154. Synthesis and Steroidogenic Activity of Covalently Dimerized Corticotropin (ACTH) Fragments[1])

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(6.VI.88)

The chemical synthesis by classical methods in solution of a dimer of the sequence 1 to 24 of adrenocorticotropin (ACTH) is described. The two monomers were covalently linked through their C-termini, using lysine amide as spacer. Dimerization did not improve significantly the potency of the agonist ACTH-(1–24), while it strongly potentiated the antagonistic effect of the fragments ACTH-(11–24) and ACTH-(7–24) in the stimulation of steroidogenesis in isolated adrenal cells. The results seem to imply a microaggregation of the receptors at the adrenal cell surface.

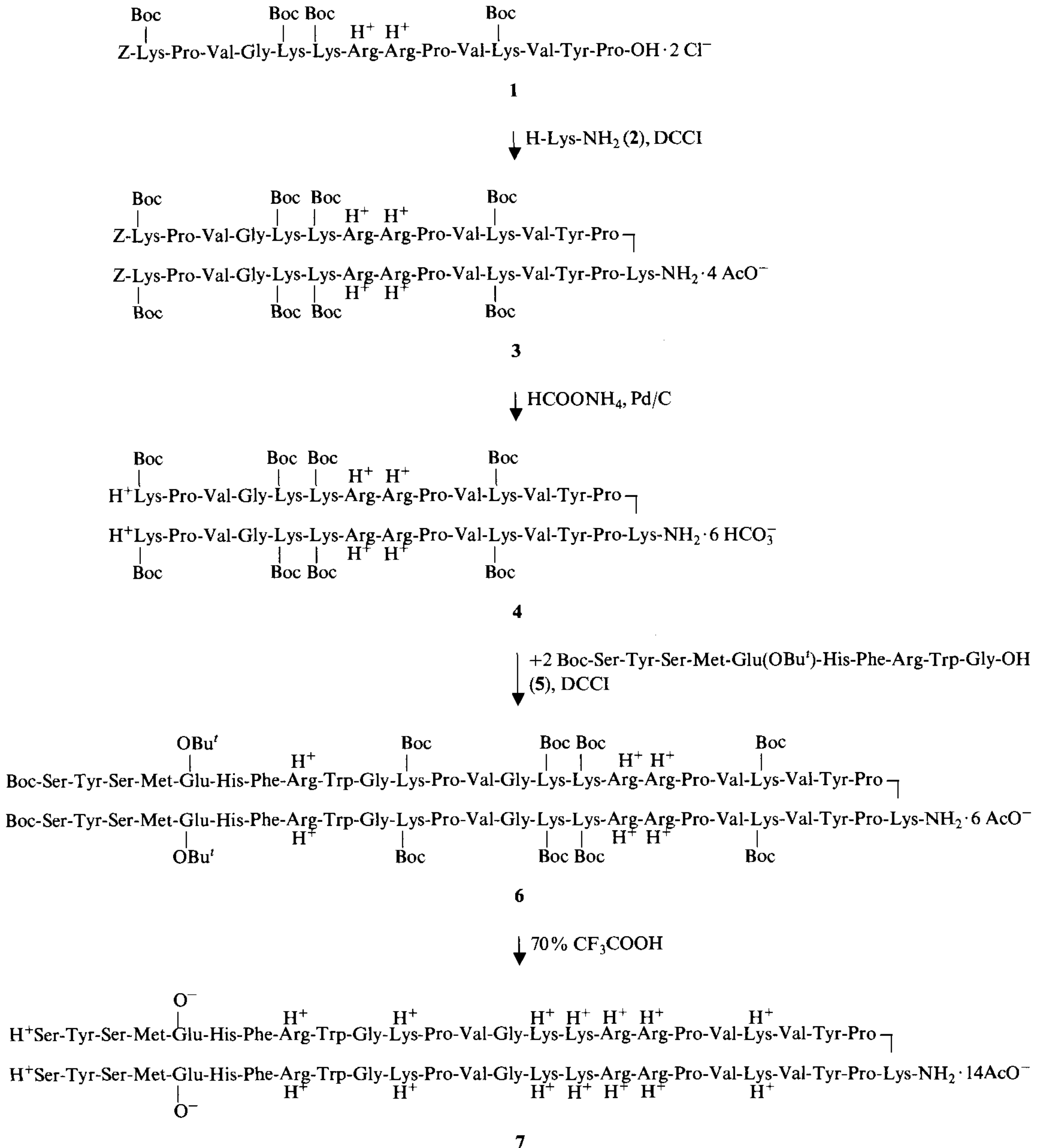
Introduction. – Covalent dimers of peptide hormones and neuropeptides have been shown in a few cases to display enhanced potency [2–4] and selectivity [5]. In particular, the inhibitory potency of the corticotropin fragment ACTH-(11–24) was strongly increased when the fragment ACTH-(11–24) was dimerized *via* the 11- or the 24-position [6]. In these experiments, a log dose/response was measured in isolated adrenal cells for the agonist ACTH-(1–24) in the presence of a constant concentration (10^{-5} M) of one of the synthetic antagonists. The potentiation was explained in two ways: 1) either a microaggregation of the hormone receptors at the cell surface would be induced by the dimeric antagonists which would prevent the access of the agonist to the receptors; 2) or an accumulation of the inhibitors at the negatively charged cell membrane would take place due to the higher net positive charge of the antagonists (10 and 14, respectively) compared to the monomer (net charge 6). No conclusive explanation could be provided, partly because the dimer of the corresponding agonistic sequence has not been available so far.

