possibly other toxic heavy elements and optimal conditions for their administration have been established.

Acknowledgment. The authors gratefully acknowledge the valuable scientific advice received from Professor C. D. Poulter and the expert technical assistance of Mr. R. K. Olsen and Mmes. G. X. Bi and H. Wang. This work was supported by Grant CA47659 from the National Cancer Institute.

Registry No. 1, 112-24-3; **2a**, 56166-94-0; **2a**·4HCl, 137433-54-6; **2b**, 137203-77-1; **2b**·4HCl, 137433-55-7; **3a**, 137203-78-2; **3b**, 137203-79-3; **4a**, 137203-80-6; **4a**·4HCl, 137433-56-8; **4b**, 137203-81-7; **4b**·4HCl, 137433-57-9; Pu, 7440-07-5; Am, 7440-35-9; ^{239}Pu, 15117-48-3; ^{241}Am, 14596-10-2; $CH_3(CH_2)_{11}Br$, 143-15-7; $CH_3(CH_2)_{21}Br$, 6938-66-5; $BrCH_2COOEt$, 105-36-2.

Supplementary Material Available: Mass spectrum of **4b** (2 pages). Ordering information is given on any current masthead page.

Synthesis and Biological Activities of Fatty Acid Conjugates of a Cyclic Lactam α-Melanotropin

Fahad Al-Obeidi,[†] Victor J. Hruby,*[,†] Nader Yaghoubi,[†] Mohamed M. Marwan,[‡] and Mac E. Hadley[‡]

Departments of Chemistry and Anatomy, University of Arizona, Tucson, Arizona 85721. *Received February 13, 1991*

Four fatty acid conjugates of a cyclic lactam-bridged α-MSH fragment analogue were synthesized and their potencies and biological activities compared in several melanotropin bioassays. Palmitoyl, myristoyl, decanoyl, and hexanoyl conjugates of H-Asp-His-D-Phe-Arg-Trp-Lys-NH_2 were prepared. In the in vitro mouse melanoma cell assay, each of the conjugates was 10–100 times more potent than α-MSH or the substrate peptide in elevating tyrosinase activity. The shorter conjugates of hexanoic and decanoic acid were as potent as α-MSH in the lizard skin bioassay, whereas the longer myristoyl and palmitoyl analogues were about 100 times less potent. The potency of the myristoyl and palmitoyl conjugates increased with time in contact with the skins. These observations may be related to the more lipid-like nature of these peptide–fatty acid conjugates. Each of the conjugates exhibited prolonged melanotropic activity in the lizard skin bioassays and in the mouse S91 melanoma tyrosinase bioassay, since the biological response continued following removal of the conjugates from the incubation media. The prolonged residual melanotropic activity resulted from conjugation of the fatty acids to the MSH fragment analogue since the analogue itself did not exhibit prolonged activity.

Introduction

α-Melanotropin, α-MSH (Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2), is a tridecapeptide that is biosynthesized and secreted from the pars intermedia of the pituitary gland.[2] This hormone plays an essential role in the adaptive color changes of many animals through its action on integumental melanocytes.[3] The melanotropic peptide appears to regulate other physiological functions as well.[4-6]

Because of an extensive hepatic first-pass elimination, peptides usually are not orally active and often require parenteral administration to be therapeutically effective. A potential route of administration of peptides is through the skin. It has been shown that the skin lacks the proteolytic enzymes which are responsible for the enzymatic degradation of peptides.[7] In addition, the use of iontophoresis as a facilitating technique in transdermal delivery of peptides shows promising results.[8] The majority of drugs that have been successfully delivered through skin are lipophilic, charged, and of small molecular size. Certain α-MSH analogues can be transdermally delivered through the skin of mice[9-11] and humans.[12]

We have attempted to design α-MSH analogues that possess structural characteristics more favorable for delivery either across the skin or across other epithelia. These peptides are derivatives of a previously reported superpotent melanotropin, Ac[Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH_{4-10}-NH_2.[13-15] All of these peptides have a general structure, FA-[Asp^5,D-Phe^7,Lys^{10}]α-MSH_{5-10}-NH_2 (FA = fatty acid), with various chain lengths of lipophilic hydrocarbons at their N-terminal, a fixed ring size (23-membered), and a charge (2+ if His imidazole is counted). The preparation of these cyclic lactam α-melanotropin

* Author to whom correspondence should be addressed.
[†] Department of Chemistry.
[‡] Department of Anatomy.

(1) Symbols and abbreviations are in accord with the recommendations of the IUPAC–IUB Commission of Biochemical Nomenclature (*J. Biol. Chem.* **1972**, *247*, 977). All optically active amino acids are of the L variety unless otherwise stated. Other abbreviations include the following: α-MSH, α-melanotropin, α-melanocyte stimulating hormone; Nle, norleucine (2-aminohexanoic acid); Bom, benzyloxymethyl; 2,4-Cl_2-Z, 2,4-dichlorobenzyloxycarbonyl; DCC, dicyclohexylcarbodiimide; DIC, diisopropylcarbodiimide; 2,6-Cl_2-Bzl, 2,6-dichlorobenzyl; Fmoc, fluorenyloxymethylcarbonyl; Fmo, fluorenylmethyl ester, pMBHA resin, *p*-methylbenzhydrylamine resin; HOBt, *N*-hydroxybenzotriazole; For, formyl; Tos, tosyl; N^{α}-Boc, N^{α}-*tert*-butyloxycarbonyl; 1-BuOH, 1-butanol; TEA, triethylamine; TFA, trifluoroacetic acid; DIEA, diisopropylethylamine; DCM, dichloromethane; DMAP, 4-*N*,*N*-dimethylpyridine; FA, fatty acid; Pml, palmitic; Mrl, myristic; Dcl, decanoic; Hxl, hexanoic.

