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SYNTHESIS AND BIOLOGICAL ACTIVITIES OF TWO ACTH-ANALOGUES CONTAINING L-NORARGININE IN POSITION 8

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Two new ACTH-analogues, an octadecapeptide amide and a tetracosapeptide containing the lower homologue of arginine (norarginine) in position 8, have been synthesized by the generally accepted method. Special attention was paid to the synthesis of the required tetrapeptide representing the 7–10 sequence, which was obtained either by direct introduction of L-nitronorarginine or by amidination of the γ-amino function in a protected peptide containing α, γ-diaminobutyric acid.

Biological activity determination showed that the shortening of the arginine side chain in position 8 results in the formation of active ACTH-analogues.

The role of the arginine residue in the information locus of ACTH has been investigated previously by replacement of the basic amino acid by ornithine (Tesser & Rittel, 1969) and lysine (Chung & Li, 1967). Both alterations led to an almost complete loss of steroidogenic potency and melanocyte-stimulating activity; in the ornithine-containing analogue the lipolytic activity was completely lost. It was concluded from these results that the presence of a guanidino function in the side chain of the eighth amino acid is essential for biological activity.

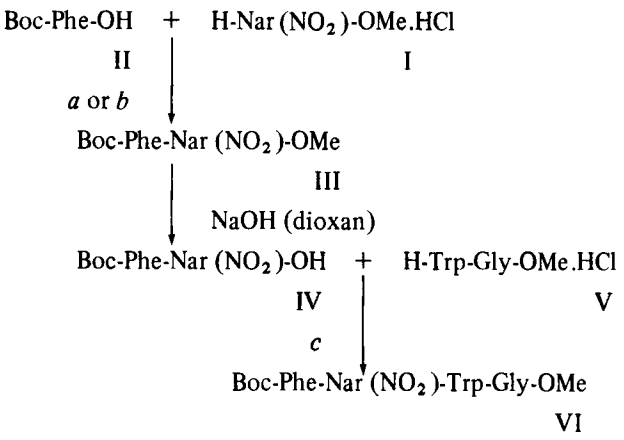
The influence of the length of the side chain of this residue was then investigated by the preparation (Tesser *et al.*, 1973*a*) and evaluation of biological activity (Tesser *et al.*, 1973*b*) of ACTH-analogues containing the higher homologue of arginine, *viz.* L-homoarginine in position 8. These peptides showed a surprisingly high biological activity: in none of the tests performed did the loss of activity due to the substitution exceed a factor 4.

In continuation of this work we have now synthesized ACTH-peptides containing the lower homologue of arginine, L-α-amino-γ-guanidinobutyric acid or norarginine*, in position 8 in order to investigate whether shortening of the arginine side chain also results in effectors which fulfil the demands for excitation of ACTH-receptors. Since norarginine has not been applied previously in peptide syntheses the special problems arising during its introduction in synthetic peptides were rather extensively investigated. (During our investigations studies on bradikinin analogues containing norarginine have been published by Arold & Gersch, 1975, 1976.)

Synthetic design

Two routes can be envisaged for the incorporation of norarginine into peptides: direct introduction of free or protected norarginine, or introduction of a diaminobutyric acid

* By analogy with the accepted abbreviation Har for L-homoarginine, the symbol Nar was chosen for L-norarginine. Other abbreviations used are those recommended by the IUPAC-IUB Commission for Biochemical Nomenclature (1973), *Biochem. J.* **131**, 1–20.



a: with P $(OC_6H_5)_3$ according to Mitin & Glinskaya (1969)
b: with DCC/HONSu (Wünsch & Drees, 1966; Weygand *et al.*, 1966)
c: with DCC/HOBt (Konig & Geiger, 1970)

Scheme 1

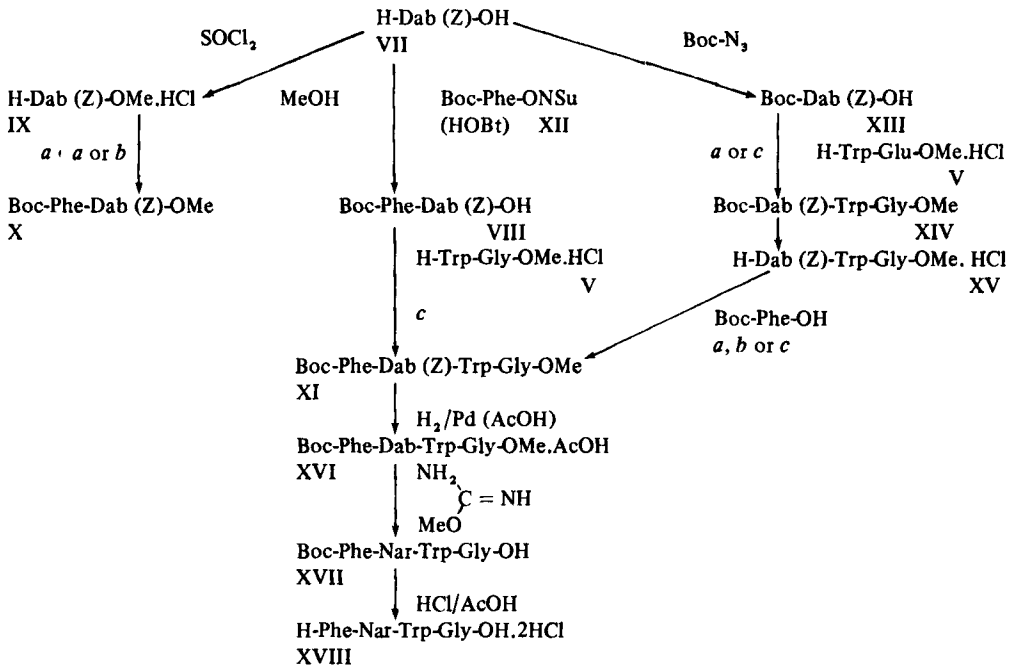
Synthesis of a protected tetrapeptide ester comprising the modified 7–10 sequence of ACTH, starting with protected nitronorarginine.

