268. Synthesis and Biological Properties of *p*-Azidophenylalanine[13]-α-melanotropin, a Potent Photoaffinity Label for MSH Receptors[1])

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Dedicated to Professor *Theodor Wieland*

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Summary

p-Azidophenylalanine[13]-α-melantropin ([Pap[13]]-α-MSH) was synthesized in homogeneous solution by the fragment condensation method, and its biological activity was determined in three different assay systems. The pigment-dispersing activity relative to α-MSH was 65%, measured with melanophores of *Rana pipiens* or of *Xenopus laevis* tadpoles. The tyrosinase-stimulating activity was 50%, determined with cultured mouse melanoma cells. UV. irradiation of solutions containing $\leq 10^{-4}$M [Pap[13]]-α-MSH at 338 nm (intensity: 10^{-3} W·cm^{-2}) led to complete photolysis of the photolabel within <20 min. Under these conditions [Pap[13]]-α-MSH was covalently inserted into MSH-receptors which produced a longlasting pigment dispersion in *Xenopus* melanphores (see [3]). The extent of this prolonged stimulation depended on the hormone concentration used during photolysis. $1.8 \cdot 10^{-9}$M [Pap[13]]-α-MSH which produced a full initial response failed to prolong the effect, whereas $1.2 \cdot 10^{-8}$M hormone caused irreversible stimulation. It appears that only about 10% of the initially occupied receptors were covalently labelled because the log dose response curve was shifted to ~10x higher concentration after a 200 min wash period: EC_{50} immediately after photolysis was $6 \cdot 10^{-10}$M; after 200 min EC_{50} increased to $\sim 8 \cdot 10^{-9}$M.

Introduction. – Receptors for α-MSH on pigment cells and in brain tissue are widely investigated because of the role of α-MSH as a neuropeptide and because of its possible application in the treatment of melanoma (reviewed in [4]). In order to understand α-MSH receptor interactions in more detail, extensive structure-activity studies have been carried out employing pigment cell and melanoma cell systems [5]

[1]) Abbreviations are according to the *IUPAC-IUB Commission on Biochemical Nomenclature* [1] and *Houben-Weyl* [2]. Additional abbreviations are: MSH = melanotropin (melanocyte-stimulating hormone); MsOC = 2-(methylsulfonyl)-ethoxycarbonyl; Pap = *p*-azidophenylalanine; PBS = phosphate buffered saline. All chiral amino acids are in the L-configuration.

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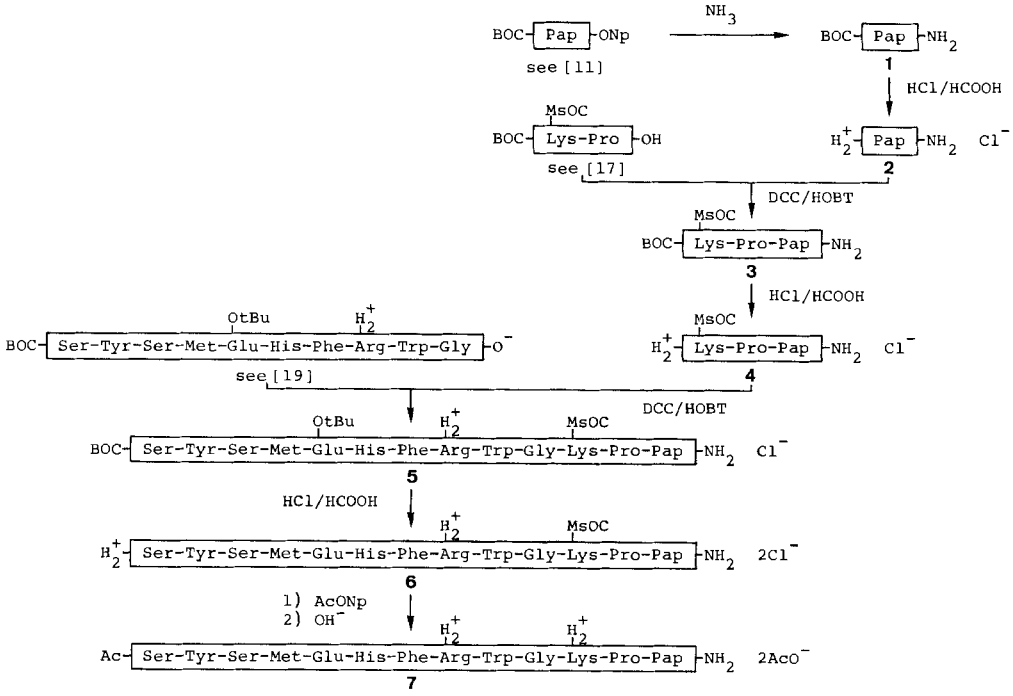
or behavioural systems [6]. Iontophoretic injection of α-MSH and cyclic AMP into melanophores [7] and the use of α-MSH covalently attached to large-size carrier molecules [8] revealed that the MSH receptors are located on the outer face of the plasma membrane of the various target cells. Further characterization of MSH receptors, *e.g.* through isolation, has not been possible until now.