The purpose of this work was, therefore, to prepare a dimer of the corticotropin sequence 1–24 (*Synacthen*® [7]) known to display the full agonistic activity of corticotropin ACTH-(1–39) in the stimulation of steroidogenesis. We describe in this communication the chemical synthesis and analytical characterization of the dimer obtained when two ACTH-(1–24) monomers were linked through their C-termini, using lysine amide as the spacer molecule. We describe further the preparation of the dimer of the antagonistic sequence ACTH-(7–24) by the same method. Finally, we report on the preliminary evaluation of the pharmacological effects of dimerized corticotropin agonists and antagonists in adrenal-cell steroidogenesis.

[1]) Abbreviations for amino acids and derivatives follow the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature [1]. Further abbreviations: DMF = *N,N'*-dimethylformamide, DCCI = *N,N'*-dicyclohexylcarbodiimide, SDS = sodium dodecyl sulfate, FPLC = fast protein liquid chromatography.

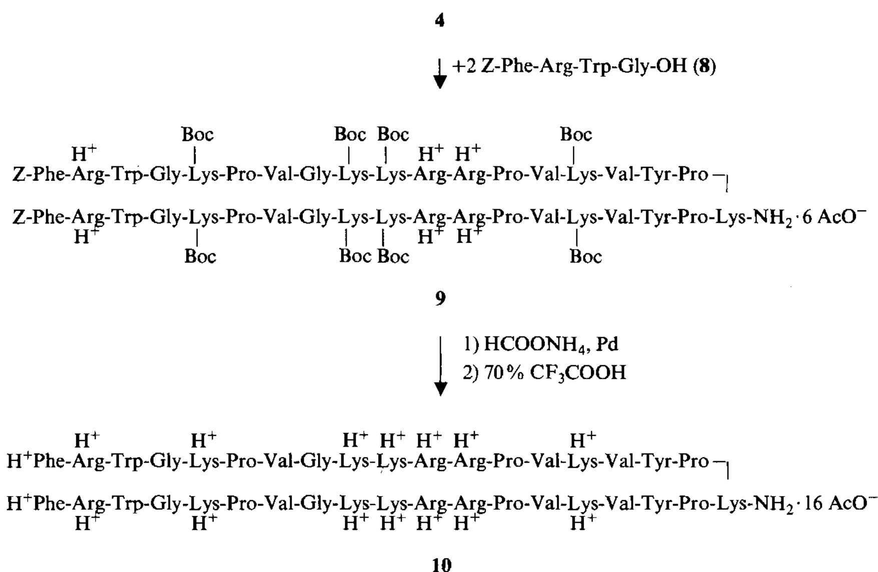
Synthesis. – The goal compound **7** was obtained by dimerization of the fragment ACTH-(11–24), followed by a one-step condensation of the dimer with two ACTH-(1–10) fragments, and by final deprotection under acidic conditions *(Scheme 1)*. We used

