3,4-Dihydro-8-hydroxy-9-trifluoromethyl-1(2*H*)-dibenzofuranone (19a). A solution of 700 mg of 4a,7,8,9a-tetrahydro-9a-hydroxy-4-trifluoromethylcarbazole-3,5(4*H*,6*H*)-dione (3a) in 15 ml of 20% HCl was heated at reflux temperature for 2 hr, cooled, and diluted with water. Filtration gave 370 mg (55%) of pale yellow powder, mp 240–250° dec. Crystallization from methanol gave the analytical specimen as a white powder: mp 272–274° dec; uv max 210, 238, 255 (sh), 300 mμ (ε 27,500, 15,500, 10,500, 7000); ir max 3.24, 6.04, 6.15, 6.27, 6.40 μ; nmr (DMSO-d_6) δ 10.3 (broad, O*H*), 7.72 (d, 1, $J_{6,7}$ = 10 Hz, 6-*H*), 7.02 (d, 1, $J_{6,7}$ = 10 Hz, 7-*H*), 3.05 (t, 2, *J* = 7 Hz, 3-CH_2), 2.75–1.83 (envelope).

Anal. Calcd for $C_{13}H_9F_3O_3$: C, 57.78; H, 3.36; F, 21.10. Found: C, 58.02; H, 3.31; F, 21.04,

5,6,7,8-Tetrahydro-3-hydroxy-7,7-dimethyl-5-keto-4-carbazolecarboxylic acid-1-*d*. Treatment of 250 g of 4a,7,8,9a-tetrahydro-9a-hydroxy-7,7-dimethyl-4-trifluoromethylcarbazole-3,5(4*H*,6*H*)-dione-1,4a-*d* (3c) with 25 mg of **1a** in 2.5 ml of acetic acid as described in method B for the preparation of **5b** gave 70 mg (32%) of 5,6,7,8-tetrahydro-3-hydroxy-7,7-dimethyl-5-keto-4-carbazolecarboxylic acid-1-*d*, mp 270–272° dec. The nmr spectrum of this material showed a sharp singlet at δ 6.80 (2-*H*).

2,3-Dihydro-6-hydroxy-2,2-dimethyl-5-trifluoromethyl-4(1*H*)-carbazolone-3,3,8-*d*. Treatment of 4a,7,8,9a-tetrahydro-9a-hydroxy-7,7-dimethyl-4-(trifluoromethyl)-carbazole-3,5-(4*H*,6*H*)-dione-1,4a-*d* (3c) with 60 mg of **1a** in 6 ml of acetic acid-d_4 at reflux temperature for 4 hr gave white crystals, mp 260–265° dec. The nmr spectrum of this material showed a sharp singlet at δ 6.85 (7-*H*).

3-Amino-2-(α,α,α-trifluoro-3,6-dihydroxy-*o*-tolyl)-5,5-dimethyl-2-cyclohexen-1-one (4b). A suspension of 900 mg (2.86 mmol) of 4a,7,8,9a-tetrahydro-9a-hydroxy-7,7-dimethyl-4-trifluoromethylcarbazole-3,5(4*H*,6*H*)-dione (3b) in 9 ml of acetic acid was heated rapidly to reflux temperature. Although solution did not occur, a visual change in the character of the solid was observed. After 3 min the mixture was filtered to give 760 mg (84%) of white crystals, mp 264–266° dec.

Material from a similar experiment was recrystallized from ethanol–acetone to give crystals: mp 285° dec; uv max 230, 290 mμ (ε 6300, 25,000); ir max 2.85, 2.95, 3.00, 3.40, 6.05, 6.52 μ; nmr (DMSO-d_6) δ 9.22 (s, 1, O*H*), 8.03 (s, 1, O*H*), 6.78 (s, 2, aryl *H*), 5.72 (broad s, 2, NH_2), 2.27 (s, 2, 4-CH_2), 2.05 (s, 2, 6-CH_2), 1.05, 1.02 (nonequivalent s, 6, C(CH_3)$_2$).

