

# PATENT SPECIFICATION

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## COMPLETE SPECIFICATION

### Peptides having an Adrenocorticotrophic Hormone Action and Process for their Manufacture

We, CIBA LIMITED, a body corporate organised according to the laws of Switzerland, of Basle, Switzerland, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

It has been known that the peptide chain of  $\beta$ -corticotropin can be substantially modified, especially at the carboxyl end, without losing any of the adrenocorticotrophic action. Thus, for example, the activity referred to 1 mg of peptide (about 100 international units per mg) remains unaffected when the aminoacids up to the 20th aminoacid are split off from the carboxyl end of natural ACTH. Further splitting off causes an increasing drop in activity. Thus,  $\beta^{1-19}$ -corticotropin still possesses about 30% of the ACTH activity per mg and  $\beta^{1-18}$ -corticotropin displays about 1%/mg. At the amino end the first aminoacid, serine<sup>1</sup>, can be replaced by glycine without impairing the activity (Dixon, Biochem. J. 84, page 462 [1962]). Other, relatively slight modifications reduce the activity. Taking  $\beta^{1-23}$ -corticotropin-23-amide (which contains about 100 international units per mg) as reference substance, it is found that the removal of the first aminoacid, serine<sup>1</sup>, reduces the activity by 50%. Replacement of the third aminoacid, serine<sup>3</sup>, by alanine likewise reduces the activity by 50%, whereas replacement of the second aminoacid, tyrosine, by phenylalanine, reduces the activity to 2/3 (cf. Z. Naturf. 19b, pages 858—860 [1964]). To date it has not been possible ever to raise the activity, referred to 1 mg of peptide,

over that of natural ACTH by modifying the sequence of ACTH.

In a fragment of ACTH that had MSH-activity but no ACTH-activity, namely in the pentapeptide of the aminoacid sequence 6—10 of corticotropin —H-His-Phe-Arg-Try-Gly-OH— the natural aminoacids L-histidine, L-phenylalanine, L-arginine and L-tryptophan have been replaced by corresponding D-aminoacids: the pentapeptide obtained in this manner did not display any MSH-activity (cf. Hano et al., Biochem. Biophys. Acta 90, page 201 [1964]).

Surprisingly, it has now been observed that peptides which differ from ACTH-active peptides having at least towards the N-terminus a complete ACTH-sequence in which however some aminoacids may be replaced by other natural  $\alpha$ -aminoacids provided that the ACTH-activity remains substantially unaffected, solely in that they contain as the first aminoacid an aminoacid with D-configuration, are more active than the corresponding peptides that contain no D-aminoacid in the N-terminal 1-position. Moreover, they have a long-lasting action.

It is another advantage of these new peptides that they are cheaper to synthesize than the peptides in which L-serine is the first aminoacid.

Accordingly, the present invention provides a peptide that differs from an ACTH-active peptide containing 16 to 39 aminoacid residues and having an N-terminal aminoacid residue with L-configuration, solely in that the N-terminal aminoacid residue has D-configuration, as well as acid addition salts, derivatives

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and complexes, especially metal complexes such as zinc complexes, of these peptides.

By ACTH-active peptides, there are to be understood herein, besides the adrenocorticotrophic hormones (ACTH) themselves, which are peptides containing 39 aminoacid residues, those peptides which, at least to some degree, have adrenocorticotrophic activity and which have a sequence of 16 to 39 aminoacids beginning from the N-terminus of natural ACTH, said sequence being either identical with that of ACTH or being modified by replacement of one or more of the aminoacid residues thereof by the residues of other natural  $\alpha$ -aminoacids or of their homologues.

For testing adrenocorticotrophic activity there are several methods available, for example the *in vitro* method of Saffran and Schally [Endocrinology 56, 523 (1955)] modified by Schuler et al., Schweiz. Mediz. Wochenschrift 93, 1027 (1963); in this method the formation of corticosteroids in isolated rat adrenals under the influence of ACTH-active substances is measured. In the Sayers test [Endocrinology 42, 379 (1948)] the ascorbic acid depletion of the adrenal cortex of hypophysectomized rats under the influence of ACTH-active substances is measured. In another method [Barthe et al., Nature 202, 908 (1964)] the increase in the plasma cortisol level under the influence of ACTH-active substances is determined.