Scheme 1. *Synthesis of the Dimeric Conjugate of ACTH-(1–24)*



as starting material compound **1** prepared according to methods described in [4] and designed so as to enable N^{α}-elongation to be performed. A new synthesis strategy was adopted for the preparation of the following derivatives, in which deprotections as well as condensations were performed simultaneously on the two arms of the dimers. Hence, compound **3** was obtained by direct condensation of **1** with the two free amino groups of lysine amide (**2**). Compound **3** was obtained much more easily in this way than in [4], as was also compound **4**, after selective deprotection at the N^{α}-positions by catalytic transfer hydrogenation [8]. The fragment condensation with **5** to produce **6** was also performed in one step. At this stage, all protecting groups were of the *tert*-butyl type and could be removed by treatment of **6** with CF_3COOH. The free peptide **7** and the intermediates were purified by conventional chromatographic methods.

Scheme 2. *Synthesis of the Dimeric Conjugate of ACTH-(7–24)*



The dimer **10** of the sequence ACTH-(7–24) was prepared according to a similar strategy *(Scheme 2)* in which dimer **4** was substituted with **8** in one step at its two N-terminals. Because of the presence of benzyloxycarbonyl (Z) groups in addition to *tert*-butyl protecting groups, final deprotection of **9** was performed in two steps, a reductive followed by an acidic treatment.

Due to the size of the fragments to be condensed, solubility problems in organic solvents were encountered during the whole synthesis, and the yields of condensations were generally low. Moreover, because of the two possible reaction sites in lysine amide and in the dimer **4**, extensive purifications of the crude products were necessary to separate the desired product from unreacted or monosubstituted materials using mainly column chromatography on silica gel in aqueous/organic solvent mixtures and gel filtration on *Sephadex* in aqueous NH_4OAc gradients *(cf. Exper. Part)*.

Analytical characterization of the key intermediates was performed by TLC, elemental and amino-acid analysis, and gel electrophoresis *(Table 1)*. The ratio of the lysine content to the other amino acids was a useful indicator of successful fragment condensation because of its presence in the spacer molecule. The final product **7** was a 49-residue peptide which had to be analyzed by classical methods of protein chemistry. In the gel filtration of this compound, two peaks with higher molecular weight than to be expected for **7** were observed *(Fig. 1a)*. Amino-acid analysis of *Peaks I* and *II* revealed the same composition as in *Peak III* (= **7**). Repetition of the gel filtration on *Sephadex G50* with the fraction isolated from *Peak III* produced a symmetrical elution peak with no side contaminations. We concluded that **7** had the tendency to self-aggregate in organic solvents under the conditions used for deprotection and for column chromatography, but not during gel filtration in purely aqueous solvents. Practically the same observations were made with the dimer **10** *(Fig. 1b)*. In addition to gel filtration, the purity of **7** and **10** was assessed by polyacrylamide gel electrophoresis in which the material from *Peak III* (both for **7** and **10**) was homogeneous by amino-acid analysis *(Table)* and by HPLC, the latter showing a single symmetrical elution peak.

