Synthesis and Biological Actions of Highly Potent and Prolonged Acting Biotin-Labeled Melanotropins

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Biocytin derivatives of a superpotent analogue of α-melanotropin, [Nle[4],D-Phe[7]]-α-MSH, were prepared. [N^{α}-Bct-Ser[1],Nle[4],D-Phe[7]]-α-MSH and [12-Bct-N^{α}-dodecanoyl-Ser[1],Nle[4],D-Phe[7]]-α-MSH were synthesized by solid-phase techniques, and the coupling of biotin and 12-aminododecanoic acid was achieved through their succinimido esters. These melanotropins possessed almost identical actions to [Nle[4],D-Phe[7]]-α-MSH as determined by several melanocyte bioassays. Both biocytin derivatives were highly potent agonists and exhibited prolonged biological activity as determined in the frog and lizard skin bioassays. Both biotinylated peptides were at least equipotent to α-MSH in stimulating Cloudman S91 mouse melanoma tyrosinase activity. The analogues were resistant to inactivation by α-chymotrypsin.

α-Melanocyte stimulating hormone (α-MSH, α-melanotropin)[1] is a linear tridecapeptide (Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2) synthesized and secreted by the pars intermedia of the vertebrate pituitary. A large number of pigmentary as well as extrapigmentary actions, including a role in fetal development and thermoregulation and effects on learning and attention, are associated with α-MSH, as reviewed recently.[2-4] A knowledge of the cellular specificity of α-MSH and an elucidation of the mechanism of MSH-receptor interactions would provide important information for many investigators in this area.

We recently reported that [Nle[4],D-Phe[7]]-α-MSH and a number of related fragment analogues have extraordinary potency and prolonged biological activity both in vitro and in vivo.[5-7] Our results suggested that these α-MSH analogues might be useful for incorporation of ligands with readily monitored spectroscopic and chemical properties without modifying the extraordinary biological activities of these compounds so that they could be used to examine hormone–receptor interactions. The biotin molecule seemed particularly promising for this because the avidin–biotin system can be used to construct sensitive methods for the visual localization of specific receptor on or within cells. Our objective then was to prepare biotin-labeled melanotropins that are (1) highly potent, (2) nonbiodegradable, and (3) prolonged in their biological activity as compared to α-MSH. We report here the synthesis of biotin-labeled [Nle[4],D-Phe[7]]-α-MSH analogues (Figures 1, **1b** and **1c**). A number of bioassay systems have been utilized to demonstrate that the synthesized compounds possess these important features.

Chemistry. The analogues of [Nle[4],D-Phe[7]]-α-MSH were prepared with use of *p*-methylbenzhydrylamine (*p*-MBHA) resin[5,8] by solid-phase synthetic methods[9] reported earlier,[6-10] with some modifications. It has been shown in many studies that the binding of biotin to avidin is not diminished by modification of the carboxyl group of biotin and its covalent attachment to other molecules. The results from our laboratory reveal that the N-terminal tripeptide (Ser-Tyr-Ser) is not essential for retention of full biological activity of α-MSH and related superpotent analogues.[11,12] This suggested that a structural change at this end of the peptide might not adversely affect melanotropic activity. These data led us to design [N^{α}-Bct-Ser[1],Nle[4],D-Phe[7]]-α-MSH (Figure 1b), which utilizes the carboxyl group of the biotin and the N-terminal amino group of the melanotropin for the final coupling. This compound was synthesized by using active ester coupling of succinimido biotinate (Figure 2, **2a**) to the protected [Nle[4],D-Phe[7]]-α-MSH resin. The synthesis of the compound **2a** was routed through the reaction of biotin with *N,N'*-carbonyldiimidazole followed by the action of *N*-hydroxysuccinimide with the intermediate biocytin imidazolide, as outlined in Figure 2. We further synthesized [12-Bct-N^{α}-dodecanoyl-Ser[1],Nle[4],D-Phe[7]]-α-MSH (Figure 1c) using the spacer arm of 12-aminododecanoic acid between the biotin and the [Nle[4],D-Phe[7]]-α-MSH with the aim to provide a derivative, free of any possible steric hindrance by the bulky [Nle[4],D-Phe[7]]-α-MSH moiety on the biotin molecule. The synthetic strategy of melanotropin **1c** was similar to that of the melanotropin **1b** except that it utilized one extra coupling of succinimido 12-(Boc-amino)dodecanoate (**2b**), the synthesis of which is