Anal. Calcd for $C_{15}H_{16}F_3NO_3$: C, 57.13; H, 5.16; F, 18.08; N, 4.44. Found: C, 57.36; H, 5.25; F, 18.42; N, 4.33.

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Semisynthetic Polypeptides. Transformation of Native Porcine β-Melanotropin into the Lysine-10 Analog of the Human Hormone[1]

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Abstract: To determine the utility of preparing peptide analogs by a semisynthetic approach, the lysine-10 analog of human β-melanotropin, a docosapeptide, was synthesized by treating native porcine β-MSH with a suitably blocked azide of alanylglutamyllysyllysine. In addition to the desired straight-chain semisynthetic peptide, a branched-chain product was obtained, the result of acylation of an N$^\epsilon$ site of porcine β-melanotropin.

The combination of native and synthetic peptides provides a method alternate to total synthesis for the preparation of polypeptide analogs. Such a semisynthetic approach may be particularly useful for the convenient introduction of limited amino acid substitutions in enzymes or other large molecules in order to examine the molecular basis of specificity of action or the nature of forces stabilizing tertiary structure. Semisynthesis, widely employed in the preparation of analogs of many natural products, has only rarely been applied in peptide chemistry. Among reports on the use of native substrates for the synthesis or study of well characterized peptide analogs are those recorded by Dixon, *et al.*,[4] on transamination of amino-terminal residues, and on transformation of corticotropin[5] into [Gly[1]]-corticotropin,[6] by Katsoyannis, *et al.*,[7] on the combination of native and synthetic chains of insulin, and by Hofmann, *et al.*,[8] on interactions of synthetic S-peptide analogs with native S-protein of ribonuclease. Milne and Carpenter[9] employed *t*-butyloxycarbonylamino acids for acylation of insulin in the formation of di- and triaminoacylinsulins. Only the latter study, however, was concerned with the formation of new peptide bonds and the consequent necessity for

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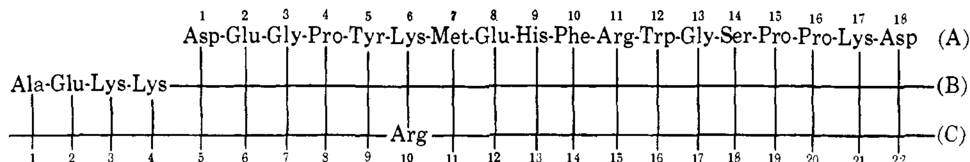

Figure 1. Amino acid sequences of porcine and human β-melanotropins. (A) β^p-MSH[13]; (B) [Lys[10]]-β^h-MSH; (C)β^h-MSH.[11] Residues are the same as column headings except where indicated. Numbering systems for the porcine and human peptides are given.

application of specific and reversible blocking groups. Initial efforts of Offord to employ enzymatically derived native subunits for the synthesis of analogs[10] prompted this report of our own research in which the naturally occurring octadecapeptide β^p-MSH[11] was used for the preparation of [Lys[10]]-β^h-MSH, a docosapeptide analog of human β-melanotropin.

The primary structures of human[12] and porcine[13] β-melanotropin (β^h-MSH and β^p-MSH) are shown in Figure 1. The human hormone is an analog of the porcine derivative in which the amino terminus of the latter is acylated with the tetrapeptide Ala-Glu-Lys-Lys and in which lysine-6 is replaced with arginine. Thus, β^h-MSH might also be termed [Arg[6]]-Ala-Glu-Lys-Lys-β^p-MSH. Specific acylation of the amino terminus of β^p-MSH with the same tetrapeptide would yield Ala-Glu-Lys-Lys-β^p-MSH, an analog of the human hormone, *i.e.*, [Lys[10]]-β^h-MSH (Figure 1). To determine the feasibility of such a reaction, BOC-Ala-Glu(O-*t*-Bu)-Lys(BOC)-Lys(BOC)-$NHNH_2$[14] was synthesized, transformed into azide, and allowed to react under various conditions with natural β^p-MSH. Preparation and characterization of the blocked tetrapeptide azide[15] and reaction products of azide with β-MSH are reported here as is the biologic characterization of products and deblocked products.

