114. Synthesis and Biological Activity of Four Adrenocorticotropin-(1-24)-tetracosapeptide Analogues with *N*-Terminal Deletions of Four to Seven Amino-Acid Residues[1])

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Summary

The syntheses of adrenocorticotropin-(5-24)-eicosapeptide, -(6-24)-nonadecapeptide, -(7-24)-octadecapeptide, and -(8-24)-heptadecapeptide are described. Their biological activities on isolated adrenal cortex cells and adipocytes, on superfused adrenal tissue, as well as on the adenylate cyclase system of isolated cell membranes are summarized.

Analogues of corticotropin-(1-24)-tetracosapeptide **1** [2], with deletions of various lengths of peptide at the *N*-terminal end of the molecule have been used for the study of structure-activity relationships [3]. Such analogues are especially interesting because they give insight into details of the organization of information within the message sequence, Glu-His-Phe-Arg-Trp, which is believed to trigger the hormonal stimulus at the receptors of adrenal cortex cells and adipocytes (review [4]).

In order to be able to extend our studies, we have prepared the analogues **5**–**8** of **1** (**6** had been synthesized by another route [5]; **5** had been prepared in this laboratory before, but its synthesis not published):

1 5 10 15 20 24

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-OH

1 Corticotropin-(1-24)-tetracosapeptide (hexaacetate)

H-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-OH

5 Corticotropin-(5-24)-eicosapeptide (hexahydrochloride)

H-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-OH

6 Corticotropin-(6-24)-nonadecapeptide (heptahydrochloride)

H-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-OH

7 Corticotropin-(7-24)-octadecapeptide (heptahydrochloride)

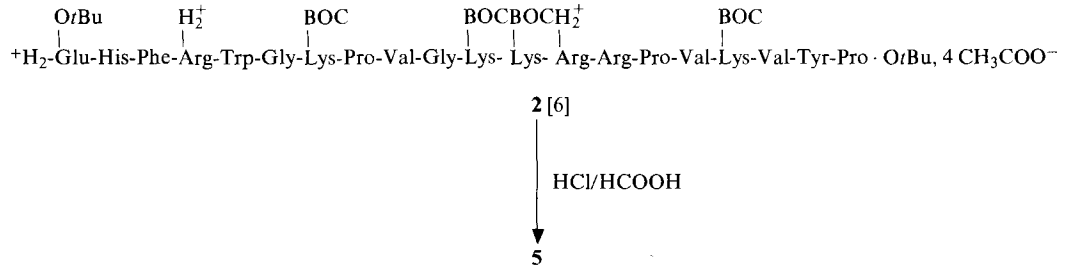
H-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-OH

8 Corticotropin-(8-24)-heptadecapeptide (heptaacetate)

[1]) Abbreviations: *cf.* [1].

The synthesis of **5** *(Scheme 1)* was simply a removal of the *t*-butoxy-carbonyl (BOC) and *t*-butyl ester groups from **2** [6] with the aid of 0.4 N HCl in formic acid (a standard procedure [1] used throughout this work and checked quantitatively by NMR.) followed by precipitation of the free peptide in a pure state as the hexahydrochloride (this is the probable number of acidic molecules bound. It was checked qualitatively, but not quantitatively, which is also true for other compounds with the exceptions explained in the Experimental Part).

Scheme 1. *Synthesis of corticotropin-(5-24)-eicosapeptide* (**5**)