Thiol groups were once reported to be essential for MSH receptor interactions [9]. Therefore it appeared promising to apply affinity labelling of MSH receptors for the subsequent isolation of the receptors. However, stimulation of melanophores with α-MSH derivatives containing bromoacetyl or diazoacetyl groups [10] or maleimidocaproyl groups (unpublished) failed to produce covalent hormone-receptor complexes. As an alternative, photoreactive α-MSH analogues containing *p*-azidophenylalanine were synthesized, persuing the concept of *Schwyzer et al.* [11] [12] of studying peptide-protein interactions by photoaffinity labelling with modified phenylalanine residues. The first α-MSH derivative of this kind [13] contained tritiated Pap in position 2, and Ser[1] and Met[4] were replaced by D-Ala[1] and Nva[4]. With this compound, photoaffinity labelling of MSH receptors on *Xenopus* melanophores was only possible when very high concentrations of the peptide were used (unpublished). It appeared that the position of the photolabel and/or the other substitutions were not ideal for an efficient labelling.

A new analogue of α-MSH containing Pap in position 13 proved to be very potent and upon UV. irradiation produced an irreversible stimulation of tail-fin

Scheme. *Synthesis of p-azidophenylalanine[13]-α-melanotropin*



melanophores of *Xenopus* tadpoles [3] [14]. Here we describe in detail the synthesis and some of the biological properties of [Pap^{13}]-α-MSH.

Synthesis of [Pap^{13}]-α-MSH. – The synthesis was carried out in homogeneous solution following similar strategy and tactics to those outlined in earlier syntheses of α-MSH [15] [16]. The alkali-labile MsOC-group was used for side-chain protection and BOC for protection of N^{α}-amino groups. The intermediates were, whenever possible or desirable, isolated, purified and characterized. All manipulations were carried out under Na light or dim lamplight.

BOC-Pap-ONp was prepared according to [11] and was converted into the amide **1** by ammonolysis in acetonitril (*Scheme*). After deprotection in HCl/HCOOH **2** was coupled to BOC-Lys(MsOC)-Pro-OH [17] according to the method of *König & Geiger* [18], and pure **3** was isolated by crystallization. BOC was removed by acidolytic cleavage, yielding **4**. Condensation of **4** with protected decapeptide (1–10) [19] to **5**, deprotection to **6**, N^{α}-acetylation and elimination of the MsOC-group was performed as described for α-MSH [16]. The final product **7** was purified by chromatography on *Sephadex* LH-20.

