1. Melanotropin Receptors I. Synthesis and Biological Activity of N^{α}-(5-Bromovaleryl)-N^{α}-deacetyl-α-melanotropin[1])

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Dedicated to Dr. *Nitya Anand,* Director of the Central Drug Research Institute, Lucknow, India, on the occasion of his 60th birthday

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Chemical synthesis and biological activities of a new α-melanotropin derivative are described. N^{α}-(5-Bromovaleryl)-N^{α}-deacetyl-α-melanotropin contains the 5-bromopentanoyl group as a chemical 'handle' in place of the acetyl group of the natural hormone. The synthesis involved a new protected intermediate which allowed the selective deprotection of either the N^{α} or N^{ε} amino group. The title compound reacted with sodium thiosulfate to give N^{α}-deacetyl-N^{α}-(5-(sulfothio)valeryl)-α-melanotropin, a key intermediate for the preparation of tobacco-mosaic virus/α-melanotropin disulfide conjugates. As a basis for the study of the conjugates, biological activities of the title compound on *Cloudman S-91* mouse melanoma cell cultures (tyrosinase stimulation, binding, and *cyclic* AMP accumulation) were determined. They proved to be quite similar to the corresponding α-melanotropin activities. Differences in bindings may be explained by stronger hydrophobic interaction of the new derivative with the lipid phase of the target cell membranes.

Introduction. – The use of covalent conjugates between neuropeptides and tobacco-mosaic virus as 'artificial antibodies' [3] to study the localization and cellular dynamics of neuropeptide receptors [4] requires in the next step of development the availability of conjugates in which the bond between the peptide and the virion can be cleaved under conditions that do not destroy cells. It was found that disulfide-linked conjugates have the required property [1] and can be prepared from mercaptosuccinyl virus [5] and sulfomercaptovaleryl derivatives ('*Bunte* Salze') of peptides.

α-Melanotropin is a neuropeptide of considerable interest with actions in the central nervous system and in cells of the skin of various species [6]. This report describes the synthesis of the 5-bromovaleryl derivative **10** of α-MSH and its conversion to the 5-(sulfothio)valeryl derivative **13** *(Fig. 1)*. For analytical purposes, the compounds were labeled with ^{125}iodine. The biological activity of **10** on *Cloudman S-91* mouse melanoma cells was investigated in detail and compared with that of the natural neuro-

[1]) Parts of this report have appeared as a thesis [1]. Nomenclature and abbreviations [2]. Additional abbreviations: MSH = melanophore stimulating hormone (melanotropin), DCC = *N,N'*-dicyclohexylcarbodiimide, DCU = *N,N'*-dicyclohexylurea, DMF = dimethylformamide, HOBt = 1-hydroxybenzotriazole, Boc = *tert*-butoxycarbonyl, OBu^t = *tert*-butoxy, Msoc = 2-(methylsulfonyl)ethoxycarbonyl, Np = 4-nitrophenyl, Z = benzyloxycarbonyl, TFA = trifluoroacetic acid. Chiral amino acids are in their L-configuration. Culture media see *Experimental.*