derivative with a temporarily blocked γ-amino group, which is converted in a later stage into a guanidinium function.

Both techniques have been attempted for the preparation of free or protected [Nar^8]-β-corticotrophin-(7–10)-tetrapeptide, H-Phe-Nar-Trp-Gly-OH, from which peptides with a sufficiently long amino acid chain for biological activity and otherwise unaltered composition might be obtained via conventional procedures.

Direct introduction of norarginine (Van Nispen & Tesser, 1972; Tesser & Van Nispen, 1971) was performed by fragment condensation as presented in Scheme 1. It deserves attention that both methods *a* and *b*, which were applied successfully and with similar results in the condensation of I and II, failed in the fragment condensation between IV and V; method *a* gave several products among which the lactam of IV was present; method *b* gave the desired tetrapeptide derivative (VI) only as a minor product (15%). According to the method of Konig & Geiger (1970) VI was obtained, however, in 75% yield.

N^{γ}-Benzyloxycarbonyl-α, γ-diaminobutyric acid (VII) was used as the starting compound for the preparation of a Nar-containing tetrapeptide (XVIII) by side chain guanidination in the final stage of the synthesis (Scheme 2). It appeared that several conventional condensation techniques are again less satisfactory when applied to N-acylated Dab-derivatives. Coupling of VIII, obtained from VII and from X as identical products, with V, using the methods *a*, *b* and *c* yielded three samples of XI, which analysed correctly but showed differences in their melting points (from 171 to 182°C) and optical rotation ($[\alpha]_D^{22}$ from −28.0 to −33.2° in MeOH). A fourth sample, obtained from X via the azide method melted unsharply between 183 and 188°C ($[\alpha]_D^{22}$ −29.5° (c = 1.0, MeOH). In the preparation of XI via the stepwise method, in which the α-amino group of Dab is present as a urethane function (Boc-NH-) in the condensation steps, these difficulties were not encountered. Condensation of XIII with V via two different methods (*a* and *c*) gave identical products (XIV). The final condensation step between XV and Boc-Phe-OH, performed by methods *a*, *b* and *c*, gave identical samples of XI. Their melting points (182.5–184.5°C) and optical rotation ($[\alpha]_D^{22}$ −33.3° (c = 1.0, MeOH) and −19.8° (c = 1.0, DMF) showed that in the preparation of XI by frag-



For *a*, *b* and *c* see Scheme 1.

Scheme 2

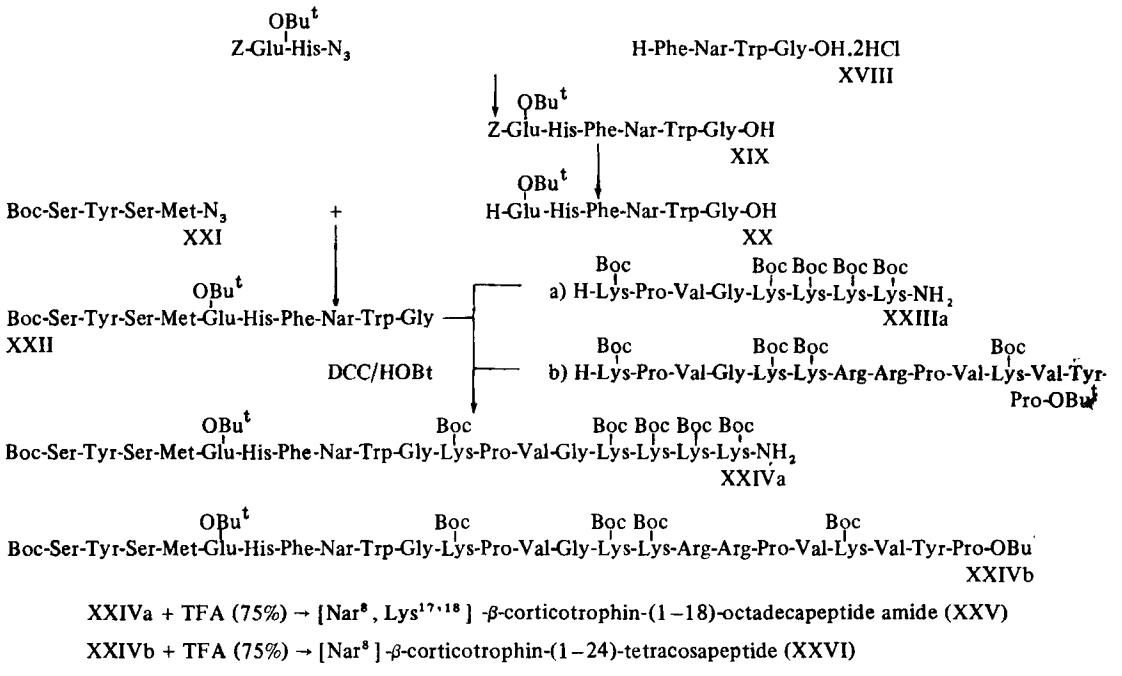
Synthesis of the modified 7–10 sequence of ACTH, starting from an α, γ-diaminobutyric acid derivative.