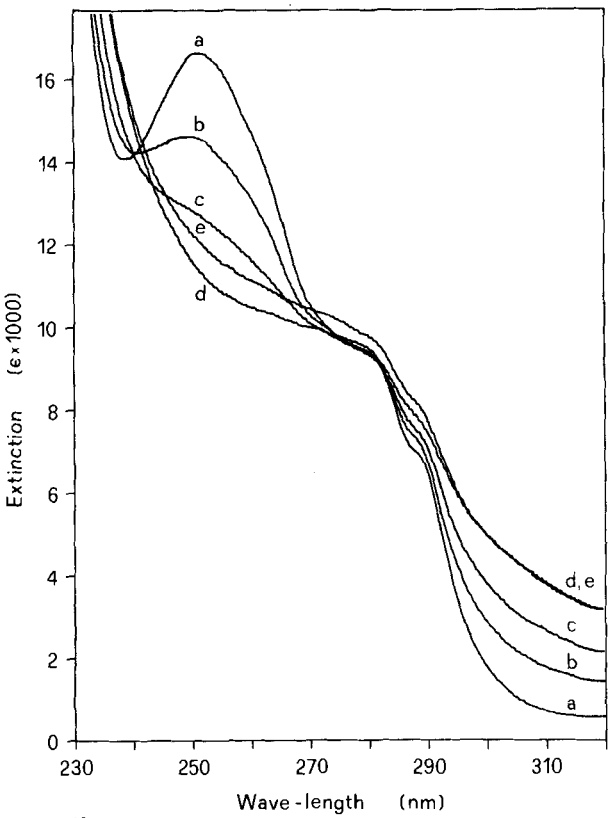


Fig. 1. *UV. spectra of [Pap^{13}]-α-MSH before and after UV. irradiation.* A solution ($6 \cdot 10^{-5}$M) of the hormone in PBS (0.1M Na-phosphate, pH 7, 0.9% NaCl) was irradiated at 338 nm for 0 min (a), 6 min (b), 12 min (c) and 18 min (d). As a control for complete photolysis, an identical sample was irradiated for 10 min with unfiltered UV. light of at least 10×higher intensity (e).

Photolysis of the *p*-azidophenyl group was studied by exposing the peptide to the same conditions as used for labelling melanophores (see below). The time course of the photolytic decomposition of [Pap^{13}]-α-MSH by UV. light (338 nm; 10^{-3} W · cm^{-2}) is depicted in *Figure 1*. After 20 min photolysis was virtually quantitative.

Biological activity of [Pap^{13}]-α-MSH. – *Figure 2* shows log dose-response curves for α-MSH and [Pap^{13}]-α-MSH in the modified [20] reflectometric *in vitro* frog skin assay [21] (A; pigment dispersion) and with cultured *Cloudman* S-91 mouse melano-

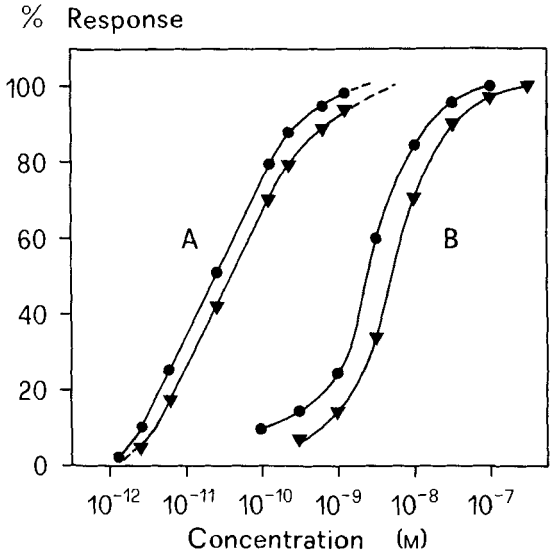


Fig. 2. *log Dose/response curves for α-MSH* (●——●) *and [Pap^{13}]-α-MSH* (▼——▼). A: Pigment dispersion in melanophores of isolated frog skin; B: tyrosinase-stimulation in *Cloudman* S-91 mouse melanoma cells. For calculation of % response see experimental part. Each point of the log dose/response curves is the mean of 8–20 measurements. SEM values are usually ± 10% (A) and ± 6% (B) (see [27]).

ma cells [22] (B; tyrosinase stimulation). In both assays the curves for the two peptides are parallel, and [Pap^{13}]-α-MSH is a full agonist with a potency of 65% (frog skin) and 50% (melanoma cells), respectively, relative to α-MSH. In the third assay system, a new microscopic melanophore assay using tail-fin pieces of *Xenopus laevis* tadpoles [23], the potency of [Pap^{13}]-α-MSH was identical with the value obtained with skin of *Rana pipiens (Table)*. The latter system, however, is much more sensitive with EC_{50} amounting to $4 \cdot 10^{-11}$M, whereas in the former system EC_{50} is $8 \cdot 10^{-10}$M. Even higher concentrations are required for tyrosinase-stimulation in cultured melanoma cells: $EC_{50} = 5 \cdot 10^{-9}$M *(Table)*.

The homology of the results obtained for α-MSH and [Pap^{13}]-α-MSH in the three different systems indicates that the two peptides are recognized in the same way by all three receptor types. At present, [Pap^{13}]-α-MSH is the most potent photoreactive α-MSH derivative known. Since it is a full agonist, active in the same concentration range as α-MSH, it is highly suitable for photoaffinity labelling.

