pieces of ca. 10 mg of fresh weight derived from *Nicotiana tabacum* L. var. Wisconsin No. 38 were implanted on the agar surface and maintained at 28 °C in darkness for 4 weeks, and then the average fresh weight was determined. The standard deviation of the activity measurements was within ±30%.

Acknowledgment. H.I. is indebted to the Ministry of Education for financial support by Grant No. 56390005 for scientific research. H.I. is also indebted to Prof. Toshio Fujita for his helpful discussions. The regression analyses were carried out on a FACOM M-382 computer of the Data Processing Center of this university.

Registry No. 1, 94993-30-3; 2, 94993-31-4; 3, 72564-68-2; 4, 72564-67-1; 5, 94993-32-5; 6, 94993-33-6; 7, 94993-34-7; 8, 72564-71-7; 9, 72564-70-6; 10, 72564-73-9; 11, 72564-69-3; 12, 72564-72-8; 13, 94993-35-8; 14, 72564-65-9; 15, 94993-36-9; 16, 72564-63-7; 17, 94993-37-0; 18, 94993-38-1; 19, 94993-39-2; 20, 72564-74-0; 21, 94993-40-5; 22, 94993-41-6; 23, 94993-42-7; 24, 94993-43-8; 25, 94993-44-9; 26, 94993-45-0; 27, 94993-46-1; 28, 94993-47-2; 29, 94993-48-3; 30, 94993-49-4; 31, 94993-50-7; 32, 94993-51-8; 33, 94993-52-9; 34, 94993-53-0; 35, 94993-54-1; 36, 94993-55-2; 37, 94993-56-3; 38, 94993-57-4; 39, 94993-58-5; 40, 94993-59-6; 41, 94993-60-9; 42, 94993-62-1; 43, 94993-63-2; 44, 94993-64-3; 45, 94993-65-4; 46, 94993-66-5; 47, 94993-67-6; 48, 94993-68-7; CH_3NH_2, 74-89-5; $CH_3(CH_2)_2NH_2$, 107-10-8; $CH_3(CH_2)_5NH_2$, 111-26-2; $CH_3(CH_2)_6NH_2$, 111-68-2; c-$C_3H_5NH_2$, 765-30-0; $CH_3CH_2(CH_3CH_2)CHNH_2$, 616-24-0; CH_2=$CHCH_2NH_2$, 107-11-9; $CH_3O(CH_2)_2NH_2$, 109-85-3; $CH_3CH_2O(CH_2)_2NH_2$, 110-76-9; $CH_3(CH_2)_7NH_2$, 111-86-4; $C_6H_5NH_2$, 62-53-3; F-*p*-$C_6H_4NH_2$, 371-40-4; Cl-*p*-$C_6H_4NH_2$, 106-47-8; Br-*p*-$C_6H_4NH_2$, 106-40-1; Et-*p*-$C_6H_4NH_2$, 589-16-2; MeO-*p*-$C_6H_4NH_2$, 104-94-9; EtO-*p*-$C_6H_4NH_2$, 156-43-4; Ac-*p*-$C_6H_4NH_2$, 99-92-3; CN-*p*-$C_6H_4NH_2$, 873-74-5; NO_2-*m*-$C_6H_4NH_2$, 99-09-2; Ac-*m*-$C_6H_4NH_2$, 99-03-6; COOH-*m*-$C_6H_4NH_2$, 99-05-8; NO_2-*p*-$C_6H_4NH_2$, 100-01-6; F-*m*-$C_6H_4NH_2$, 372-19-0; Br-*m*-$C_6H_4NH_2$, 591-19-5; I-*m*-$C_6H_4NH_2$, 626-01-7; Me-*m*-$C_6H_4NH_2$, 108-44-1; Et-*m*-$C_6H_4NH_2$, 587-02-0; HO-*m*-$C_6H_4NH_2$, 591-27-5; CN-*m*-$C_6H_4NH_2$, 2237-30-1; I-*p*-$C_6H_4NH_2$, 540-37-4; COOH-*p*-$C_6H_4NH_2$, 150-13-0; Me-*o*-$C_6H_4NH_2$, 95-53-4; 3,5-Cl_2-$C_6H_3NH_2$, 626-43-7; 3,5-Me_2-$C_6H_3NH_2$, 108-69-0; 2,5-Me_2-$C_6H_3NH_2$, 95-78-3; 3,4-Me_2-$C_6H_3NH_2$, 95-64-7; 2,4-Cl_2-$C_6H_3NH_2$, 554-00-7; Cl-*o*-$C_6H_4NH_2$, 95-51-2; 4-chloro-2-(methylthio)pyrido[2,3-*d*]pyrimidine, 72564-62-6.

Cyclic Melanotropins. 9.[1] 7-D-Phenylalanine Analogues of the Active-Site Sequence

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The cyclic melanotropin Ac-Ser[1]-Tyr[2]-Ser[3]-Cys[4]-Glu[5]-His[6]-Phe[7]-Arg[8]-Trp[9]-Cys[10]-Lys[11]-Pro[12]-Val[13]-NH_2 is a highly potent agonist as determined in several melanocyte bioassays. In linear melanotropins, a D-Phe[7] substitution leads to increased potency and often prolonged biological activity. In order to determine if this substitution would have the same effect in cyclic melanotropins, we have prepared a series of these analogues. The D-Phe[7]-substituted cyclic melanotropins Ac-[Cys[4],D-Phe[7],Cys[10]]-α-MSH_{4-10}-NH_2 and Ac-[Cys[4],D-Phe[7],Cys[10]]-α-MSH_{4-11}-NH_2 were both more potent than their cyclic L-Phe[7]-containing counterparts in either the frog or lizard skin bioassay by more than a factor of 10. Neither peptide, however, exhibited prolongation of biological activity in either assay. Substitution of D-Phe[7] into the cyclic 4–12 and 4–13 sequences led to a slight or no increase in potency in both assays relative to the L-Phe[7] counterparts, but the activity of the melanotropins was ultraprolonged in each assay. Ac-[Cys[4],D-Phe[7],Cys[10]]-α-MSH_{4-12}-NH_2 was about equipotent to Ac-[Cys[4],D-Phe[7],Cys[10]]-α-MSH_{4-13}-NH_2, again demonstrating, as with certain linear and cyclic L-Phe[7]-containing melanotropins, that the C-terminal amino acid valine is not required for biological activity or for superpotency. Similar to the linear D-Phe[7] analogues that possessed ultraprolonged melanotropic activity, the 4–12 and 4–13 cyclic D-Phe[7] analogues also displayed the phenomenon of superagonism, which is a time-dependent increase in efficacy over that produced by an equipotent concentration of the native hormone. Cyclization of certain linear melanotropins resulted in analogues with increased resistance to biological degradation by serum enzymes or purified proteolytic enzymes. Further, incorporation of a D-Phe[7] into in the cyclic analogues led to melanotropins that were totally resistant to enzymatic inactivation by trypsin.