ment condensation only method *c* had given a pure product (m.p. 181–182°C; $[\alpha]_D^{22} -33.2$ (c = 1.0, DMF)).

For the conversion of the Dab-tetrapeptide derivative into the corresponding Nar-tetrapeptide, the side chain protecting group in XI was removed by catalytic hydrogenation in acetic acid, and the resulting product (XVI) was treated with the reagents, which are currently in use for amidination . In our case the reagent of Habeeb (1960), which has been used by Bodanszky *et al.* (1965) in a related problem, did not give satisfactory results. Introduction of the guanidino function with a large excess of neutralized O-methylisouronium sulfate as applied by Borin *et al.* (1972) and Chervenka & Wilcox (1956) also failed to give pure XVII in reasonable yield. Good results were obtained, however, when the reaction was carried out in methanol with a moderate excess (20 equivalents) of *O*-methylisourea as the free base (Stieglitz & McKee, 1900), by analogy with the guanidation of simple amino acids (Kapfhammer & Muller, 1934) and dipeptides (Greenstein, 1935).

After 20 h at 0–4°C the pure norarginine tetrapeptide XVII was obtained as the free acid and subsequent treatment with hydrochloric acid in ethyl acetate gave the unprotected tetrapeptide (XVIII).

In the further synthesis of the desired analogues, [Nar^8, Lys^{17,18}]-β-corticotrophin-(1–18)-octadecapeptide amide and [Nar^8]-β-corticotrophin-(1–24)-tetracosapeptide the use of the unprotected tetrapapeptide (XVIII) appeared to be preferable to application of the protected tetrapeptide (VI). After treatment of the latter with hydrochloric acid to unmask the terminal amino function, the resulting ester underwent clean acylation with Z-Glu (OBu^t)-His-N_3, giving the pure hexapeptide ester in reasonable yield. However, after alkaline hydrolysis of the ester group a product was obtained which stubbornly resisted catalytic



Scheme 3
Synthesis of two corticotrophic peptides substituted with norarginine in position 8.

hydrogenolysis. It appeared that purification by counter-current distribution, prior to hydrogenolysis, is necessary for complete, albeit slow, elimination of both *N*-protecting groups in this type of peptides (*cf.* Tesser *et al.*, 1973*a*). The hexapeptide (XIX, Scheme 3), obtained by the same acylation method from the free tetrapeptide (XVIII), underwent hydrogenolytic cleavage of the benzyloxycarbonyl group without difficulties. The product was elongated at its *N*-terminal end with the protected tetrapeptide azide (XXI) according to the method of Hönzl & Rudinger (1961), yielding the partially protected zwitter-ionic [Nar^8] -β-corticotrophin-(1–10)-decapeptide (XXII). The desired octadecapeptide amide and tetracosapeptide derivatives were obtained by acylation of the appropriate amino components (XXIIIa and b) with the partially protected decapeptide (XXII), according to the method of Konig & Geiger (1970). After purification by counter-current distribution, the products (XXIVa and b) were deprotected with 75% trifluoroacetic acid and finally converted into the acetate form using an ion exchange resin.

Biological activity

The effect of shortening the side chain of the residue in position 8 on the biological activity has been investigated by comparison of the steroidogenic, lipolytic and melanocyte-stimulating activities of [Nar^8, Lys^{17,18}]-β-corticotrophin-(1–18)-octadecapeptide amide (XXV) and [Nar^8]-β-corticotrophin-(1–24)-tetracosapeptide (XXVI) with those of standard compounds with equal chain length but Arg at position 8. Full details and complete results of these investigations will be published elsewhere (Van Nispen *et al.*, 1977).

In vivo steroidogenesis of XXV was comparable to that of the standard; *in vitro* experiments with XXV showed *ca.* 10% activity. In *in vivo* tests of lipolytic activity XXV showed a linear dose/response relation for doses between 30 and 300 μg/kg body weight. Its potency was *ca.* 50% of that of equal doses of the standard.

In vitro lipolysis was only 5–10%.