Table. *Biological activities of* p-*azidophenylalanine*^{13}-*α-melanotropin and of α-melanotropin*

Compound	Potency	Pigment dispersion		Tyrosinase stimulation
		Rana pipiens	*Xenopus laevis*	*Cloudman* S-91 mouse melanoma cells
[Pap^{13}]-α-MSH	EC_{50}	$4 \cdot 10^{-11}$ M	$8 \cdot 10^{-10}$ M	$5 \cdot 10^{-9}$ M
	units/mmol[a])	$2.5 \cdot 10^{10}$	$2.5 \cdot 10^{10}$	$2 \cdot 10^{10}$
α-MSH	EC_{50}	$2.5 \cdot 10^{-11}$ M	$5 \cdot 10^{-10}$ M	$2.5 \cdot 10^{-9}$ M
	units/mmol[a])	$4 \cdot 10^{10}$	$4 \cdot 10^{10}$	$4 \cdot 10^{10}$

[a]) Units/mmol are normally used to compare the potencies of different melanotropic peptides. For reasons of standardization, 1 unit/ml was defined as the amount of α-MSH producing a 50% response in the *Rana pipiens* assay (see [27]). Thus, $1/EC_{50}$ is equivalent to units/mmol in the *Rana pipiens* assay. In the *Xenopus* assay, units/mmol correspond to $1/EC_{50} \cdot 20$, and in the tyrosinase assay to $1/EC_{50} \cdot 100$.

Photoaffinity labelling of MSH receptors. – Unpublished experiments with skin of *Rana pipiens* showed that this tissue is not transparent enough for efficient labelling of MSH-receptors with [D-Ala^1, Pap^2, Nva^4]-α-MSH [13] or with [Pap^{13}]-α-MSH at light intensities that do not damage the cells. Therefore, the melanophore system of *Xenopus* tadpoles was adapted for photoaffinity experiments. Tail-fins of this species are very transparent because in that tissue the melanophores are the only cells that absorb light. In a first series of photoaffinity experiments, a specific and irreversible stimulation of the melanophores was demonstrated which lasted for several hours [3]. By increasing the temperature during the wash phase, covalent hormone-receptor complexes were progressively inactivated, which made an estimation of the receptor turnover possible [3]. We then examined in more detail how the longlasting stimulation is dependent on the hormone concentration used during photolysis.

In *Figure 3* the decrease of the response after photolysis (time = 0 min) is shown in a three-dimensional graph in relation to the hormone concentration used during photolysis. The graph represents log dose/response curves recorded every 20 min during a wash period of 200 min after photolysis. The initial log dose/response curve for 0 min is shifted to a slightly lower concentration than in the case of non-irradiated melanophores ($EC_{50} = 6 \cdot 10^{-10}$ M *vers.* $8 \cdot 10^{-10}$ M). There is no explanation yet for this slight increase in sensitivity of the melanophores during photolysis.

The log dose/response curve is shifted towards ~10× higher concentrations during a 200 min wash period. A precise comparison of the curves is not possible because they are not parallel. As the curves become progressively steeper during the wash phase, the EC_{50}'s can only be estimated. Nevertheless, the results permit the conclusion that about one out of ten receptors initially occupied becomes labelled, and that a ~10× higher hormone concentration must be present during photolysis to ensure formation of as many covalent hormone-receptor complexes as there were initially non-covalent ones. (This value is 2.5 times higher than reported previously [3] from a preliminary study). An other explanation, however, assuming a higher degree of labelling and hence either a more rapid receptor turnover or desensitization or an increased inactivation of cyclic AMP, would have to postulate a relatively large number of spare receptors that would have to be labelled for the generation of the longlasting stimulation. Although the existence of spare receptors cannot be

ruled out, it appears unlikely that even at low temperatures receptor turnover is up to 10×higher than originally estimated [3]. Whether the longlasting stimulation may produce a higher phosphodiesterase activity and hence an increased inactivation of cyclic AMP (which has to be compensated by labelling of spare receptors), cannot finally be decided at present. Nevertheless, we believe that the results reflect the degree of labelling.