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(1) Symbols and abbreviations are in accord with the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature [*J. Biol. Chem.* **1972**, *247*, 977]. Other abbreviations include the following: α-MSH, α-melanotropin, α-melanocyte stimulating hormone; Nle, norleucine; 2,4-Cl_2-Z, 2,4-dichlorobenzyloxycarbonyl; DCC, dicyclohexylcarbodiimide; 2,6-Cl_2-Bzl, 2,6-dichlorobenzyl; *p*-MBHA resin, *p*-methylbezhydrylamine resin; HOBT, *N*-hydroxybenzotriazole; For, formyl; Tos, tosyl; Bct, biocytin; N^{α}-Boc, N^{α}-*tert*-butyloxycarbonyl; 1-BuOH, 1-butanol; Pyr, pyridine. All optically active amino acids are of L variety unless otherwise stated.

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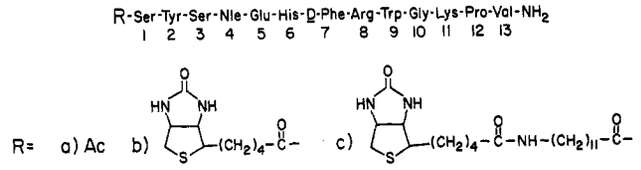

Figure 1. Structures of (a) [Nle^4,D-Phe^7]-α-MSH, (b) [N^α-Bct-Ser^1,Nle^4,D-Phe^7]-α-MSH, and (c) [12-Bct-N^α-dodecanoyl-Ser^1,Nle^4,D-Phe^7]-α-MSH.

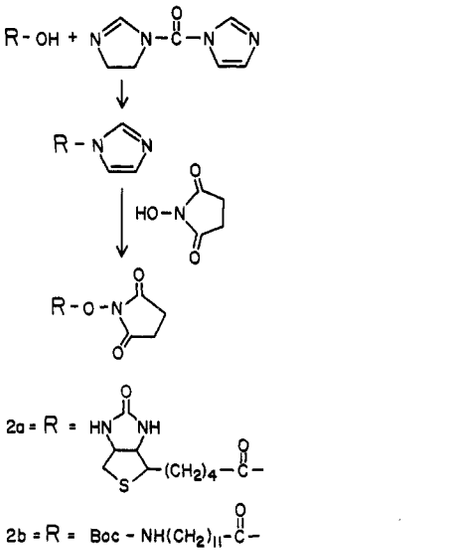

Figure 2. Schematic route for the synthesis of succinimido biotinate (**2a**) and succinimido 12-(Boc-amino)dodecanoate (**2b**).

delineated in Figure 2. The analogues were purified by ion exchange chromatography on (carboxymethyl)cellulose, followed by gel filtration on Sephadex G-25. The homogeneity and structure of each compound was ascertained through amino acid analysis, TLC in three solvent systems, IR, and NMR data. The details of the synthetic methods and the analytical data are provided in the Experimental Section.

Results and Discussion

[Nle^4,D-Phe^7]-α-MSH provided us with the first definitive stereostructural analogue of α-MSH exhibiting exceptionally prolonged melanotropic activity in vitro[6] and in vivo.[7] In addition, the analogue was highly potent in the in vitro tyrosinase assay system.[6] This peptide along with other analogues developed in our laboratory[13,14] has provided important insights relative to the structural requirements of the hormone for receptor binding and transduction.[15-17] The present paper extends this work by developing biotin-labeled analogues of [Nle^4,D-Phe^7]-α-MSH which should provide useful probes for answering basic biological questions related to the nature of cellular and tissue distributions of melanotropin receptors in target tissues of the native hormone.