The ACTH-active peptides, from which the new peptides differ by the above-mentioned changed configuration, are, for example, peptides that contain the first 16 to 39, especially the first 20 to 25, aminoacids of the ACTH-molecule. In these peptides some aminoacids of the natural sequence may have been exchanged for others, provided that the ACTH-activity remains substantially unaffected as is the case, for example, when the second aminoacid, tyrosine, is replaced by phenylalanine, or the third aminoacid, serine, by glycine, or the fourth aminoacid, methionine, by norvaline, leucine or  $\alpha$ -aminobutyric acid, or the fifth aminoacid, glutamic acid, by glutamine, or the 17th and 18th aminoacid, arginine, by ornithine or lysine. Also the first aminoacid — which according to the present invention is present in the D-configuration — may be the D-form of a natural  $\alpha$ -aminoacid other than serine, primarily, for example, D-alanine, D-proline or D-threonine. Of the new peptides, special mention is deserved by those which contain 20 to 25 aminoacids, but differ from the sequence of the first 20 to 25 aminoacids of ACTH in that the first aminoacid is D-serine, and particularly those in which the first aminoacid is D-serine and in which the arginine<sup>17,18</sup>-radicals are replaced by ornithine radicals and/or the serine<sup>3</sup>-radical by glycyl, and/or the glutamyl<sup>5</sup>-radical by glutaminyl.

Acid addition salts are primarily salts of physiologically tolerable acids such as hydro-

chloric or acetic acid, or above all sparingly soluble salts such as sulphates, phosphates or sulphonates.

Relevant derivatives are, for example, esters such as lower alkyl esters, for example methyl, ethyl, propyl or tertiary butyl ester, or benzyl esters which may be unsubstituted or substituted, for example by nitro groups, halogen atoms, lower alkyl groups or lower alkoxy groups, and furthermore hydrazides and amides, especially peptide amides in which the C-terminal carboxyl group is amidated. "Complexes" are intended herein to mean complex-like compounds whose structure has not so far been explored and which are formed when certain inorganic or organic substances are added to peptides having an adrenocorticotrophic action, primarily those which impart a prolonged action to the peptides. Such inorganic substances are compounds derived from metals, such as calcium, magnesium, aluminum, cobalt, and especially zinc, primarily sparingly soluble salts, such as phosphates or pyrophosphates, as well as hydroxides of these metals. Organic substances which prolong the activity of the peptides are, for example, non-antigenic gelatine, for example oxypolygelatine, polyvinylpyrrolidone and carboxymethyl cellulose, also sulphonic acid esters, or phosphoric acid esters of alginic acid, dextrin, polyphenols and polyhydric alcohols, primarily polyphlorethine phosphate and phytinic acid, as well as polymers and copolymers of aminoacids, for example protamine, and especially of aminoacids containing a preponderance of acid  $\alpha$ -aminoacids such as glutamic acid or aspartic acid.

As mentioned above, the new compounds have a considerably greater ACTH-activity than the corresponding compounds with L-configuration at the first aminoacid. They are therefore intended for use in human and veterinary medicine, for example, instead of the natural hormone. Special mention is deserved by D-Ser<sup>1</sup>- $\beta^{1-24}$ -corticotropin D-Ser<sup>1</sup>, -Orn<sup>17,18</sup>- $\beta^{1-24}$ -corticotropin and D-Ser<sup>1</sup>, Gly<sup>3</sup>- $\beta^{1-24}$ -corticotropin, because of their strong and prolonged ACTH-action. Also Ala<sup>1</sup>- $\beta^{1-24}$ -corticotropin is distinguished by an especially strong action.

