249. Divergent Melanophore-Dispersing and Tyrosinase-Stimulating Activity of Synthetic Leucine^9-α-melanotropin[1])

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Summary

Leucine^9-α-melanotropin ([Leu^9]α-MSH) was synthesized in homogeneous solution by a fragment condensation approach, and it was assayed for its melanophore-dispersing and its tyrosinase-stimulating activity with a reflectometric *in vitro* frog skin assay and with cultured mouse melanoma cells, respectively. In both assay systems, parallel log dose-response curves were obtained for [Leu^9]α-MSH and α-MSH; however, in the frog skin assay the activity of the title compound was $1 \cdot 10^{10}$ Units/mmol, *i.e.* 25% of the activity of α-MSH, whereas its tyrosinase-stimulating potency was only 1% compared to α-MSH ($EC_{50} = 2.5 \cdot 10^{-7}$ M). This indicates a major difference in the recognition/stimulation process of the receptors of the two cell types.

Introduction. – The functional role of a number of peptide fragments and of side chain groups of α-melanotropin (α-MSH) interacting with amphibian melanophores has been investigated in our institute during the last few years [4]. It appeared that α-MSH may be divided into three regions with regard to its function as a melanophore-dispersing agent: (1) the *N*-terminal potentiating sequence, Ac · Ser-Tyr-Ser-Met-(Glu-), (2) the 'classical' central message sequence, (-Glu)-His-Phe-Arg-Trp-, and (3) the hitherto unknown *C*-terminal message sequence, -Gly-Lys-Pro-Val · NH_2 [5]. Within the two message sequences, -Phe-Arg- and -Lys-Pro- appeared to be the key elements for triggering the stimulus [6]. Much in contrast to ACTH where tryptophan^9 is known to be important for eliciting steroidogenesis in adrenal cells or lipolysis in rat adipocytes [7] [8], α-MSH is very little affected in its potency to induce pigment migration in frog melanophores when this residue is modified [1] [9]. With respect to position 9 of both hormones, there seems to be a major

[1]) Parts of this work have appeared in a preliminary form [1]. Abbreviations are according to the *IUPAC-IUB Commission on Biochemical Nomenclature* [2] and *Houben-Weyl* [3]. Additional abbreviations are: ACTH = corticotropin, MSH = melanotropin; DMF = *N*,*N*-dimethylformamide, DCC = *N*,*N'*-dicyclohexylcarbodiimide, DCU = dicyclohexylurea, HOBT = 1-hydroxybenzotriazole; Ac = acetyl, BOC = *t*-butyloxycarbonyl, MsOC = 2-(methylsulfonyl)-ethoxycarbonyl, OTsE = 2-(4-tolylsulfonyl)-ethoxy. All chiral amino acids are in the L-configuration.

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difference of the recognition pattern between amphibian (frog) melanophore α-MSH-receptors and mammalian adrenal ACTH-receptors which is either due to the considerable species difference of the two biological systems or just a consequence of different evolutionary processes of receptor development in the two cell populations within the same (or related) species. In order to clarify this question, we have extended our structure-function studies of α-MSH to a mouse melanoma cell line in culture [10] which responds to the hormone with increased tyrosinase activity, *cyclic* AMP production, and pigment formation [11].

In this report, we describe the synthesis of leucine^9-α-melanotropin ([Leu^9]α-MSH) and its melanophore-dispersing and tyrosinase-stimulating activity in frog melanophores and *Cloudman* S-91 mouse melanoma cells, respectively. Leucine was chosen instead of tryptophan^9 in order to replace this aromatic side chain by an aliphatic one with similar lipophilicity.

Synthesis of leucine^9-α-melanotropin. – The synthesis was carried out in homogeneous solution, and the intermediates were, whenever possible or desirable, isolated, purified, and characterized. Similar strategy and tactics as outlined in earlier syntheses of α-MSH [12] [13] were followed using OTsE as carboxy and MsOC as side chain amino protecting groups.