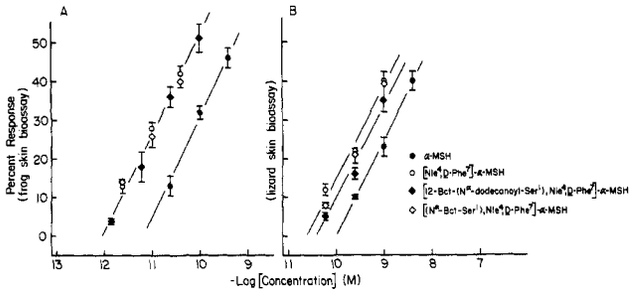

Figure 3. Relative potencies of [N^α-Bct-Ser^1,Nle^4,D-Phe^7]-α-MSH (◊), [12-Bct-N^α-dodecanoyl-Ser^1,Nle^4,D-Phe^7]-α-MSH (♦), [Nle^4,D-Phe^7]-α-MSH (○), and α-MSH (●) as determined in the in vitro (A) frog and (B) lizard skin bioassays. Each value represents the mean ± SE, response (darkening) of the skins (N = 6) to the melanotropins at the concentrations noted.

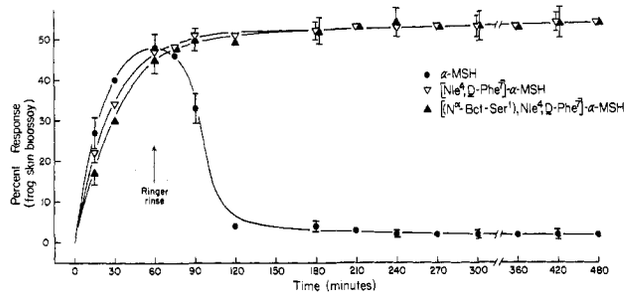

Figure 4. In vitro demonstration of the prolonged melanotropic action of [N^α-Bct-Ser^1,Nle^4,D-Phe^7]-α-MSH (▲) as compared to [Nle^4,D-Phe^7]-α-MSH (▽), and α-MSH (●), as determined in the frog skin bioassay. Each value represents the mean response (darkening) of the skins (N = 6) to the melanotropins at the times indicated. The standard errors are shown for every other point.

The methods of choice for the coupling of biotin to the amino groups of peptides and proteins have been the dicyclohexylcarbodiimide couplings and the the active ester reactions. It is claimed[18] that the active ester procedure gives a product of superior purity to that obtained by the carbodiimide method[19] since the material synthesized by the latter method was contaminated with dicyclohexylurea as shown by mass spectroscopy. Furthermore, the by-products obtained during the active ester methods are generally easily removable. Some difficulties were encountered in the preparation of *p*-nitrophenyl active esters of biocytin and biocytin-containing peptides in satisfactory pure state[20] and in good yield[19,21] because these and certain other active ester methods presumably produce acylation of the biotin ring.[22] We attempted the synthesis of [N^α-Bct-Ser^1,Nle^4,D-Phe^7]-α-MSH (Figure 1b) and [12-Bct-N^α-dodecanoyl-Ser^1,Nle^4,D-Phe^7]-α-MSH (Figure 1c) through the succinimido ester couplings both for biotin and 12-aminododecanoic acid. The coupling of these esters to the protected melanotropin resin was best achieved in the presence of *N*-hydroxybenzotriazole (HOBT). For the preparation of the succinimido ester derivative we tried several methods, but the best results were obtained following the route delineated in Figure 2. These procedures provided the desired compounds in good yields and high purity, as determined by various analytical methods (see

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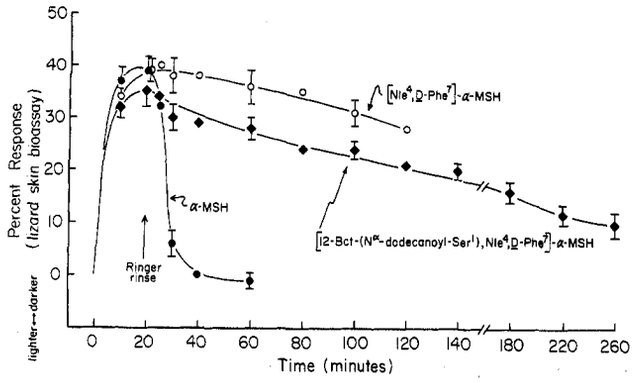

Figure 5. In vitro demonstration of the prolonged melanotropic action of [12-Bct-N^{α}-dodecanoyl-Ser^1,Nle^4,D-Phe^7]-α-MSH as determined in the lizard skin bioassay. Each value represents the mean response (darkening) of the skins to the melanotropins at the times indicated. Representative standard errors of the means are provided for every other point.