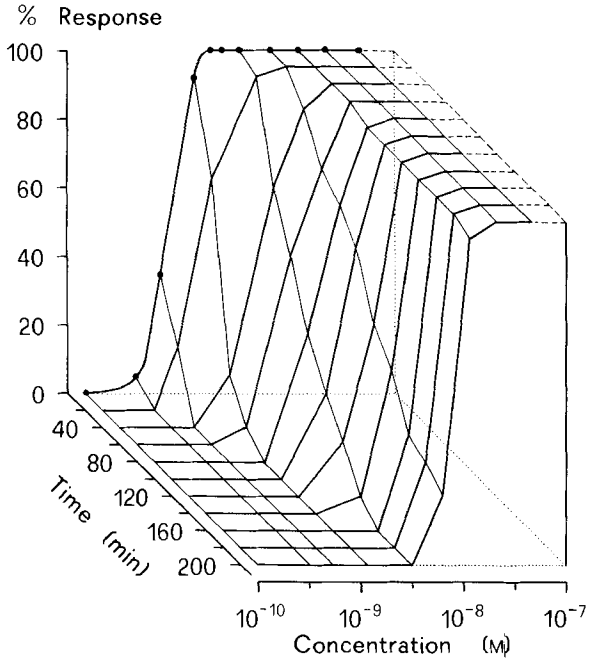


Fig. 3. *Time course of the log dose/response curve after photolysis.* Tail-fin melanophores of *Xenopus* tadpoles were covalently labelled with different concentrations of [Pap^{13}]-*a*-MSH (20 min preincubation with the hormone followed by a 20 min UV. irradiation in the presence of the hormone). The log dose/response curve was recorded just after photolysis (●——●) and then every 20 min during a 200 min wash period with buffer.

Discussion. – The principle of photoaffinity labelling has been treated extensively in recent review articles [24]. Aryl azides as the source for reactive nitrenes have been widely used for labelling of hormone receptors (*cf.* [3] [24]). An elegant and convenient way of introducing aryl azides into peptides has proved to be the use of Pap replacing Phe or Tyr [11]. In the case of α-MSH, Pap may replace Tyr^2, Trp^9, Val^{13} (and perhaps Phe^7) without substantial reduction of biological activity. Substitution of Val^{13} with Pap^{13} is particularly suitable because the C-terminal residue has been thought to interact very closely with the receptor [25]. This hypothesis is now supported by the results obtained with [Pap^{13}]-α-MSH.

UV. irradiation at 338 nm produced complete photolysis of the azidophenyl group of [Pap^{13}]-α-MSH within 20 min. This confirms earlier findings (*e. g.* [12]) that photolysis of Pap is possible with wave-lengths ranging from 330–360 nm. These wave-lengths do not harm melanophores in tail-fin pieces as long as the light intensity is kept to the minimum required for photolysis (10^{-3} W·cm^{-2}).

The application of [Pap[13]]-α-MSH together with intact melanophores of *Xenopus* tadpoles represents a unique system for studying the temporal involvement of various factors during MSH-signal transduction. In this way, Ca^{2+} has recently been shown to play a dual role in the generation of the hormonal response, i. e. Ca^{2+} is essential for the binding of the hormone to the receptor and for the transduction of the signal from the receptor to the adenylate cyclase or for the activation of this enzyme [14]. Such studies have hitherto rarely been reported for intact cell systems.

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Experimental Part

Peptide Synthesis. – *General remarks.* All solvents and reagents were obtained from commercial sources and were of analytical grade. Melting points (m. p.) were determined in open capillaries and are uncorrected. 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 = 1-butanol/pyridine/AcOH/water 42:24:4:30, CMA = $CHCl_3$/CH_3OH/AcOH 95:5:3, EBPAW = ethyl acetate/1-butanol/pyridine/AcOH/water 42:24:21:6:10. TLE. was performed with *Merck* cellulose plates using the following buffers: pH 3.6 = AcOH/pyridine/water 100:10:890 and pH 6.4 = 4:100:900. The compound spots were detected with I_2-vapour and with ninhydrin and *Reindel-Hoppe* reagents. UV. spectra were recorded on a *Pye Unicam* SP8-100 spectrophotometer. Amino acid and elemental analyses were carried out as described in [26].

BOC-Pap-NH_2 (**1**). To a solution of 2.15 g (5 mmol) BOC-Pap-ONp [11] in 50 ml dry acetonitril 150 ml NH_3 were added, and the mixture was kept under pressure for 24 h and then evaporated. The product was crystallized from acetonitril/ether. Yield: 1.49 g (98%). M. p. 158°; Rf 0.55 (CMA), 0.67 (BAW), 0.92 (BPAW 1); $[\alpha]_D^{25} = -23.7°$ ($c = 0.6$, MeOH).