The new peptides are manufactured by the methods known for the manufacture of long-chain peptides, especially the carbodiimide method, the activated ester method, the azide method or the mixed anhydride method, with the use of D-aminoacid as N-terminal aminoacid, linking the aminoacids together in the indicated sequence either as such or after first having formed small peptide units. For example: One of the aminoacid or peptide molecules in the form of an ester may be linked with a further aminoacid or peptide molecule containing a protected amino group, in the presence of a condensing agent such as a carbodiimide or a phosphorous acid ester

halide; or the aminoacid or peptide ester containing a free amino group may be reacted with an aminoacid or a peptide containing an activated carboxyl group (and a protected amino group), for example an acid halide, azide, anhydride, imidazolidine or isoxazolidine for example from N-ethyl-5-phenyl-isoxazolum-3'-sulphonate; see Woodward et al., J. Am. Chem. Soc. 89, page 1011 [1961]) or with an activated ester such as cyanomethyl ester, carboxymethyl thiol ester or nitrophenyl ester. Conversely an aminoacid or a peptide containing a free carboxyl group (and a protected amino group) may be reacted with an aminoacid or a peptide containing an activated amino group (and a protected carboxyl group), for example a phosphite amide.

Any free, functional groups not participating in the reaction are advantageously protected, especially by means of groups that are easy to eliminate by hydrolysis or reduction, the carboxyl group advantageously by esterification, for example with methanol, tertiary butanol, benzyl alcohol or para-nitrobenzyl alcohol, or amide formation or the amino group, for example, by introducing the tosyl, trityl, formyl, trifluoroacetyl, phthalyl or carbobenzoxy group or coloured protective groups such as the para - phenylazo - benzyloxycarbonyl group or the para - (para' - methoxy - phenylazo) - benzyloxycarbonyl group, or especially the tertiary butyloxycarbonyl group. The amino group in the guanido grouping of arginine can be protected by the nitro group, though it is not absolutely necessary for the amino group of arginine to be protected during the reaction. The amino group of histidine can be protected by the benzyl or trityl group.

The conversion of a protected amino or imino group into a free group and the conversion of a functionally modified carboxyl group into a free carboxyl group in the course of the present process is performed in a known manner by treatment with a hydrolyzing or, respectively, reducing agent.

According to a preferred process the tripeptide containing the first 3 aminoacids, for example H-D-Ser-Tyr-Ser-OH (the abbreviated forms indicate the L-aminoacids, unless D is specifically shown) or the tetrapeptide, which further contains the 4th aminoacid, is condensed with the heptapeptide or hexapeptide of the following aminoacids up to aminoacid 10, preferably by the azide method, whereupon the decapeptide is condensed with the whole of the residual peptide sequence, thus for example, for the manufacture of the hexadecapeptide with the hexapeptide of aminoacids 11-16, the nonadecapeptide with the nonapeptide of aminoacids 11-19, the eicosapeptide with the decapeptide of aminoacids 11-20, the heneicosapeptide with the undecapeptide of aminoacids 11-21, the docosapeptide with the dodecapeptide of

aminoacids 11-22, the tricosapeptide with the tridecapeptide of aminoacids 11-23, the tetracosapeptide with the tetradecapeptide of aminoacids 11-24, the pentacosapeptide with the pentadecapeptide of aminoacids 11-25, the octacosapeptide with the octadecapeptide of aminoacids 11-28, the nonatriacontapeptide with the nonacosapeptide of aminoacids 11-39 or the corresponding peptides having a constitution which differs from that of the natural ACTH-sequence only as far as the kind of some aminoacids is concerned.