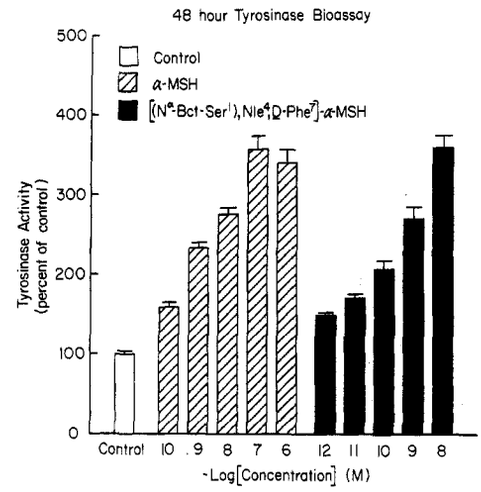

Figure 6. Relative potencies of α-MSH and [N^{α}-Bct-Ser^1,Nle^4,D-Phe^7]-α-MSH, as determined in the S91 mouse melanoma tyrosinase assay. Each value is the mean ± SE, response (tyrosinase activity) of the cultured cells (N = 3) to the melanotropins at the concentrations indicated. The other biocytin derivative, [12-Bct-N^{α}-dodecanoyl-Ser^1,Nle^4,D-Phe^7]-α-MSH, was also equipotent to α-MSH in stimulating melanoma cell tyrosinase activity (data not shown).

the Experimental Section for details).

Biological activities of the biotin-labeled melanotropins were determined in several bioassay systems. In the in vitro frog skin assay (Figure 3A) the biocytin melanotropins (**1b** and **1c**) were about 50 times more potent than the native hormone, α-MSH, and were essentially equipotent to [Nle^4,D-Phe^7]-α-MSH. In the in vitro lizard skin bioassay the biocytin analogues also were superpotent agonists, being equipotent to [Nle^4,D-Phe^7]-α-MSH (Figure 3B). Both biocytin melanotropins exhibited prolonged biological activity in both the frog (Figure 4) and the lizard (Figure 5) bioassays. Repeated rinsing of the skins in Ringer solution *in the absence* of the melanotropins did not lead to lightening of the skins as in the case of α-MSH (Figures 4 and 5). The results were similar to that of [Nle^4,D-Phe^7]-α-MSH, in which case the skins remained darkened due to continued melanosome dispersion within melanophores.

The relative activities of α-MSH, [Nle^4,D-Phe^7]-α-MSH and the biocytin analogues in stimulating S91 melanoma cell tyrosinase activity were then studied (Figure 6). It was observed that the biocytin analogues were at least equipotent to α-MSH (Figure 6a) in activating tyrosinase.

We then determined the relative stability of [Nle^4,D-Phe^7]-α-MSH and the biocytin analogues to enzymatic inactivation. [Nle^4,D-Phe^7]-α-MSH and each of the [Nle^4,D-Phe^7]-substituted analogues were totally resistant to inactivation by α-chymotrypsin during a 1-h incubation in the presence of the enzyme. In contrast, α-MSH at the same concentration was totally inactivated after a 30-min incubation in the presence of α-chymotrypsin. It should be noted that the enzymatic stability of the biotin analogues as well as [Nle^4,D-Phe^7]-α-MSH is not related to their in vitro prolonged activity. For example, we have synthesized melanotropins that are totally resistant to enzymatic inactivation but do not exhibit any prolonged biological activity.

These results suggest that (1) the synthetic methods employed to prepare biotin-labeled MSH analogues can be extended to prepare biotin conjugates of other peptides and proteins, (2) the coupling of biotin or other similar substitution at the N-terminal of analogues of α-MSH does not lead to loss of biological activity of the peptide, and (3) the N-terminal of α-MSH and related analogues probably can be utilized for coupling of other important molecules, for example, cytotoxic agents, to α-MSH analogues.

Although polypeptide hormones have been investigated from numerous points of view as natural substances of vital importance in the living organism, much is still to be done on the delineation of the functions and characteristics of various hormone receptors. We have prepared biotin-labeled melanotropin analogues that activate melanotropin receptors in several bioassays. The system should provide an accurate method for demonstrating the distribution of receptor regions in various tissues. The biotin-labeled ligands should also prove useful in following the mobilization and possible internalization of α-MSH and analogues at the receptor. In addition, avidin-solid support columns may be prepared for affinity chromatography of receptor-bound biotinyl ligands that would be a powerful step in the purification of surface proteins for structure–function studies and for the preparation of antibodies.