(2) Hadley, M. E. *Endocrinology*; Prentice-Hall, Inc.: Englewood Cliffs, New Jersey, 1983; p 160.
(3) For a comprehensive review see *The Melanotropic Peptides*; Hadley, M. E., Ed.; CRC Press: Boca Raton, FL, 1989; Vols. I–III.
(4) O'Donohue, T. L.; Dorsa, D. M. The Opiomelanotropinergic Neuronal and Endocrine Systems. *Peptides (Fayetteville, N.Y.)* **1982**, *3*, 353–395.
(5) Hadley, M. E., Ed. *The Melanotropic Peptides: Source, Synthesis, Chemistry, Secretion, and Metabolism*; CRC Press: Boca Raton, FL, 1988; Vol. I.
(6) Sawyer, T. K.; Hadley, M. E.; Hruby, V. J.; Castrucci, A. M. L.; Staples, D. J.; Farah, J.; O'Donohue, T. L. α-Melanocyte-Stimulating Hormone Structure–Activity Studies: Comparative Analysis of Melanotropic and CNS Bioactivities. *Synapse (N.Y.)* **1988**, *2*, 288–292.
(7) Pannatier, A.; Jeener, P.; Tetsu, B.; Etter, J. C. The Skin as a Drug Metabolizing Organ. *Drug Metab. Rev.* **1978**, *8*, 319–343.
(8) Chien, Y. W.; Siddiqui, O.; Shi, W.-M.; Lelawongs, P.; Liu, J.-C. Direct Current Iontophoretic Transdermal Delivery of Peptide and Protein Drugs. *J. Pharm. Sci.* **1989**, *78*, 376–383.

0022-2623/92/1835-0118$03.00/0

Table I. Comparative Biological Activities of Fatty Acid Conjugates of H-[Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{5-10}$-NH$_2$

peptide	structure	peptide activity[a,b]	
		lizard skin	melanoma tyrosinase
α-MSH		1.0 (-)	1.0 (-)
I	H-[Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{5-10}$-NH$_2$	0.20 (-)	1.0 (±)[c]
II	Ac-[Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{5-10}$-NH$_2$	1.0 (-)	1.0 (±)
III	Hxl-[Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{5-10}$-NH$_2$	1.0 (+)	10–100 (+)
IV	Dcl-[Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{5-10}$-NH$_2$	1.0 (+)	10–100 (+)
V	Mrl-[Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{5-10}$-NH$_2$	0.010 (+)	10–100 (+)
VI	Pml-[Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{5-10}$-NH$_2$	0.010 (+)	10–100 (+)

[a] All peptide activities were tested at a range of 10^{-9} to 10^{-12} M concentrations and compared to the half-maximal effective dose of α-MSH in the lizard skin (10^{-9} M) bioassays. In the mouse melanoma tyrosinase bioassay, potencies refer to the minimal effective dose of the peptide to elicit a response. In each bioassay, the potency of α-MSH is taken as 1.0. [b] Indicates that the response is prolonged (+) or not prolonged (-). [c] (±) Somewhat prolonged.

fatty acid conjugates will be described in this paper as well as their biological activities as determined in several bioassays.

Results

The partially protected, cyclic hexapeptide resin precursor, H-Asp-His(Bom)-D-Phe-Arg(Tos)-Trp(For)-Lys-pMBHA resin, was assembled and cyclized on the resin using an orthogonal solid-phase synthetic methodology similar to that previously reported in our laboratory.[14,16] Following cyclization, the protected peptide resin was acylated with the desired fatty acid by the diisopropylcarbodiimide (DIC)/*N*-hydroxybenzotriazole (HOBT) method (see Experimental Section). The cyclic peptide conjugate was cleaved from the resin, and the remaining protecting groups were removed by liquid HF at 0 °C. The peptides were purified by reverse-phase high-pressure liquid chromatography (RP-HPLC). The purity of the peptides and their properties were assessed by fast atom bombardment mass spectrometry, high-pressure liquid chromatography, thin-layer chromatography in three

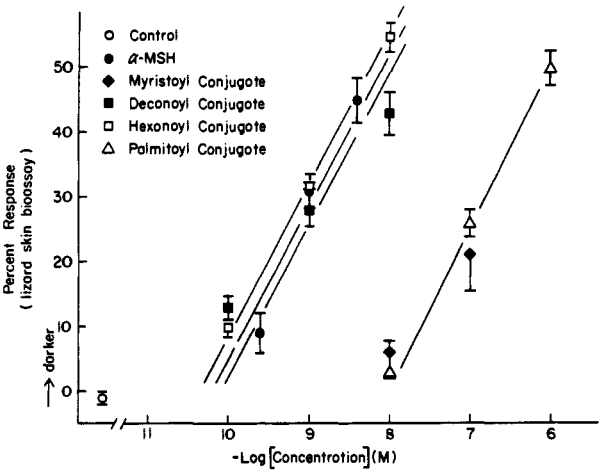

Figure 1. In vitro demonstration of the potencies of four FA-melanotropic peptide conjugates as determined in the lizard skin bioassay. Note that although drawn parallel, the response to the decanoyl conjugate is actually depressed because of the slower response achieved within the 20-min bioassay. This is even more evident for the palmitoyl conjugate as noted in Figure 2. Each value is the mean (±SE) response (darkening) of the skins (n = 5) to the melanotropins at the concentrations noted.