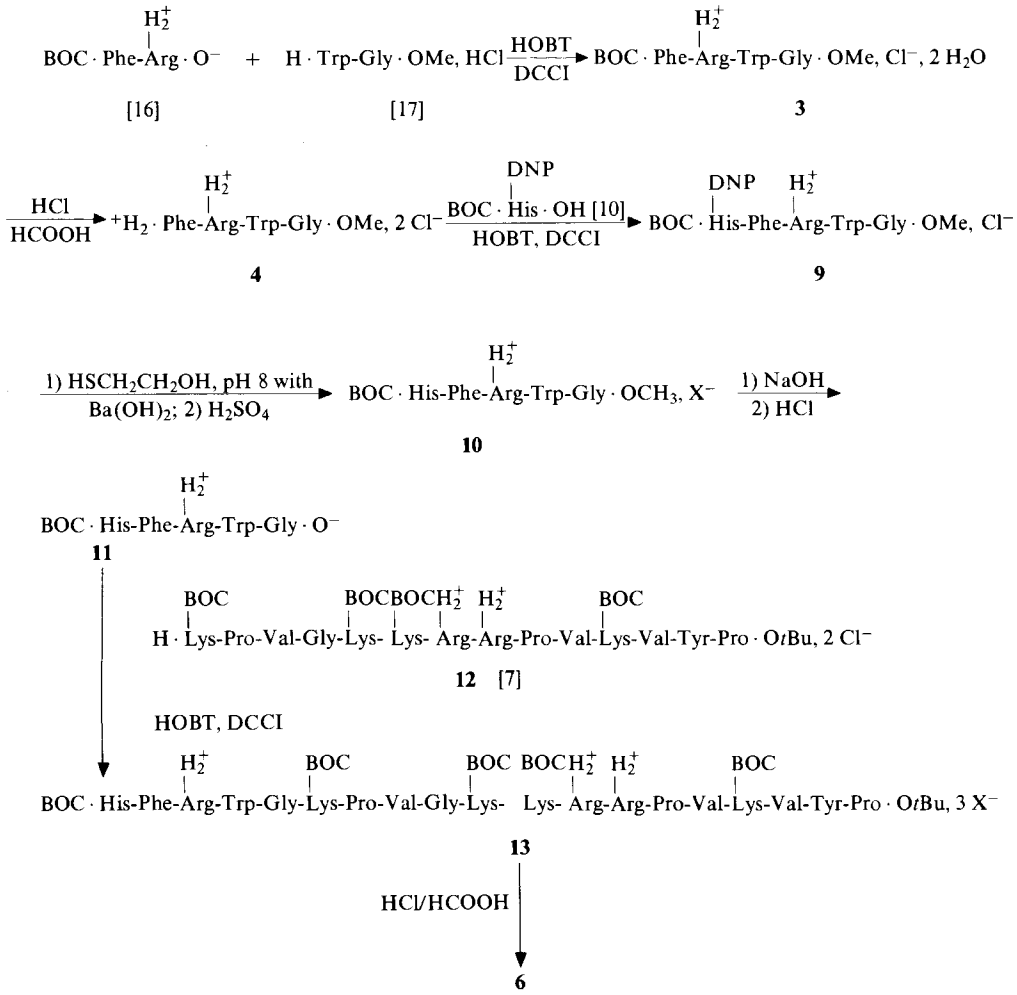


The other syntheses *(Schemes 2–4)* were carried out by preparing the suitably protected *N*-terminal peptides (**11, 14, 17**) and condensing them with the *C*-terminal tetradecapeptide dihydrochloride **12** [7]. Peptide bond formation was always performed with 1-hydroxy-benzotriazole (HOBT) and dicyclohexyl carbodiimide (DCCI) [8]. The protected large peptides obtained in this manner (**13, 15, 18**) were purified by counter-current distribution in a solvent system in which the tetradecapeptide reactant **12** had a larger distribution coefficient (*K*) than the products. After removal of the protective groups, the end product **6** was pure, **7** and **8** had to be purified by chromatography on carboxymethylcellulose (CM). The identities were checked by amino-acid analysis.

For the preparation of the *N*-terminal small peptides, either the methyl ester or the 2-tolylsulfonyl-ethyl ester [9] was used for protecting the glycine carboxyl group (the advantage of the latter is its facile removal by β-elimination). Histidine was introduced as the N^{α}-BOC-N^{im}-2,4-dinitrophenyl-(DNP) derivative [10]. The DNP group was selectively removed with 2-mercaptoethanol and barium hydroxide. Arginine was always introduced without special protection except protonation of the guanido group.

The corticotropin analogues **5**, **6**, **7**, and **8** were tested for their ability to stimulate adenylate cyclase in membrane preparations from bovine adrenal cortex cells [11] [12] and rat epididymal adipocytes [13]. This activity was compared with the effects on lipolysis in rat adipocytes [13] and (in part) to steroidogenesis and cyclic adenosine-3′,5′-monophosphate (cAMP) accumulation in isolated rat adrenal cells [3]. The results can be summarized as follows: **8** was inactive in all tests; **7**, **6**, and **5** were partial agonists for adenylate cyclase in both cell membrane preparations, but only **5** stimulated lipolysis, whereas **5** and **6** stimulated steroidogenesis. In adrenal cortex cells, **6** inhibited cAMP accumulation and **5** was a partial agonist in this respect [3].