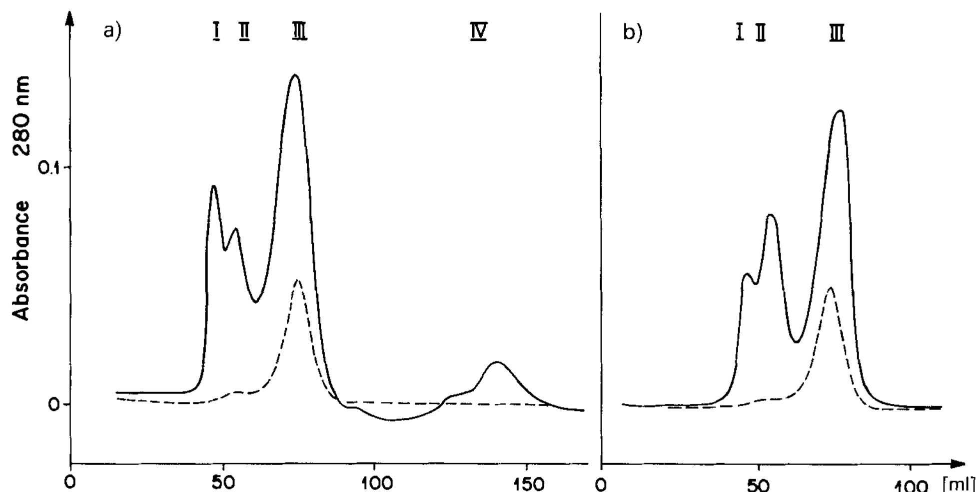


Fig. 1. *Gel filtration* (——) *of the partially aggregated dimers on* Sephadex G50 *in* 0.2M NH_4OAc *and rechromatography* (– – –) *of* Peak III (in each case) *under the same conditions*. a) Dimer **7**; b) dimer **10**.

Biological Results. – We report on preliminary results obtained with bis[ACTH-(1–24)] **7** and bis[ACTH-(7–24)] **10**, and compare them with those obtained earlier [6] for the two dimers **11** and **12** *(Fig. 2)*.

Experiments with the dimer **10** of ACTH-(7–24): The dimer was devoid of agonistic activity up to a concentration of 10^{-4}M and was a stronger antagonist than the dimers **11** and **12** of ACTH-(11–24). A concentration of 10^{-6}M of **7** was sufficient to fully suppress the agonistic effect of $3 \cdot 10^{-10}$M ACTH-(1–24), while a concentration of 10^{-5}M of **12** was necessary to obtain the same antagonistic effect [6].

Experiments with bis[ACTH-(1–24)] **7**: A log dose/response curve for the stimulation of steroidogenesis in isolated adrenal cells was measured for **7** in the concentration range 10^{-13}–10^{-5}M and compared on the same preparation with the effect of the monomer

Table. *Physical and Analytical Data of the Products and of the Key Intermediates*

	$[\alpha]_D^{23}$[a])	R_f (TLC)[b])			Amino-acid or elemental analysis[c])[d])											
		CMWA1	CMWA2	BAW	Arg	Glu	Gly	His	Lys	Met	Phe	Pro	Ser	Trp	Tyr	Val
2	+17.5	(n.d.)[d])			C 33.20 (33.04) H 8.00 (7.86) N 19.56 (19.26) Cl 32.90 (32.51)											
3	−59.7	0.37	0.93	0.21	4.0(4)	–	2.0(2)	–	9.7(9)	–	–	6.8(6)	–		1.6(2)	5.7(6)
4	−59.1	0.12	0.66	0.10	(n.d.)[e])											
6	−59.8	0.16	0.37	0.11	6.1(6)	2.3(2)	4.1(4)	2.2(2)	8.2(9)	2.1(2)	2.4(2)	(n.d.)	4.3(4)	(n.d.)	4.1(4)	5.5(6)
7	−71.9	(n.d.)[f])			6.4(6)	2.1(2)	4.0(4)	2.2(2)	9.0(9)	2.0(2)	2.0(2)	(n.d.)	3.1(4)	(n.d.)	3.9(4)	5.9(6)
9	−38.6	0.24	0.80	0.20	6.5(6)	–	4.2(4)	–	8.6(9)	–	2.2(2)	(n.d.)	–	(n.d.)	1.8(2)	5.6(6)
10	−90.0[g])	(n.d.)[f])			6.4(6)	–	3.9(4)	–	9.1(9)	–	1.8(2)	(n.d.)	–	(n.d.)	1.9(2)	6.0(6)

[a]) Specific rotation in AcOH, $c = 1$.
[b]) R_f values on silica-gel plates in the solvent systems $CHCl_3/MeOH/H_2O/AcOH$ 700:250:47:3 (CMWA1); $CHCl_3/MeOH/H_2O/AcOH$ 535:360:100:5 (CMWA2), and $BuOH/AcOH/H_2O$ 72:7:21 (BAW).
[c]) Theoretical values in parentheses.
[d]) n.d. = not determined.
[e]) Not determined since **4** is only deprotected **3**.
[f]) Not determined, replaced by polyacrylamide gel electrophoresis.
[g]) In H_2O, $c = 1$.