$C_{14}H_{19}N_5O_3$ (305.34) Calc. C 55.07 H 6.27 N 22.94% Found C 55.27 H 6.32 N 22.79%

H-Pap-$NH_2 \cdot HCl$ (**2**). After treating 305 mg (1 mmol) of **1** with 10 ml 0.12N HCl in HCOOH for 15 min, the acid was evaporated *i. V.* and the product was repetitively dissolved in methanol and evaporated in order to remove traces of acid. Crystallization from methanol/ether yielded 227 mg (94%) **2**. M. p. 82°; Rf 0.35 (BAW), 0.76 BPAW 1); $[\alpha]_D^{25} = -8.2°$ ($c = 1$, EtOH).

$C_9H_{12}ClN_5O$ (241.68) Calc. C 44.73 H 5.00 N 28.98% Found C 44.68 H 4.98 N 28.79%

BOC-Lys(MsOC)-Pro-Pap-NH_2 (**3**). A solution of 494 mg (1 mmol) BOC-Lys(MsOC)-Pro-OH [17] and 228 mg (1 mmol) of **2** in 10 ml DMF was treated with 126 μl (1 mmol) *N*-ethylmorpholin, 270 mg (2 mmol) HOBT and 227 mg (1.1 mmol) DCC, and kept at 22° for 36 h. After addition of a few drops of glacial acetic acid, the DCU was filtered off and the solvent evaporated. The residue, dissolved in $CHCl_3$, was consecutively extracted 3 × with 5% $KHSO_4$-solution/5% K_2SO_4-solution 1:2, 5% aqueous $NaHCO_3$-solution, and saturated NaCl-solution. The organic solvent was dried with anhydrous Na_2SO_4, filtered and evaporated *i. V.* The product was crystallized from ethyl acetate/petrol ether. Yield: 480 mg (71%) of pure **3**. M. p. 91–94°; Rf 0.55 (BAW), 0.83 (BPAW 1); $[\alpha]_D^{25} = -50.0°$ ($c = 1$, MeOH).

$C_{29}H_{44}N_8O_9S$	Calc.	C 51.16	H 6.51	N 16.46	S 4.71%
(680.79)	Found „	51.24	„ 6.70	„ 16.29	„ 4.53%

H-Lys-(MsOC)-Pro-Pap-$NH_2 \cdot HCl$ (**4**). After treating 684 mg (1 mmol) of **3** with 10 ml 0.12N HCl in HCOOH as described for **2**. repetitive precipitation from methanol/ether yielded 487 mg (79%) homogeneous **4**. M. p. 178–184°; Rf 0.11 (BAW), 0.40 (BPAW 1); $[\alpha]_D^{25} = -12.0°$ ($c = 0.84$, MeOH).

$C_{24}H_{37}ClN_8O_7S$ (617.13) Calc. C 46.71 H 6.04 N 18.16% Found C 46.68 H 6.12 N 18.01%

BOC-Ser-Tyr-Ser-Met-Glu(OtBu)-His-Phe-Arg-Trp-Gly-Lys(MsOC)-Pro-Pap-$NH_2 \cdot HCl$(**5**). The suspension of 364 mg (0.25 mmol) BOC-Ser-Tyr-Ser-Met-Glu(O*t*Bu)-His-Phe-Arg-Trp-Gly-OH [19] and 170 mg (0.275 mmol) of **4** in 5 ml DMF was treated with 68 mg (0.5 mmol) HOBT and 62 mg (0.30 mmol) DCC

and kept at 22° for 48 h. The DCU was filtered off and the solvent evaporated. The residue was purified on *Sephadex* LH-20 (elution with DMF/water 9:1). Yield: 430 mg (84%). M. p. 198–202° (decomp.); Rf 0.35 (BAW), 0.58 (BPAW 1); $[\alpha]_D^{25} = -30.3°$ ($c = 0.33$, AcOH). – UV. (1N AcOH): $\lambda_{max} =$ 252 nm ($\varepsilon = 15800$).

Amino acid analysis: Ser 1.81, Tyr 0.96, Met 0.94, Glu 1.07, His 0.95, Phe 0.98, Arg 1.08, Trp 0.92, Gly 1.00 (R), Lys 1.08, Pro 1.10.