Scheme 2. *Synthesis of corticotropin-(6-24)-nonadecapeptide* (**6**)

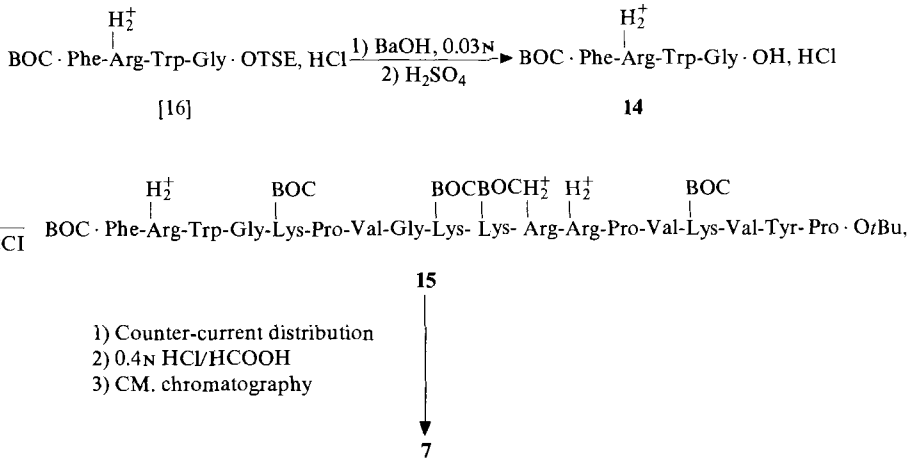


Hence, in order to be active in isolated cell and cell membrane preparations, corticotropin-(11-24)-tetradecapeptide must be complemented at the *N*-terminus with at least the following fragments of the message sequence: Glu-His-Phe-Arg-Trp-Gly- for cAMP accumulation, steroidogenesis, and lipolysis; His-Phe-Arg-Trp-Gly- for steroidogenesis without cAMP accumulation; Phe-Arg-Trp-Gly- for adenylate cyclase stimulation [14].

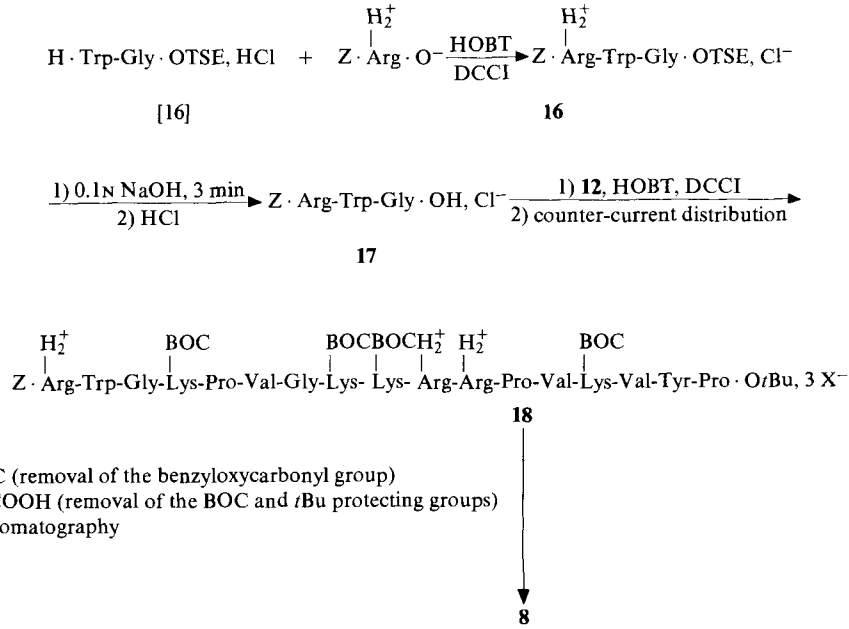
The compounds were also investigated by Prof. *Murray Saffran*, Dept. of Biochemistry, Medical College of Ohio, Toledo, Ohio, in his classical *in vitro* test for steroidogenesis using superfused rat adrenal tissue [15]. Only **5** proved to be able to promote steroidogenesis; its potency was found to be at least 1000 times lower than that of **1** (the potentiator sequence Ser-Tyr-Ser-Met- is missing). **6**, **7**, and **8** had no effect except an occasionally observed inhibitory one.