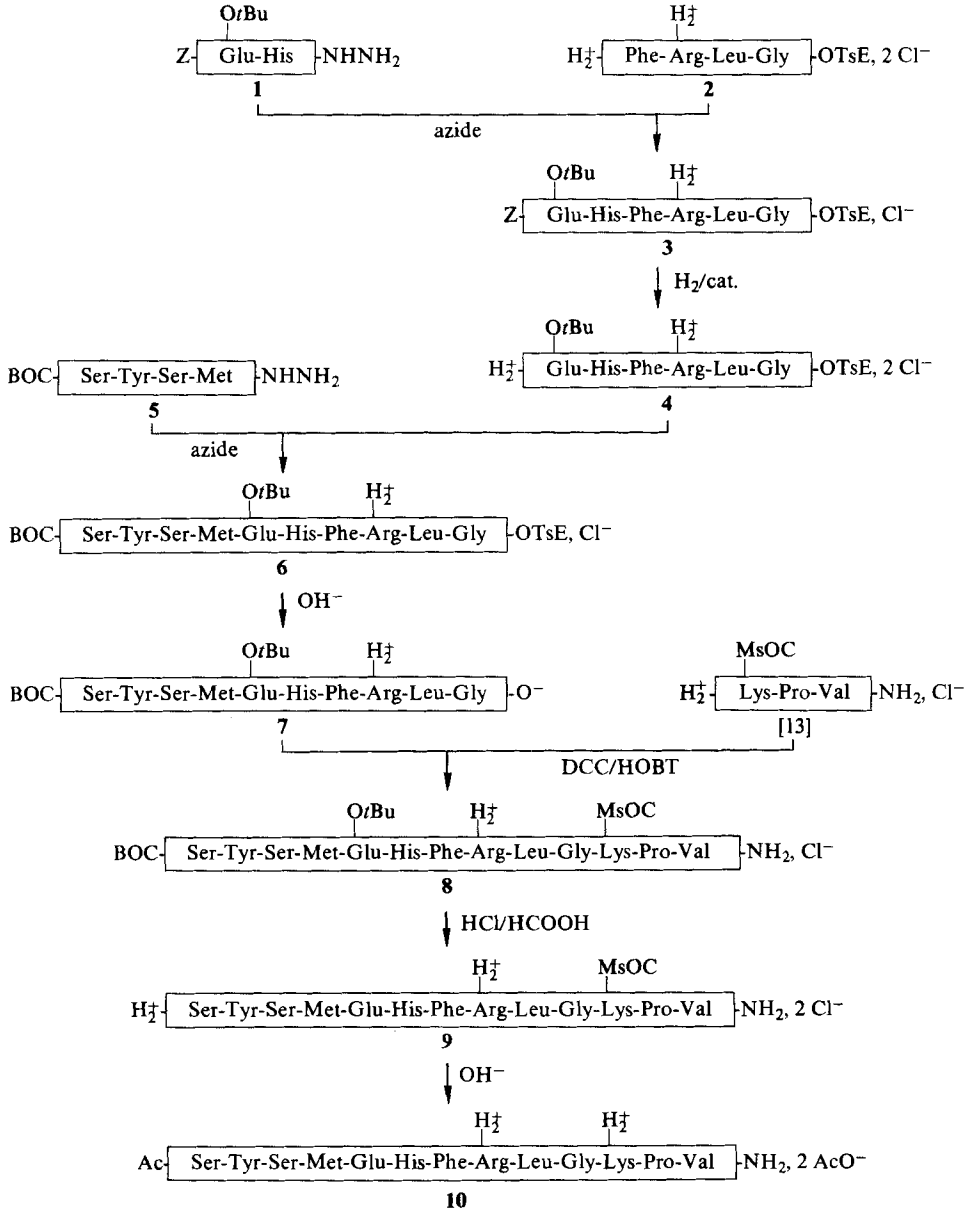
The protected peptide fragments **1** and **2** were prepared as described in [13]. Condensation of **1** (*via* its azide) with **2** yielded **3** which was isolated in homogeneous form by partition chromatography on *Sephadex* G-25. Hydrogenolytic cleavage of the Z-group was not affected by the presence of the TsE-group and was complete within 90 min in 80% acetic acid. The protected decapeptide **6** was synthesized in a similar way as **3** by azide coupling in order to minimize racemization of methionine[4]. As in the case of the hexapeptide **3**, the TsE-group enhanced the solubility of the decapeptide **6** in organic solvents and facilitated the purification of the peptide by countercurrent distribution. β-Elimination of the TsE-group was complete within 10 min yielding homogeneous **7**.