Fig. 2. *Structure formula* (see [1]) *of the dimeric agonist* **7** (this work) *and of the dimeric antagonists* **10** (this work) *and* **11** *and* **12** [4] [6]

ACTH-(1–24). The two responses were not significantly different neither in terms of potency (ED_{50}: $3.0 \pm 1.5 \cdot 10^{-11}$ and $5.0 \pm 1.5 \cdot 10^{-11}$ M, resp.) nor of intrinsic activity.

Experiments with the dimers **11** and **12** of ACTH-(11–24): Dimer **11** when applied at constant concentration (10^{-5} M) in the assay displaced the log dose/response curve of ACTH-(1–24) 750 times to higher ED_{50}. The effect was even higher for dimer **12** under the same conditions which increased the ED_{50} by a factor of 4514 [4].

Discussion. – The stronger antagonistic effect of dimerized ACTH-(7–24) compared to dimerized ACTH-(11–24) was in agreement with the observations made with the corresponding monomers in the same pharmacological test where ACTH-(11–24) was a weaker antagonist of *Synacthen* than ACTH-(7–24) [9].

The main observation to be rationalized was the lack of potentiating effect when the agonist ACTH-(1–24) was dimerized compared to the large effect observed when the dimerization was performed on the antagonistic fragment ACTH-(11–24).

If microaggregation is induced by the dimers, the strong adverse effect of the antagonistic dimers upon the activity of the agonist ACTH-(1–24) may still be explained like in [4]: microaggregated receptors would no longer be accessible for the agonist. In contrast, microaggregation caused by the dimer-agonist **7** would necessarily follow binding and not impair (neither potentiate) the stimulation of the receptor sites. This possible mechanism cannot be rejected at the present stage of our experiments.

A second possible explanation is based on the fact that polycations such as our both monomeric and dimeric ACTH derivatives accumulate at the negatively charged cell membrane according to a *Boltzmann* distribution [10] and that surface accumulation of these polycations is lowered by the addition of a polyvalent cationic salt. Accordingly, the high concentration (10^{-5} M) of the dimer-antagonists would decrease the effective concentration of the agonist ACTH-(1–24) at the cell surface and correspondingly shift the log dose/response to the right as observed. The shift would be more pronounced with dimer **12** (net charge 14) than with dimer **11** (net charge 10) and of course than with the monomer ACTH-(11–24) (net charge 6), as also observed. However, the apparently unchanged potency of the agonist in its dimeric form **7**, in spite of a large increase of the

net charge (14 compared to 6 in the monomer) and of the correspondingly strong electrostatic surface accumulation, is not easily explained in the frame of this model. A loss of the inherent biological activity of the agonistic sequence, when dimerized, would have to be postulated which would compensate the positive effect of the induced electrostatic accumulation at the cell surface. The latter hypothesis will have to be checked before a conclusion about the validity of the electrostatic-accumulation model in this case can be reached.

This work was supported by *Ciba-Geigy AG*, Basel, and by the *Swiss National Science Foundation* (grant 3.205-0.85).

Experimental Part

Materials. Boc-Lys(Boc)-ONp was a product of *Bachem AG*, CH-4416 Bubendorf. The protected corticotropin fragments Z-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-OBu^t · 2 HCl, Boc-Ser-Tyr-Ser-Met-Glu(OBu^t)-His-Phe-Arg-Trp-Gly-OH, and Z-Phe-Arg-Trp-Gly-OMe were generous gifts of *Ciba-Geigy AG*, Basel.