$C_{92}H_{128}ClN_{24}O_{24}S_2$ (2053.78)

H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys(MsOC)-Pro-Pap-NH_2·2 HCl (**6**). After treating 369 mg (0.18 mmol) of **5** with 8 ml 0.12N HCl in HCOOH for 20 min, the solution was evaporated *i. V.* and traces of acid removed by repetitive evaporation of methanol/water 1:1. Precipitation from DMF with ether yielded 330 mg (95%) of **6**. Rf 0.12 (BAW), 0.36 (BPAW 1). – UV. (1N AcOH): $\lambda_{max} = 252$ ($\varepsilon = 15900$).

$C_{83}H_{114}Cl_2N_{24}O_{22}S_2$ (1935.02)

Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Pap-NH_2·2 AcOH (**7**). The solution of 310 mg (0.16 mmol) of **6** in 4 ml DMF/pyridine 3:1 was treated with 4-nitrophenylacetate (45 mg, 0.25 mmol) and *N*-ethylmorpholine (18.4 mg, 0.16 mmol). After 36 h at 22°, the solution was filtered through *Sephadex* LH-20 (elution with DMF/water 9:1), and the homogeneous fractions were precipitated with ether. The solution of 275 mg (0.14 mmol) of protected **7** in 5 ml DMF/CH_3OH 1:1 was treated with 5 ml 0.1N $Ba(OH)_2$ for 5 min at 20°. The reaction was stopped by addition of 0.1N H_2SO_4 at 0°, $BaSO_4$ was removed by centrifugation, and the solution was evaporated *i. V.* The residue was dissolved in 1N AcOH and passed through weakly basic ion changer (*Merck* II). Purification by chromatography on *Sephadex* LH-20 (elution with AcOH/H_2O 1:99). Precipitation of the pure fractions from water/acetone yielded 164 mg (61%) of homogeneous **7**. Rf 0.13 (EBPAW), 0.34 (BPAW 1); 0.48 (BPAW 2); TLE. R(Arg) 0.48 (pH 6.4), 0.62 (pH 3.6); $[\alpha]_D^{25} = -56.0°$ ($c = 0.48$, AcOH/H_2O 1:99). – UV. (PBS): $\lambda_{max} = 252$ nm ($\varepsilon = 16500$).

Amino acid analysis: Ser 1.89, Tyr 1.02, Met 0.92, Glu 1.11, His 0.93, Phe 0.99, Arg 1.09, Trp 0.93, Gly 1.00 (R), Lys 1.03, Pro 1.12.

$C_{85}H_{117}N_{24}O_{23}S$ (1875.09)

Bioassys. – *Melanophore-stimulating activities* were determined microscopically with tail-fin pieces from *Xenopus laevis* tadpoles according to *de Graan et al.* [23], and photometrically with the modified [20] test system of *Shizume et al.* [21] using isolated skin of the leopard frog, *Rana pipiens*. In this assay, log dose/response curves were obtained by calibrating the individual skins with $2.5 \cdot 10^{-11}$M α-MSH (1 unit/ml), washing with buffer, incubating with the different hormone concentrations, washing with buffer, and incubating wit $2.5 \cdot 10^{-9}$M α-MSH (100 units/ml) in order to produce a maximal reflectance change [27]. The response to a given hormone concentration corresponds to the measured reflectance change expressed as % of the maximal reflectance change. In the *Xenopus* assay, each measurement represents the average melanophore index (MI) of 50–100 melanophores (MI 1: 0% response; MI 3: 50% response; MI 5: 100% response [23]).

Tyrosinse-stimulating activities were determined with a *Cloudman* S-91 mouse melanoma cell line in culture by measuring the release of ^3H_2O from the cultures incubated with the peptides in the presence of (3′,5′-$^3H_2$)-tyrosine (see [22]).

Photoaffinity labelling. – UV. irradiation experiments were carried out with a Xenon XBO 450 lamp *(Zeiss)* fitted with a 6 cm water filter, an interference filter (338 nm, *Balzers*), and a UV. reflecting mirror. Excised tail-fin pieces (2 × 2 mm) were washed in buffer and incubated with different concentrations of [Pap[13]]-α-MSH for 40 min at 20°, as described in [3]. During the first 20 min the pieces were kept under normal lamplight, and during the second 20 min they were irradiated with a UV. beam of an intensity of 10^{-3} W·cm^{-2} (control pieces were kept under normal lamplight). The pieces were then kept in buffer at 15°, and the MI was determined every 20 min.

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