α-Melanocyte stimulating hormone (α-melanotropin, α-MSH) is a linear tridecapeptide that is synthesized in cells of the pars intermedia and the brain.[2] α-MSH stimulates melanin biosynthesis and melanosome dispersion within integumental melanophores,[3,4] and it appears to have numerous other physiological functions as well.[5,6] It has been proposed that a reverse turn is important for the biological activity of α-MSH at certain receptors partially on the basis of the much higher activity of linear analogues containing a D-Phe in the 7-position[7,8] (D-amino acids can stabilize reverse turns[9]). The importance of a reverse turn to the increased biological activity of α-MSH also has been suggested by the superpotency of cyclic

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I.	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	α-MSH
II.	Ac-Ser-Tyr-Ser-Cys-Glu-His-Phe-Arg-Trp-Cys-Lys-Pro-Val-NH_2	[Cys⁴,Cys¹⁰]-α-MSH
III.	Ac-Cys-Glu-His-Phe-Arg-Trp-Cys-NH_2	Ac-[Cys⁴,Cys¹⁰]-α-MSH_{4-10}-NH_2
IV.	Ac-Cys-Glu-His-Phe-Arg-Trp-Cys-Lys-NH_2	Ac-[Cys⁴,Cys¹⁰]-α-MSH_{4-11}-NH_2
V.	Ac-Cys-Glu-His-Phe-Arg-Trp-Cys-Lys-Pro-NH_2	Ac-[Cys⁴,Cys¹⁰]-α-MSH_{4-12}-NH_2
VI.	Ac-Cys-Glu-His-Phe-Arg-Trp-Cys-Lys-Pro-Val-NH_2	Ac-[Cys⁴,Cys¹⁰]-α-MSH_{4-13}-NH_2
VII.	Ac-Cys-Glu-His-D-Phe-Arg-Trp-Cys-NH_2	Ac-[Cys⁴,D-Phe⁷,Cys¹⁰]-α-MSH_{4-10}-NH_2
VIII.	Ac-Cys-Glu-His-D-Phe-Arg-Trp-Cys-Lys-NH_2	Ac-[Cys⁴,D-Phe⁷,Cys¹⁰]-α-MSH_{4-11}-NH_2
IX.	Ac-Cys-Glu-His-D-Phe-Arg-Trp-Cys-Lys-Pro-NH_2	Ac-[Cys⁴,D-Phe⁷,Cys¹⁰]-α-MSH_{4-12}-NH_2
X.	Ac-Cys-Glu-His-D-Phe-Arg-Trp-Cys-Lys-Pro-Val-NH_2	Ac-[Cys⁴,D-Phe⁷,Cys¹⁰]-α-MSH_{4-13}-NH_2

Figure 1. Primary structures of the melanotropins studied.

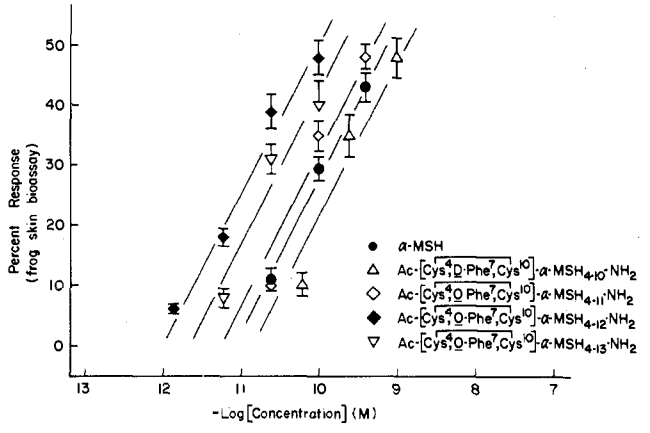

Figure 2. Comparative in vitro dose–response curves of a number of cyclic [Cys⁴,D-Phe⁷,Cys¹⁰] melanotropins as determined by the frog (*Rana pipiens*) skin bioassay. Each value represents the mean (± SE) darkening response of the skins (N = 6 or more in all experiments) to the melanotropins at the concentrations noted.

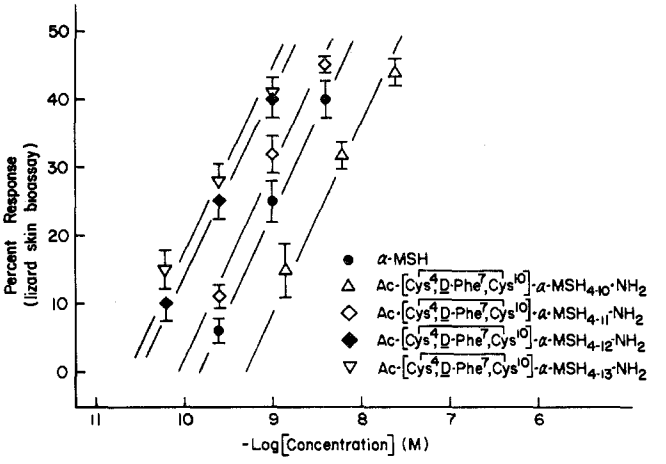

Figure 3. As in Figure 2. Lizard (*Anolis carolinensis*) skin bioassay.