There is a discrepancy between the action of **6** in this test and in isolated adrenal cells. The difference could be due to the large intrinsic error of the superfusion assay (that would make the detection of a partial agonist almost impossible) or even to proteolytic degradation of the peptide by amino-peptidases [11]. However, the issue is still open.

Scheme 3. *Synthesis of corticotropin-(7-24)-octadecapeptide* (**7**)



Scheme 4. *Synthesis of corticotropin-(8-24)-heptadecapeptide* (**8**)



Experimental Part

General remarks. M.p. were determined in open capillaries and are uncorrected. *Counter-current distributions* were carried out in an apparatus of *Quickfit & Quartz Ltd.*, Stone, England. The solvent system was the same as used in the preparation of protected corticotropin-(1-24)-tetracosapeptide [2]. *Column chromatography* was performed with columns (2×90 cm) of carboxymethylcellulose (CM, 0.76 meq./g, *Serva*, Heidelberg). The products were eluted with an ammonium acetate gradient between 0.1 and 0.6M. The fractions were lyophilized and excess ammonium acetate removed by sublimation at 45° in a high vacuum. The *amino-acid analyses* were performed on a *Beckman* 121 C Analyzer equipped with an automatic *Autolab* I integrator, after hydrolysis of the peptides in 6N HCl at 110° for 24 h according to *Moore & Stein* [18]. For *abbreviations* of solvents and reagents, as well as for further methods (including thin-layer chromatography, TLC.) *cf.* [16]. In *TLC.*, the fully deprotected final products were found to migrate best on cellulose in the system BPAW (1-butanol/pyridine/formic acid/water 40:24:6:30). The spots were made visible by the new modification [19] of the well-known *Reindel-Hoppe* chlorine/tolidine reaction.

1. Synthesis of corticotropin-(5-24)-eicosapeptide (**5**, *Scheme 1*). – *H-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-OH, hexahydrochloride* (**5**). 67 mg of **2** [TLC.: (S) Rf 0.45 (BEW 1), 0.65 (BPEW 2)] was treated with HCl in formic acid (0.4N, 1 ml) for 15 min at RT. The product (**5**) was precipitated with much dry ether, isolated by centrifugation, and washed thrice with ether: 52 mg, pure aspect on TLC.-Rf (S): 0.13 (BPEW 2), (C) 0.55 (BPAW). $[\alpha]_D^{25} = -89.4°$ ($c = 0.5$, 0.1M acetic acid). Amino acid analysis: Arg 2.96 (3), Lys 4.01 (4), His 1.0 (1) (reference), Phe 0.97 (1), Tyr 0.96 (1), Val 2.98 (3), Glu 1.03 (1), Gly 2.09 (2), Pro 3.20 (3), Trp 0.4 (1).

2. Synthesis of corticotropin-(6-24)-nonadecapeptide (**6**, *Scheme 2*). – *BOC · Phe-Arg-Trp-Gly · OMe, HCl, $2H_2O$* (**3**). H · Trp-Gly-OMe · HCl [17] (462 mg, 1.5 mmol), BOC · Phe-Arg [16] (605 mg, 1.5 mmol), and HOBT (227 mg, 1.65 mmol) were dissolved in DMF (15 ml). DCCI (340 mg, 1.65 mmol) was then added and the reaction mixture kept 1 h at 0° and 18 h at RT. Dicyclohexylurea was removed by filtration after addition of a few drops of acetic acid and cooling to 4°. The solvent was evaporated and the residue subjected to the usual isolation procedure (ethyl acetate). The compound was purified twice by dissolving in 2-propanol and precipitation with diisopropyl ether: 680 mg (66%). TLC.-Rf (S) 0.50 (BEW 1), 0.62 (BPEW 2). $[\alpha]_D^{25} = -18.7°$ ($c = 1$, methanol).