Experimental Section

General Methods. Capillary melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. Thin-layer chromatography (TLC) was performed on silica gel G plates with 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) ethyl acetate/pyridine/HOAc/H_2O 5:5:1:3), (D) 2-propanol/25% aqueous NH_3/H_2O (3:1:1), (E) chloroform/methanol (9.5:0.5), (F) chloroform/methanol/HOAc (6.5:3.0:5), and (G) benzene/chloroform (4:6). The load size was 30–60 μg, and chromatographic lengths were 12–16 cm. Detection was made by ninhydrin, iodine vapors, and UV light. In all cases, unless otherwise specified, single symmetrical spots were observed for purified materials. Amino acid analyses[23] were obtained on a Beckman 120C amino acid analyzer following hydrolysis for 22 h at 110 °C in one of the following reagents: (1) 6 N HCl containing 0.2% phenol, (2) 3 M mercaptoethanesulfonic acid (MESA),[24] or (3) 4 N methanesulfonic acid (MSA) containing 0.2% 3-(2-aminoethyl)indole.[25] Hydrolysis of the biotin-labeled melanotropin was also done with use of papain followed by leucine aminopeptidase enzyme digestions[26] for 24 h at 37 °C. No corrections were made for destruction of amino acids during hy-

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drolysis. Infrared (IR) spectra were obtained with a Perkin-Elmer 337 spectrophotometer. Nuclear magnetic resonance (NMR) spectra were obtained on Varian T-60 or Bruker WH-250 instruments. Peptide syntheses were done with a Model 250 automated Peptide Synthesizer (Vega Biochemicals, Tucson, AZ).

N^{α}-Boc-protected amino acids and amino acid derivatives were purchased from Peninsula (San Carlos, CA), Bachem (Torrance, CA), Sigma (St. Louis, MO), and Vega, or were prepared following the standard procedures and were of the L configuration except for Boc-phenylalanine, which was of the D configuration. Before use, all amino acid derivatives were tested for homogeneity by TLC and by the ninhydrin test.[27] Biotin was purchased from Sigma, *N*-hydroxysuccinimide from Pierce (Rockford, IL), *N,N'*-carbonyldiimidazole from Aldrich (Milwaukee, WI), and di-*tert*-butyl dicarbonate from Vega. These chemicals were also tested for homogeneity by TLC before use. The *p*-methylbenzhydrylamine (*p*-MBHA) resin (0.44 mg/g, 1% divinylbenzene cross-linked polystyrene) was obtained from United States Biochemical Corp. (Cleveland, OH). Solvents used for gel filtration, TLC, and other chromatographic methods were purified as previously reported.[28]

The frogs, *Rana pipiens*, used in bioassay studies, were obtained from Kons Scientific Co. (Germantown, WI) and the lizards, *Anolis carolinensis*, were obtained from the Snake Farm (LaPlace, LA). Ham's F-10 medium, fetal calf serum, and penicillin/streptomycin solutions were purchased from GIBCO Laboratories (Santa Clara, CA). Horse serum was obtained from Flow Laboratories (McLean, VA). Radioactive L-[3',5'-^{3}H]tyrosine (specific activity 54.6 Ci/mmol) was purchased from New England Nuclear (Boston, MA). The Cloudman S91 3960 (CCL 53.1) murine melanoma cell line was obtained from the American Type Culture Collection Cell Repository.