melanotropins such as [Cys⁴,Cys¹⁰]-α-MSH, Ac-[Cys⁴,Cys¹⁰]-α-MSH_{4-13}-NH_2, and Ac-[Cys⁴,Cys¹⁰]-α-MSH_{4-12}-NH_2.[10-12] Recently, we investigated the role of the C-terminal tripeptide Lys^{11}-Pro^{12}-Val^{13} in the melanotropic action of cyclic analogues of α-MSH.[12] We determined the contribution of the individual residues of the C-terminal tripeptide to the superpotency of cyclic melanotropins by the stepwise elongation of the central heptapeptide Ac-[Cys⁴,Cys¹⁰]-α-MSH_{4-10}-NH_2 to include the 4–11, 4–12, and 4–13 sequences and found that Val^{13} was not required for the biological activity or superpotency of the cyclic melanotropins.[12]

To further investigate the effect of a D-Phe^7 substitution on the overall biological potency and prolonged activity of the linear and cyclic melanotropins, we have prepared a series of cyclic analogues that contain a D-Phe^7 residue: Ac-[Cys⁴,D-Phe⁷,Cys¹⁰]-α-MSH_{4-10}-NH_2, Ac-[Cys⁴,D-Phe⁷,Cys¹⁰]-α-MSH_{4-11}-NH_2, Ac-[Cys⁴,D-Phe⁷,Cys¹⁰]-α-MSH_{4-12}-NH_2, and Ac-[Cys⁴,D-Phe⁷,Cys¹⁰]-α-$MSH_{4-13}NH_2$ (Figure 1). We then examined their biological activities in order to determine whether the increase in biological potency and prolongation seen in linear D-Phe^7 analogues over their L-Phe^7 counterparts was also observed in the cyclic D-Phe^7 analogues. The results obtained provide new insight into the relative importance of the D-Phe^7 substitution itself vs. its reverse turn stabilizing ability on biological potency.

Table I. Biological Activities of D-Phe^7-Substituted Melanotropins

compound	frog[a]	lizard[a]
I, α-MSH	1.00	1.00
II, Ac-[Cys⁴,Cys¹⁰]-α-MSH_{4-10}-NH_2	0.06	0.003
III, Ac-[Cys⁴,Cys¹⁰]-α-MSH_{4-11}-NH_2	0.16	0.07
IV, Ac-[Cys⁴,Cys¹⁰]-α-MSH_{4-12}-NH_2	10.0	1.5
V, Ac-[Cys⁴,Cys¹⁰]-α-MSH_{4-13}-NH_2	30.0	0.6
VI, [Cys⁴,Cys¹⁰]-α-MSH	10.0	2.0
VII, Ac-[Cys⁴,D-Phe⁷,Cys¹⁰]-α-MSH_{4-10}-NH_2	0.75	0.5
VIII, Ac-[Cys⁴,D-Phe⁷,Cys¹⁰]-α-MSH_{4-11}-NH_2	2.5	3.0
IX, Ac-[Cys⁴,D-Phe⁷,Cys¹⁰]-α-MSH_{4-12}-NH_2	20.0	6.0
X, Ac-[Cys⁴,D-Phe⁷,Cys¹⁰]-α-MSH_{4-13}-NH_2	6.0	6.0

[a] Potencies are calculated along the linear portion of the dose–response curves and standardized to α-MSH = 1.00 (I). Potencies for compounds II–VI were previously reported in ref 12. The minimum effective dose is much greater for compounds IV–VI in the frog skin bioassay.[12]

Results

The cyclic peptides (Ac-[Cys⁴,D-Phe⁷,Cys¹⁰]-α-

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MSH_{4-10}-NH_2, Ac-[Cys^4,D-Phe^7,Cys^{10}]-α-MSH_{4-11}-NH_2, Ac-[Cys^4,D-Phe^7,Cys^{10}]-α-MSH_{4-12}-NH_2, and Ac-[Cys^4,D-Phe^7,Cys^{10}]-α-MSH_{4-13}-NH_2) were synthesized and purified by methods similar to those used previously for cyclic melanotropins.[10,11] Then they were tested for their biological activity in the frog (*Rana pipiens*) (Figure 2) and the lizard (*Anolis carolinensis*) (Figure 3) skin bioassays (see Experimental Section). Ac-[Cys^4,D-Phe^7,Cys^{10}]-α-MSH_{4-10}-NH_2 was a full agonist, slightly less potent than α-MSH in both assay systems, but considerably more potent than the corresponding L-Phe^7 analogue (Table I). As the amino acids Lys^{11} and Pro^{12} were successively added to the C terminus, a progressive increase in potency was observed in both the frog and lizard skin bioassays. The addition of Val^{13} resulted in no further increase of melanotropic activity in the lizard skin bioassay (Table I), whereas in the frog skin bioassay, the 4–13 sequence actually showed a decrease in biological activity in the cyclic D-Phe^7 series, with a 3–4 fold lower potency than the 4–12 analogue.

Previously we reported that the biological activity of linear melanotropins was enhanced by the presence of a D-Phe (rather than L-Phe) in the 7-position.[3,4] We again observed this situation in the case of the cyclic analogues, an effect that was especially predominant in the shorter sequences Ac-[Cys^4,D-Phe^7,Cys^{10}]-α-MSH_{4-10}-NH_2 and Ac-[Cys^4,D-Phe^7,Cys^{10}]-α-MSH_{4-11}-NH_2 (Table I). In fact, in these cases the D-Phe^7 analogues were 10–100 times more potent than the corresponding D-Phe^7 peptides. For example, Ac-[Cys^4,D-Phe^7,Cys^{10}]-α-MSH_{4-11}-NH_2 was more potent than α-MSH on both assays and 15 and 40 times more potent than the corresponding L-Phe^7 cyclic analogue on the frog and lizard skin bioassay, respectively. The longer 4–12 and 4–13 D-Phe^7 peptides did not show this increase in potency but instead showed similar potencies as the corresponding L-Phe^7 analogues with one exception, Ac-[Cys^4,D-Phe^7,Cys^{10}]-α-MSH_{4-13}-NH_2, which was 10 times more active than the L-containing analogue on the lizard skin bioassay.