$C_{34}H_{51}ClN_8O_9$ (751.28) Calc. C 54.35 H 6.84 N 14.91% Found C 54.65 H 6.45 N 14.69%

BOC · His-Phe-Arg-Trp-Gly · OH (**11**). **3** (680 mg, 0.9 mmol) was treated for 15 min at RT. with HCl in formic acid (0.4M, 4 ml). The product was precipitated with much dry ether, isolated by centrifugation, and washed thrice with dry ether: 500 mg (0.8 mmol), TLC.-Rf (S) 0.40 (BEW 1), 0.48 (BPEW 2), ninhydrin-positive. The tetrapeptide **4** so obtained was dissolved in DMF (8 ml) together with BOC · His (DNP) [10] (340 mg, 0.8 mmol). The following reagents were then added: *N*-ethylmorpholine (0.1 ml), HOBT (110 mg), and DCCI (184 mg), the latter at 0°. After 1 h at 0° and 15 h at RT., the dicyclohexylurea was filtered off at 4° and the solvent evaporated. The solid residue was isolated by the usual procedure (chloroform). An oily residue was obtained that was crystallized from methanol/ether: 424 mg (54%) of **9**. TLC.-Rf(S): 0.52 (BEW 1), 0.59 (BPEW 2); m.p. 175° decomp.; $[\alpha]_D^{25} = -11.6°$ ($c = 0.5$, methanol). For removal of the DNP protecting group, the crystalline compound was dissolved in 2-mercaptoethanol/methanol/water 1:1:1 (10 ml). The solution was adjusted to pH 8 with barium hydroxide (0.5M, 6.5 ml) and kept for 1 h at RT. After neutralization with 3.25 ml 1M H_2SO_4, the precipitate of barium sulfate was removed by filtration, the organic solvents evaporated and the residue, after dilution with water, lyophilized. This intermediate product (**10**) was pure on TLC.-Rf(S): 0.35 (BEW 1). For ester hydrolysis, the product was dissolved in methanol/dioxane, 1:1 (5 ml) and filtered from a small insoluble residue. The clear solution was then treated with NaOH (0.5N, 1.7 ml) and kept for 15 min at RT. The pH was adjusted to 7 with 0.2N HCl and the organic solvent evaporated. The concentrated solution was then diluted with water to about 10 ml and the peptide precipitated at its isoelectric point (pH ~ 4.5) with HCl (0.2N): 263 mg (75%). TLC.-Rf(S): 0.26 (BEW 1), 0.36 (BPEW 2), single spot. $[\alpha]_D^{25} = -17.6°$ ($c = 0.5$, methanol/water 1:1). Amino-acid analysis: His 0.96 (1), Phe 1.06 (1), Arg 1.04 (1), Gly 0.94 (1). A significant peak (~ 0.4) demonstrated qualitatively the presence of Trp.

H-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr · Pro-OH, heptahydrochloride (**6**). A solution of **11** (300 mg, 0.37 mmol) in DMF (6 ml) was prepared at 45° and mixed with a solution of **12** [7] (800 mg, 0.37 mmol) in the same solvent (10 ml). Condensation was effected by

adding HOBT (55 mg, 0.41 mmol) and DCCI (116 mg, 0.56 mmol). After 19 h at 45°, the mixture was cooled to 4° and filtered from a precipitate of dicyclohexylurea. The crude product (900 mg) was precipitated with ether and isolated by filtration. It was then subjected to counter-current distribution: after 300 transfers, a pure fraction (430 mg **13**) was isolated from tubes 85–105 (K = 0.45). TLC.-Rf(S): 0.37 (BEW 1), 0.75 (BPEW 2), single spots. An aliquot of the material (60 mg) was treated for 15 min at RT. with HCl in formic acid (0.4N, 0.8 ml) in order to remove the protecting groups. The polypeptide **6** was precipitated with much dry ether, collected by centrifugation and washed thrice with ether: 42 mg, pure aspect on TLC.-Rf(S): 0.32 (BPEW 2), (C) 0.57 (BPAW). $[\alpha]_D^{25} = -84.8°$ ($c = 0.5$, 0.1M acetic acid). Amino-acid analysis: Pro 2.74 (3), Gly 2.22 (2), Val 2.86 (3), Tyr 1.11 (1), Phe 0.90 (1), His 1.04 (1), Lys 4.10 (4), Arg 3.11 (3), Trp 0.4 (1).