Solid-Phase Peptide Synthesis of Melanotropins. The protected [Nle^4,D-Phe^7]-α-MSH tridecapeptide resin was synthesized by solid-phase methods similar to those employed previously for the synthesis of α-MSH[29] and [Nle^4,D-Phe^7]-αMSH[6] but with some modifications. Side-chain functional groups of N^{α}-Boc-protected amino acids were protected as follows: tyrosine, *O*-2,6-dichlorobenzyl; glutamic acid, γ-benzyl ester; lysine, N^{ϵ}-2,4-dichlorobenzyloxycarbonyl; arginine, N^{g}-*p*-tolylsulfonyl; histidine, N^{im}-*p*-tolylsulfonyl; tryptophan, N^{i}-formyl; and serine, *O*-benzyl. Threefold excess of the N^{α}-Boc-protected amino acid derivatives were successively coupled to a substituted *p*-methylbezhydrylamine (*p*-MBHA) resin with a 2.4-fold excess of dicyclohexylcarbodiimide (DCC). A 3-fold excess of *N*-hydroxybenzotriazole (HOBT) was also added to suppress racemization.[30] In certain cases, the preformed symmetrical anhydride of Boc-protected amino acids, which were prepared by 3.5 mmol of Boc-amino acid and 1.75 mmol of DCC in dichloromethane (CH_2Cl_2), were also used for the stepwise couplings. In still other cases, for example, Boc-D-Phe, the coupling was achieved by employing mixed anhydride, prepared by 3.6 mmol each of Boc-D-Phe, *N*-methylmorpholine, and isobutyl chloroformate in dimethylformamide (DMF). Solvents like CH_2Cl_2 and DMF were used as general solvents for the synthesis of peptide, but for coupling of the last amino acid, serine, a more polar solvent, trifluoroethanol (15% in CH_2Cl_2), was used for completion of the reaction. Removal of the N^{α}-Boc protecting groups after every coupling, neutralization of the peptide resin salt, and addition of the next amino acid residue to the growing peptide chain followed the program reported recently from our laboratory.[11] The resultant Boc-Ser(Bzl)-Tyr(2,4-Cl_2-Bzl)-Ser(Bzl)-Nle-Glu(Bzl)-His(Tos)-D-Phe-Arg(Tos)-Trp(For)-Gly-Lys(2,4-Cl_2-Z)-Pro-Val-*p*-MBHA resin (**3**) was utilized to prepare the biotin-labeled melanotropins, as described below.

Succinimido Biotinate (2a). Biotin and *N*-hydroxysuccinimide were dried overnight over P_2O_5 in vacuo. The 0.506 g (2.07 mmol) of biotin was dissolved in 10 mL of DMF with gentle heating at 78 °C. Biocytin imidazolide was generated by the addition of 0.336 g (2.07 mmol) of *N,N'*-carbonyldiimidazole, followed by further heating until CO_2 evolution ceased. The stirring was continued at room temperature for 2 h during which time the intermediate precipitated out. It was converted to the succinimido ester by the addition of 0.238 g (2.07 mmol) of *N*-hydroxysuccinimide in 10 mL of DMF. The mixture was stirred overnight in a sealed flask during which time the precipitate disappeared. The solvent was removed in vacuo and the residue was recrystallized as a fine white powder from 2-propanol and then with DMF–ether: yield 0.64 g (90%); mp 209–211 °C dec (lit. mp 208–210 °C); R_f 0.44 in solvent system E. NMR (Me_2SO-d_6, 250 MHz) was in good agreement with the ^{1}H NMR analysis of the biotin molecule reported recently,[31] except that a triplet for α-CH_2 protons was shifted downfield to 2.6 ppm (as against 2.20 ppm assigned in biotin) because of the adjacent succinimido group. An additional singlet appeared at 2.79 ppm and was assigned to 4 H of the succinimido group. Further characterization by IR spectroscopy with the sample in a KBr pellet confirmed the presence of the desired functional groups.

[N^{α}-Bct-Ser^1,Nle^4,D-Phe^7]-α-MSH (1b). The Boc group of 0.75 g (0.079 mmol, 0.105 mmol/g) of the protected tridecapeptide resin **3** was removed by trifluoroacetic acid containing 2% anisole and neutralized by 10% of *N,N*-diisopropylethylamine in CH_2Cl_2. Then 80.9 mg (3-fold excess) of succinimido biotinate (**2a**) was allowed to react with the above resin in the presence of 20.4 mg (2-fold excess) of HOBT. The finish protected peptide was cleaved from the resin, and all protecting groups, with the exception of the formyl group on tryptophan, were removed with anhydrous liquid hydrogen fluoride (HF) containing anisole. The title product was prepared by deformylation on the formylated peptide in aqueous solution by addition of 4 N NaOH to a pH of 11.5 for 3 min as previously reported.[29] The deformylation reaction was terminated by the addition of glacial acetic acid to a final pH of 4.5. The ion-exchange chromatography of the biotin-labeled peptide was done on a (carboxymethyl)cellulose column (2 × 17.8 cm). The main peak obtained (tubes 80–91) was collected, concentrated, and desalted in a Sephadex G-25 column in 30% aqueous acetic acid. After lyophilization there was obtained 1.109 mg (yield 10%) of the desired compound. Amino acid analysis gave the following molar ratios: Lys, 1.02; His, 1.07; Gly, 1.05; Val, 1.07; D-Phe, 1.02; Tyr, 0.85; Arg, 0.99; Ser, 1.83; Glu, 1.04; Pro, 0.94; Nle, 1.02; and Trp, 0.82. The peptide gave a characteristic pink color upon reaction with *p*-(dimethylamino)-cinnamaldehyde, indicating the presence of biotinyl moiety.[20]