Experimental Section

Z-Glu-(O-*t*-Bu)-Lys(BOC)-Lys(BOC)-OMe (I). Z-Lys(BOC)-Lys(BOC)-OMe[16] (6.2 g, 10 mmol) in methanol (100 ml) was hydrogenated over palladium oxide (0.5 g) in a continuous stream of hydrogen for 2.5 hr. The product, after filtration and evaporation *in vacuo* (R_f 0.53),[17] was dissolved in methylene chloride (75 ml). Z-Glu(O-*t*-Bu)[19] (3.4 g, 10 mmol), prepared *via* the dicyclohexylamine salt of Z-Glu-OEt,[20] was added and the solution cooled to −15°. After addition of dicyclohexylcarbodiimide (DCC) (2.10 g, 10 mmol),[21] the mixture was left at −15° for 30 min and 2° for 17 hr. The reaction mixture was filtered and the filtrate was extracted twice at 2° with water (50 ml) containing sufficient citric acid monohydrate to bring the pH to about 3, and then twice with a half-saturated aqueous sodium chloride solution, 0.25 *M* in sodium bicarbonate. After drying over calcium sulfate, the organic phase was evaporated *in vacuo* and the residue, 7.6 g, was recrystallized twice from chloroform–hexane (1:9): yield 6.2 g (76%); mp 151–152°; $[\alpha]^{19}D$ −18.7° (*c* 3.70, MeOH) [lit.[15] mp 153–156°; $[\alpha]^{25}D$ −37.0° (MeOH)]; R_f 0.22;[22] quantitative amino acid analysis:[23] $Lys_{2.12}Glu_{0.90}$. *Anal.* Calcd for $C_{40}H_{65}N_5O_{12}$: C, 59.5; H, 8.1; N, 8.7. Found: C, 59.1; H, 8.1; N, 8.7.

BOC-Ala-Glu(O-*t*-Bu)-Lys(BOC)-Lys(BOC)-OMe (II). Peptide I, 3.2 g, 4 mmol, in methanol (100 ml) was hydrogenated over palladium oxide (0.3 g) for 2.5 hr in a continuous stream of hydrogen; thin layer chromatography showed that reaction was complete, R_f 0.76.[22] To the product of hydrogenation, after filtration and evaporation *in vacuo*, BOC-Ala[24] (0.76 g, 4 mmol) and DCC (0.8 g, 4 mmol) in methylene chloride (100 ml) were added and allowed to react as described above. The neutral product was obtained as above, and crystallized from ether–hexane (2:3): yield 2.7 g (81%); mp 91–96°; $[\alpha]^{21}D$ −28.6° (*c* 1.19, MeOH) [lit.[15] mp 91–95°; $[\alpha]^{25}D$ −30.9° (MeOH)]; R_f 0.63;[22] quantitative amino acid analysis: $Lys_{2.07}Glu_{1.03}Ala_{0.89}$. *Anal.* Calcd for $C_{40}H_{72}N_6O_{13}$: C, 56.9; H, 8.6; N, 10.0. Found: C, 56.9; H, 8.7; N, 10.1.

BOC-Ala-Glu(O-*t*-Bu)-Lys(BOC)-Lys(BOC)-$NHNH_2$ (III) and Azide (IV). A solution of II (5.92 g, 7 mmol) in 1 *M* methanolic hydrazine (70 ml) was kept at 5° for 18 hr, evaporated to dryness *in vacuo*, and stored over concentrated sulfuric acid under vacuum. The residue was crystallized from methylene chloride–hexane (2:1): yield 4.5 g (77%); mp 159–161°; $[\alpha]^{21}D$ −27.5° (*c* 0.9, MeOH) [lit.[15] mp 165–167°; $[\alpha]^{23}D$ −25.0° (MeOH)]; R_f 0.53;[22] quantitative amino acid analysis: $Lys_{2.01}Glu_{1.03}Ala_{0.97}$. *Anal.* Calcd for $C_{39}H_{72}N_8O_{12}$: C, 55.4; H, 8.6; N, 13.3. Found: C, 54.9; H, 8.7; N, 13.1.