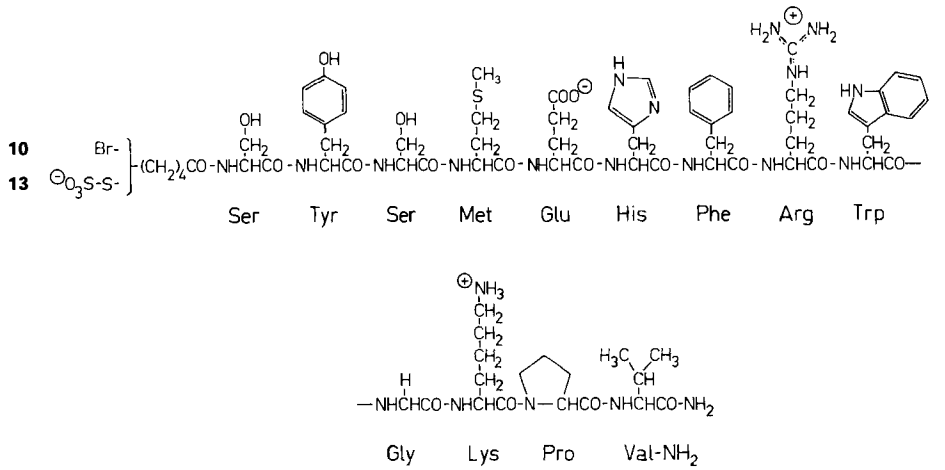


Fig. 1. *Primary structures of new α-MSH derivatives*

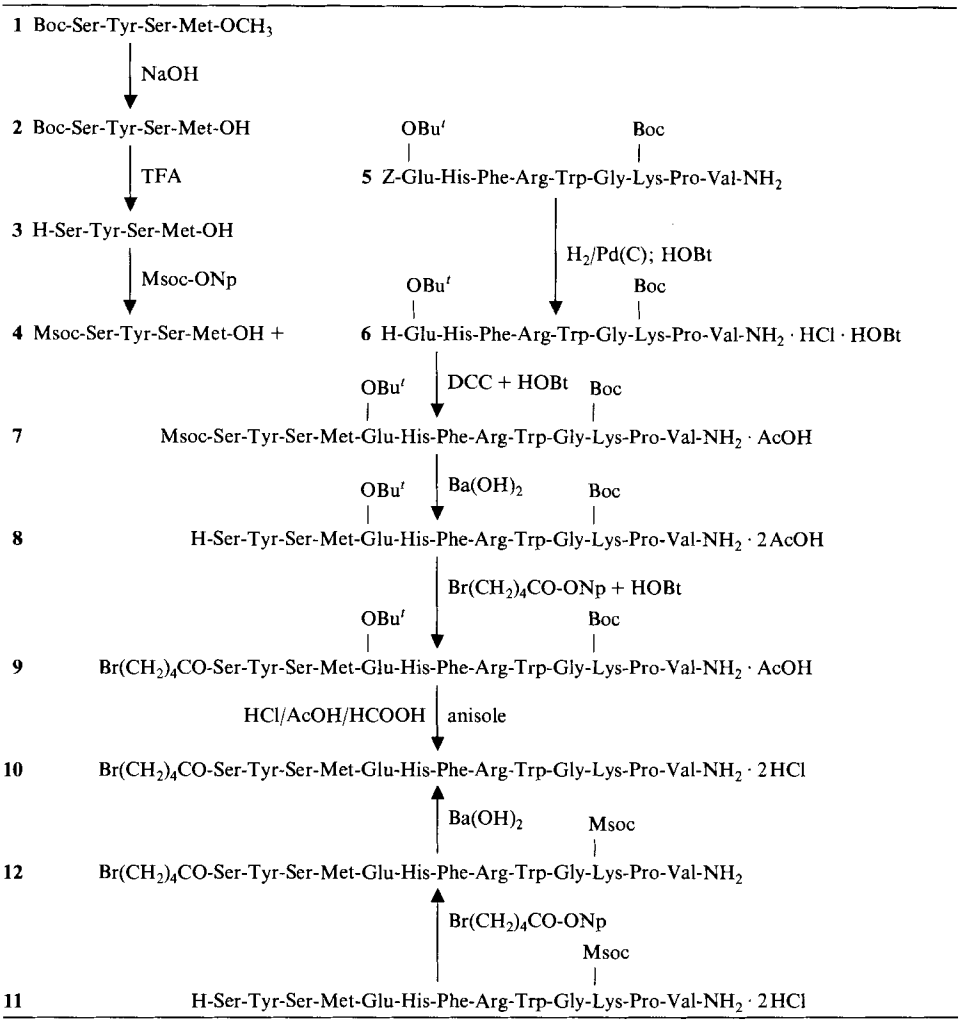
peptide, α-MSH, as a preliminary for the study of the tobacco mosaic virus disulfide conjugates.

Synthesis of N^{α}-(5-Bromovaleryl)-N^{α}-deacetyl-α-melanotropin (10). – Two approaches are illustrated in the *Scheme*. One (steps **11**→**12**→**10**) was based on an earlier synthesis of α-MSH [7] wherein the Lys (Msoc)-11 protection is removed by alcaline β-elimination in the last step. This worked here for the relatively stable 5-bromovaleryl derivative. However, for more reactive handles, such as bromoacetyl or maleimido groups [5] [8], deblocking in the final stage must be effected by mild acid treatment. We, therefore, reversed the type of protection, using Msoc for the N^{α}-atom and protecting groups derived from *t*-butyl alcohol for the side chains [9]. The key intermediates **7** and **8** can serve a wider range of handles than **11**.

Suitable educts for the second approach (steps **1**→**10**) were the Boc-tetrapeptide methyl ester **1** [10] and the Z-protected nonapeptide **5** [11]. The former was saponified to the free acid **2** which was treated with TFA to remove the N-terminal protection. The crystalline tetrapeptide salt **3**·TFA was reacted with 4-nitrophenyl 2-(methylsulfonyl)ethyl carbonate [7] [12] to yield crystalline, analytically pure Msoc-tetrapeptide **4**. Its isolation was rather difficult because of a pronounced solubility in H_2O and organic solvents.

Compound **4** was condensed with the mixed HOBt·HCl salt **6** of partially protected nonapeptide amide (produced from **5** by catalytic hydrogenation in the presence of HOBt), using the DCC/HOBt method without base. This procedure is known to minimize side reactions, particularly epimerization at the carboxyl component [13]. The protected tridecapeptide **7** was obtained in good yield (89–90%). However, its complete separation from nonpeptide contaminants proved rather difficult (partition chromatography) so that the crude material was used in the next step, removal of the Msoc group by alcaline β-elimination (*ca.* 0.1N $Ba(OH)_2$, 10 min, room temp.). $O^{5,5}$-(*tert*-Butyl)-$N^{6,11}$-(*tert*-butoxycarbonyl)-$N^{2,1}$-deacetyl-α-MSH (**8**) was purified by gel-permeation chromatography and characterized by amino-acid analysis.

Scheme. *Preparation of* N^{α}*-(5-Bromovaleryl)-*N^{α}*-desacetyl-α-MSH* (**10**)



Introduction of the 5-bromovaleryl group *via* active ester required an excess of HOBt in the reaction mixture in order to avoid the very disturbing acylation of the serine OH-group [14]. Gel-permeation chromatography removed traces of contaminants from **9** *(Fig. 2)*. Finally, the cleavage of the Boc and OBu^t protection by HCl had to be carried out in the presence of HCOOH, because in AcOH alone, the HCl salt precipitated and included varying amounts of peptide that still contained $Glu(OBu^t)$ as demonstrated by TLC. In the amino-acid analysis of the final product **10**, the serine content was invariably on the low side despite a chromatographically very pure product. Presumably this is caused by side reactions of the N^{α}-substituent during acid hydrolysis.

Compound **10** was labeled in Tyr-2 with ^{125}iodine by the mild procedure described for α-MSH [15]. Mixtures of **10** and [^{125}I]iodo-**10** reacted readily with sodium thiosul-

fate to produce the '*Bunte* Salz' **13**, N^{α}-deacetyl-N^{α}-(5-(sulfothio)valeryl)-α-MSH *(Fig. 1)* containing its radiolabeled derivative [^{125}I]iodo-**13**. This reaction was characterized by the electrophoretic pattern *(Fig. 3)* at pH 3.6 showing that a positively charged, large peptide (**10**, net charge 3 +) lost one third of its positive charge as indicated by the ratio of the electrophoretic mobilities (about 47:77 = 0.61). The '*Bunte* Salze' were successfully used to prepare tobacco mosaic virus disulfide conjugates (see [1] and the following report [16]).