Condensation of the decapeptide **7** with the *C*-terminal tripeptide {H · Lys(MsOC)-Pro-Val · NH_2, HCl} to **8**, deprotection to **9**, N^{α}-acetylation, and elimination of the MsOC-group was performed as described for α-MSH [13]. The final product **10** was purified by chromatography on *Sephadex* LH-20.

Melanophore-dispersing activity of leucine^9-α-melanotropin. – From a preliminary investigation of [Leu^9]α-MSH with isolated skin pieces of the frog, *Rana pipiens*, we have reported a melanophore-dispersing activity of $1 \cdot 10^{10}$ Units/mmol, *i.e.* 25% of the potency of natural α-MSH [1]. A recent reinvestigation confirmed our previous finding. The results with the modified [4a] reflectometric *in vitro* frog skin assay [15] are displayed in *Figure 1*. Reflectance changes are expressed as % of the maximally attainable reflectance change (of each skin piece) in the presence of a large excess of α-MSH (100 Units/ml). In this way, log dose-response curves are generated which show linear characteristics between 10% and 60% reflectance change. This is in fact just the range in which the assay is usually performed (see [1]). In addition to the reflectance measurements, the skin pieces were routinely examined under the microscope in order to check the degree of melanosome-dispersion.

Both peptides, α-MSH and [Leu^9]α-MSH, have parallel log dose-response curves with EC_{50} values of $2.5 \cdot 10^{-11}$ M for α-MSH and 10^{-10} M for [Leu^9]α-MSH, respectively. In this assay system, the relative molar melanophore-dispersing potency of α-MSH ($4 \cdot 10^{10}$), taken as $1/EC_{50}$, corresponds exactly to the value normally used to express the activity of α-MSH: $4 \cdot 10^{10}$ Units/mmol. The

Scheme. *Synthesis of leucine^9-α-melanotropin*



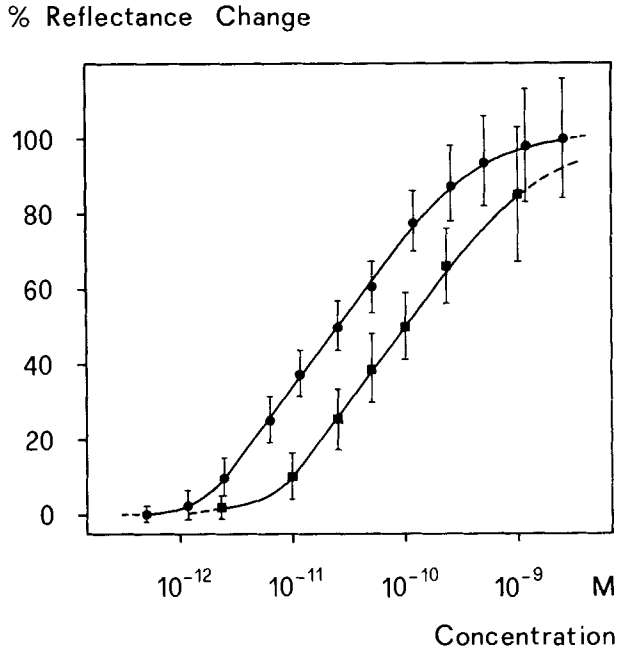


Fig. 1. *Melanophore-dispersing activity of α-MSH* (●——●) *and [Leu⁹]α-MSH* (■——■). Each point of the log dose-response curves is the mean of 8–20 measurements. Values in the range between 60% and 100% reflectance change were determined after calibrating the skins with $2.5 \cdot 10^{-11}$M (1 Unit/ml) α-MSH (since dispersion is irreversible at high MSH-concentrations). $2.5 \cdot 10^{-9}$M α-MSH was set as 100% because generally no further darkening was observed at even higher concentrations.

corresponding value for [Leu⁹]α-MSH is 4× smaller: $1 \cdot 10^{10}$ Units/mmol. (In contrast to the activity found with frog melanophores, [Leu⁹]α-MSH exhibits a much lower potency in the *Anolis carolinensis* or the *Xenopus laevis* assay systems: ~2% and ~0.2%, respectively [14].)