Optical purity of amino acids in III was determined by the ion exchange method of Manning and Moore.[25] Assuming color values similar to those reported, percentages of D-amino acids found were 1.9, 0.2, and 0.4 for alanine, glutamic acid, and lysine, respectively, amounts usually produced by acid hydrolysis.[25,26]

Azide (IV) was prepared by mixing hydrazide (III) (250 mg, 0.3 mmol) in 50% aqueous acetic acid (7.8 ml) with sodium nitrite (25 mg in 2.4 ml of H_2O) for 2 min at −5°. The product was extracted into ether, washed neutral, dried over calcium sulfate, and isolated in oily form by evaporation of solvent *in vacuo*—all at 5°; yield 165 mg (65%).

BOC-Ala-Glu(O-*t*-Bu)-Lys(BOC)-Lys(BOC)-β^p-MSH (V). β^p-MSH of greater than 98% purity, as judged by amino acid analysis, was prepared from a β-MSH "concentrate"[27] by ion exchange chromatography on DEAE-Sephadex A-25 with Tris buffer, pH 8.0, μ = 0.01. A portion of the analysis of an acid hydrolysate of this material is given in Table I. β^p-MSH (50 mg) in 2.0 ml of cold water and azide (IV) (165 mg) in 2 ml of cold pyridine were mixed and left for 4 hr at 5°. The mixture was diluted with 8 ml of water and extracted three times with 10 ml of ether, titrated to pH 4.9 with acetic acid, reextracted three times with ether, and lyophilized. Products were isolated by an ion exchange chromatography on a column of DEAE-sephadex (1.2 × 95 cm) developed with a buffer of 0.02 *M* NH_4OAc, pH 4.9. The effluent, monitored at 220 mμ, was collected in 5-ml aliquots at a rate of 15.6 ml/hr (Figure 2). Material from peaks A, B, and C (2.3, 4.1, and 23.0 mg, respectively) was obtained in dry form by lyophilization.

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Table I. Determination of the Site of Acylation by Amino Acid Analysis[a]

	β-MSH		Mono-N-acyl-β-MSH			DNP-N$^{\alpha}$-acyl-β-MSH (V)		DNP-N$^{\epsilon}$-acyl-β-MSH	
	Theor	Exper	Theor	Exper		Theor	Exper	Theor	Exper
				(B)	(A)		(B)		(A)
Lys	2	2.09	4	3.9	4.0	2	2.2	3	2.7
His	1	1.00	1	1.1	1.1				
Arg	1	1.00	1	1.0	0.84	1	1.0	1	0.91
Asp	2	1.95	2	2.1	1.9	2	1.9	1	1.3
Glu	2	2.05	3	3.1	2.8	3	2.9	3	3.0
Ala	0	0.00	1	0.87	0.86	1	0.92	1	0.94

[a] Acid hydrolysates were prepared and analyzed on a Spinco 120 B analyzer.[23] A total analysis of β-MSH is not given to conserve space. However, the level of extraneous amino acids in our purified β^{p}-MSH, for example, alanine, was less than 0.1 residue.

Products were identified by quantitative amino acid analysis; unequivocal identification of acylation sites was made by comparison of amino acid analyses of products and dinitrophenylated[28] products. By this means N$^{\alpha}$-, N$^{\epsilon}$-, or polyacylated derivatives are readily distinguishable (Table I). Clearly, component B (Figure 2) is the desired N$^{\alpha}$-acylated product (V) while component A is a mono-N$^{\epsilon}$-acyl derivative. Component C of the chromatograph, unaltered β^{p}-MSH, was recovered in 50% yield.