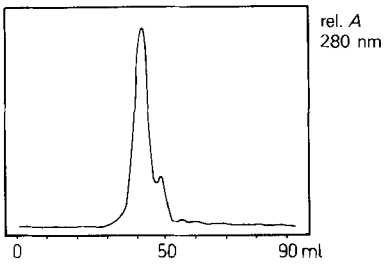


Fig. 2. *Purification of* **9** *by gel filtration through* Sephadex LH-20 *with MeOH* (elution profile, see *Exper. Part*)

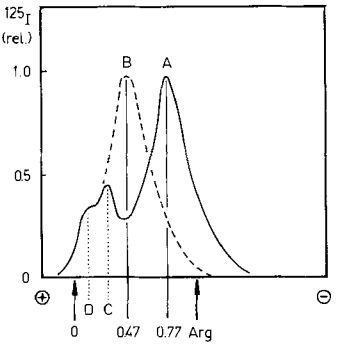


Fig. 3. *Thin-layer electrophoresis at pH 3.6 of* [^{125}*I*]*iodo-***10** *(solid line) and* [^{125}*I*]*iodo-***13** *(dashed line) scanned with an* Actigraph III TLC Scanner (Nuclear Chicago). Ordinate: relative cpm; Abscissa: distance travelled between anode and cathode; O = starting point; Arg = position of arginine; D, C = radioactive nonpeptide contaminants of [^{125}I]iodo-**10**; A = [^{125}I]iodo-**10**, B = [^{125}I]iodo-**13**, revealed with peptide reagents. Electrophoretic mobility is relative to Arg.

Biological Activity of 10 in *Cloudman S-91* Mouse Melanoma Cell Cultures. – Current methods of α-MSH bioassay are based mainly on amphibian skin melanophores (skin darkening, melanin disperison) and mammalian melanocytes (activation of tyrosinase and adenylate cyclase). We compared **10** with α-MSH (prepared according to [7]) for tyrosinase stimulation (procedure of *Pomerantz* [17]) and *cyclic* AMP accumulation (procedure of *Hamprecht et al.* [18]) in melanocyte tumor cell cultures (*Cloudman S-91* mouse melanoma).

As shown in *Fig. 4*, **10** is a full agonist for tyrosinase stimulation and a partial agonist for *cyclic* AMP accumulation (intrinsic activity about 75% of α-MSH). In the tyrosinase assay, **10** was 9.8 times and in the *cyclic* AMP assay 1.9 times less potent than α-MSH. In both cases, the ED_{50} values for *cyclic* AMP accumulation were considerably larger than those for the specific hormonal effect, in agreement with other systems [19]. A change of the initial pH of the incubation media from 7.4 to 8.1 by adding 20 µl of 1N NaOH just before the agonist did not affect the ED_{50} values but increased the maximal rate of enzyme activity (intrinsic activity, efficacy) 1.4 to 1.5 times, although the basal rate (without agonist) remained unchanged.

The stimulation-time course of the two assays was quite typical [19]: a transient burst of *cyclic* AMP accumulation in the minute range *(Fig. 5)* was followed by a

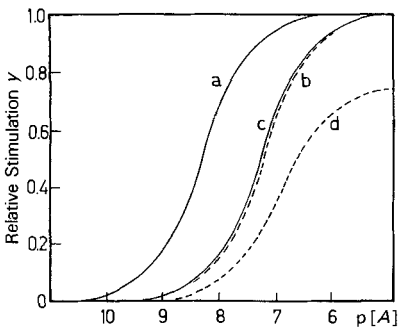


Fig. 4. Cloudman S-91 *melanoma cell cultures: log(dose)/response curves calculated with* Eqn. 1 (*see* Exper. Part) *for tyrosinase stimulation (solid lines) and* cyclic *AMP accumulation (dashed lines) with α-MSH* (a,b) *and* **10** (c,d). Abscissa: negative decadic log of agonist concentration p[*A*]. Ordinate: fractional effect stimulation *Y*, relative to maximal effects of α-MSH, measured 24 or 48 h after agonist application (tyrosinase assay), or after 10 min (*cyclic* AMP assay). ED_{50} values in the tyrosinase assay were $5.37 \cdot 10^{-9}$M (α-MSH) and $5.27 \cdot 10^{-8}$M (**10**) and in the *cyclic* AMP assay $5.6 \cdot 10^{-8}$M (α-MSH) and $1.05 \cdot 10^{-7}$M (**10**).

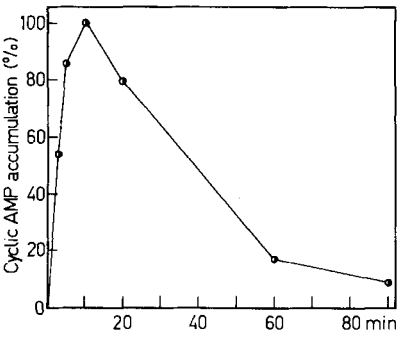


Fig. 5. Cloudman S-91 *melanoma cell cultures: time course of* cyclic *AMP accumulation after addition of 10^{-6}M α-MSH or* **10**. The values relative to maximal stimulation in each case represent the means of 6 experiments and were not significantly different for α-MSH and **10**; standard deviations were between 1 and 8%.

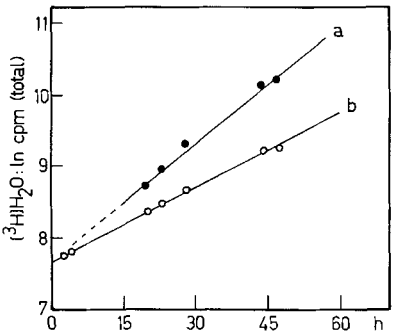


Fig. 6. Cloudman S-91 *melanoma cell cultures: time course of tyrosinase stimulation by 10^{-7}M α-MSH applied at time 0 (dots) compared with the basal, unstimulated tyrosinase activity (circles).* A short lag period of up to 4 h was observed for α-MSH stimulation; **10** behaved very similarly to α-MSH (not shown).