XXVI exhibited lower activities in both respects: Steroidegenesis was 3–10% *in vivo*, 10% *in vitro;* lipolysis was extremely low *in vivo*, but *ca.* 30% *in vitro*, in comparison with the appropriate standard.

The MSH activity of both analogues XXV and XXVI, measured on lizard skins (Burgers, 1961) was 20–30% that of β-corticotrophin-(1–24). In conclusion, it can be stated, that both analogues have a significant activity. Comparison with previous results (Tesser *et al.*, 1973*b*) shows, however, that shortening of the side chain bearing the indispensable guanidine function (position 8) is a more serious structural deviation than elongation.

EXPERIMENTAL PROCEDURES

Melting points are uncorrected. Specific rotations were measured on a Perkin-Elmer 141 or 241 apparatus, or with a Zeiss polarimeter. U.V.-spectra were recorded with a Beckman DK-2A spectrophotometer. Thin-layer chromatography was on silicagel (TS; Merck F254), on alumina (TA; Merck 254, type E) and on cellulose (TC; Merck) using the following solvent systems: A = benzene-acetone (1:1), B = butanol-acetic acid-water (10:1:3), C = chloroform-methanol (9:1), D = chloroform-methanol (1:1), E = chloroform-methanol-acetic acid (95:20:3), F = butanol-acetic acid-water (4:1:1), G = isopropyl alcohol-25% ammonia-water (8:1:1), H = propanol-25% ammonia (3:2), I = chloroform-methanol-17% ammonia (5:5:1), J = ethyl acetate-pyridine-acetic acid-water (62:21:6:11), K = butanol-pyridine-acetic acid-water (38:24:8:30), L = butanol-pyridine-formic acid-water (40:24:6:30).

Boc-Phe-Nar-(NO_2)-OMe (III). 5 mmol of Boc-Phe-OH, 5 mmol of H-Nar-(NO_2)-OMe. HCl (I), 5 mmol of *N*-ethylmorpholine, 7.5 mmol of imidazole and 7.5 mmol of triphenyl phosphite were dissolved in that order, in approximately 15 ml of dimethylformamide. The solution was agitated by rotation of the vessel and the temperature was maintained at 40°. After 16 h the solvent was evaporated, the residue was dissolved in ethyl acetate and the solution was extracted with 5% sodium hydrogen carbonate solution, water, 5% citric acid solution, water and saturated sodium chloride solution. The organic layer was dried (sodium sulfate), filtered and evaporated. The residual oil crystallized upon rubbing it under ether. Yield 71%, m.p. 122–124°; TS: R_f = 0.21 (A), = 0.70 (B), = 0.45 (C); $[\alpha]_D^{25} = -4.8°$ (c = 1.0, EtOAc), $= -9.5°$ (c = 1.0, MeOH); ϵ = 15,920 (λ_{max} 269.0 nm in DMF/0.2 N HCl, 1:1 v/v), = 1,540 (λ_{max} 261.5 nm in TFA). Anal. calc. for $C_{20}H_{30}N_6O_7$ (466.49): C, 51.49; H, 6.48; N, 18.02. Found: C, 51.3; H, 6.5; N, 18.0.

Acylation of I with Boc-Phe-ONSu gave the same product (III) in 69% yield.

Boc - Phe -Nar (NO_2) -OH (IV). A solution of III in dioxan was stirred for 0.5 h with at least 2 equivalents of sodium hydroxide solution (final water concentration 25%). The solvents were evaporated, the residue was dissolved in ethyl acetate and the solution extracted with 5% citric acid solution. The upper layer was washed with water and saturated sodium chloride solution, dried and evaporated. Yield 92%; m.p. 109–113° (dec); TS: R_f = 0.50 (B); $[\alpha]_D^{25} = -3.2°$ (c = 1.0, MeOH); ϵ = 13,920 (λ_{max} 268.5 nm in DMF/0.2 N HCl, 1:1 v/v), = 1,790 (λ_{max} 260.5 nm in TFA); nitroarginine content (Gamper, 1962): 40% (calc. 45.1%). Anal. calc. $C_{19}H_{28}N_6O_7$ (452.47): C, 50.44; H, 6.24; N, 18.57. Found: C, 50.1: H, 6.2; N, 18.2.

Boc - Phe - Nar (NO_2) - Trp-Gly-OMe (VI). To a solution of 4.28 mmol of IV in 15 ml of dimethylformamide, 4.28 mmol of H-Trp-Gly-OMe. HCl, 0.54 ml of *N*-ethylmorpholine, 6.50 mmol of HOBt were added with stirring and finally at 0° 4.71 mmol of dicyclohexylcarbodiimide. After 2 h at 0° and 2 h at room temperature the reaction mixture was filtered and the filtrate was evaporated. The residue was dissolved in ethyl acetate and worked up as described for III. Recrystallization from methanol/ether gave pure VI. Yield 70% m.p. 120–122°; TS: R_f = 0.74 (B), = 0.30 (C); $[\alpha]_D^{28} = -20.5°$ (c = 1.0, MeOH); ϵ = 22,310 (λ_{max} 270.0 nm in DMF/0.2 N HCl, 1:1 v/v), = 7,370 (λ_{max} 263.5 in TFA); nitronorarginine content: 30% (calc. 28.9%). Anal. calc. for $C_{33}H_{43}N_9O_9.H_2O$ (727.78): C, 54.46; H, 6.23; N, 17.32. Found: C, 54.6; H, 6.2; N, 17.4.