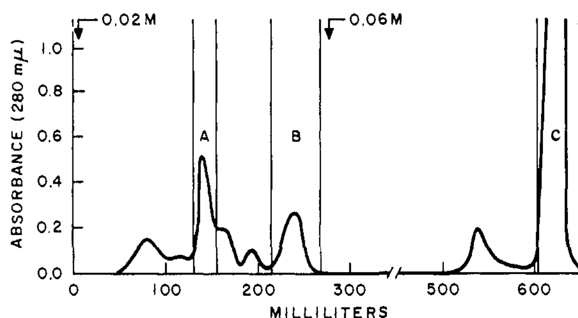

Figure 2. Isolation of [Lys[10]]-β^{human}-MSH. Chromatography was performed on a 1.2 × 95 cm column of CM-52 (Whatman) with ammonium acetate at pH 4.9. Concentrations of buffer are indicated in the figure.

[10-Lysine]-β^{h}-melanotropin (VI). Peptide V, 1 mg, was deblocked in anhydrous formic acid (1 ml) for 2.5 hr at room temperature,[29] lyophilized, and then relyophilized from dilute acetic acid. The product appeared homogeneous by thin layer chromatography, R_f 0.20[30] (R_f V = 0.41, R_f β-MSH = 0.08).

Melanotropic Activities of Semisynthetic Products. Products were assayed by the *in vitro* frog skin technique.[32] Specific activities are given relative to that of β^{p}-MSH (1 × 10^{10} U/g): β^{p}-MSH, 1.0; BOC-Ala-Glu(O-*t*-Bu)-Lys(BOC)-Lys(BOC)-β^{p}-MSH, 0.04; semisynthetic [Lys[10]]-β^{h}-MSH, 0.43; synthetic β^{h}-MSH, 0.47.[33]

Discussion

Semisynthetic human [10-lysine]-β^{h}-MSH was prepared by coupling a suitably blocked synthetic tetrapeptide to native porcine β-MSH. Purity and identity of the desired product were established by quantitative amino acid analyses of blocked, deblocked, blocked–dinitrophenylated, and deblocked–dinitrophenylated derivatives. Overall yields of about 4% were obtained, an order of magnitude often achieved in the total synthesis of peptides of similar size and complexity. The technique of combining synthetic and native peptides, when the latter are available, appears to be a practical means for obtaining analogs.

Sufficient semisynthetic material was prepared by these procedures to allow characterization of biological activity, the major reason for analog synthesis. Results of these experiments were reported above. The tenfold enhancement of melanotropic activity of peptide VI, relative to V, after deblocking with formic acid, indicates that observed biologic effects of V or VI did not arise from contamination with β^{p}-MSH. Also, it should be noted that β^{h}-MSH and [10-lysine]-β^{h}-MSH exhibit virtually identical melanotropic activities *in vitro*.

In addition to the desired straight chain semisynthetic peptide, a branched-chain product was obtained, the result of acylation of an N$^{\epsilon}$ site of porcine β-MSH. That the branched product was a mono-N$^{\epsilon}$-acyl derivative was established by dinitrophenylation; whether acylation occurred at lysine-6 or -17 or both was not determined. Melanotropic activities of the blocked–branched and deblocked–branched docosapeptides were, respectively, 5 and 10% that of β^{p}-MSH, and these compounds were not studied further.

Extending reaction times of blocked tetrapeptide azide with β^{p}-MSH failed to increase yields of docosapeptide but resulted instead in the formation of polyacyl products and further consumption of β^{p}-MSH. In spite of a large α–ε-amino pK difference, protonation alone is an insufficient deterrent to N$^{\epsilon}$ acylation in β-MSH. Apparently high nucleophilicity compensates for low concentration of unprotonated N$^{\epsilon}$-amino species at neutral pH. Techniques to enhance specificity of acylation, based on this property, are being studied.

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