Tyrosinase-stimulating activity of leucine⁹-α-melanotropin. – Tyrosinase activity of cultured *Cloudman* S-91 mouse melanoma cells was determined by measuring ^3H_2O release from (3′,5′-$^3H_2$)-L-tyrosine added to the culture medium (details see [16]). Maximal ^3H_2O release obtained with a 10^{-7}M α-MSH solution was set as 100% of the response. The log dose-response curves for α-MSH and [Leu⁹]α-MSH are displayed in *Figure 2*. They are fairly parallel, but differ in their shape from those obtained with the frog skin assay because of completely different test systems. EC_{50} values are $2.5 \cdot 10^{-9}$M for α-MSH and $2.5 \cdot 10^{-7}$M for [Leu⁹]α-MSH. The relative molar tyrosinase-stimulating potency, expressed as $1/EC_{50} \cdot 100$ [10], amounts to $4 \cdot 10^{10}$ for α-MSH and to $4 \cdot 10^{8}$ for [Leu⁹]α-MSH, the latter being a 100 times less potent agonist than the former.

Discussion. – [Leu⁹]α-MSH displays a similar activity in the *Rana* melanophore-assay system as the compounds where tryptophan⁹ has been replaced by phenylalanine or pentamethylphenylalanine [9b]; it is 25 times more potent than in the

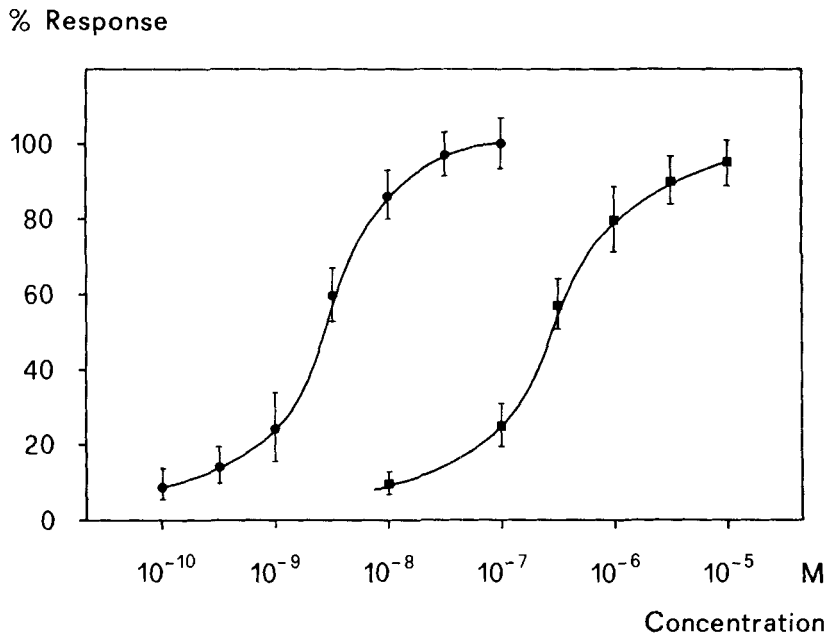


Fig. 2. *Tyrosinase stimulation of α-MSH* (●——●) *and [Leu⁹]α-MSH* (■——■). Each point of the log dose-response curves is the mean of 8–12 measurements.

tyrosinase assay with cultured mouse melanoma cells (and more than 100 times more potent than in the *Xenopus* melanophore assay). These results could be explained by a different degradation process of [Leu⁹]α-MSH compared to α-MSH in the different assay-systems. However, a first series of experiments investigating binding and antagonist properties of [Leu⁹]α-MSH have shown that this α-MSH analogue may in fact displace α-MSH from the target cells (melanoma cells, *Xenopus melanophores*), thus showing a relatively higher affinity than would have been expected from the log dose/response curves [14a] [16]. Therefore, the reason for the divergent potencies of [Leu⁹]α-MSH reported above may be explained by mixed agonist/antagonist properties of this compound, the latter being studied in more detail at present.