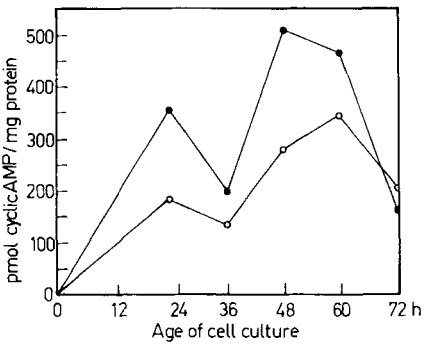


Fig. 7. Cloudman S-91 *melanoma cell cultures: influence of age upon* cyclic *AMP accumulation stimulated for 10 min as in* Fig. 5 *by 10^{-6}M α-MSH (dots) or* **10** *(circles).* Means of 2–5 experiments for each time and compound, standard deviations about 10%.

long-lasting stimulation of tyrosinase activity *(Fig. 6)* after a lag period of up to 4 h. *Fig. 7* shows the *cyclic* AMP accumulation as a function of the cell-culture age, proving that the decrease after 10 min *(Fig. 5)* is not caused by ageing.

Binding of α-MSH and 10 to *Cloudman S-91* Mouse Melanoma Cell Cultures. – The labeled [^{125}I]iodo-α-MSH and [^{125}I]iodo-**10** used in these studies were extensively

purified by *BioGel-P-6* chromatography until the peaks C, and D of *Fig. 3* were lacking. At $5 \cdot 10^{-9}$M, *ca.* 16 times as many molecules of **10** as of α-MSH were bound per cell *(Fig. 8)*. The time for half-maximal binding was somewhat greater for **10**, but still less than 3 min. Very characteristic of **10** is the maximum of binding after about 30 min, followed by a sharp decrease. The binding of **10** was also characterized by a pH optimum at *ca.* 7.4, only 72% being bound at pH 6.9 and 45% at 8.2. The maximum of binding at 30 min was strongly influenced by the temperature: 100% at 37–40°, 83% at 22°, and 20% at 0°.

Another characteristic difference between **10** and α-MSH is shown in *Fig. 9*. Bound α-MSH can be washed out with BSS (see *Exper. Part*) at 37° in a biphasic process: 50% after 2 min and 97% after 30 min; only about 20% of **10** can be washed out in 2 min and 60% remain bound after 30 min.

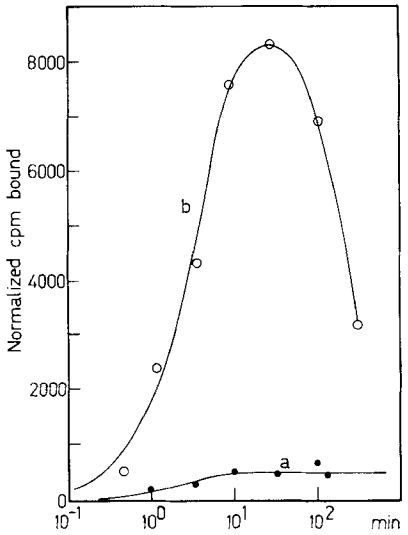


Fig. 8. Cloudman S-91 *melanoma cell cultures: time course of the binding of α-MSH (a) and* **10** *(b), 10^{-9}M each.* The values on the ordinate were normalized to $2 \cdot 10^{6}$ cells and $5 \cdot 10^{5}$ cpm per experiment.

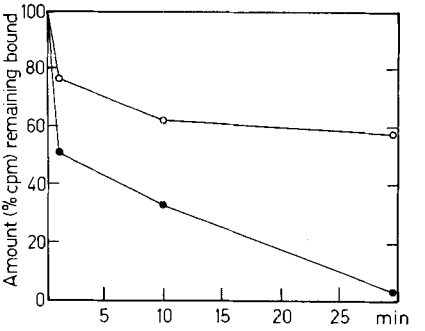


Fig. 9. Cloudman S-91 *melanoma cell cultures: relative amounts of α-MSH (dots) and* **10** *(circles) remaining bound after different times of contact with fresh BSS at 37°*

The behaviour of **10** in the binding experiments is hard to explain. Perhaps a strong hydrophobic interaction with the lipid phase of the target cell membrane [20–23] is involved, followed by internalization and metabolic elimination processes. The different behaviour of **10**, α-MSH, and the corresponding tobacco mosaic virus conjugates [1] may be important for understanding α-MSH-receptor interactions [16].

Support of this work by the *Swiss National Science Foundation* and by research grants of the ETH is greatly appreciated. Some of the synthetic experiments were explored by Miss *Gabriela Wegmann* as a requirement for her diploma examination.

Experimental Part

General. BSS ('Balanced Salt Solution' or *Dulbecco's* PBS) and PBS ('Phosphate Buffered Solution'), both commonly used for cell cultures, are described in [25]. DTT (dithiothreitol = 1,4-dimercapto-2,3-butanediol) was from *Fluka*. Evaporation of solv. from reaction mixtures was carried out in *Büchi* rotary evaporators at 0.1–20 mbar and at moderate bath temp. Products were dried in a vacuum desiccator over NaOH pellets; for micro-analyses, they were heated to 40° during 24 to 48 h at 0.01 mbar over P_2O_5 as desiccant. Radioactive samples were prepared with the necessary precautions in a type-B or -C laboratory and were counted in a *Nuclear Chicago* γ-counter, *Mod. 4230*. Solv. mixtures are in volume parts. TLC and thin-layer electrophoresis (TLE) were carried out on *Merck* silica gel *(G 60, F 254)* or cellulose plates, resp. For TLE an apparatus of *Camag* was used (about 1 h at 25 V/cm). The TLC solv. systems were: BAW 1 = 2-BuOH/AcOH/H_2O 72:7:21, BAW 2 = 1-BuOH/AcOH/H_2O 67:10:23, BPAW 1 = 1-BuOH/pyridine/AcOH/H_2O 50:12:12:25, BPAW 2 = same, but 42:24:4:30, CME = $CHCl_3$/MeOH/AcOH 95:5:3, EPAW = EtOAc/pyridine/HCOOH/H_2O 63:21:10:6. The electrophoresis buffer pH 3.6 was AcOH/pyridine/H_2O 100:10:890. The compound spots were revealed by fluorescence quenching, I_2 vapor, ninhydrin, *Reindel-Hoppe, Ehrlich* (Trp), *Pauli* (Tyr), and *Sakaguchi* (Arg) reagents [24]. In addition, a charring procedure was used to show peptides: the plates were sprayed with a soln. of 20 g of $(NH_4)_2SO_4$ and 4 ml of conc. H_2SO_4 in 100 ml of H_2O and then heated at about 200° for 10 min (brown spots). M.p. were determined in open capillaries and are uncorrected. Optical rotations were measured with a *Perkin-Elmer* polarimeter, Mod. *141*. Microanalyses were performed in the Laboratorium für Organische Chemie, ETHZ (Mr. *D. Manser*). Amino-acid analyses were carried out in this institute by Mrs. *M. Wirth* (Laboratory of Prof. Dr. *H. Zuber*) according to *Stein and Moore* with *Beckman 120 B* and *121* analyzers. The samples were hydrolyzed with 6N HCl for 24 h at 110°.