Methods. Classical peptide synthesis was applied in solution according to the methods described *e.g.* in [11] using differential protection and selective deprotection schemes and chemically specific fragment condensation for chain elongation. TLC: *Merck* F_{254} precoated plates of silica gel in the solvent systems given in the *Table*; detection by UV_{254} light and after spraying with ninhydrin, CF_3COOH/ninhydrin, and the *Reindel-Hoppe* reagent [12]. SDS polyacrylamide gel electrophoresis: gradient of polyacrylamide and of the cross-linker using the procedure of *DeWald et al.* [13]. $[\alpha]_D$: *Perkin-Elmer 241*-polarimeter. Elemental analysis was performed in the microanalytical laboratory of the ETHZ (*D. Manser*). For amino-acid analysis, peptides were hydrolyzed with 6N HCl in sealed, evacuated tubes at 110° for 20 h, and analyzed according to *Moore* and *Stein* [14] with a *Biotronik LC 6001* apparatus (courtesy of Prof. *B. Gutte*).

Evaluation of the biological activity was performed according to the procedure described by *Capponi et al.* [15] in the laboratory of Prof. *M. Vallotton*, Division of Endocrinology, University Hospital of Geneva. The dose-dependent stimulation by ACTH-(1–24) and by **7** of corticosterone production in isolated adrenal fasciculata cells was measured. For characterization of the antagonists **10–12**, the log dose/response of ACTH-(1–24) was measured in the presence of a constant concentration of the antagonist.

H-Lys-NH$_2$ · 2 HCl (**2**). Boc-Lys(Boc)-ONp (5 g, 10.7 mmol) was dissolved in MeCN (90 ml) into which gaseous NH_3 was introduced as a slow stream at r.t. After 45 min, a turbidity appeared and after additional 15 min, the solvent was evaporated to ¼ of its volume. The product was precipitated by the addition of Et_2O (100 ml), separated by decantation, and dried *in vacuo*. After dissolution in dioxane (10 ml), the product was treated with 4N HCl (in AcOEt; 10 ml) and kept for 30 min at r.t. Partial evaporation of the solvent and filtration afforded 1.07 g (50%) of **2**. TLC: homogeneous. M.p. 250°.

N^2,N^6-*Bis[Z-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro]-Lys-NH$_2$ · 4 AcOH* (**3**). For condensation, the following compounds were dissolved in DMF (6.5 ml) in the given order: Z-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-OH · 2 HCl (**1**; 750 mg, *ca.* 0.3 mmol), HOBt (48 mg, 0.35 mmol), L-lysine amide (24 mg, 0.12 mmol), Et_3N (34 µl, 0.24 mmol), and DCCI (70 mg, 0.35 mmol). The mixture was stirred for 18 h at r.t., after which unreacted lysine amide could no longer be detected by TLC. Filtration of dicyclohexylurea and evaporation of the solvent were followed by separation of **3** from unreacted **1** and from other side-products by chromatography on silica gel (column ∅ 4 × 40 cm) in CMWA1 (*cf.* the *Table*): 300 mg (54%) of **3**. TLC: homogeneous.

N^2, N^6-*Bis[H-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro]-Lys-NH$_2$ · 6 H$_2$CO$_3$* (**4**). A soln. of **3** (100 mg, 0.022 mmol) 95% AcOH (5 ml) was treated with ammonium formate (100 mg) and 10% Pd/C (100 mg). A N_2 stream was passed through the soln. to remove air, the reaction vessel closed, and the mixture vigorously stirred for 2 h at r.t. After filtration, the solvent was evaporated and the residue taken up in 4 ml of 90% CF_3CH_2OH/DMF 1:1 from which the product was precipitated by addition of 0.1M $NaHCO_3$ (20 ml). The product was gathered by centrifugation, washed twice with H_2O and dried in the exsiccator *in vacuo*: 75 mg (80%) of **4**.