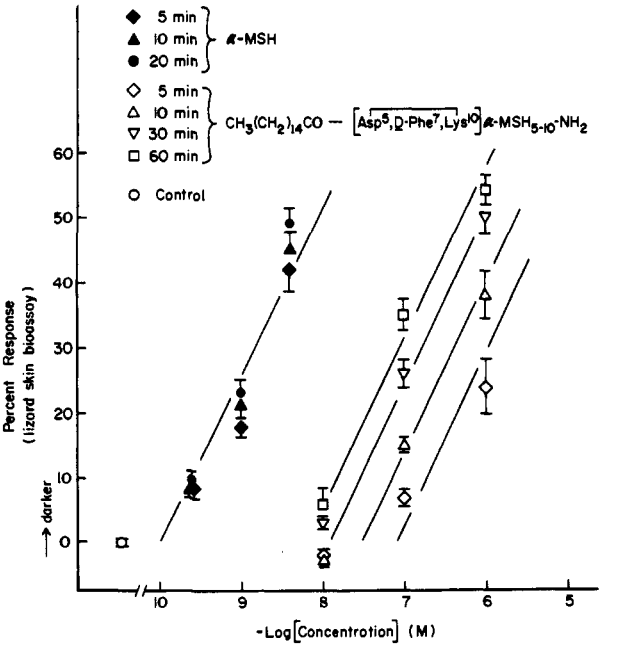

Figure 2. In vitro demonstration of the increasing potency of the Pml conjugate with time in the lizard skin bioassay compared to α-MSH. Each value is the mean (±SE) response (darkening) of the skins (n = 5) to melanotropins at the concentrations noted.

(9) Hadley, M. E.; Wood, S. H.; Lemus-Wilson, A. M.; Dawson, B. V.; Levine, N.; Dorr, R. T.; Hruby, V. J. Topical Application of a Melanotropic Peptide Induces Systemic Follicular Melanogenesis. *Life Sci.* **1987**, *40*, 1889–1895.

(10) Levine, N.; Lemus-Wilson, A.; Sterling, H. W.; Abdel Malek, Z. A.; Al-Obeidi, F.; Hruby, V. J.; Hadley, M. E. Stimulation of Follicular Melanogenesis in the Mouse by Topical and Injected Melanotropins. *J. Invest. Dermatol.* **1987**, *89*, 269–273.

(11) Dawson, B. V.; Hadley, M. E.; Kreutzfeld, K.; Dorr, R. T.; Hruby, V. J.; Al-Obeidi, F.; Don, S. Transdermal Delivery of a Melanotropic Peptide Hormone Analogue. *Life Sci.* **1988**, *43*, 1111–1117.

(12) Dawson, B. V.; Hadley, M. E.; Kreutzfeld, K. L.; Don, S.; Levine, N., Eytan, T.; Hruby, V. J. In Vitro Trandermal Delivery of a Melanotropic Peptide Through Human Skin. *J. Invest. Dermatol.* **1990**, *94*, 432–435.

(13) Al-Obeidi, F.; Hruby, V. J.; Pettitt, M.; Hadley, M. E. Design of a New Class of Superpotent Cyclic α-Melanotropins Based on Quenched Dynamic Simulations. *J. Am. Chem. Soc.* **1989**, *111*, 3413–3416.

(14) Al-Obeidi, F.; Castrucci, A. M. L.; Hadley, M. E.; Hruby, V. J. Potent and Prolonged Acting Cyclic Lactam Analogues of α-Melanotropin: Design Based on Molecular Dynamics. *J. Med. Chem.* **1989**, *32*, 2555–2561.

(15) Hadley, M. E.; Marwan, M. M.; Al-Obeidi, F.; Hruby, V. J.; Castrucci, A. M. L. Linear and Cyclic α-Melanotropin[4–10]-Fragment Analogues That Exhibit Superpotency and Residual Activity. *Pigment Cell Res.* **1989**, *2*, 478–484.

(16) Hruby, V. J.; Al-Obeidi, F.; Sanderson, D. G.; Smith, D. D. Synthesis of Cyclic Peptides by Solid Phase Methods. In *Innovations and Perspective in Solid Phase Synthesis*; Epton, R., Ed.; SPCC Ltd.: Birmingham, 1990; pp 197–203.

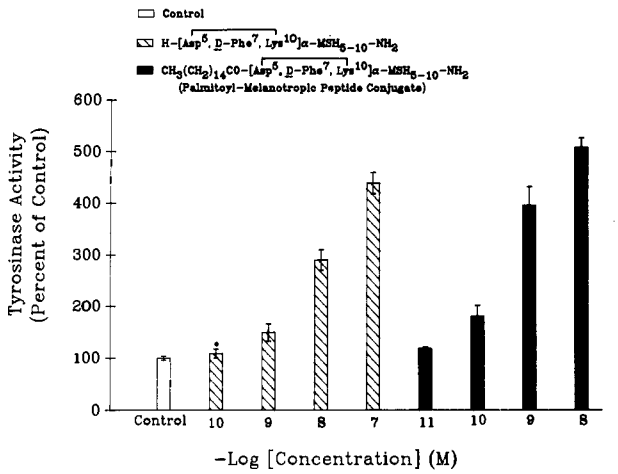

Figure 3. In vitro demonstration of the potency of the Pml-melanotropin conjugate compared to the non-fatty acid conjugated cyclic α-MSH hexapeptide analogue in the mouse S91 melanoma cell tyrosinase bioassay. Each value is the mean (±SE) of three replicated (n = 6) samples.

solvent systems, optical rotation, and amino acid analysis (see Experimental Section).

The melanotropic activities and potencies of the cyclic lactam FA-[Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{5-10}$-NH$_2$ (FA = palmitic, Pml; myristic, Mrl; decanoic, Dcl; hexanoic, Hxl) conjugates were compared to the native hormone, α-MSH, in two bioassay systems (Figure 1; Table I). The Pml and Mrl conjugates were about $^1/_{100}$ as potent as α-MSH in the lizard skin bioassay, whereas the shorter two conjugates had potencies very similar to MSH (Figure 1). As shown in Figure 2, the Pml conjugate (and Mrl conjugate, Figure 5) exhibited increased activity with time in the lizard skin bioassay, whereas with α-MSH the maximal darkening response was generally complete within 20 min (Figure 2). The Pml conjugate was about 10–100 times more potent than the unconjugated hexapeptide, H-[Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{5-10}$-NH$_2$ (Figure 3), and α-MSH (data not shown; see ref 17) in the melanoma tyrosinase bioassay.[17] The other three melanotropin conjugates were similar to potency to the Pml conjugate (Table I).