*N-[2-(Methylsulfonyl)ethoxycarbonyl]-*L*-seryl-*L*-tyrosyl-*L*-seryl-*L*-methionine* (**4**). *N(*tert*-Butoxycarbonyl)-*L*-seryl-*L*-tyrosyl-*L*-seryl-*L*-methionine methyl ester* (**1**; 400 mg, 0.66 mmol; prepared according to [10]) was saponified in MeOH (5 ml) with 1N NaOH (1.3 ml) during 45 min at r.t. The solv. was evaporated and the residue diluted with H_2O (10 ml) and acidified to pH 3 with 1N HCl. The product was extracted into $CHCl_3$ (4 × 50 ml), the org. phase dried and evaporated. The oily residue was solidified by treatment with Et_2O/hexane 1:1 and the resulting powder dried. Yield about 85% of hygroscopic, crude *N-(*tert*-butoxycarbonyl)-*L*-seryl-*L*-tyrosyl-*L*-seryl-*L*-methionine* (**2**). TLC: R_f 0.62 (**2**), small amounts of impurities at 0.13, 0.28, and 0.71 (BAW 1).

The Boc group was removed by dissolving **2** (330 mg, 0.56 mmol) in TFA (8 ml) and keeping the soln. for 25 min at 20°. The acid was removed by evaporation and the oily residue triturated with Et_2O/hexane 1:1. *L-Seryl-*L*-tyrosyl-*L*-seryl-*L*-methionine* (**3**) crystallized from EtOH/$(i\text{-}Pr)_2O$ as trifluoroacetate salt. Yield about 65% (first crop of crystals), m.p. 198–200° (dec.). TLC: R_f 0.42, very slight impurities at 0.54, 0.20, and 0.01 (BAW 1).

For N^{α}-protection, **3** (244 mg, 0.418 mmol) was dissolved in DMF (5 ml) and treated with $Et(i\text{-}Pr)_2N$ (158 µl, 2 equiv.) followed by solid 4-nitrophenyl 2-(methylsulfonyl)ethyl carbonate [7] [12] (133 mg, 0.460 mmol) that dissolved upon stirring. After 15 h at r.t., the solv. was evaporated and the residue dissolved in H_2O (7 ml). The aq. phase was washed with EtOAc (2 × 4 ml), acidified to pH 2 with HCOOH, frozen, and lyophilized for 4 h to remove H_2O and $Et(i\text{-}Pr)_2N \cdot HCOOH$. The residual solid foam was dissolved in a small amount of EtOH and precipitated with Et_2O. The precipitate was crystallized from EtOH/Et_2O and further purified either by twice recrystallizing from the same solv. or (preferably) by gel filtration through *Sephadex LH-20* with 1% aq. AcOH. Slight amounts of impurities emerged before the main peak and were easily removed. Yields of anal. and chromatographically pure **4** between 40 and 60%; m.p. 139–140°. TLC: R_f 0.3 (BAW 1), 0.52 (BPAW 1), and 0.59 (BPAW 2). $[\alpha]_D^{25} = -5.1°$ (c = 1, MeOH). Anal. calc. for $C_{24}H_{37}N_4O_{12}S_2$ (637.6): C 45.20, H 5.85, N 8.79; found: C 45.28, H 5.64, N 8.77.

*O^5-(*tert*-Butyl)-*L*-glutam-1-yl-*L*-histidyl-*L*-phenylalanyl-*L*-arginyl-*L*-tryptophylglycyl-N-(*tert*-butoxycarbonyl)-*L*-lysyl-*L*-prolyl-*L*-valinamide · HCl · HOBt* (**6**). The hydrochloride of *N-benzyloxycarbonyl-O^5-(*tert*-butyl)-*L*-glutam-1-yl-*L*-histidyl-*L*-phenylalanyl-*L*-arginyl-*L*-tryptophyl-glycyl-N^6-(*tert*-butoxycarbonyl)-*L*-lysyl-*L*-prolyl-*L*-valinamide* (**5**), was prepared according to [11]. Its hydrogenation, however, was carried out in MeOH in the presence of 1 equiv. of HOBt [13] to give the mixed HCl/HOBt salt **6**. The pure product was obtained in 70% yield by gel filtration through *Sephadex LH-20* with MeOH. TLC: behaviour identical with that of the dihydrochloride described earlier [11], R_f 0.57 (BPAW 1), 0.13 (EPAW).