N^2, N^6-*Bis[Boc-Ser-Tyr-Ser-Met-Glu(OBu^t)-His-Phe-Arg-Trp-Gly-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro]-Lys-NH$_2$ · 6 AcOH* (**6**). A soln. of Boc-Ser-Tyr-Ser-Met-Glu(OBu^t)-His-Phe-Arg-Trp-Gly-OH (**5**; 294 mg, 0.2 mmol) in DMF (6 ml) was obtained, after cooling to 0°, by the addition of 1N HCl (220 µl) to the initial suspension. For condensation, the soln. was treated with HOBt (30 mg)

and with the separately dissolved **4** (230 mg, 0.05 mmol) in DMF (2.3 ml) in the presence of Et_3N (14 µl). After further addition of DCCI (45 mg), the soln. was stirred for 20 h at 45°, after which **4** could no longer be detected by TLC. After removal of dicyclohexylurea by filtration and evaporation of the solvent, the mixture of products was fractionated by chromatography on silica gel (column ∅ 4 × 50 cm) in CMWA1 *(cf.* the *Table)*. The central fraction of product-containing peak (105 mg) was still slightly contaminated with **5** (*cf.* amino-acid analysis, *Table*).

N^2,N^6-*Bis[H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro]-Lys-*NH_2*·14 AcOH* (**7**). The soln. of **6** (60 mg) in 70% CF_3COOH (2 ml) was kept for 3½ h at 30°. The product was then precipitated by addition of cold peroxide-free Et_2O (15 ml) and gathered by filtration. The free peptide was then passed through a *Sephadex-G50* column (∅ 1.5 × 50 cm) in 0.2M NH_4OAc and the fractions corresponding to 4 distinct peaks *(Fig. 1)* collected and lyophilized at 45°/0.01 Torr in order to remove the NH_4OAc completely. Analysis of the 4 fractions was performed first by polyacrylamide gel electrophoresis in the presence of SDS and in comparison with markers of defined molecular weight. *Peak III* was shown to contain the product with the correct molecular weight ($M = 6500$), while *Peaks I* and *II* probably contained aggregates of **7** and *Peak IV* residual deprotected **5**. Purity of **7** was further checked by amino-acid analysis and by reversed-phase HPLC (on *Nucleosil C8 (Macherey & Nagel)* in a gradient of MeCN (0 to 35% in 60 min) in 0.1% CF_3COOH in H_2O): 20 mg of **7** from *Peak III*.

N^2,N^6-*Bis[Z-Phe-Arg-Trp-Gly-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro]-Lys-*NH_2*·6 AcOH* (**9**). A soln. of Z-Phe-Arg-Trp-Gly-OMe (600 mg, 0.8 mmol) in MeOH/dioxane 1:1 (10 ml) was treated with 0.5N NaOH (3.4 ml) at r.t. for 15 min. After neutralisation (with 0.2N HCl) to pH 7, the precipitate of **8** was isolated by filtration (526 mg, 88%). To the soln. of **8** (150 mg, 0.2 mmol) in DMF (6 ml) were added benztriazol-1-ol (30 mg), **4** (200 mg, *ca.* 0.05 mmol), Et_3N (14 µl), and DCCI (50 mg) in the given order. The mixture was stirred for 14 h at r.t., then the precipitate of dicyclohexylurea filtered off and the solvent evaporated. The residue was further purified by chromatography on silica gel (column ∅ 2.8 × 50 cm) in CMWA1 *(cf.* the *Table)*. From the pure fractions, 120 mg (46%) of **9** could be isolated.

N^2,N^6-*Bis[H-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro]-Lys-*NH_2 *· 16 AcOH* (**10**). To the soln. of 120 mg (0.023 mmol) of **9** in AcOH (6 ml), ammonium formate (120 mg) and 10% Pd/C (120 mg) were added. The mixture was stirred vigorously for 2½ h at r.t. After removal of the catalyst by filtration and of the solvent by evaporation, the residue was dissolved in 3 ml of 95% CF_3CH_2OH/DMF 1:1 and precipitated by addition of 15 ml of 0.1M $NaHCO_3$. The product was washed twice with H_2O and gathered by centrifugation. A small amount of Z-containing material could still be detected which was removed by chromatography on silica gel under the same conditions as for the purification of **9**. The 53 mg of TLC-pure material was dissolved in 70% aq. CF_3COOH and kept for 3½ h at 30°. Precipitation of the crude product by addition of Et_2O (30 ml) was followed by chromatography on *Sephadex G50* (column ∅ 1.5 × 55 cm) in 0.2M NH_4OAc. From the pure fractions, 15 mg (30%) of **10** could be recovered.

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