The Pml conjugate exhibited prolonged activity in the two bioassays. In the lizard skin bioassay, the melanotropic effects of α-MSH were fully reversed within 20 min after hormone removal from the bathing medium. In contrast, with the Pml-melanotropin, it was observed that skin darkening was increased with time, even after removal of this molecule from the incubation medium (Figure 4). Similar prolonged physiological activity was observed in the lizard skin bioassay by the other fatty acid conjugates. The Mrl conjugate, like the Pml conjugate exhibited a creeping potency with time (Figure 5). The prolonged activity resulted from incorporation of the FA into the peptide conjugate since the substrate fragment analogue, H-[Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{5-10}$-NH$_2$, lacked prolonged melanotropic activity.

Following exposure of melanoma cells to the Pml conjugate for 24 h, the cells were rinsed four times, and medium lacking the peptide was added to the cells for the following 24 h. The medium was changed every 24 h thereafter for the next 7 days of incubation. Tyrosinase activity of melanoma cells was enhanced over that of the control cells even though the melanotropin had been "removed" from the incubation medium (Figure 6). The Mrl-, Dcl-, and Hxl-melanotropin conjugates also proved to be prolonged acting in the mouse melanoma tyrosinase bioassay.

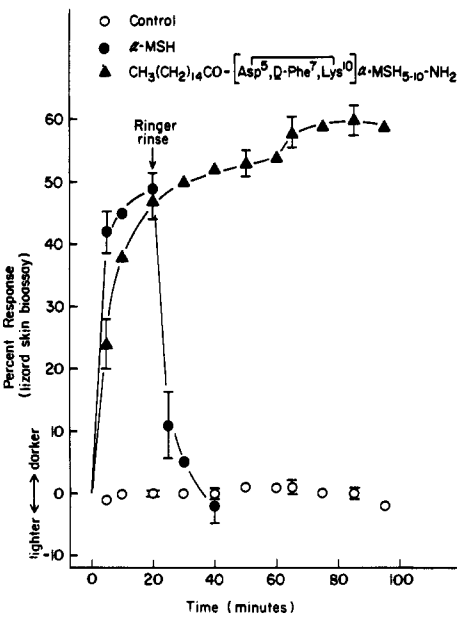

Figure 4. In vitro demonstration of the prolonged (residual) melanotropic activity of the Pml conjugate in the lizard skin bioassay. The darkening response of the skins to the Pml conjugate (10^{-6} M) compared to α-MSH (4×10^{-9} M) is shown. Note the "creeping potency" of the conjugate. Although the concentration of the FA conjugate is much higher than that of α-MSH, a similar prolonged response is noted at equimolar concentrations of peptides in Figure 5. Each value is the mean (±SE) response of the skins (n = 6) to the melanotropins.

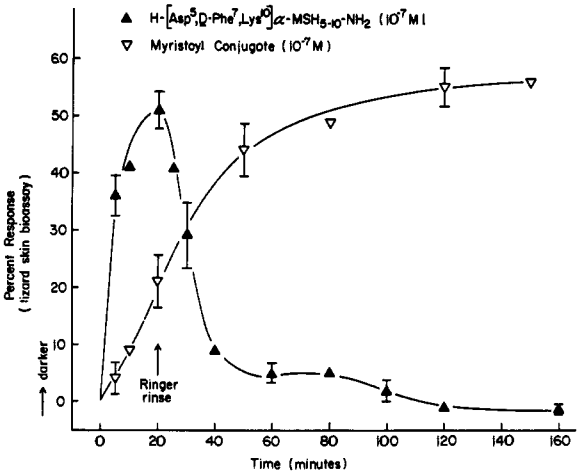

Figure 5. In vitro demonstration that the prolonged melanotropic activity of the FA conjugate (myristoyl, in the present case) of H-[Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{5-10}$-NH$_2$ is due to the FA moiety and not to the peptide substrate to which it is attached. Note the slow onset of melanotropin activity and the continued darkening of the skins after removal of the peptide from the bathing solution. Each value is the mean (±SE) response (darkening) of the lizard skins (n = 5) to the melanotropins at the concentrations noted.

Discussion

We have synthesized a large number of [Nle^4,D-Phe^7]-substituted analogues of α-MSH.[18,19] The analogues

(17) Marwan, M. M.; Abdel Malek, Z. A.; Kreutzfeld, K. L.; Hadley, M. E.; Wilkes, B. C.; Hruby, V. J.; Castrucci, A. M. L. Stimulation of S91 Melanoma Tyrosinase Activity by Superpotent α-Melanotropins. *Mol. Cell. Endocrinol.* **1985**, *41*, 171–177.

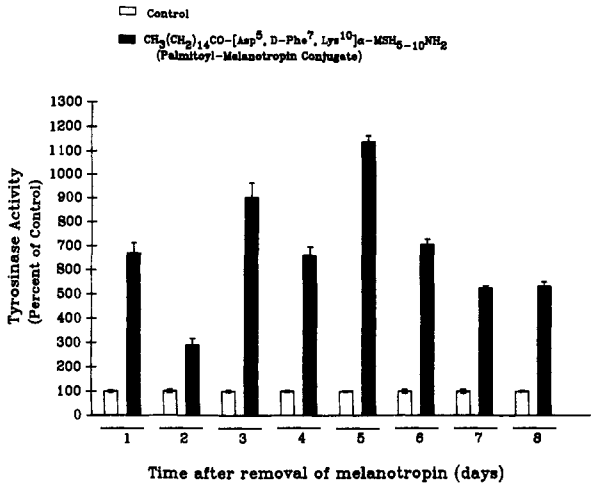

Figure 6. In vitro demonstration of the prolonged activity of the Pml-melanotropin conjugate in the S91 mouse melanoma cell tyrosinase bioassay. Each value (n = 6) is the mean (±SE) response (tyrosinase activity) to the melanotropin.

generally exhibit superpotency, prolonged biological activity, and resistance to inactivation by proteolytic enzymes.[20-24] [Nle^4,D-Phe^7]α-MSH-substituted fragment analogues also often exhibit these unique biological characteristics.[23] We recently synthesized a number of linear and cyclic, lactam-bridged [Nle^4,Asp^5,D-Phe^7,Lys^{10}]-substituted analogues of α-MSH, including 4-10 and 4-13 fragment analogues.[13-15] Some of these analogues were also superactive, prolonged acting, and resistant to inactivation by proteolytic enzymes. Thus, we have utilized the H-[Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{5-10}$-NH$_2$ cyclic lactam-bridged peptide, which does not have prolonged biological activity (Figure 5) as a substrate for conjugation to fatty acids.