3. Synthesis of corticotropin-(7-24)-octadecapeptide (**7**, *Scheme 3*). – *BOC · Phe-Arg-Trp-Gly · OH, HCl* (**14**). A solution of barium hydroxide (0.2M, 2.25 ml, 1.5 equiv.) was added slowly to a solution of BOC · Phe-Arg-Trp-Gly · OTse, HCl [16] (254 mg, 0.3 mmol) in 30 ml of dioxane/water 1:1, so that the pH never exceeded 12. After 30 min at room temperature, the solution was exactly neutralized with sulfuric acid (2.25 ml 0.2N) and the precipitate of barium sulfate removed by filtration. The solution was lyophilized and the residue extracted with methanol in order to get rid of some residual barium sulfate. Evaporation of the solvent and trituration of the product in dry ether afforded solid **14** which was crystallized from warm 2-propanol after the addition of HCl (0.03 ml 10N HCl, 1 equiv.): 170 mg (67%), m.p. 180–185° (decomp.). TLC.-Rf(S): 0.47 (BEW 1), 0.55 (BPEW 2). $[\alpha]_D^{25} = -21.3°$ ($c = 0.5$, methanol). Amino-acid analysis (hydrolysis with 6N HCl/thioglycolic acid, 10:1): Arg 1.0 (1), Phe 0.98 (1), Gly 1.08 (1), Trp 0.90 (1).

$C_{33}H_{45}ClN_8O_7$	Calc.	C 56.42	H 6.37	Cl 4.99	N 15.88%
(701.23)	Found	„ 56.86	„ 6.47	„ 4.49	„ 16.02%

H-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-OH, heptaacetate (**7**). **14** (50 mg, 0.075 mmol) was dissolved in 1 ml DMF by the addition of 82.5 µl of 1N HCl. This solution was added to the solution of **12** dihydrochloride (163.5 mg, 0.075 mmol) in DMF (1 ml), and the mixture treated with a solution of HOBT (11.1 mg, 0.082 mmol) and DCCI (23.2 mg, 0.1 mmol) in DMF (0.4 ml). After 20 h at 45°, the mixture was cooled to 0°, the dicyclohexylurea was filtered off and the solvent evaporated. The residue was triturated in ethyl acetate and the insoluble material (210 mg) subjected to counter-current distribution. After 125 transfers, 55 mg of the desired octadecapeptide derivative **15**, identified by TLC., was isolated from the tubes 18 to 40 (K = 0.32). TLC.-Rf(S): 0.30 (BEW 1), 0.59 (BPEW 2). The product of tubes 41 to 72 (100 mg) still contained **12** as contaminant. Amino-acid analysis of the pure fraction after hydrolysis with thioglycolic acid: Trp 0.83 (1), Lys 4.41 (4), Arg 2.62 (3), Pro 3.22 (3), Gly 1.85 (2), Val 2.78 (3), Tyr 0.93 (1), Phe 0.94 (1). The pure **15** (52 mg) was treated with HCl in formic acid (0.4N, 0.4 ml) for 20 min at RT. The product was precipitated with much dry ether, separated by centrifugation, and washed thrice with dry ether. Upon chromatography on carboxymethyl cellulose, the main product appeared at ionic strength ~0.4: 25 mg **7**, single spot on TLC.-Rf(S): 0.23 (BPEW 2), (C) 0.60 (BPAW). $[\alpha]_D^{25} = -86.9°$ ($c = 0.5$, 0.1N acetic acid). Amino-acid analysis: Lys 4.39 (4), Arg 2.72 (3), Pro 3.22 (3), Gly 1.85 (2), Val 2.82 (3), Tyr 0.97 (1), Phe 0.97 (1). A significant Trp-peak demonstrated qualitatively the presence of this amino-acid.

4. Synthesis of corticotropin-(8-24)-heptadecapeptide (**8**, *Scheme 4*). – *Z · Arg-Trp-Gly · OH, HCl* (**17**). A solution of Trp-Gly · OTSE, HCl [15] (3 g, 6.25 mmol) in DMF (20 ml) was mixed with a solution of Z · Arg and HOBT (844 mg, 6.25 mmol) in pyridine (20 ml), cooled to 0° and treated with DCCI (1.42 g, 6.9 mmol). The mixture was stirred for 1 h at 0° and 16 h at RT. The solvents were removed and the residue dissolved in DMF. Water was added and the precipitate collected by centrifugation. The pellet was extracted with chloroform, the chloroform extract evaporated to dryness, and the residue dissolved and precipitated from 2-propanol/diisopropyl ether: 2.5 g (56%) of a colourless powder, m.p. 140–141°. TLC.-Rf(S): 0.55 (BEW 1), 0.73 (EBPEW), one spot only, *Sakaguchi* – positive. $[\alpha]_D^{25} = -15.2°$ ($c = 0.5$, methanol). This product (**16**) was hydrolysed as follows: 733 mg (1 mmol) were dissolved in dioxane/methanol 1:1 (5 ml) and then treated with 0.2N NaOH (5 ml). After 3 min at RT., the mixture was neutralized with HCl. The precipitate was collected by filtration, dissolved in warm methanol, and reprecipitated with ether: 520 mg **17** (94%). TLC.-Rf(S): 0.38 (BEW 1), 0.51 (BPEW 2), single spot $[\alpha]_D^{25} = -19.7°$ ($c = 0.5$, methanol).