*N-[2-(Methylsulfonyl)ethoxycarbonyl]-*L*-seryl-*L*-tyrosyl-*L*-seryl-*L*-methionyl-O^5-(*tert*-butyl)-*L*-glutam-1-yl-*L*-histidyl-*L*-phenylalanyl-*L*-arginyl-*L*-tryptophyl-glycyl-N^6-(*tert*-butoxycarbonyl)-*L*-lysyl-*L*-prolyl-*L*-valinamide · AcOH* (**7**). To a cold (0°) soln. of **4** (159.2 mg, 0.250 mmol) in DMF (12 ml) were added successively **6** (370.5 mg, 0.250 mmol), HOBt (40 mg, 0.28 mmol), and DCC (55 mg, 0.27 mmol). After 1 h at 0° and 40 h at 20°, the

precipitated DCU was removed by filtration, the filtrate evaporated, and the residue treated with excess Et_2O: 450 mg of solid, crude **7** (apparent yield 92%). Purification was best effected by partition chromatography in 40-mg portions over *BioGel A-0.5* (2 by 28 cm) swollen with the lower phase of 1-BuOH/AcOH/H_2O 4:1:54 and equilibrated with the upper phase. Elution with the upper phase gave a peak between 80 and 140 ml of effluent followed by tailing material (UV detection). The fractions between 95 and 120 ml contained pure material in the acetate form: one spot on TLC, R_f 0.26 (EPAW), 0.62 (BPAW 1). The impurities (10–15%) contained in the crude material did not impair the next step (removal of the Msoc group) and the partially deprotected tridecapeptide amide **8** was easier to purify.

L-*Seryl*-L-*tyrosyl*-L-*seryl*-L-*methionyl*-O^5-(tert-*butyl*)-L-*glutam-1-yl*-L-*histidyl*-L-*phenylalanyl*-L-*arginyl*-L-*tryptophyl-glycyl*-N^6-(tert-*butoxycarbonyl*)-L-*lysyl*-L-*prolyl*-L-*valinamide*·*2AcOH* (**8**). Crude **7** (100 mg, 50.8 μmol) was dissolved in warm MeOH (4 ml, 30°) by stirring for 30 min. To the soln. 0.2N $Ba(OH)_2$ (3 ml) was added at 20°. After 10 min, the mixture was cooled to 4° and acidified to pH 3–4 with AcOH, whereupon the MeOH was quickly evaporated (bath at 25°). The residual soln. was subjected to gel filtration over a column of *Sephadex LH-20*. The products were eluted with 1% aq. AcOH at a flow rate of 30 ml/h and monitored by UV absorption. A sharp peak of pure **8** emerged between 150 and 210 ml of effluent (65 mg, R_f 0.16 EPAW, 0.56 BPEW 1, and 0.15 BAW 1); it was followed by shoulders (250 ml) and a peak (330 ml) containing low molecular weight impurities. Amino-acid analysis of **8** (molar ratios found; Trp not determined): Ser 1.85, Glu 1.1, Gly 1.1, Val 1.1, Met 1.1, Tyr 1.2, Phe 1.1, Lys 1.1, Arg 1.1, Pro 1.0.

4-Nitrophenyl 5-Bromovalerate. A soln. of 5-bromovaleric acid (*Fluka;* 362 mg, 2 mmol) in EtOAc (5 mol) was treated with 4-nitrophenol (285 mg, 2.05 mmol) followed by DCC (454 mg, 2.2 mmol). After 15 h at 20°, DCU was removed by filtration and the filtrate washed with acid ($KHSO_4/K_2SO_4$ 1:2, 5% soln.), dil. base (5% $NaHCO_3$), and sat. NaCl soln. Drying with $MgSO_4$ and evaporation yielded a yellowish oil that crystallized with dry Et_2O. Recrystallization from EtOAc/Et_2O afforded 4-nitrophenyl 5-bromovalerate (421 mg, 70%), m.p. 60–62°. TLC: R_f 0.91 (CME). Stable upon storage in dry state. Anal. calc. for $C_{11}H_{12}BrO_4N$ (302.13): C 43.73, H 4.00, N 4.64, Br 26.45; found: C 43.70, H 4.07, N 4.75, Br 26.45.

N-(*5-Bromovaleryl*)-L-*seryl*-L-*tyrosyl*-L-*seryl*-L-*methionyl*-O^5-(tert-*butyl*)-L-*glutam-1-yl*-L-*histidyl*-L-*phenylalanyl*-L-*arginyl*-L-*tryptophyl-glycyl*-N^6-(tert-*butoxycarbonyl*)-L-*lysyl*-L-*prolyl*-L-*valinamide*·*AcOH* (**9**). A soln. of crude **8** (10 mg, 5.26 μmol) in anh. DMF (100 μl) was treated with 5.26 μl of a freshly prepared soln. of 4-nitrophenyl 5-bromovalerate (1.5M) and HOBt (2M) in DMF, followed by 6.5 μl of 10% soln. of *N*-ethylmorpholine in DMF. The mixture was stirred for 3 h at r.t. (disappearance of **8** as revealed by TLC) and the product precipitated with anh. Et_2O, filtered, washed thoroughly with Et_2O, and dried. TLC with BPAW 1 revealed complete reaction of **8** (R_f 0.70) which was replaced by a spot at R_f 0.79. The impurities contained in **8** (very slight spots at R_f *ca.* 0.65 and 0.75) remained unaltered. The product (about 8 mg) was put on a 1.6 by 34 cm column of *Sephadex LH-20* that had been equilibrated with MeOH. With MeOH, at a flow rate of 24 ml/h, **9** (7.2 mg, 67%) was obtained in fractions 38–46 ml of the eluant *(Fig. 2)*. The trailing impurities visible on TLC (R_f 0.65 and 0.75, see above) had been eliminated, mainly by removal of the peaks at 47 to 60 ml of the eluant. TLC (**9**): R_f 0.79 (BPAW 1).