The FA-melanotropic peptide conjugates were 10–100 times more potent in enhancing mouse melanoma cell enzyme (tyrosinase) activity than α-MSH or the substrate fragment melanotropin, H-[Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{5-10}$-NH$_2$ (Table I). In the lizard skin bioassays, the shorter Hxl and Dcl conjugates were equipotent to α-MSH, whereas the longer Pml and Mrl conjugates were about 100 times less potent. The fragment melanotropin was about 10 times less potent than α-MSH in these bioassays. These results demonstrate that, in general, conjugation of the melanotropin fragment to a FA enhances the duration of biological response but not always the potency of the peptide.

Although certain D-Phe^7-substituted melanotropins exhibit prolonged (residual) bioactivity, certain short fragments such as H-[Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{5-10}$-NH$_2$ (Figure 5) do not. It is important to note, therefore, that following attachment of the fragment melanotropin to a FA the conjugates all exhibited prolonged melanotropic activity. The results, therefore, suggest that the actions of a readily reversible agonist can be converted to a highly prolonged acting agonist by these modifications. These observations may have relevance for the conversion of other hormones into more potent and long-acting agonists. In this regard, it is interesting to note that Hashimoto et al.[25] have reported that palmitoyl insulin conjugates had a longer duration of hypoglycemic effect than insulin.

It is an important observation that a short but active sequence of a peptide hormone can be attached to a longer fatty acid and not only still be active but, in fact, become even more potent. With respect to a melanotropin this is not too surprising in that ligands such as biotin (short- and long-arm)[23] and fluorescein isocyanate[24] have been conjugated to α-MSH analogues without loss of potency. We have previously demonstrated that the minimal message sequence of α-MSH is the central 6-9 sequence -His-Phe-Arg-Trp-.[26,27] Apparently, as long as the availability of this tetrapeptide sequence is not compromised when conjugated to a much larger ligand, this message sequence is available for receptor interactions.

Why are FA conjugates generally more active and prolonged acting than α-MSH or the substrate fragment analogue H-[Asp^5,D-Phe^7,Lys^{10}]α-MSH$_{5-10}$-NH$_2$? Based upon the observation of "creeping" potency and irreversible activity even after removal of the FA–melanotropin peptide conjugates from contact with the cells, it is suggested that the conjugates, unlike α-MSH, may have become sequestered to the plasma membrane compartment of the cell wherein they are able to continuously stimulate the processes involved in receptor signal transduction.

These observations have important implications for the design of vehicles for the more efficacious delivery of hormones to their receptor sites. In addition, conjugation of a larger ligand to the melanotropin message sequence may allow for the attachment of diagnostic or therapeutic ligands to the conjugate.

Experimental Section

Materials. TLC was done on silica gel G plates using the following solvent systems: (A) 1-butanol/acetic acid/pyridine/water (5:5:1:4); (B) ammonium hydroxide/water/2-propanol (1:1:3); (C) ethyl acetate/acetic acid/pyridine/water (5:5:1:4). The

(18) Sawyer, T. K.; Sanfilippo, P. J.; Hruby, V. J.; Engel, M. H.; Heward, C. B.; Burnett, J. B.; Hadley, M. E. [Nle^4, D-Phe^7]-α-Melanocyte Stimulating Hormone: A Highly Potent α-Melanotropin with Ultralong Biological Activity. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 5754–5758.

(19) Sawyer, T. K.; Hruby, V. J.; Wilkes, B. C.; Draelos, M. T.; Hadley, M. E.; Bergsneider, M. Comparative Biological Activities of Highly Potent Active Site Analogues of α-Melanotropin. *J. Med. Chem.* **1982**, *25*, 1022–1027.

(20) Castrucci, A. M. L.; Hadley, M. E.; Sawyer, T. K.; Hruby, V. J. Enzymological Studies of Melanotropins. *Comp. Biochem. Physiol. B: Comp. Biochem.* **1984**, *78B*, 519–524.

(21) Castrucci, A. M. L.; Hadley, M. E.; Hruby, V. J. Melanotropin Enzymology. In *The Melanotropic Peptides*; Hadley, M. E., Ed.; CRC Press: Boca Raton, FL, 1988; pp 171–182.

(22) Hruby, V. J.; Wilkes, B. C.; Cody, W. L.; Sawyer, T. K.; Hadley, M. E. Melanotropins: Structural, Conformational, and Biological Considerations in the Development of Superpotent and Superprolonged Analogs. *Pep. Protein Rev.* **1984**, *3*, 1–64.

(23) Chaturvedi, D. N.; Knittel, J. J.; Hruby, V. J.; Castrucci, A. M. L.; Hadley, M. E. Synthesis and Biological Actions of Superpotent and Prolonged Acting Biotin-Labeled Melanotropins. *J. Med. Chem.* **1984**, *27*, 1406–1410.

(24) Chaturvedi, D. N.; Hruby, V. J.; Castrucci, A. M. L.; Hadley, M. E. Synthesis and Biological Evaluation of the Superagonist [N$^\alpha$-Chlorotriazinylaminofluoresceine-Ser^1,Nle^4,D-Phe^7]-α-MSH. *J. Pharm. Sci.* **1985**, *74*, 237–241.

(25) Hashimoto, M.; Takada, K.; Kiso, Y.; Muranishi, S. Synthesis of Palmitoyl Derivatives of Insulin and Their Biological Activities. *Pharm. Res.* **1989**, *6*, 171–176.