12-(Boc-amino)dodecanoic Acid (4). This compound was prepared from 2.0 g (9 mmol) of 12-aminododecanoic acid and 2.23 g (10.23 mmol) of di-*tert*-butyl dicarbonate following the conditions reported previously.[33] The recrystallization from ethyl acetate–hexane provided 2.23 g (76% yield) of the desired product having mp 80–81 and R_f 0.66 in the solvent system E. The compound was ninhydrin negative, which showed the absence of free amino group. NMR ($CDCl_3$, 60 MHz) also showed the expected pattern of the signals. The 9 H of the Boc group appeared as a singlet at 1.5 ppm.

Succinimido 12-(Boc-amino)dodecanoate (2b). This compound was prepared from 2.0 g (6.34 mmol) of 12-(Boc-amino)-dodecanoic acid (**4**), 1.13 g (6.98 mmol) of *N,N'*-carbonyldiimidazole, and 0.8 g (6.98 mmol) of *N*-hydroxysuccinimide following the conditions reported for the synthesis of succinimido biotinate (**2a**). On recrystallization from ethyl acetate–petroleum ether, 1.88 g (72% yield) of the desired compound was obtained: mp 107.5–108 °C; R_f 0.61 in the solvent system E. Satisfactory elemental analyses were obtained for ($C_{21}H_{36}N_2O_6$) C, H, and N. The NMR ($CDCl_3$, 250 MHz) showed disappearance of carboxyl proton signal from 10.5 ppm and appearance of an additional singlet at 2.84 ppm for 4 H of the succinimido group. The IR spectroscopic data of the title compound in a KBr pellet further confirmed the presence of the desired functional groups.

[12-Bct-N^{α}-dodecanoyl-Ser^1,Nle^4,D-Phe^7]-α-MSH (1c). The 0.50-g (0.22 mmol, 0.44 mmol/g) sample of the protected tride-

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capeptide resin **3** was used to prepare the title compound. Succinimido 12-(Boc-amino)dodecanoate (**2b**) and then succinimido biotinate (**2a**) were used to incorporate N-terminal 12-biocytin-N^{α}-dodecanoyl moiety into the peptide in the following manner: (1) washing with four 20-mL portions of CH_2Cl_2 for 1 min each, (2) cleavage of the Boc group by addition of 20 mL of 45% TFA in CH_2Cl_2 containing 2% anisole and shaking for 2 min, (3) another addition of 20 mL of 45% TFA in CH_2Cl_2 containing 2% anisole and shaking for 20 min, (4) washing with three 20-mL portions of dichloromethane for 1 min each, (5) neutralization by addition of two 20-mL portions of 10% *N,N*-diisopropylethylamine in CH_2Cl_2 and shaking for 2 min each, (6) washing with four 20-mL portions of DMF for 1 min each, (7) addition of 3-fold excess of succinimido 12-(Boc-amino)dodecanoate (**2b**) in 10 mL of DMF and 3-fold excess of HOBT in 10 mL of DMF and shaking until ninhydrin negative, (8) washing with three 20-mL portions of DMF for 1 min each, (9) washing with three 20-mL portions of ethanol for 1 min each, and (10) washing with four 20-mL portions of CH_2Cl_2 for 1 min each. The procedure was repeated except that at step 7 a 3-fold excess each of succinimido biotinate and HOBT in 20 mL of DMF was added and the mixture shaken until ninhydrin negative. The protected peptide was then cleaved from the resin, and all protecting groups (except the N^{i}-formyl group of tryptophan) were removed by treatment with anhydrous liquid HF containing anisole for 45 min at 0 °C. After the reaction, HF and anisole were evaporated in vacuo. The dried product was washed with three 30-mL portions of ethyl acetate, and the peptide was extracted with three 30-mL portions each of 30% acetic acid, 1 N acetic acid, and H_2O. The combined aqueous extracts were lyophilized. The formylated peptide was deformylated by adding 4 N NaOH to pH 11.5 for 3 min, and the reaction was terminated by addition of glacial acetic acid to a final pH of 4.5. The crude peptide was purified by ion-exchange chromatography on a (carboxymethyl)cellulose column (2 × 23.3 cm) using a discontinuous gradient of 250 mL of 0.01 M ammonium acetate (pH 4.5) and then 250 mL each of 0.1, 0.2, and 0.4 M ammonium acetate (pH 6.8). The fraction containing the major peak (tubes 91–119) was lyophilized to give a cream color powder, which was subjected to gel filtration on Sephadex G-25 in 30% aqueous acetic acid. Lyophilization yielded 6.58 mg (yield 14.7%) of white title product. Amino acid analysis of this compound gave the following molar ratios: Tyr, 0.95; Phe, 0.93; Nle, 0.99; Val, 1.08; Gly, 1.02; Pro, 0.95; Trp, 0.93; Glu, 1.09; Ser, 1.90; Arg, 1.06; His, 0.95; and Lys, 1.05. The peptide gave a characteristic pink color upon reaction with *p*-(dimethylamino)cinnamaldehyde, indicating the presence of biotinyl moiety.[20] NMR (D_2O + CD_3COOD, 250 MHz) was in good agreement with the ^1H NMR of the $ACTH_{1-10}$ (which is a desacetylated α-MSH_{1-10}) and of biotin as reported recently.[31,34] Other peaks were also reasonably assignable to individual protons of Val, Pro, Lys, and the 12-aminododecanoic acid moieties.