N^{α}-(*5-Bromovaleryl*)-N^{α}-*deacetyl-α-MSH* = N-(*5-Bromovaleryl*)-L-*seryl*-L-*tyrosyl*-L-*seryl*-L-*methionyl*-L-*glutam-1-yl*-L-*histidyl*-L-*phenylalanyl*-L-*arginyl*-L-*tryptophyl-glycyl*-L-*lysyl*-L-*prolyl*-L-*prolyl*-L-*valinamide*·*2HCl* (**10**). a) Anisole (0.5 μl) and 1N HCl in AcOH (24.6 μl) were added successively to a soln. of **9** (5 mg, μmol) in 98 100% HCOOH (100 μl) contained in the flask of a *Büchi Rotavap,* and the mixture was stirred by rotation for 20 min at r.t. The solv. were then evaporated and the product solidified with anh. Et_2O and dried: 4.2 mg (91%). TLC revealed 1 spot only, with positive reactions to ninhydrin, *Sakaguchi, Ehrlich, Pauli,* and *Reindel-Hoppe* reagents, R_f 0.16 (BAW 1), 0,46 (BPAW 1). Amino-acid analysis of **10** (Trp not determined; relative molar ratios, Met as reference): Ser 1.89, Glu 1.14, Gly 1.13, Val 1.10, Met 1.00, Tyr 0.90, Phe 1.05, Lys 1.11, His 1.04, Arg 1.05, Pro 1.14.

b) An identical product was obtained by reacting **11** [7] (164 mg) in a similar manner with 4-nitrophenyl 5-bromovalerate, purifying N-(*5-bromovaleryl*)-L-*seryl*-L-*tyrosyl-seryl*-L-*methionyl*-L-*glutam-1-yl*-L-*histidyl*-L-*phenylalanyl*-L-*arginyl*-L-*tryptophyl-glycyl*-N^6-(*2-(methylsulfonyl)ethoxycarbonyl)*-L-*lysyl*-L-*prolyl*-L-*valinamide* (**12**) over *Sephadex LH-20* with DMF, and cleaving the Msoc group with alcali: **12** (75 mg, 38.5 μmol) was dissolved in 0.1N $Ba(OH)_2$ (6 ml) and kept for 5 min at r.t. The mixture was then neutralized with 0.2N H_2SO_4 (3 ml) and the $BaSO_4$ removed by filtration. The filtrate was acidified slightly with HCl, evaporated, and the residue dissolved in H_2O. Isolation by chromatography over *Sephadex LH-20* (3.8 by 70 cm) with 1% aq. AcOH and lyophilisation of the pure fractions: 79 mg (49%) of **10**. TLE: R_f 0.77 relative to Arg (pH 3.6). Amino-acid molar ratio relative to Met: Ser 1.48, Glu 1.20, Gly 1.20, Val 1.17, Met 1.00, Tyr 1.00, Phe 1.13, Lys 1.17, His 1.05, Arg 1.11, Pro 1.24.

$N^{2,1}$-(5-Bromovaleryl)-$N^{2,1}$-deacetyl-$C^{3,2}$-[^{125}I]iodo-α-MSH. Iodination of **10** was carried out with equimolar amounts of chloramine T and sodium [^{125}I]iodide according to the procedure of *Eberle and Hübscher* for iodinating α-MSH [15]. Purification was, however, modified to resemble the procedure of *Greenwood et al.* [26]: The column material *(Sephadex G-10)* was saturated twice with buffer containing bovine-serum albumin and then washed thrice with protein-free buffer (0.05M sodium phosphate, pH 7.0). Development and elution were carried out with buffer alone. Final purification was effected by chromatography of about 30×10^6 cpm through *BioGel P-6* (200–400 mesh; 1 by 16 cm) with BSS containing 0.5% bovine-serum albumine and 0.02% NaN_3.

$N^{2,1}$-Deacetyl-$N^{2,1}$-(5-(sulfothio)valeryl)-α-MSH/$N^{2,1}$-Deacetyl-$N^{2,1}$-(5-(sulfothio)valeryl)-$C^{3,2}$-[^{125}I]iodo-α-MSH (**13**/[^{125}I]*iodo*-**13**). A soln. of **10** (5.2 mg, 2.8 μmol) in DMF (75 μl) was treated with a 116 mM soln. of $Na_2S_2O_4$ in 125 mM phosphate buffer, pH 8.1 (236 μl, 10 equiv. of thiosulfate), immediately followed by a soln. of [^{125}I]iodo-**10** (that had not been purified by *BioGel P-6* chromatography) in 1% aq. AcOH (180 μl, $2.1 \cdot 10^8$ cpm) and by 1N NaOH (31.5 μl). The reaction was complete after 1 h at r.t. as judged by TLC: R_f 0.46 (educt), 0.40 (product; BPAW 1). The reaction was also followed by TLE at pH 3.6: R_f (relative to Arg) 0.77 (educt) and 0.47 (product), *Fig. 3*.

Melanoma Cell Cultures. Monolayer cell cultures of the murine line *Cloudman S-91,* Clone *M-3* (CCL 53.1; *American Type Culture Collection,* Rockeville, Md., USA) were established in a conventional manner using 25- or 75-cm^2 culture flasks (*Becton Dickinson,* Basel), *NCTC-135* culture medium supplemented with 10% horse serum (both *Gibco,* Basel), cysteine hydrochloride monohydride monohydrate (12.4 mg/l), $NaHCO_3$ (2.2 g(/l), penicillin G (33.4 mg/l, *Fluka,* Buchs), and streptomycin sulfate (50 mg/l; *Dolder,* Basel) and saturated with CO_2. The cultures were grown at 37° in an atmosphere of 95% air and 5% CO_2. Every 3–4 days, the monolayers were disrupted, diluted, and transferred to new flasks in portions containing between 1 and 3 million cells. Cell disaggregation was carried out with EDTA (ethylenediamine tetraacetate), which proved to be superior to trypsinization in our case: The culture medium was decanted by aspiration, the cells washed with BBS and then treated with 3 ml of a 2 mM EDTA solution containing penicillin G (336 mg/l), streptomycin sulfate (100 mg/l), NaCl (5.33 g/l), KCl (300 mg/l), $Na_2HPO_4 \cdot 2H_2O$ (73 mg/l), KH_2PO_4 (20 mg/l), and phenol red (500 mg/l; *Fluka,* Buchs) which was adjusted to pH 8.4 with 1N NaOH. The cells were neatly disaggregated after 5 min at 37° without having to scrub with a 'rubber policeman'.