Another biological phenomenon observed for linear Nle^4,D-Phe^7 α-MSH fragments of the 4–11 or longer sequences was prolongation of melanotropic activity.[7,8] None of the synthesized cyclic L-Phe^7 analogues (Table I) were prolonged[13] but neither were the Ac-[Cys^4,D-Phe^7,Cys^{10}]-α-MSH_{4-10}-NH_2 and Ac-[Cys^4,D-Phe^7,Cys^{10}]-α-MSH_{4-11}-NH_2 fragment analogues. However, both Ac-[Cys^4,D-Phe^7,Cys^{10}]-α-MSH_{4-12}-NH_2 and Ac-[Cys^4,D-Phe^7,Cys^{10}]-α-MSH_{4-13}-NH_2 exhibited ultraprolonged activity (Figure 4) in both assay systems.

The resistance of melanotropins to enzymatic degradation is greatly increased in the cyclic series of analogues,[14-16] and further substitution of L-Phe^7 by its D enantiomer results in cyclic D-Phe^7 melanotropins that are nonbiodegradable by trypsin. The relative resistance of the 4–13 cyclic analogue to trypsin was chosen to illustrate this trend (Figure 5). Ac-[Nle^4]-α-MSH_{4-13}-NH_2 is rapidly inactivated, whereas Ac-[Cys^4,Cys^{10}]-α-MSH_{4-13}-NH_2 is slowly degraded and Ac-[Cys^4,D-Phe^7,Cys^{10}]-α-MSH_{4-13}-NH_2 is completely stable even after 120 min (Figure 5) in the presence of the enzyme.

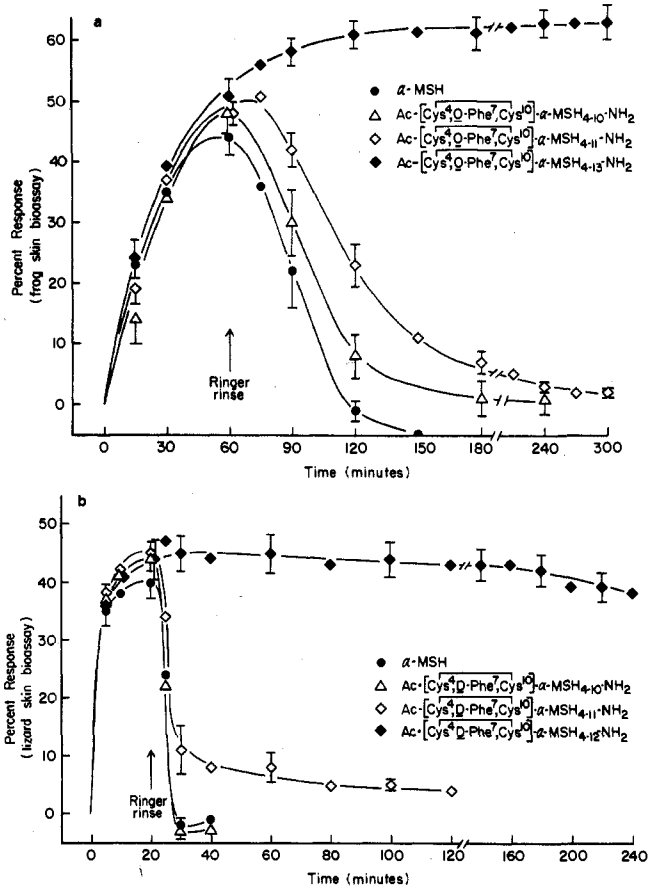

Figure 4. In vitro demonstration of the prolonged activity (frog skin darkening) of Ac-[Cys^4,D-Phe^7,Cys^{10}]-α-MSH_{4-13}-NH_2 (♦), compared to Ac-[Cys^4,D-Phe^7,Cys^{10}]-α-MSH_{4-10}-NH_2 (△), Ac-[Cys^4,D-Phe^7,Cys^{10}]-α-MSH_{4-11}-NH_2 (◇), and α-MSH (●). Each value represents the mean (± SE) response of the skins (N = 6) to the melanotropins, at the times noted. (b) In vitro demonstration of the prolonged activity (lizard skin darkening) of Ac-[Cys^4,D-Phe^7,Cys^{10}]-α-MSH_{4-12}-NH_2 (♦) compared to Ac-[Cys^4,D-Phe,Cys^{10}]-α-MSH_{4-10}-NH_2 (△), Ac-[Cys^4,D-Phe^7,Cys^{10}]-α-MSH_{4-11}-NH_2 (◇), and α-MSH (●). Each value represents the mean (± SE) response of the skins (N = 6) to the melanotropins at the time indicated.

Superagonism has been previously observed with certain linear D-Phe^7-containing melanotropins, such as [Nle^4,D-Phe^7]-α-MSH.[8] This term has been used to denote an increase in the efficacy of the biological response to an analogue over that produced by the native hormone at concentrations of the analogue and hormone chosen to elicit the same biological response after a given incubation time (60 min). Among the cyclic D-Phe^7 melanotropins, only the 4–12 (not shown) and 4–13 sequences (Figure 6) exhibit this characteristic.