$C_{27}H_{34}ClN_7O_6$	Calc.	C 55.14	H 5.83	Cl 6.02	N 16.67%
(588.06)	Found	„ 55.86	„ 5.99	„ 6.13	„ 16.89%

H-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-OH, heptaacetate (**8**). A solution of **17** (276 mg, 0.5 mmol) in DFM/1N HCl (10 ml + 0.5 ml) was mixed with a solution of **12** dihydrochloride [7] (1090 mg, 0.5 mmol) in DMF (6 ml) at 45° and treated with HOBT (74 mg, 0.55 mmol) and DCCI (155 mg, 0.75 mmol). The mixture was stirred for 18 h at 45° and, after adding a few drops of acetic acid, cooled to 0°. Dicyclohexylurea was removed by filtration. The filtrate was evaporated to a small volume and added dropwise into a larger volume of dry ether. The resulting precipitate was isolated by filtration and reprecipitated from methanol/ethyl acetate: 1.2 g of a colourless powder. TLC. revealed a small amount of protected tetradecapeptide reactant **12** besides a larger amount of protected heptadecapeptide, Z · Arg-Trp-Gly-Lys(BOC)-Pro-Val-Gly-Lys(BOC)-Lys(BOC)-Arg-Arg-Pro-Val-Lys(BOC)-Val-Tyr-Pro-O*t*Bu (**18**) in an ionized state with unspecified counter-ions. This product (400 mg) was subjected to counter-current distribution. After 300 transfers, only a partial separation had been achieved. The desired heptadecapeptide derivative **18** [TLC.-Rf(S): 0.40 (BEW 1)] had a distribution coefficient $R = 0.51$ (calculated from tube numbers), whereas the tetradecapeptide educt, [Rf 0.30 (BEW 1)] had $R = 0.57$. The comparatively pure fractions were separated, the material isolated and used in the next step. This consisted in removal of the benzyloxycarbonyl group by catalytic hydrogenation of **18** (105 mg) in methanol/acetic acid (12 ml + 0.1 ml) with Pd/C catalyst (20 mg) in the usual manner (30 h, RT.). During this time, the catalyst was replaced twice with equal amounts of fresh catalyst. At the end, the solution was filtered and concentrated *in vacuo*, and then added dropwise to ether. On TLC.(S), the product **8** [Rf 0.28 (BEW 1)] migrated very close to the tetradecapeptide educt **12** [Rf 0.30]; however, no trace of the benzyloxycarbonyl derivative **18** could be detected.

The still impure **8** was treated for 20 min at RT. with 0.4N HCl in formic acid (1 ml). The solution was then diluted with much dry ether, and the resulting precipitate collected by centrifugation. The product was washed thrice with ether: yield 72.5 mg (~80–85%). An aliquot (30 mg) was chromatographed on carboxymethylcellulose. A small amount of tetradecapeptide impurity [TLC.-Rf(C): 0.64 (BPAW)] preceded the main product (**8**), which was eluted at ionic strength ~0.35. Recovery: 19 mg **8**, TLC. – single spot: Rf(C) 0.53 (BPAW), Rf(S) 0.30 (BPEW 2). $[\alpha]_D^{25} = -79.1°$ ($c = 0.5$, 0.1N acetic acid). Amino-acid analysis: Pro 3.29 (3), Gly 1.94 (2), Val 3.09 (3), Tyr 1.08 (1), Lys 3.87 (4), Arg 2.76 (3); no attempt was made to determine Trp quantitatively. The appearance of a significant Trp-peak (about 40% of the theoretical value) demonstrated its presence qualitatively.

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