The finding that [Leu⁹]α-MSH exhibits divergent melanophore-dispersing and tyrosinase-stimulating activities in pigment cells of different species reveals that their α-MSH-receptors may be triggered by slightly different recognition/stimulation processes due to different receptor structures. It is indeed well probable that during evolution receptor structures may have been modified whilst the gene of the corresponding hormone remained unchanged. However, a precise statement will only be possible after examining a rather large series of synthetic peptides in the various assay systems on one hand, and after isolation and structural analysis of *e.g.* the *Rana* and *Xenopus* MSH on the other.

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Experimental Part

General. Removal of solvents from dissolved products was carried out in a rotatory evaporator at reduced pressure. Solvents were of highest purity and whenever necessary redistilled or checked for peroxide. The composition of solvent mixtures are indicated in volume parts. M.p. were determined in open capillaries and are uncorrected. Microanalyses were performed in the Laboratorium für Organische Chemie, ETHZ *(D. Manser).* Amino acid analyses were carried out in this institute by Mrs. *Z. Zanivan* (Laboratory of Prof. Dr. *H. Zuber*) according to *Stein & Moore* with *Beckman* Mod. 120B and 121 analyzers. The samples were hydrolyzed with 6N HCl containing 0.4% thioglycolic acid. UV. spectra were recorded with a *Beckman* Acta V spectrometer. Optical activity was determined with a *Perkin-Elmer* 141 polarimeter. Thin-layer chromatography (TLC.) was carried out with *Merck* silica gel plates using the following solvent systems: BAW = 2-butanol/AcOH/water 72:7:21, BPAW 1 = 1-butanol/pyridine/AcOH/water 50:12:12:25, BPAW 2 = solvents of BPAW 1, but 42:24:4:30. Thin-layer electrophoresis (TLE.) was performed with *Merck* cellulose plates using an apparatus of *Camag* and the following buffers: pH 3.6 = AcOH/pyridine/water 100:10:890, pH 6.5 = AcOH/pyridine/water 4:100:900. The compound spots on TLC. and TLE. plates were detected with ninhydrine and *Reindel-Hoppe* reagents.

Z · Glu(OtBu)-His-Phe-Arg-Leu-Gly · OTsE, HCl (**3**). A solution of **1** (410 mg, 0.84 mmol) [13] in dry DMF (4 ml) was kept at −18° and treated with 4.38N HCl in dioxane (0.57 ml, 2.5 mmol) and *t*-butyl nitrite (0.12 ml, 1 mmol) for 20 min. A solution of **2** (600 mg, 0.8 mmol) in 2 ml DMF [13] was added, and the mixture was neutralized with *N*-ethyldiisopropylamine (322 mg, 2.5 mmol) and kept at −15° for 1 h, and 0° for 24 h. The solvent was evaporated, and the residue was purified by partition chromatography on *Sephadex* G-25 with the system AcOH(1%)/1-butanol. Evaporation and crystallization from methanol/ether yielded 670 mg (72%) of pure **3**. M.p. 192°; Rf 0.40 (BAW), 0.61 (BPAW 2), 0.65 (BPAW 1); $[\alpha]_D^{25} = -29.1°$ ($c = 1$, methanol).

Amino acid analysis: Glu 1.08, His 0.95, Phe 0.99, Arg 1.03, Leu 1.00(R), Gly 1.01.