Discussion

We have previously reported on the importance of the individual residues of the C-terminal tripeptide Lys^{11}-

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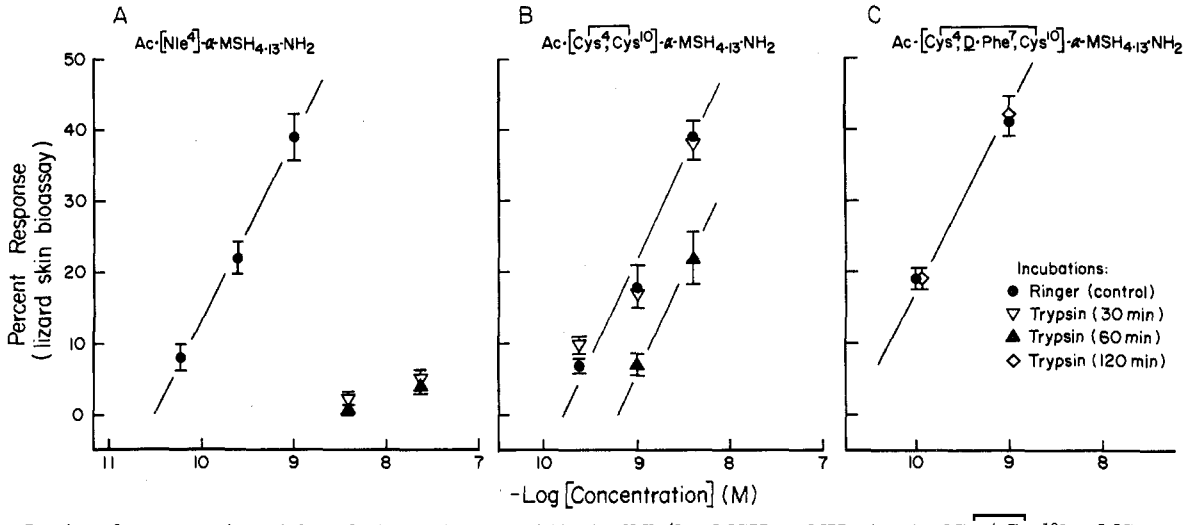

Figure 5. In vitro demonstration of the relative resistance of (A) Ac-[Nle^4]-α-MSH$_{4-13}$-NH$_2$, (B) Ac-[Cys^4,Cys^{10}]-α-MSH$_{4-13}$-NH$_2$, and (C) Ac-[Cys^4,D-Phe^7,Cys^{10}]-α-MSH$_{4-13}$-NH$_2$ to incubation in the presence of trypsin, as determined by the lizard skin bioassay. Each value represents the mean (± SE) darkening response of the skins (N = 6) at the concentrations noted.

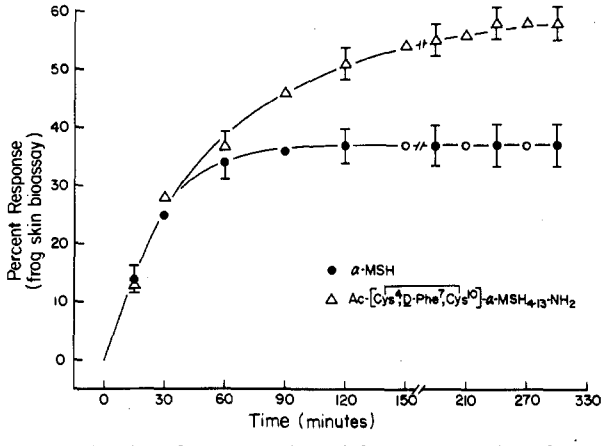

Figure 6. In vitro demonstration of the superagonism shown by Ac-[Cys^4,D-Phe^7,Cys^{10}]-α-MSH$_{4-13}$-NH$_2$ (1.25×10^{-11} M) compared to α-MSH (5.00×10^{-11} M) in the frog skin bioassay. Each value represents the mean (± SE) darkening response to the skins (N = 6) to the melanotropins, at the times indicated.

Pro^{12}-Val^{13} to the biological potency of cyclic L-Phe^7 α-MSH fragments.[12] We have also investigated the importance of substituting a D-Phe^7 for L-Phe^7 to the biological potency and prolongation of biological activity in linear α-MSH analogues.[7,8] We have now examined the effect of the D-Phe^7 substitution in several cyclic melanotropins.

As would be predicted from the cyclic L-Phe^7 analogues,[12] the sequential addition of Lys^{11} and Pro^{12} to the C terminus of the central cyclic heptapeptide (Ac-Cys^4-Glu^5-His^6-D-Phe^7-Arg^8-Trp^9-Cys^{10}-NH$_2$) results in an increase in biological potency. Interestingly, as seen in L-Phe^7 compounds, incorporation of Val^{13} onto the C terminus did not lead to an increase in the biological potency of the 4–12 sequence. This again illustrates that only the 4–12 sequence of α-MSH is needed for superpotency in cyclic melanotropins.[12]

Examination of the relative potencies of the cyclic L-Phe^7 and D-Phe^7 analogues upon addition of the same amino acid residues to the C terminus leads to an interesting trend. In the shorter 4–10 and 4–11 sequences, the D-Phe^7 analogues were about 10–100 times more potent than the corresponding L-Phe^7 analogues, but in the longer sequences (4–12 and 4–13) the D-Phe^7 and L-Phe^7 analogues were essentially equipotent on the lizard skin bioassay. On the frog skin assay, Ac-[Cys^4,D-Phe^7,Cys^{10}]-α-MSH$_{4-12}$-NH$_2$ and Ac-[Cys^4,D-Phe^7,Cys^{10}]-α-MSH$_{4-13}$-NH$_2$ were about 4 and 10 times more potent than the corresponding L-Phe^7 compound, respectively.