H-Dab (Z)-OH (VII). A solution of 50 mmol of H-Dab-OH.HCl and 25 mmol of copper sulfate pentahydrate in 40 ml of water was prepared and its pH was adjusted to 10.5 in a pH-stat, loaded with 4 N sodium hydroxide. At constant pH about 60 mmol of benzyloxycarbonyl chloride were added slowly, with stirring. After 5 h the blue copper complex of VII (67%) was filtered and washed with water, acetone and ether. A suspension of the complex in water was treated with 3 equivalents of ethylene diamine tetra acetate (EDTA) and HCl to pH 2.5 (Eckstein *et al.*, 1973). Addition of a large amount of water gave a clear solution which was neutralized with NaOH solution. Concentration of the solution to approximately 10% of its original volume gave a precipitate of VII. The crude product was recrystallized from ethanol-water (1:1) containing a small amount of EDTA. Yield 71%; m.p. 216–218°C; $[\alpha]_D^{27} = 21.0°$ (c = 0.4, 2N HCl). Anal. calc. for $C_{12}H_{16}N_2O_4$ (252.27): C, 57.13; H, 6.39; N, 11.10. Found: C, 57.2; H, 6.3; N, 11.3.

H-Dab (Z)-OMe.HCl (IX). Esterification of VII by the method of Vogler & Lanz (1960) yielded IX, but the product contained a small amount of H-Dab-OMe.2HCl. The by-product was removed by filtration of the crude ester through silicagel with $CHCl_3$-MeOH (9:1) as the eluent. The filtrate was concentrated, the residue dissolved in methanol and the solution was diluted with ether. Yield 73%; m.p. 157–158°C; $[\alpha]_D^{22} = +15.5°$ (c = 1.0, MeOH); TS: $R_f = 0.40$ (B), = 0.33 (C), = 0.55 (D). Anal. calc. for $C_{13}H_{18}N_2O_4$.HCl (302.76): C, 51.57; H, 6.33; N, 9.25. Found: C, 51.4; H, 6.2; N, 9.2;

Boc-Phe-Dab (Z)-OMe (X). Equimolecular amounts of IX, Boc-Phe-ONSu and triethylamine were dissolved in dimethylformamide (25 ml for 6.37 mmol of each reactant). After 3 h water was added and the precipitated oil was extracted into ethyl acetate. The organic layer was washed with an aqueous solution of 3-dimethylaminopropyl amine, water, citric acid solution and water, dried (sodium sulfate) concentrated and diluted with petroleum ether. On cooling X crystallized in 81% yield. M.p. 117.5–118.5°C; $[\alpha]_D^{27} = -21.7°$ (c = 1.0, MeOH); TS: $R_f = 0.60$ (A), = 0.74 (B). Anal. calc. for $C_{27}H_{35}N_3O_7$ (513.39): C, 63.14; H, 6.87; N, 8.18. Found: C, 63.0; H, 7.0; N, 8.1. The product was also obtained using the phosphite method (*cf.* preparation of III).

Boc-Phe-Dab-(Z)-OH (VIII). Aqueous sodium hydroxide (12.1 ml, 1M) was added to a suspension of 6 mmol of X in 37 ml of dioxan-water (2:1) with stirring. After 20 min the solution was acidified with citric acid and extracted with ethyl acetate. The product (VIII) was precipitated by addition of petroleum ether. Yield 90%; m.p. 111.5–112.5°; $[\alpha]_D^{24} = -24.9°$ (c = 1.0, EtOAc); TS: $R_f = 0.67$ (B). Anal. calc. for $C_{26}H_{33}N_3O_7$ (499.56): C, 62.51; H, 6.66; N, 8.41. Found: C, 62.5; H, 6.8; N, 8.5.

Boc-Dab(Z)-OH(XIII). 5 mmol of N^{γ}-benzyloxycarbonyl-α, γ-diaminobutyric acid (VII), suspended in 40 ml dioxan-water (1:1), were acylated with butyloxycarbonyl azide, at pH = 10.5, during 24 h, according to the general method of Schnabel (1967). The product, a colourless oil, was obtained in 80% yield. $[\alpha]_D^{23}$ = −14.7° (c = 2.0, MeOH), = −20.5° (c = 1.0, DMF), = −12.5° (c = 1.0, AcOH); TS: $R_f = 0.67$ (B).

Addition of 5 mmol of dicyclohexylamine to a solution of 4 mmol of the oily product in 10 ml of ether gave upon cooling the crystalline DCA salt. Yield 88% m.p. 99–102°C; $[\alpha]_D^{23}$ = 0° (c = 1.0, MeOH or DMF), = −8.3° (c = 1.0, AcOH); TS: $R_f = 0.68$ and 0.60 (dec) (B). Anal. calc. for $C_{29}H_{47}N_3O_6$ (533.71): C, 65.26 H, 8.88; N, 7.88. Found: C, 65.4; H, 9.2; N, 7.7.