$C_{55}H_{77}ClN_{11}O_{13}S$ (1167.80) Calc. C 56.57 H 6.65 N 13.19% Found C 56.27 H 6.63 N 12.86%

H · Glu(OtBu)-His-Phe-Arg-Leu-Gly · OTsE, 2 HCl (**4**). A solution of **3** (820 mg, 0.7 mmol) in 80% acetic acid (10 ml) was hydrogenated in the presence of Pd/C (200 mg) for 1.5 h. The catalyst was removed by filtration through *celite,* and the solvent was evaporated and dried. Acetate ions were exchanged against chloride by adding pyridinium hydrochloride (2.2 mol-equiv.) to a methanolic solution of **4**. Crystallization from methanol/ether yielded 715 mg (92%) of pure **4**. M.p. 166°; Rf 0.41 (BAW), 0.60 (BPAW 1); $[\alpha]_D^{25} = -24.0°$ ($c = 0.33$, methanol).

Amino acid analysis: Glu 1.07, His 0.96, Phe 1.01, Arg 1.05, Leu 1.00(R), Gly 1.02.

$C_{47}H_{72}Cl_2N_{11}O_{11}S$ (1070.13)

BOC · Ser-Tyr-Ser-Met-Glu(OtBu)-His-Phe-Arg-Leu-Gly · OTsE, HCl (**6**). A solution of **5** (300 mg, 0.5 mmol) [17] in dry DMF (4 ml) was kept at −20° and treated with 5.0N HCl in dioxane (0.25 ml, 1.25 mmol) and *t*-butyl nitrite (72 µl, 0.6 mmol). After 20 min at −15°, **4** (535 mg, 0.5 mmol, dissolved in 3 ml DMF) and *N*-ethyldiisopropylamine (225 mg, 1.75 mmol) were added. The solution was kept at −15° and 0° for 2 and 16 h, respectively, and was evaporated i.V. Purification by counter-current distribution in the system 1N AcOH/1-butanol (125 steps). Pure **6** was obtained from tubes 92–108. Precipitation from DMF/ether as amorphous solid yielded 410 mg (51%). M.p. 200–202°; Rf 0.67 (BPAW 1), 0.69 (BPAW 2); $[\alpha]_D^{25} = -18.9°$ ($c = 0.33$, AcOH). – UV. (1N AcOH): $\lambda_{max} = 269$ nm ($\varepsilon = 1540$), 275 nm ($\varepsilon = 1685$), shoulder at 283 nm ($\varepsilon = 1060$).

Amino acid analysis: Ser 1.80, Tyr 1.04, Met 0.95, Glu 1.10, His 0.96, Phe 1.03, Arg 1.07, Leu 1.00(R), Gly 1.02.

$C_{72}H_{107}ClN_{15}O_{20}S$ (1602.31)

BOC · Ser-Tyr-Ser-Met-Glu(OtBu)-His-Phe-Arg-Leu-Gly · OH (**7**). A solution of **6** (200 mg, 125 µmol) in methanol/dioxane 1:1 (4 ml) was treated with 0.1N NaOH (4 ml) for 10 min. Upon neutralization to pH 8 with 0.1N HCl **7** precipitated as white amorphous solid which was filtered off, thoroughly washed with water, and dried. Yield: 150 mg (85%) of homogeneous **7**. M.p. 120–130°; Rf 0.61 (BPAW 1), 0.60 (BPAW 2); $[\alpha]_D^{25} = -23.1°$ ($c = 0.33$, AcOH). – UV. (1N AcOH): $\lambda_{max} = 276$ nm ($\varepsilon = 1350$).

Amino acid analysis: Ser 1.78, Tyr 1.01, Met 0.94, Glu 1.08, His 0.95, Phe 0.99, Arg 1.08, Leu 1.00(R), Gly 1.04.