In order to fully analyze the effects of the D-Phe^7 substitution on the potency of cyclic melanotropins, it is necessary to note the differences between the potencies of the cyclic fragments with both L- and D-Phe in the 7-position in conjunction with the corresponding linear Nle^4,D-Phe^7 fragments. In particular, it is important to consider the potencies of the cyclic 4–10 and 4–11 sequences vs. the 4–12 and the 4–13 sequences in both bioassays with either enantiomer of phenylalanine in the 7-position. Prior to this investigation, it was shown that the D-Phe^7 substitution in linear melanotropins (with Nle in the 4-position) of the 4–10 or longer sequences resulted in a substantial increase of potency in both assays in all cases investigated.[7,8] Upon examination of the biological activities of the cyclic D-Phe^7-containing melanotropins, it is apparent that the 4–10 or the 4–11 sequences follow this trend while the longer 4–12 and 4–13 sequences deviate from this trend significantly. The increase in potency of the cyclic 4–10 and 4–11 D-Phe^7 sequences as compared to the corresponding L-Phe^7 analogues tends to indicate that this property is not simply the ability of a D-amino acid to help stabilize a reverse turn as had been suggested in the case of linear D-Phe^7-containing melanotropins. Therefore, the increase in potency must be related to the D-Phe^7 residue itself, or some other structural–conformational perturbation imposed upon the molecule by the D-amino acid. Further examination indicates that the D-Phe^7 substitution does not have the same effect in the longer cyclic analogues. This effect may be related directly to the frog or lizard skin receptor itself and not to the melanotropin. In other words, it is possible that the receptors require a certain minimal concentration of a melanotropin present in order for initiation of transduction of melanocyte dispersion whatever the binding activity. If the superpotent cyclic melanotropins with an L-Phe in the 7-position have binding potencies that approach this concentration, incorporation of D-Phe^7 might not cause any significant further increase in the potency of cyclic melanotropins.

An interesting aspect of melanotropic activity that we have observed, particularly in D-Phe^7-containing analogues, has been prolongation of the biological response even after

removal of the analogue from the incubation medium. In particular, both linear and cyclic (L-Phe^7) compounds containing the 4–10, 4–11, 4–12, and 4–13 sequences show no significant prolongation of melanotropic biological activity, but the substitution of a D-Phe^7 into the same sequences changed these results rather dramatically. For example, the linear peptide Ac-[Nle^4,D-Phe^7]-α-MSH$_{4-10}$-NH$_2$ was not prolonged in the frog skin bioassay,[7] but the analogue prepared by the addition of Lys^{11} (Ac-[Nle^4,D-Phe^7]-α-MSH$_{4-11}$-NH$_2$) was prolonged. Interestingly, in the cyclic analogues, both Ac-[Cys^4,D-Phe^7,Cys^{10}]-α-MSH$_{4-10}$-NH$_2$ and surprisingly the 4–11 analogue, Ac-[Cys^4,D-Phe^7,Cys^{10}]-α-MSH$_{4-11}$-NH$_2$, were not prolonged, but both the 4–12 and 4–13 cyclic D-Phe^7 melanotropins were prolonged. It therefore appears that, in linear analogues of α-MSH (with Nle^4), the 4–11 sequence is required for prolongation, but in cyclic analogues, the 4–12 sequence is required, provided that the 4–10 amino acids of the native hormone and a D-Phe in the 7-position are present in both cases.

The preparation of α-MSH analogues stable to enzymatic degradation is particularly important for in vivo studies. In previous publications we have reported on the resistance of linear [Nle^4,D-Phe^7] melanotropins to in vitro enzymatic degradation by serum enzymes[3,8,14-16] and stability to rat brain homogenate.[16] Interestingly, incorporation of a cystine bridge into the fragments of α-MSH leads to considerable stability for the analogues to frog and rat sera (data not shown) and to trypsin. Furthermore, incorporation of a D-Phe^7 leads to analogues very stable in the presence of purified enzymes as already reported[8,14-16] for linear D-Phe^7-containing analogues, such as [Nle^4,D-Phe^7]-α-MSH.[3,17] Since trypsin cleaves specifically at the carbonyl of Arg^8 and Lys^{11} of α-MSH to inactivate the molecule, the increased stability of the cyclic 4–12 and especially 4–13 sequences to trypsin may be related to a conformational effect,[18] which could prevent the required enzyme-substrate interaction.

In order to understand the phenomenon of superagonism, it is important to define the bioassay carefully. As seen in Figure 6, different concentrations of α-MSH (5.00×10^{-11} M) and Ac-[Cys^4,D-Phe^7,Cys^{10}]-α-MSH$_{4-13}$-NH$_2$ (1.25×10^{-11} M) were chosen to elicit the same biological response after 60 min. After this time, α-MSH does not produce any further skin darkening, but the D-Phe^7 compound continues to further darken the frog skin. This excess skin darkening is defined as superagonism and the phenomenon of superagonism appears to be time dependent. Interestingly, the minimal effective doses previously reported for cyclic L-Phe^7 [11,12] analogues are also time dependent, and therefore, it is possible that these responses are linked to the same biochemical event, though each is being observed differently.

Experimental Section

General Methods. Capillary melting points were determined on a Thomas-Hoover melting point apparatus and were uncorrected. Thin-layer chromatography (TLC) was performed on silica gel G plates in three solvent systems: (A) 2-propanol/25% aqueous NH_3/H_2O (3:1:1); (B) *sec*-butyl alcohol/HOAc/pyridine/H_2O (15:3:10:12); (C) 1-butanol/pyridine/HOAc/H_2O (5:5:1:4), and detected with iodine vapors and ninhydrin. Single spots were obtained unless otherwise noted. Amino acid analyses were obtained with a Beckman 120C amino acid analyzer following hydrolysis for 22 h at 110 °C with 3.5 M mercaptoethanesulfonic acid and subsequent dilution with citrate buffer (pH 2.2) or hydrolysis with 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole and subsequent neutralization with 3.5 N NaOH, as indicated. No corrections were made for destruction of amino acids during hydrolysis. Fast atom bombardment mass spectra were obtained on a Varian 311A spectrometer equipped with an Ion Tech Ltd. source with xenon as the bombarding gas. Reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on a Spectra-Physics SP-8700 liquid chromatograph equipped with a SP-8400 continuously variable wavelength UV detector, utilizing a Vydac 830822 column (25 × 0.46 cm i.d.) packed with C-18 reversed-phase (16 μm) material.