(26) Castrucci, A. M. L.; Hadley, M. E.; Sawyer, T. K.; Wilkes, B. C.; Al-Obeidi, F.; Staples, D. J.; De Vaux, D. E.; Dym, O.; Hintz, M. F.; Riehm, J. P.; Rao, K. P.; Hruby, V. J. α-Melanotropin: The Minimal Active Sequence in the Lizard Skin Bioassay. *Gen. Comp. Endocrinol.* **1989**, *73*, 157–163.

(27) Hruby, V. J.; Wilkes, B. C.; Hadley, M. E.; Al-Obeidi, F.; Sawyer, T. K.; Staples, D. J.; de Vaux, A. E.; Dym, O.; Castrucci, A. M. L.; Hintz, M. F.; Riehm, J. P.; Rao, K. R. α-Melanotropin: The Minimum Active Sequence in the Frog Skin Bioassay. *J. Med. Chem.* **1987**, *30*, 2126–2130.

peptides were detected on TLC plates using UV light and iodine vapor. The final purification was effected by a preparative RP-HPLC on a C_{18} bonded silia column (Vydac 218TP1-1010, 1.0 × 25 cm). They were eluted with a linear acetonitrile gradient (15–60%) over 45 min at a flow rate of 3 mL/min, with a constant concentration of TFA (0.1% v/v). The linear gradient was generated with a Spectra-Physics SP 8800 ternary pump system. The separations were monitored at 280 nm with ABI Spectra-Flow 757 or Spectra-Physics absorbance detectors, and the peaks were integrated with a Spectra-Physics SP4270 integrator. Amino acid analyses were obtained by a Beckman 7500 amino acid analyzer after hydrolysis for 22 h at 110 °C in a 1:1 mixture of concentrated HCl and propionic acid containing 0.5% w/v phenol and were not corrected for losses on hydrolysis. All amino acid values were within 10% variation from the calculated values. Optical rotation values were measured on an Autopol III (at 589 nm) in 30% acetic acid. The pMBHA resin (0.31 mmol NH_2/g) was purchased from Advanced Chem Tech (Louisville, KY 40201). N^{α}-Boc-protected amino acids and amino acid derivatives were purchased from Bachem (Torrance, CA) or were prepared by published procedures.[28] All amino acids were of the L configuration except for phenylalanine which was of the D configuration. The reactive side chains of the amino acids were protected as follows: Lys, with fluorenylmethoxycarbonyl (Fmoc); Asp with fluorenylmethyl ester (Fmo);[29,30] His, with benzyloxymethyl (Bom);[31] Arg, with tosyl (Tos); Trp, with formyl (For). Palmitic, myristic, decanoic, and hexanoic acids (99% purity) were obtained from Aldrich Chemical Co. (Milwaukee, WI). All reagents and solvents were ACS grade or better and were used without further purification. The purity of the finished peptides was checked by TLC in at least three solvents and analytical RP-HPLC at 280 and 223 nm and in all cases were greater than 96% pure as determined by these methods. The structures of the pure peptides were confirmed by fast atom bombardment (FAB) mass spectrometry, by amino acid analysis, and in the case of peptide I, by 250-MHz 1D and 2D proton nuclear magnetic resonance (NMR) spectroscopy.

Peptide Synthesis. The peptides were synthesized using a Vega 1000 peptide synthesizer or a manual synthesizer by using the previously published solid-phase procedure.[14,32]

Synthesis of H-Asp-His-D-Phe-Arg-Trp-Lys-NH_2 (I). The protected peptide resin to the title compound was prepared from 3.0 g of pMBHA (0.31 mmol of NH_2/g of resin) by first coupling N^{α}-Boc-Lys (N^{ϵ}-Fmoc) to the resin using DIC/HOBt in DCM as a coupling reagent for 1 h. The remaining amino groups were then blocked by acetylation using acetic anhydride. The other amino acid residues were then coupled 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) and N^{α}-Boc-Asp(N^{β}-Fmo) using the same coupling reagents as above. Each coupling reaction was achieved with a 3-fold excess of DIC and HOBt and a 2.4-fold excess of amino acid derivative. After coupling the last amino acid, the Fmo and Fmoc protecting groups were removed by treating the peptide resin with 50% piperidine in NMP for 1 h. The peptide resin was washed with DMF (3 × 40 mL), DCM (3 × 40 mL), 10% DIEA (3 × 40 mL), DCM (3 × 40 mL), 1:1 dioxane/water (1 × 40 mL), and DMF (3 × 40 mL) and suspended in 30 mL of NMP and mixed with a 6-fold excess of BOP reagent[33,34] in the presence of an 8-fold excess of TEA or DIEA for 6 h. The coupling was repeated twice until the resin gave a negative ninhydrin test. After cyclization, the N^{α}-Boc protecting group was removed, the amino group was neutralized, and the resin was washed with DMF (1 × 30 mL) and DCM (2 × 30 mL) and then dried in vacuo to yield 3.86 g of product. A 0.65-g sample of the peptide resin was cleaved by anhydrous HF (6 mL) in the presence of 0.8 mL of anisole and 0.6 mL of 1,2-ethanedithiol. After workup and lyophilization, a crude peptide (I) (84 mg, 57% HPLC pure) was obtained. A 54-mg sample of this peptide was purified by HPLC to give 23.8 mg. The analytical data for the peptide are given in Table II, and the biological potencies are given in Table I. Amino acid analysis of I was as follows: Asp 0.96 (1.0); His 0.94 (1.0); Phe 1.0 (1.0); Arg 0.97 (1.0); Trp (not determined); Lys 0.92 (1.0).