Boc-Dab (Z)-Trp-Gly-OMe (XIV). This product was obtained from XIII and V by application of Konig & Geiger's method (*cf.* preparation of VI). The product crystallized from methanol/ether. Yield 74%; m.p. 152–153°C; $[\alpha]_D^{21} = -28.8°$ (c = 0.9, MeOH); TS: $R_f = 0.44$ (A), = 0.76 (B). Anal. calc. for $C_{31}H_{39}N_5O_8$ (609.68): C, 61.07; H, 6.45; N, 11.49. Found: C, 60.7; H, 6.6; N, 11.4.

A product having identical physical constants was obtained by application of Mitin's method (Mitin & Glinskaya, 1969); *cf.* the preparation of III.

H-Dab (Z) - Trp - Gly - OMe.HCl (XV). The preceding ester (XIV) was treated with 20 equivalents of hydrochloric acid and the progress of the elimination of the Boc-group was followed by t.l.c. (A). As soon as XIV had disappeared, N_2 was bubbled through the solution (5 min). The solution was then concentrated *in vacuo* at room temperature, cooled and XV was precipitated by addition of dry ether. Yield 90%; m.p. 142–146°C (dec. at about 180°C); $[\alpha]_D^{26} = +9.7°$ (c = 1.0, MeOH), = 3.6° (c = 1.0, DMF): TS: $R_f = 0.50$ (B). Anal. calc. for $C_{26}H_{32}N_5O_6Cl$ (546.02): C, 57.19; H, 5.91; N, 12.83. Found: C, 56.7; H, 5.9; N, 12.8.

Boc-Phe-Dab (Z)-Trp-Gly-OMe (XI)

By stepwise condensation. A solution of XV (4.77 mmol), containing some residual hydrochloric acid, in DMF (20 ml) was adjusted to pH 4.5 with N-ethylmorpholine. An additional equivalent of the base and 4.77 mmol of Boc-Phe-ONSu were then added, and the solution was kept at room temperature for 5 h. Water was added with cooling and the precipitate was filtered, washed with water, dissolved in methanol, and reprecipitated by addition of ether. Yield 75%; m.p. 179–181°C; $[\alpha]_D^{22} = -33.2°$ (c = 1.0, MeOH), = −18.5° (c = 1.0, DMF). Anal. calc. for $C_{40}H_{48}N_6O_9$ (756.86): C, 63.48; H, 6.39; N, 11.10. Found: C, 63.2; H, 6.4; N, 11.0; Identical samples were obtained via the condensation methods used in the preparation of III and VI.

By fragment condensation. V and VIII were coupled by the method described for the preparation of VI. A slight contamination of dicyclohexylurea was eliminated by treatment of the product with methanol-ether. Yield 75%; m.p. 181–182°C; $[\alpha]_D^{25} = -33.2°$ (c = 1.0, MeOH). Anal. found: C, 63.1; H, 6.6; N, 11.1.

Boc - Phe - Dab - Trp-Gly-OMe.AcOH (XVI). Hydrogenation of XI in 90% acetic acid over 10% palladium on carbon was complete in about 30 min. The catalyst was removed by filtration through hyflo, and the filtrate was evaporated. The residue was freed from acetic acid by re-evaporation with isopropyl alcohol, and dissolved in the minimum of isopropyl alcohol. Addition of diisopropyl ether precipitated XVI in 90% yield. TS: $R_f = 0.44$ (B), = 0.17 (E), = 0.55 (F). The product was used without further purification.

Boc-Phe-Nar-Trp-Gly-OH (XVII). 1.40 mmol of XVI were added to 7.5 mol of a freshly prepared methanolic solution of *O*-methylisourea (about 4 mmol/ml), freshly prepared from the hydrogen sulfate (Steiglitz & McKee, 1900). The solution was left for 24 h at 0°, concentrated and cooled. Water was added slowly (about 10 times the volume of the solution) and the resulting precipitate was isolated by centrifugation, washed with water and dried. It was purified by chromatography on Kieselgel 60 (Merck) using isopropyl alcohol-ammonia-water (8:1:1) as the eluent. Fractions containing the Sakaguchi positive product (XVII) were evaporated. The residue was dissolved in methanol, and the turbid solution filtered through hyflo. Concentration of the solution and addition of ethyl acetate precipitated pure XVII. Yield 40%; m.p. 185°C (dec); $[\alpha]_D^{21} = -17.6°$ (c = 0.6, MeOH); TS: $R_f = 0.52$ (F), = 0.32 (G), = 0.50 (H). Amino acid analysis (after hydrolysis in 6 N HCl during 24 h at 105°): Trp 0.77 (1), Nar 1.04 (1), Gly 1.09 (1), Phe 1.00 (1). Anal. calc. for $C_{32}H_{42}N_8O_7 . 2H_2O$ (686.77): C, 55.96; H, 6.75; N, 16.32. Found: C, 56.3; H, 6.8; N, 16.0.