In Vitro Frog and Lizard Skin Bioassays. The biotin-labeled analogues were compared to α-MSH and [Nle^4,D-Phe^7]-α-MSH with respect to their ability to stimulate melanosome dispersion in vitro with use of the frog (*Rana pipiens*) and lizard (*Anolis carolinensis*) skin bioassays as described previously.[35-37] Changes in skin color are monitored by a photovolt reflectometer and recorded as differences from the initial base (zero) value. In response to melanotropins, melanosomes within integumental melanophores migrate from a perinuclear position into the dendritic processes of the pigment cells. This centrifugal organellar movement results in darkening of the skins. Subsequent removal of the melanotropins (Ringer rinse, in figures) usually result in a rapid perinuclear (centripetal) reaggregation of melanosomes, leading to a lightening of the skins back to their original reflectance value.

Tyrosinase Bioassay. Cloudman S91 3960 (CCL 53.1) melanoma cells were maintained in Ham's F-10 medium supplemented with 10% horse serum and 2% fetal calf serum, both heat inactivated at 56 °C for 30 min. Cells were incubated at 37 °C in an atmosphere of 5% CO_2–95% air. To determine hormone-induced tyrosinase activity, cells were seeded at a density of 2 × 10^5 cells/25 cm^2 flask and were exposed to the melanotropins for 48 h. Twenty-four hours before the end of the experiment, the media were replaced by fresh media containing 1 μCi of [^{3}H]tyrosine/mL and the melanotropins at the concentrations indicated. At the end of the experiment, the cells were harvested with Tyrode's solution and counted with the aid of a hemacytometer. The labeled media were then assayed for tyrosinase activity according to a modification of the method of Pomerantz.[38] This assay is based on 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 and as a percent of the control cells taken as 100%.

Enzyme Studies. The stabilities of the biotin-labeled and [Nle^4,D-Phe^7] analogues were compared to α-MSH stability by incubating the melanotropins for 30 and 60 min in α-chymotrypsin. The melanotropins (1 mL of a 5 × 10^{-5} M solution) were incubated at room temperature in an equal volume or Ringer (control) or α-chymotrypsin (10 μ/mL) to provide a 2.5 × 10^{-5} M substrate concentration of the melanotropins. The activities of the melanotropin were then determined by the lizard skin bioassay.

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Registry No. **1b**, 91295-35-1; **1c**, 91311-00-1; **2a**, 35013-72-0; **2b**, 91295-36-2; **4**, 18934-81-1; biotin, 58-85-5; 12-aminododecanoic acid, 693-57-2; di-*tert*-butyl dicarbonate, 24424-99-5; tyrosinase, 9002-10-2.

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