Table II. Physicochemical Properties of the Fatty Acid Conjugates of H-[Asp[5],D-Phe[7],Lys[10]]α-MSH_{5-10}-NH_2

peptide	$[\alpha]^{23}_{589}$, deg (in 30% HOAc)	R_f, TLC solvents[a] A	B	C	FAB-MS (calc)	k'[b]
I	−40.0 (c = 0.015)	0.37	0.74	0.23	854 (854)	2.25
II		0.32	0.74	0.19	912 (912)	2.8
III	−51.2 (c = 0.041)	0.50	0.30	0.43	968 (968)	3.1
IV	−34.1 (c = 0.044)	0.51	0.32	0.47	1024 (1024)	6.5
V	−25.5 (c = 0.047)	0.52	0.34	0.48	1080 (1080)	9.9
VI	−63.8 (c = 0.04)	0.57	0.37	0.55	1108 (1108)	12.7

[a] R_f values on thin-layer chromatograms of silica gel were observed in the following solvent systems: (A) 1-butanol/HOAc/pyridine/H_2O (5:5:1:4); (B) ammonium hydroxide/H_2O/2-propanol (1:1:3); (C) 1-butanol/HOAc/H_2O (4:1:5 upper phase only). [b] HPLC k' = [(peptide retention time − solvent retention time)/solvent retention time] in solvent system of 15% acetonitrile in 0.1% trifluoroacetic acid and gradient to 60% acetonitrile over 45 min. An analytical Vydac C_{18} column was used with flow rate 1.5 mL/min.

Synthesis of Ac-Asp-His-D-Phe-Arg-Trp-Lys-NH_2 (II). A 0.54-g sample of N^{α}-Boc-Asp-His(N^{π}-Bom)-D-Phe-Arg(N^{g}-(Tos)-Trp(N^{i}-For)-Lys-pMBHA resin (0.31 mmol/g substitution) was N^{a}-Boc-deprotected, neutralized, and acetylated with 2-fold acetic anhydride in the presence of 0.5 mmol of HOBt in DCM for 35 min. The resin was washed with DCM, DMF, and DCM and dried in vacuo to yield 0.52 g. HF cleavage and workup as for I gave 0.16 g of crude peptide. A 21.7-mg sample of the crude peptide was HPLC-purified to give 9.8 mg of pure peptide. The analytical data for the title peptide are given in Table II, and the biological potencies are given in Table I.

Synthesis of Hxl-Asp-His-D-Phe-Arg-Trp-Lys-NH_2 (III). To 0.25 g of peptide resin H-Asp-His(N^{π}-Bom)-D-Phe-Arg(N^{g}-Tos)-Trp(N^{i}-For)-Lys-pMBHA in 1:1 DCM/NMP was added 300 mg (2.58 mmol) of hexanoic acid, 3 mL of HOBt (1 mmol/mL solution in DMF), and 3 mL of DIC (1 mmol/mL in DCM) and shaken for 45 min. The completion of the reaction was monitored by ninhydrin test. The completed peptide resin then was washed with DCM and DMF and dried in vacuo to give 0.28 g. After HF cleavage and extraction of the cleaved peptide with glacial and 30% acetic acid followed by lyophilization, 49.3 mg (54% pure by HPLC) of the crude peptide was obtained. The peptide was then purified by HPLC to give 20 mg of white powder (75.2%). The analytical data for the peptide are given in Table II, and the

(28) Stewart, J. M.; Young, J. D. *Solid Phase Peptide Synthesis*, 2nd Ed.; Pierce Chemical Co.: Rockford, IL, 1984.
(29) Al-Obeidi, F.; Sanderson, D. G.; Hruby, V. J. Synthesis of β- and γ-Fluorenylmethyl Esters Respectively of N^{α}-Boc-L-Aspartic Acid and N^{α}-Boc-L-Glutamic Acid. *Int. J. Pept. Protein Res.* **1990**, *35*, 215–218.
(30) Bolin, D. R.; Wang, C.-T.; Felix, A. M. Preparation of N-*t*-Butyloxycarbonyl-O^{ω}-9-Fluorenylmethyl Esters of Aspartic and Glutamic Acids. *Org. Prep. Proced. Int.* **1989**, *21*, 67–74.
(31) Pipkorn, R.; Ekberg, B. Histidine Racemization in the Synthesis of an Analog of the Luteinizing Hormone Releasing Factor. *Int. J. Pept. Protein Res.* **1986**, *27*, 583–588.
(32) Cody, M. L.; Wilkes, B. C.; Muska, B. J.; Hruby, V. J.; Castrucci, A. M. L.; Hadley, M. E. Cyclic Melanotropins, Part VI: Importance of the C-Terminal Tripeptide (Lys-Pro-Val). *J. Med. Chem.* **1984**, *27*, 1186–1190.
(33) Castro, B.; Dormoy, J. R.; Evin, G.; Seive, C. Reactifs de Couplage Peptidique IV (1)-L'-Hexafluorophosphate de Benzotriazolyl N-Oxytrisdimethylamino Phosphonium (B.O.P.). *Tetrahedron Lett.* **1975**, 1219–1220.
(34) Nguyen, D. L.; Castro, B. BOP Reagent: Rising a "Major" for Peptide Coupling. In *Peptide Chemistry*; Shiba, T., Sakakibara, S., Eds.; Protein Research Foundation: Osaka, 1987; pp 231–240.

biological potencies are given in Table I.

Synthesis of Dcl-Asp-His-D-Phe-Arg-Trp-Lys-NH_2 (IV). According to the same procedure for III with substitution of decanoic acid for hexanoic acid, 0.21 g of peptide resin of IV was prepared. HF cleavage and workup gave 37 mg of crude peptide IV. A 19.9-mg sample of crude peptide was HPLC-purified to give 9.8 mg (87%) of pure peptide IV. The analytical data for the peptide are given in Table II, and the biological potencies are given in Table I.

Synthesis of Mrl-Asp-His-D-Phe-Arg-Trp-Lys-NH_2 (V). By a similar approach as outlined for II, with substitution of myristic acid for hexanoic acid, 0.28 g of peptide resin V was prepared. HF cleavage and workup gave 56 mg of crude peptide V. HPLC purification of 25 mg of this peptide gave 13.2 mg of pure product V. The analytical data for the peptide are given in Table II, and the biological potencies are given in Table I.