H - Phe - Nar - Trp - Gly - OH.2HCl (XVIII). The above compound was treated with a solution of hydrochloric acid in acetic acid (1.6 N, 20 equivalents). Ether was added after 10 min and the resulting precipitate was filtered and washed thoroughly with ether. Yield 92%; m.p. 187–189°C; $[\alpha]_D^{21} = +5.4°$C (c = 1.23, 90% AcOH); TS: $R_f = 0.24$ (F), = 0.48 (H). The peptide hydrochloride was dried over P_2O_5 at 80° and then equilibrated with air moisture. U.V. spectrum: A = 0.621 at λ_{max} = 280.0 nm (conc: 3.92 mg in 50 ml 0.1 N HCl). Peptide content: 78% (Beaven & Holiday, 1952). Anal. calc. for $C_{27}H_{36}N_8O_5Cl_2 . 4H_2O$ (695.60): C, 46.62; H, 6.38; N, 16.11; Cl, 10.19. Found: C, 46.5; H, 6.3; N, 15.7; Cl, 9.9.

Z-Glu (OBu^t) - His - Phe-Nar-Trp-Gly-OH (XIX). A solution of 0.7 mmol of Z-Glu (OBu^t)-His-N_2H_3 (Schwyzer & Kappeler, 1961) in 10 ml of

dimethylformamide was treated at −15°C with 4 equivalents of dry hydrochloric acid in ethyl acetate. The hydrazide was then allowed to react for 20 min, also at −15°C, with 0.15 ml of *tert.* butyl nitrite. The solution was carefully neutralized with *N*-ethylmorpholine and mixed with 8 ml of a solution containing XVIII (0.46 mmol) in dimethylformamide to which 2 equivalents of *N*-ethylmorpholine were previously added. The mixture was stirred for 40 h at 0°, concentrated and then cooled. Addition of water yielded a precipitate, which was recrystallized from acetic acid/ether. Yield 58%; m.p. 208–210°C (dec); TS: $R_f = 0.41$ (F), = 0.53 (H), = 0.64 (I); $[\alpha]_D^{21} = -17.0°$ (c = 1.0, 90% AcOH). Anal. calc. for $C_{50}H_{62}N_{12}O_{11}$. AcOH.H_2O (1085.19): C, 57.55; H, 6.32; N, 15.49. Found: C, 56.5; H, 6.1; N, 15.5.

H-Glu(OBu^t)-His-Phe-Nar-Trp-Gly-OH (XX). For removal of the *N*-protecting function, compound XIX was dissolved in 90% acetic acid. Palladium on carbon (10%) was added and hydrogen was bubbled through the solution for 3 h. Then the catalyst was removed and replaced by a fresh quantity whereupon the hydrogenation was resumed for a further 3 h. The suspension was then filtered again and the filtrate, after concentration, was diluted with ether. Yield 85%; $R_f = 0.10$ (F), = 0.59 (I). The compound, which evidently contained a minor by-product with lower chromatrographic mobility, was used without purification in the next step.

Boc-Ser-Tyr-Ser-Met-Glu (OBu^t)-His-Phe-Nar-Trp-Gly-OH (XXII). A solution of 0.5 mmol of Boc-Ser-Tyr-Ser-Met-N_2H_3 (Iselin & Schwyzer, 1961) in 5 ml of dimethylformamide was treated with 0.75 ml of 2 M hydrochloric acid in dry ethyl acetate, maintaining the temperature at −15°C. The hydrazide was converted into the azide by addition of 0.60 mmol of *tert.* butyl nitrite during 15 min at the same temperature. The mixture was then neutralized and added to a solution of XXI (0.3 mmol) in 40 ml of dimethylformamide. After 20 h at 0° the solvent was evaporated *in vacuo*, and the concentrate was treated with acetonitrile. The precipitate that formed was filtered, washed with acetonitrile and dried. It was purified by dissolution in dimethylformamide followed by precipitation with acetonitrile. Yield 79%; m.p. 183–187°C; $[\alpha]_D^{21} = -16.1°$ (c = 1.0, DMF), $= -19.0°$ (c = 0.7, 90% AcOH); TS: $R_f =$ 0.10 (J); = 0.26 (B), = 0.37 (F), = 0.60 (K).

Boc-Ser-Tyr-Ser-Met-Glu (OBu^t)-His-Phe-Nar-Trp-Gly-Lys (Boc)-Pro-Val-Gly-Lys-(Boc)-Lys (Boc)-Lys (Boc)-Lys (Boc)-NH$_2$ (XXIVa). The acylation of XXIIIa with XXII was performed according to Riniker & Rittel (1970), but *N*-hydroxybenzotriazole was used, rather than *N*-hydroxysuccinimide. The crude product, as obtained by precipitation with ether was subjected to counter-current distribution in the system methanol-chloroform-carbon tetrachloride-buffer (10:7:4:3; buffer composition: 7.7 g of ammonium acetate and 57.1 ml of acetic acid made up to 1000 ml with water). After 160 transfers, 135 mg (48%) of a pure product were obtained (K = 0.86, $r_{max} = 74$). M.p. 195–200°C (dec); $[\alpha]_D^{21} = -29.7°$ (c = 0.43, 90% AcOH); TS: $R_f = 0.48$ (J), = 0.61 (F), = 0.76 (K); U.V. spectrum: A = 0.393 at $\lambda_{max} = 278.2$ nm (90% AcOH, conc. 0.217 mg/ml).

