII). This peptide was prepared according to method B (Scheme I). Cyclization of 21 mg of the linear precursor peptide Ac-Nle-Asp-His-D-Phe-Arg-Trp-Dab-NH_2[12] gave 8.9 mg (38% yield) of the title peptide. The biological activities and the analytical data are given in Tables I and II, respectively.

Ac-[Nle[4],Asp[5],D-Phe[7],Dpr[10]]-α-MSH_{4-10}-NH_2 (VIII). The title peptide was prepared by cyclization in solution (method B, Scheme I) as well as by cyclization on resin (method A, Scheme I). A 16.8-mg sample of the linear precursor peptide Ac-Nle-Asp-D-Phe-Arg-Trp-Dpr-NH_2[12] was cyclized in solution to give 6.5 mg (35% yield) of the title peptide. Alternatively, cleavage of 0.66 g of the peptide resin (0.35 mmol/g substitution) to the title compound [Ac-Nle-Asp-His(N^{π}-Bom)-Arg(N^G-Tos)-Trp-(N^i-For)-Dab-pMBHA-resin] gave 113 mg (85% yield) of crude peptide. A 20-mg sample of the crude peptide was HPLC purified to give 12.8 mg (64% yield) of a white powder of the title peptide. The biological activities and the analytical data are given in Tables I and II, respectively.

Bioassays. Bioassays using frog (*R. pipiens*) and lizard (*A. carolinensis*) skins were performed by published procedures.[30-32] In these bioassays skins become dark in response to melanin granule dispersion within melanocytes in response to a melanotropin. This darkening response can be conveniently monitored by photoreflectance methods. The potency of each peptide was determined from dose–response curves comparing the melanotropic activity of the analogues with that of the native hormone, α-MSH. Prolonged residual biological activities of the peptides were monitored for 6–24 h after removal of all peptide from the bathing fluid by several washes.[7,11,32] Selected dose–response curves are shown in Figure 1, and Table I lists the potencies and prolonged activities (positive or negative) of all of the cyclic

(24) Castro, B.; Dormoy, J. R.; Evin, G.; Seive, C. *Tetrahedron Lett.* **1975**, 1219.
(25) Nguyen, D. L.; Castro, B. *Peptide Chemistry*; Shiba, T., Sakakibara, S., Eds.; Protein Research Foundation: Osaka, Japan, 1987; pp 231–240.
(26) Danzel, B.; Rivier, J.; Goodman, M. *Biopolymers* **1977**, *16*, 2587.
(27) Bradley, S. F.; Paleveda, W. J.; Arison, B. H.; Freidinger, R. M.; Nutt, R. F.; Veber, D. F. *Peptides: Synthesis, Structure and Function*; Hruby, V. J., Rich, D. H., Eds.; Pierce Chemical Co.: Rockford, IL, 1983; pp 126–129.
(28) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 595.
(29) Matsueda, G. R. *Int. J. Pept. Protein Res* **1982**, *20*, 26.
(30) Shizume, K.; Lerner, A. B.; Fitzpatrick, T. B. *Endocrinology* **1954**, *54*, 553.
(31) Huntington, T.; Hadley, M. E. *Endocrinology* **1974**, *95*, 472.
(32) Castrucci, A. M. L.; Hadley, M. E.; Hruby, V. J. *Gen. Comp. Endocrinol.* **1984**, *55*, 104.

peptides in both assay systems.

Acknowledgment. This research was supported by U.S. Public Health Service Grants DK 17420 and AR 36021 and by National Science Foundation Grant DMB 8712133.

Registry No. 1, 23632-66-8; 2, 122235-68-1; 3, 122235-69-2; 4, 117014-32-1; II, 121076-34-4; II (open chain)·HCl, 122235-71-6; III, 122235-72-7; III (open chain), 121062-05-3; IV, 121062-07-5; IV (open chain)·HCl, 122269-68-5; V, 121062-08-6; V (open chain), 117499-53-3; VI, 121076-35-5; VI (open chain), 117499-55-5; VII, 122330-53-4; VII (open chain), 117499-56-6; VIII, 122330-54-5; VIII (open chain), 117499-57-7; BOC-Dpr(Fmoc)-OH, 122235-70-5; BOC-Asn-OH, 7536-55-2; Z-Asp-OH, 1152-61-0; BOC-Arg-(Tos)-OH, 13836-37-8; BOC-D-Phe-OH, 18942-49-9; BOC-His-(N^π-BOM)-OH, 79950-65-5; BOC-Trp(For)-OH, 47355-10-2; BOC-Asp(OBzl)-OH, 7536-58-5; BOC-Nle-OH, 6404-28-0; BOC-Lys(Fmoc)-OH, 84624-27-1.

Synthesis and Structure–Activity Relationships of a Series of Anxioselective Pyrazolopyridine Ester and Amide Anxiolytic Agents[1]

Thomas M. Bare,*,† Charles D. McLaren,† James B. Campbell,† Judy W. Firor,† James F. Resch,† Claudia P. Walters,† Andre I. Salama,‡ Brad A. Meiners,‡ and Jitendra B. Patel‡

Departments of Medicinal Chemistry and Pharmacology, ICI Pharmaceuticals Group, A Business Unit of ICI Americas Inc., Wilmington, Delaware 19897. *Received February 6, 1989*

A series of 1-substituted 4-amino-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxylic acid esters and amides were synthesized and screened for anxiolytic activity in the shock-induced suppression of drinking (SSD) test. The compounds were also tested for their ability to displace [^{3}H]flunitrazepam (FLU) from brain benzodiazepine (BZ) binding sites. Many compounds were active in these screens and, additionally, demonstrated a selectivity for the type 1 BZ (BZ_1) receptor over the type 2 BZ (BZ_2) receptor as indicated by Hill coefficients significantly less than unity and by analysis of [^{3}H]FLU binding results from different brain regions. Based on the results of structure–activity studies of these compounds, a hypothesis was proposed to explain the structural features necessary for optimal interaction with brain BZ receptors. A detailed pharmacological evaluation of one of the most potent behaviorally active compounds (**27**) demonstrated it to be BZ_1 selective; also, in comparison to diazepam, **27** showed minimal sedative and alcohol interactive properties at therapeutically effective doses.