Synthesis of Pml-Asp-His-D-Phe-Arg-Trp-Lys-NH_2 (VI). A 0.31-g sample of H-Asp-His(N^{τ}-Bom)-D-Phe-Arg(N^{g}-Tos)-Trp(N^{1}-For)-Lys-NH_2 peptide resin was coupled with 640 mg (2.5 mmol) of palmitoyl chloride in the presence of 2.5 mmol of HOBt and 1.5 mmol of pyridine in DCM for 30 min. The peptide resin was washed with DCM (2 × 30 mL), DMF (2 × 30 mL), and DCM (2 × 30 mL) and dried in vacuo to give 0.33 g. HF cleavage and workup as outlined for II gave 72 mg of crude peptide. HPLC purification of 23 mg gave 11.8 mg of pure peptide VI. The analytical data for the peptide are given in Table II, and the biological potencies are given in Table I.

Bioassays. Melanoma Tyrosinase Bioassays. The Cloudman S91 (3960, CCL 53.1) melanoma cell line was obtained from the American Type Culture Collection Cell Repository. Ham's F-10 medium, fetal calf serum, horse serum, and penicillin/streptomycin solutions were purchased from Gibco Laboratories (Santa Clara, CA). Radioactive L-3,5-[^{3}H]tyrosine (specific activity 59.0 Ci/mmol) was purchased from New England Nuclear (Boston, MA). The melanoma cell medium was a mixture of F-10 medium supplemented with 10% heat-inactivated (56 °C, 30 min) horse serum, 2% fetal calf serum (heat-inactivated), and 1% penicillin/streptomycin solution.

Melanoma cells were seeded into 25 cm^3 flasks at a density of 2×10^5 cells in 4 mL of medium. Incubation followed for 24 h at 37 °C in a humid atmosphere of 5% CO_2/95% air. After 24 h, the medium was removed and replaced with medium containing a melanotropin. The cells were reincubated for 24 h (48 h with 24 h medium changes for 72 h assay), and the medium was again replaced with medium containing 1 μCi [^{3}H]tyrosine/mL as well as a melanotropin. After 24 h, the cells were harvested and counted with a hemacytometer to determine cell number (and proliferation), and the media were collected for tyrosinase assay.

To determine the prolonged actions of melanotropins on tyrosinase activity, cells were treated for 24 h after seeding. At the end of the exposure period, the melanotropins were removed by four rinses of the flasks with Ham's F-10 medium lacking the melanotropin (as described in the figure legends). [^{3}H]Tyrosine (1 μCi) was added to the flasks 24 h prior to termination of the experiment, at which time the cells were harvested and counted. Tyrosinase activity was determined for 7 sequential days (24-h periods) subsequent to melanotropin removal. The medium was changed every 24 h with fresh addition of labeled tyrosine.

The tyrosinase assay utilized is a modification of the charcoal absorption method of Pomerantz,[35] and is based upon the measurement of the amount of ^3H_2O released during the conversion of [^{3}H]tyrosine to L-DOPA, a reaction catalyzed by tyrosinase. Tyrosinase activity is expressed per 10^6 cells. We have previously described our methods in detail.[17,36]

Lizard Skin Bioassay. The lizard (*Anolis carolinenses*) skin bioassay was utilized to determine the relative potencies of the synthetic melanotropins.[37,38] The assay measures the amount of light reflected from the surface of the skins in vitro. In response to melanotropic peptides, melanosomes within integumental melanocytes migrate from a perinuclear position into the dendritic processes of the pigment cells. This centrifugal organellar dispersion results in a change in color (darkening) of the skins which is measured by a Photovolt reflectometer and is expressed as the percent response compared to the initial (time zero) reflectance value. Subsequent removal of a melanotropin such as α-MSH usually results in a rapid perinuclear (centripetal) reaggregation of melanosomes within melanocytes leading to a lightening of the skins back to their original (base) value.

Acknowledgment. This work was supported, in part, by U.S. Public Health Service (AM 17420, V.J.H.) and by a grant from the Arizona Disease Control Research Commission (M.E.H.).

Registry No. I, 137668-62-3; II, 137668-63-4; III, 137668-64-5; IV, 137668-65-6; V, 137668-66-7; VI, 137668-67-8; α-MSH, 37213-49-3; N^{α}-Boc-Lys(N^{ϵ}-Fmoc), 84624-27-1; N^{α}-Boc-Trp(N^{1}-For), 47355-10-2; N^{α}-Boc-Arg(N^{g}-Tos), 13836-37-8; N^{α}-Boc-D-Phe, 18942-49-9; N^{α}-Boc-His(N^{τ}-Bom), 79950-65-5; N^{α}-Boc-Asp(β-Fmo), 117014-32-1; Hxl, 142-62-1; Dcl, 334-48-5; Mrl, 544-63-8; palmitoyl chloride, 112-67-4.

(35) Pomerantz, S. H. The Tyrosine Hydroxylase Activity of Mammalian Tyrosinase. *J. Biol. Chem.* **1966**, *241*, 161–168.
(36) Abdel Malek, Z. A.; Kreutzfeld, K. L.; Marwan, M. M.; Hadley, M. E.; Hruby, V. J.; Wilkes, B. C. Prolonged Stimulation of S91 Melanoma Tyrosinase by [Nle^4,D-Phe^7]-Substituted α-Melanotropins. *Cancer Res.* **1985**, *45*, 4735–4740.
(37) Castrucci, A. M. L.; Hadley, M. E.; Hruby, V. J. Melanotropin Bioassays: In Vitro and In Vivo Comparisons. *Gen. Comp. Endocrinol.* **1984**, *55*, 104–111.
(38) Hadley, M. E.; Castrucci, A. M. Melanotropin Bioassays. In *The Melanotropic Peptides*; Hadley, M. E., Ed.; CRC Press: Boca Raton, FL, 1988; pp 1–13.