[Nar^8, Lys^{17,18}]-β-corticotrophin-(1–18)-octapeptide amide (XXV). Deprotection of XXIVa was performed by treatment with 75% trifluoroacetate was converted into the acetate solution was then cooled to −30°, and peroxide-free ether was added dropwise with stirring. After 10 min the precipitate was filtered, washed with ether and dried. The trifluoroacetate was converted into the acetate using a weakly basic ion exchange resin (Merck, II, acetate form). Thin-layer chromatography showed that the filtrate contained some unreacted octapeptide amide. This impurity could be separated by preparative high-voltage paper electrophoresis. The pure octadecapeptide amide was isolated by elution of the pertinent zone and lyophilization. $[\alpha]_D^{21} = -56.4°$ (c = 0.50, 1% AcOH), $[\alpha]_{578} = -58.8°$, $[\alpha]_{546} = -67.0°$, $[\alpha]_{436} = -116.4°$; TA: $R_f = 0.25$ (K); TC: $R_f = 0.44$ (K); U.V. spectra (0.1 N HCl, conc. 0.291 mg/ml): A = 0.627 at λ_{max} 278.8 nm; in 0.1 N NaOH (conc. 0.227 mg/ml): A = 0.526 at λ_{max} 281.5 nm and 0.516 at λ_{max} 288.2 nm; Tyr/Trp ratio (Bencze & Schmid,

1957): 0.9. Amino acid analysis after complete hydrolysis (6 N HCl, 24 h 110°C): Lys 4.4 (5), His 1.06 (1), NH_3 1.55 (1), Nar 0.97 (1), Ser 1.46 (2), Glu 0.95 (1), Pro 1.03 (1), Gly 2.00 (2), Val 1.02 (1), Met 0.97 (1), Tyr 0.90 (1), Phe 0.94 (1).

Boc - Ser - Tyr - Ser - Met - Glu (OBu^t) - His - Phe - Nar - Trp - Gly - Lys (Boc) - Pro - Val - Gly - Lys (Boc) - Lys (Boc) - Arg - Arg - Pro - Val - Lys (Boc) - Val - Tyr - Pro - OBu^t (XXIVb). Using the reaction conditions applied in the condensation of XXII with XXIIIa, the same carboxyl-component was condensed with XXIIIb (Kappeler & Schwyzer, 1961). Crude XXIVb, obtained by precipitation with ether after a reaction period of 20 h, was purified by counter-current distribution, using the system methanol-chloroform-carbon tetrachloride-buffer (8:5:2:4, buffer composition 19.25 g of ammonium acetate and 28.5 ml of acetic acid made up to 1 l with water). After 243 transfers 260 mg (45%) of pure XXIVb were obtained ($K = 0.47$ $r_{max} = 78$); m.p. 175–180° (dec); $[\alpha]_D^{21} = -52.7°$ (c = 0.60, MeOH); TS: $R_f = 0.16$ (J), = 0.29 (B), = 0.43 (F), = 0.74 (K); U.V. spectrum: (90% AcOH, conc. 0.18 mg/ml): A = 0.385 at λ_{max} 277.6 nm.

[Nar^8] - β - corticotrophin - (1 – 24) - tetracosapeptide (XXVI). Deprotection with 75% trifluoroacetic acid yielded a homogeneous product. $[\alpha]_D^{21} = -85.0°$ (c = 0.60, 1% AcOH), $[\alpha]_{578} = -88.7°$, $[\alpha]_{546} = -101.2°$, $[\alpha]_{436} = -176.2°$, $[\alpha]_{365} = -295°$; TA: R_f = 0.60 (K); TC: $R_f = 0.49$ (K), = 0.60 (L); U.V. spectra (0.1 N HCl, conc. 0.307 mg/ml): A = 0.661 at λ_{max} 275.9 nm, (0.1 N NaOH, conc. 0.245 mg/ml): A = 0.716 at λ_{max} 288.5 nm and 0.691 at λ 282.0 nm; Tyr/Trp ratio (Bencze & Schmid, 1957): 2.0. Peptide content (Gamper, 1962): 72.5%. Amino acid analysis after complete hydrolysis (6 N HCl, 24 h at 110°): Trp 0.64 (1), Lys 3.67 (4), His 0.99 (1), NH_3 0.55 (none), Nar + Arg 3.03 (3), Ser 1.56 (2), Glu 1.00 (1), Pro 2.94 (3), Gly 2.00 (2), Val 3.20 (3), Met 1.18 (1), Tyr 2.02 (2), Phe 1.01 (1).

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