Tyrosinase-stimulating Activity of α-MSH and **10**. Tyrosinase activity was measured according to *Pomerantz* [17] (see also [27]) by estimating the amount of [^{3}H]H_2O released during the enzymic oxidation of 3',5'-ditritio-L-tyrosine. Rapidly growing cell cultures in 25-cm^2 flasks inoculated with 1.5 to $2.0 \cdot 10^6$ cells in 10 ml of medium were treated, after one replication (about 24 h), with 5 to 6 different concentrations of α-MSH or **10** (dissolved in 0.01N HCl and diluted with BSS) and 10 μCi of 3',5'-ditritio-L-tyrosine (*Amersham,* England; dissolved in H_2O and evaporated twice before use in order to remove [^{3}H]H_2O). After 24 and 48 h, aliquots of the supernatant medium (500 μl) were withdrawn, treated with 500 μl of a charcoal suspension (150 g/l) for 30 min at r.t., and centrifuged for 15 min at *ca.* 5000 rpm; 250 μl of the supernatant were homogenized with 10 ml of *Beckman EP* scintillation fluid and the radioactivity determined in an *Isocap 300 Nuclear Chicago* γ-counter. Maximal stimulation of tyrosinase activity was always achieved with $1 \cdot 10^{-7}$M α-MSH; the difference between this value and the basal tyrosinase activity (without hormone) was taken as 100% efficacy. The log(dose)/response curves were found to be almost identical for different cell cultures, even for those prepared from different ampoules of the type culture. Each experiment (5–6 different concentrations) was subjected to nonlinear least squares regression analysis [28] and the peptide concentration for half-maximal stimulation (ED_{50}) determined. The final values of ED_{50} were the means of 2–4 individual curves. The simple saturation model of *Eqn. 1* was used:

$$Y = A/(ED_{50} + A) \tag{1}$$

wherein Y is the biologic activity and A the agonist concentration.

Binding Experiments. After *ca.* 1 replication cycle (24 h after inoculation, 3–4 million cells per flask), the medium was removed, the cultures were washed twice with warm BSS and then treated with the peptide soln. (2 ml, $5 \cdot 10^{-9}$M; $1 \cdot 10^6$ cpm of α-MSH, $0.5 \cdot 10^6$ cpm of **10**) at 37°. After different incubation times, the peptide soln. was removed, and the cells were rapidly washed 3 times with ice-cold BSS (2 ml, *ca.* 20 s total exposure time). The cells were then disaggregated with 2 ml of PBS soln. containing EDTA (1mM) and *Triton X-100* (1%). After 2–3 min, the cell suspension was pipetted into vials for γ-counting. Unspecific binding was estimated with culture flasks containing culture medium without cells following the same sequence of operations. The binding data were normalized to a total of $0.5 \cdot 10^6$ cpm of added agonist and $2 \cdot 10^6$ cells per experiment. The association-time constant B_t was calculated by least squares regression analysis using the monomolecular binding model describing the occupacy Y of receptor in the presence of a large excess of agonist, *Eqn. 2*,

$$Y = B_{max}\,(1-e^{-Bt})\qquad(2)$$

wherein B_{max} is the maximal amount of agonist bound, and t is the time.

In experiments designed for studying the dissociation of bound peptide from the cells, the culture – after washing with BSS at 0° – was incubated for different lengths of time with 2 ml of BSS at 37°, followed again by 3 times washing with BSS at 0°, cell dissaggregation, and counting.

Stimulation of Cyclic *AMP Accumulation in* Cloudman S-91 *Melanoma Cell Cultures.* The estimation of *cyclic* AMP accumulated within the cells at different times after application of the peptide agonist was carried out according to the EtOH-extraction procedure of *Hamprecht* [18] without addition of phosphodiesterase inhibitors. The values were normalized to the protein content before extraction with EtOH (about 125% of that found after extraction) and, for the log(dose)/response curves, to 10 min stimulation. The basal *cyclic* AMP accumulation (without stimulation) was 2.3 ± 1 pmol *cyclic* AMP per mg of protein (mean of all experiments), whereas the stimulation produced up to 500 or more pmol/mg. The experiments were evaluated according to *Eqn. 1.* Cell cultures inoculated with 1.9–2.4 million cells per 25-cm^2 flask were grown for different times (usually 24 to 48 h, in some cases up to 72 h). Then, the culture medium was removed, and the cells were washed with 3 ml of fresh, complete medium at 37°. The cells were covered with 3 ml of fresh medium and 30 µl of a peptide soln. of known concentration added. Incubation was continued for different times at 37° when the medium was removed by aspiration and the cells cooled and covered with 1 ml of abs. EtOH at 0°. After 10 min at 4°, the EtOH was transferred into a 3-ml vial, the cells were washed with 1 ml of fresh EtOH at 0°. The combined extract and washing were evaporated in a vacuum centrifuge (*Speed Vac Concentrator Mod. RH 20-12, Savant Instruments, Sarasin,* Basel). The dry residue was dissolved in 500 µl of H_2O and the soln. kept at − 25 to − 80° if necessary. A maximum of 110 µl could be processed in the following radio-immuno assay (basal production was estimated with 100 µl, all other values with 10 µl of the soln.).

The assay was carried out with the radio-isotope-dilution procedure utilizing a *cyclic* AMP binding protein available as assay kit (*Boehringer-Mannheim AG,* Rotkreuz, Switzerland). For every test combination a separate calibration curve was determined. Control experiments included the following *i)* Proportionality: each value was determined with 5, 10, and 20 µl of 'extract'. *ii)* Masking: to 10 µl of extract, a defined amount of *cyclic* AMP was added and the result determined by substraction of the added amount. *iii)* Value after elimination of *cyclic* AMP: 100 µl of extract was treated with 100 µl of a charcoal suspension (150 g/l) during 20 min at r.t., centrifuged, and used in the assay.

For every test combination, the protein content of the EtOH-treated cells was determined according to *Lowry* [29] and *Oyama* [30] using bovine-serum albumin as reference. Both methods described by *Lowry* (for soluble and for insoluble proteins) were used as described in detail by *Wunderlin* [1].

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