Table II. Solid-Phase Synthesis Methodology[a]

reagent	repetitions	time, min
(1) CH_2Cl_2	4	1
(2) 45% TFA, 2% anisole/CH_2Cl_2	1	2
(3) CH_2Cl_2	3	1
(4) 10% DIEA/CH_2Cl_2	3	2
(5) CH_2Cl_2	4	1
(6) N^{α}-Boc-amino acid, DCC, HOBt/CH_2Cl_2	1	60–120
(7) CH_2Cl_2	3	1
(8) EtOH	3	1
(9) CH_2Cl_2	4	1

[a] An illustration of the cycle used for synthesizing the peptides reported in this paper. After steps 5 and 9, a few milligrams of the resin was removed and a ninhydrin test[19] was used to monitor to coupling. In most cases only one coupling was required for each amino acid except when the ninhydrin test after step 9 was positive. In this case either the amino acid was recoupled or the reactive site was acetylated with *N*-acetylimidazole.

N^{α}-Boc-protected amino acids and amino acid derivatives were purchased from Vega Biochemicals (Tucson, AZ), Peninsula Laboratories (San Carlos, CA), and Bachem (Torrance, CA) or were prepared by using documented procedures. Before use, all amino acid derivatives were tested for purity by TLC, in solvent systems A–C, melting point, and the ninhydrin test.[19] Solvents used for gel filtration, TLC, and ion-exchange chromatography were purified by redistillation before use. The *p*-methylbenzhydrylamine resin (*p*-MBHA) (1% divinylbenzene cross-linked polystyrene) was prepared by previously reported methods with an amine substitution[4] as indicated.

Solid-Phase Peptide Synthesis of Melanotropins. The cyclic α-MSH analogues reported were synthesized by solid-phase methods similar to those used previously for the synthesis of cyclic analogues of α-MSH. α-MSH, [Cys^4,Cys^{10}]-α-MSH, Ac-[Cys^4,Cys^{10}]-α-MSH$_{4-10}$-NH$_2$, Ac-[Cys^4,Cys^{10}]-α-MSH$_{4-11}$-NH$_2$, Ac-[Cys^4,Cys^{10}]-α-MSH$_{4-12}$-NH$_2$, and Ac-[Cys^4,Cys^{10}]-α-MSH$_{4-13}$-NH$_2$ were prepared and purified as previously reported.[2,11,12] N^{α}-Boc-protected amino acid derivatives were successively coupled to a *p*-methylbenzhydrylamine resin with a 3-fold excess of the Boc-protected amino acid derivative, a 3-fold excess of *N*-hydroxybenzotriazole (HOBt), and a 2.4-fold excess of dicyclohexylcarbodiimide (DCC). Cleavage of the N^{α}-Boc protecting group was performed by treatment with 45% trifluoroacetic acid containing 2% anisole in dichloromethane. Side-chain functionalities were protected as follows: arginine, N^g-tosyl; glutamic acid, γ-*O*-benzyl ester; lysine, N^{ϵ}-2,4-dichlorobenzyloxycarbonyl; tryptophan, N^i-formyl; histidine, N^{im}-tosyl; and cysteine, *S*-4-methylbenzyl. It has been reported that HOBt removes the tosyl protecting group from histidine under the conditions used for synthesis;[20] therefore HOBt was not used in the presence of histidine in the peptide fragments.

A cycle for the incorporation of each amino acid residue into the growing peptide chain is shown in Table II. After coupling all of the amino acid residues to the resin, the amino terminal of each peptide was acetylated with an excess of *N*-acetylimidazole in CH_2Cl_2. The finished protected peptide was cleaved from the

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Table III. Analytical Data for D-Phe[7]-Substituted Cyclic Melanotropins

compd	amino acid analysis[a]								
	Cys	Glu	His	Phe	Arg	Trp	Lys	Pro	Val
VII	1.99	1.03	0.95	0.98	0.99	1.06			
VIII	1.96	1.05	0.98	0.97	0.97	0.97	1.08		
IX	1.90	1.05	1.03	1.01	1.02	0.93	1.02	1.05	
X	1.75	1.00	0.94	0.96	1.09	0.96	1.03	1.06	1.04

compd	TLC[b]			HPLC (k')[c]		
	A	B	C	A	B	mass spectra, MH^+
VII	0.70	0.66	0.60	2.98	4.00	1020
VIII	0.47	0.49	0.48	1.47	1.94	1148
IX	0.46	0.48	0.47	2.04	3.00	1245
X	0.60	0.55	0.54	3.15	4.94	1344

[a] The peptides were hydrolyzed as follows: VII and VIII, 3.5 M mercaptoethanesulfonic acid; IX and X, 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole. [b] (A) 2-Propanol/NH_3/H_2O (3:1:1), (B) *sec*-butanol/HOAc/pyridine/H_2O (15:3:10:12), (C) 1-butanol/pyridine/HOAc/H_2O (5:5:1:4). [c] (A) Vydac (830822) C-18 column (16 μm, 25 × 0.46 cm), 82% 0.1% TFA/18% CH_3CN, 1.5 mL/min; (B) Vydac (830822) C-18 column (16 μm, 25 × 0.46 cm), 68% 0.1% TFA/32% MeOH, 1.5 mL/min.

resin, and all protecting groups were removed with anhydrous liquid HF (0 °C for 50–60 min) containing 10% anisole and 5% 1,2-dithioethane.[21] Cyclization was carried out in dilute solution via oxidation with potassium ferricyanide as reported in the literature.[2,12,13]