While the benzodiazepines (BZs) are currently the agents of choice for the treatment of anxiety, they produce a number of undesirable side effects[2] including ataxia, sedation, and a synergistic effect with ethanol and other central nervous system (CNS) depressants. Recent research efforts in the anxiolytic area have been aimed at discovering anxioselective agents,[3] i.e., compounds which at therapeutically effective doses show a reduced propensity to cause one or more of the unwanted ancillary activities associated with BZ usage. These research efforts are indeed beginning to reach fruition as evidenced by the appearance of a number of structurally novel agents purporting to exhibit anxioselective activity.[4]

Our program to discover a non-BZ anxioselective anxiolytic was aided by the discovery in 1977 of high affinity, saturable, and stereospecific binding sites in the CNS for the BZs.[5,6] The ability of the BZs to displace [^{3}H]diazepam or [^{3}H]flunitrazepam (FLU) highly correlates with their respective pharmacological[5-7] and clinical[8] properties. Consequently, the BZ binding sites have been postulated to mediate the therapeutic actions of the BZs.[5,6,9] While initial studies indicated these binding sites to be homogeneous, subsequent findings suggested the existence in the brain of two main subtypes.[10,11] The type 1 or BZ_1 receptors are located preferentially in the cerebellum while the type 2 or BZ_2 receptors are located, along with BZ_1 receptors, in the hippocampus, cortex, and other brain regions.[12,13] While the BZs appear to bind equally well to both BZ_1 and BZ_2 receptors[14] (Hill coefficient = 1[15]), certain novel potential anxiolytic compounds have been shown to demonstrate a selective affinity for the type 1 receptor (Hill coefficient in the cortex significantly < 1). For example, the triazolopyridazine CL 218,872 was shown to bind selectively to the BZ_1 receptor[10,11] and to lack the marked ataxic and depressant side effects associated with

† Department of Medicinal Chemistry.
‡ Department of Pharmacology.

(1) Preliminary accounts of part of this work were presented earlier: (a) Bare, T. M.; Campbell, J. B.; Frazee, W. J.; Giles, R. E.; Goldberg, M. E.; Hesp, B.; Malick, J. B.; Meiners, B.; Patel, J.; Resch, J. F.; Salama, A.; U'Prichard, D. C.; Warawa, E. J. *Abstracts of a Symposium on Non-Benzodiazepine Anxiolytics and Hypnotics*; Rome, Italy, Feb. 16, 1987; p 11. (b) Bare, T. M.; Patel, J. B.; Salama, A. I.; Meiners, B. A. *Abstracts of Papers*, 194th National Meeting of the American Chemical Society, New Orleans, LA; August 30–September 4, 1987, American Chemical Society: Washington, DC, 1987; Division of Medicinal Chemistry, Abstract No. 88.
(2) Bellantuono, C.; Reggi, V.; Tognoni, G.; Garattini, S. *Drugs.* **1980**, *19*, 195.
(3) Williams, M. *J. Med. Chem.* **1983**, *26*, 619.
(4) For a recent review, see: Bare, T. M.; Resch, J. F.; Patel, J. B. Anxiolytics/Sedative Hypnotics. In *Annual Reports in Medicinal Chemistry*; Bailey, D. M., Ed.; Academic Press, Inc.: New York, 1987; Vol. 22, pp 11–20.
(5) Squires, R. F.; Braestrup, C. *Nature (London)* **1977**, *266*, 732.
(6) Mohler, H.; Okada, T. *Science* **1977**, *198*, 849.
(7) Lippa, A. S.; Klepner, C. A.; Yunger, L.; Sano, M. C.; Smith, W. V.; Beer, B. *Pharmacol. Biochem. Behav.* **1978**, *9*, 853.
(8) Mohler, H.; Okada, T. *Br. J. Psychiatry* **1978**, *133*, 261.
(9) Chang, R. S. L.; Snyder, S. H. *Eur. J. Pharmacol.* **1978**, *48*, 213.
(10) Klepner, C. A.; Lippa, A. S.; Benson, D. I.; Sano, M. C.; Beer, B. *Pharmacol. Biochem. Behav.* **1979**, *11*, 457.
(11) Dubnick, B.; Lippa, A. S.; Klepner, C. A.; Couplet, J.; Greenblatt, E. N.; Beer, B. *Pharmacol. Biochem. Behav.* **1983**, *18*, 311.
(12) Young, W. S.; Niehoff, D.; Kuhar, M. J.; Beer, B.; Lippa, A. S. *J. Pharmacol. Exp. Ther.* **1981**, *216*, 425.
(13) Richards, J. G.; Mohler, H. *Neuropharmacology* **1984**, *23*, 233.
(14) Braestrup, C.; Squires, R. F. *Eur. J. Pharmacol.* **1978**, *48*, 263.
(15) For a discussion of the relationship of Hill coefficients and receptor multiplicity, see: (a) Braestrup, C.; Nielsen, M. *Trends Neurosci.* **1980**, *301*. (b) Lippa, A. S.; Meyerson, L. R.; Beer, B. *Life Sci.* **1982**, *31*, 1409.

0022-2623/89/1832-2561$01.50/0 © 1989 American Chemical Society