$C_{63}H_{97}ClN_{15}O_{18}S$ (1420.07)

BOC · Ser-Tyr-Ser-Met-Glu(OtBu)-His-Phe-Arg-Leu-Gly-Lys(MsOC)-Pro-Val · NH$_2$, HCl (**8**). A solution of **7** (142 mg, 0.1 mmol) and H · Lys(MsOC)-Pro-Val · NH_2, HCl (56 mg, 0.11 mmol) [13] in dry DMF was treated with HOBT (27 mg, 0.2 mmol) and DCC (25 mg, 0.12 mmol), and kept at 22° for 48 h. After addition of a few drops of glacial acetic acid, the DCU was filtered off, and the solvent was evaporated. The residue was purified on *Sephadex* LH-20 (elution with DMF/water 9:1). Yield: 160 mg (81%) of pure **8**. Rf 0.64 (BPAW 2), 0.68 (BPAW 1); $[\alpha]_D^{25} = -34.8°$ ($c = 0.25$, AcOH). – UV. (1N AcOH): $\lambda_{max} = 275$ nm ($\varepsilon = 1600$).

Amino acid analysis: Ser 1.82, Tyr 0.98, Met 0.95, Glu 1.06, His 0.97, Phe 0.99, Arg 1.04, Leu 0.97, Gly 1.00(R), Lys 1.05, Pro 1.05, Val 0.99.

$C_{83}H_{131}ClN_{20}O_{24}S_2$ (1892.66)

H · Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Leu-Gly-Lys(MsOC)-Pro-Val · NH$_2$, 2 HCl (**9**). The BOC and *t*-butyl groups were removed by treatment of **8** (151 mg, 80 µmol) with 0.12N HCl in HCOOH (3.5 ml) for 15 min. The solvent was evaporated and the residue triturated with ether. Yield: 133 mg (95%). Rf 0.38 (BPAW 2), 0.44 (BPAW 1).

Ac · Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Leu-Gly-Lys-Pro-Val · NH$_2$, 2 AcOH (**10**). **9** (133 mg, 75 µmol) was acetylated in DMF (0.6 ml) + pyridine (0.2 ml) with 4-nitrophenylacetate (10 mg, 55 µmol) and *N*-ethylmorpholine (3.6 mg, 32 µmol). After 36 h at 22°, the solution was filtered through *Sephadex* LH-20 (elution with DMF/water 9:1). The MsOC group was removed by treating the product with 0.1N $Ba(OH)_2$-solution containing 40% DMF and 10% methanol. Purification by chromatography on *Sephadex* LH-20 (elution with 1N AcOH). Lyophilization of the pure fractions yielded 119 mg (93%) of pure **10**. Rf 0.34 (BPAW 2), 0.40 (BPAW 1); TLE.: R(Arg) 0.46 (pH 6.5), 0.60 (pH 3.6); $[\alpha]_D^{25} = -61.0°$ ($c = 0.2$, 1N AcOH). – UV. (1N AcOH): $\lambda_{max} = 276$ nm ($\varepsilon = 1620$).

Amino acid analysis: Ser 1.83, Tyr 1.03, Met 0.92, Glu 1.06, His 1.01, Phe 0.99, Arg 1.04, Leu 1.02, Gly 1.03, Lys 1.04, Pro 1.08, Val 1.00(R).

$C_{76}H_{118}N_{20}O_{23}S$ (1711.97)

Bioassay. The melanotropic activity was determined with the modified [4a] reflectometric test system of *Schizume et al.* [15] using isolated skin of the leopard frog, *Rana pipiens.*

Tyrosinase-stimulating activities were determined with a *Cloudman* S-91 mouse melanoma cell line in culture. The cells were maintained in a rapidly growing monolayer culture (doubling time: 24 h) in 25 cm^2 *Falcon* tissue culture flasks (inoculation: 10^6 cells per flask in 10 ml of NCTC-135 medium with 10% horse serum). Sterile solutions of the peptides were added together with 10 µCi of (3′,5′-$^3H_2$)-tyrosine *(Amersham)* to the flasks when they contained $2 \cdot 10^6$ cells. The activity of tyrosinase was determined by measuring the release of ^3H_2O after 24 h (elimination of ^{3}H-tyrosine from the samples by treatment with charcoal). A maximal response of about 200% above the basal level of ^3H_2O of non-stimulated cells was obtained with a 10^{-7}M α-MSH solution (see also [16]).

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