Ac-[Cys[4],D-Phe[7],Cys[10]]-α-MSH$_{4-10}$-NH$_2$ (VII). Starting with 1.20 g of N^{α}-Boc-Cys(*S*-*p*-MeBzl)-*p*-MBHA resin (0.48 mmol of N^{α}-Boc-Cys(*S*-*p*-MeBzl) total), the protected peptide resin to the title peptide was obtained after stepwise coupling of the following N^{α}-Boc-protected amino acids (in order of addition): N^{α}-Boc-N^{i}-For-Trp, N^{α}-Boc-N^{g}-Tos-Arg, N^{α}-Boc-D-Phe, N^{α}-Boc-N^{im}-Tos-His, N^{α}-Boc-γ-*O*-Bzl-Glu, and N^{α}-Boc-*S*-*p*-MeBzl-Cys. After coupling of the last amino acid and removal of the N^{α}-Boc group, the peptide was acetylated with a 10-fold excess of *N*-acetylimidazole in 25 mL of CH_2Cl_2. The resultant Ac-Cys(*S*-*p*-MeBzl)-Glu(*O*-γ-Bzl)-His(N^{im}-Tos)-D-Phe-Arg(N^{g}-Tos)-Trp(N^{i}-For)-Cys(*S*-*p*-MeBzl)-*p*-MBHA resin was dried in vacuo. A portion (1.25 g) of the protected peptide was cleaved from the resin along with all the protecting groups by treatment with anhydrous HF (13.0 mL), anisole (1.4 mL), and 1,2-dithioethane (0.70 mL) (58 min, 0 °C). After evaporation of the HF, anisole, and 1,2-dithioethane in vacuo, the dried product was washed with EtOAc (3 × 30 mL) and extracted successively with 30% HOAc (3 × 30 mL), 10% HOAc (3 × 30 mL), and distilled water (3 × 30 mL) under a stream of argon. The combined aqueous extracts were lyophilized to a white powder, which was cyclized immediately by diluting with 1200 mL of distilled deaerated water, adjusting the pH to 8.4 with 20% NH_4OH, and adding 96 mL (100% excess) of 0.01 N $K_3Fe(CN)_6$. After 30 min at room temperature, the reaction was terminated by the addition of 10% AcOH until a pH of 5.0 was reached. Excess ferro- and ferricyanide ions were removed by the addition of Bio-Rad AG3-X4A (Cl^- form) anion-exchange resin. After stirring for 30 min and subsequent gravity filtration, the solution was lyophilized to give 319.0 mg of the crude peptide fragment. A portion of the crude peptide (176.0 mg) was dissolved in a minimal amount of 30% HOAc and eluted on a Sephadex G-15 column with 30% HOAc. Two peaks (280-nm detection) were found immediately after the void volume. The first peak was collected, lyophilized (36.5 mg), dissolved in 3 mL of 0.01 N NH_4OAc, pH 4.5, and chromatographed on a (carboxymethyl)cellulose column (CMC) (2.0 × 18.0 cm) with a discontinuous gradient (200 mL each) of 0.01 N (pH 4.5) and 0.1 and 0.2 N NH_4OAc (pH 6.8). The major peak (280-nm detection) eluted during the 0.1 N NH_4OAc (pH 6.8) fraction and was lyophilized to give 15.0 mg of the title compound. Analytical data are found in Table III.

Ac[Cys[4],D-Phe[7],Cys[10]]-α-MSH$_{4-11}$-NH$_2$ (VIII). Starting with 0.60 g N^{α}-Boc-N^{ϵ}-2,4-Cl_2-Z-Lys-*p*-MBHA resin (0.26 mmol of N^{α}-Boc-2,4-Cl_2-Z-Lys total), the title peptide was prepared by stepwise coupling of the following N^{α}-Boc-protected amino acids (in order of addition): N^{α}-Boc-Cys(*S*-*p*-MeBzl), N^{α}-Boc-Trp-(N^{i}-For), N^{α}-Boc-Arg(N^{g}-Tos), N^{α}-Boc-D-Phe, N^{α}-Boc-His-(N^{im}-Tos), N^{α}-Boc-Glu(*O*-γ-Bzl), and N^{α}-Boc-Cys(*S*-*p*-MeBzl). Acetylation of the N terminus of the protected peptides was performed with a 10-fold excess of *N*-acetylimidazole in 25 mL of CH_2Cl_2. The resulting substituted *p*-MBHA resin (1.04 g) was dried in vacuo. Cleavage, deprotection, and cyclization was performed as in peptide VII. The peptide was purified by preparative RP-HPLC on a Vydac 830822 column (16 μm, 25.0 × 0.46 cm) with 90% 0.1% trifluoroacetic acid buffer and 10% CH_3CN as the mobile phase (11.4 mg). Analytical data are found in Table III.

Ac-[Cys[4],D-Phe[7],Cys[10]]-α-MSH$_{4-12}$-NH$_2$ (IX). For synthesis of IX, see ref 7. Analytical data are found in Table III.

Ac-[Cys[4],D-Phe[7],Cys[10]]-α-MSH$_{4-13}$-NH$_2$ (X). Starting with 2.00 g of N^{α}-Boc-Val-*p*-MBHA resin (0.56 mmol of N^{α}-Boc-Val total), the title peptide was prepared by stepwise coupling of the following N^{α}-Boc-protected amino acids (in order of addition): N^{α}-Boc-Pro, N^{α}-Boc-Lys(N^{ϵ}-2,4-Cl_2-Z), N^{α}-Boc-Cys(*S*-*p*-MeBzl), N^{α}-Boc-Trp(N^{i}-For), N^{α}-Boc-Arg(N^{g}-Tos), N^{α}-Boc-D-Phe, N^{α}-Boc-His(N^{im}-Tos), N^{α}-Boc-Glu(*O*-γ-Bzl), and N^{α}-Boc-Cys(*S*-*p*-MeBzl). Acetylation of the N terminus of the protected peptide was performed with a 6-fold excess of *N*-acetylimidazole in 25 mL of CH_2Cl_2. The substituted *p*-MBHA resin (2.28 g) was dried in vacuo. Cleavage, deprotection, cyclization, and purification was performed as in peptide VII (15.8 mg). Analytical data are found in Table III.

Frog and Lizard Skin Bioassays. The biological activities of α-MSH and the cyclic analogues were determined by their ability to stimulate melanosome dispersion in vitro in the frog and lizard bioassays as previously described.[22-24] All the solutions were prepared via serial dilutions from a stock solution (10^{-4} M). The frogs (*Rana pipiens*) used in these studies were obtained from Kons Scientific, Germantown, WI, and the lizards (*Anolis carolinensis*) were from the Snake Farm, La Place, LA.

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