The coupling method employed in the afore-mentioned condensation is preferably the carbodiimide method or the method of the activated esters, primarily by means of the para-nitrophenyl ester. In the latter case the para-nitrophenyl ester of the decapeptide need not be isolated as such but can be formed during the actual condensation from the decapeptide containing a free carboxyl group, para-nitrophenol an dicyclohexyl carbodiimide. Thus, the decapeptide takes the form of an  $\alpha$ -amino protected peptide having a free carboxyl group or a para-nitrophenyl ester group. The  $\alpha$ -amino group of the decapeptide is preferably protected by the tertiary butyloxycarbonyl group. In the peptide fragment to be condensed with the decapeptide the terminal carboxyl group is preferably in the form of the tertiary butyl ester group or it is an amide group. The side-chain amino groups present in the peptide fragments to be condensed are advantageously protected by the tertiary butyloxycarbonyl group, and the side-chain carboxyl groups by the tertiary butyl ester group. N-tertiary butyloxycarbonyl derivatives of certain amino acids and peptides are described and claimed in British Specification No. 872,332. Peptides and derivatives thereof containing  $\alpha,\omega$ -diaminocarboxylic acid radicals, especially lysine radicals, of which the  $\omega$ -amino group is protected by the tertiary butyloxycarbonyl group, are described and claimed in British Specification No. 957,892. In the last stage of the process these protective groups can be eliminated by means of trifluoroacetic acid.

According to another preferred process the tetrapeptide containing the first four aminoacids, primarily H-D-Ser-Tyr-Ser-Met-OH, is condensed with the whole of the residual peptide sequence, thus, for example, for the manufacture of the nonadecapeptide with the pentadecapeptide of aminoacids 5-19, the eicosapeptide with the hexadecapeptide of aminoacids 5-20, the heneicosapeptide with the heptadecapeptide of aminoacids 5-21, the docosapeptide with the octadecapeptide of aminoacids 5-22, the tricosapeptide with the nonadecapeptide of aminoacids 5-23, the tetracosapeptide with the eicosapeptide of aminoacids 5-24, the pentacosapeptide with the heneicosapeptide of aminoacids 5-25 etc. or with the corresponding peptides modified

in the sequence as to the nature of some amino-acids. The coupling method employed in this condensation is advantageously the azide method. The  $\alpha$ -amino group of the tetrapeptide hydrazide or azide is preferably protected by the tertiary butyloxycarbonyl group. The peptide which is to be condensed with the tetrapeptide may be the free peptide or an ester, especially the tertiary butyl ester, or the  $\omega$ -amide. In this peptide the side-chain amino groups are preferably protected by the tertiary butyloxycarbonyl group, while side-chain carboxyl groups are protected by the tertiary butyl ester group.

After having formed by condensation of the peptide fragments the whole peptide (hexadecapeptide, heptadecapeptide, etc. up to the nonatriacontapeptide), whose  $\alpha$ -amino group and side-chain amino groups are protected by the tertiary butyloxycarbonyl group and whose terminal carboxyl group and side-chain carboxyl groups are protected by the tertiary butyl ester group, all these protective groups may be eliminated simultaneously by acid hydrolysis, for example with trifluoroacetic acid. If the terminal carboxylic group is not the tertiary butyl ester group but an amide group, then peptide amides are obtained. Peptide hydrazidates are obtained, for example, by treating a peptide containing a terminal lower alkyl ester group with hydrazine hydrate.

Depending on the reaction conditions used the new compounds are obtained in the form of bases or of their salts. The bases can be obtained from the salts in the usual manner. When bases are reacted with acids capable of forming physiologically tolerable salts, they form salts, for example salts with inorganic acids such as hydrohalic acids, for example hydrochloric or hydrobromic acid, perchloric, nitric or thiocyanic acid, or sulphuric or phosphoric acids; or organic acids such as formic, acetic, propionic, glycolic, lactic, pyruvic, oxalic, malonic, succinic, maleic, fumaric, malic, tartaric, citric, ascorbic, hydroxymaleic, dihydroxymaleic, benzoic, phenylacetic, 4-aminobenzoic, 4-hydroxybenzoic, anthranilic, cinnamic, mandelic, salicylic, 4-aminosalicylic, 2-phenoxybenzoic, 2-acetoxybenzoic, methanesulphonic, ethanesulphonic, hydroxyethanesulphonic, benzenesulphonic, paratoluenesulphonic, naphthalenesulphonic or sulphanilic acid.

The peptides of the present invention or their physiologically tolerable acid addition salts, derivatives or complexes may be used in the form of pharmaceutical preparations containing these compounds in admixture or conjunction with an organic or inorganic pharmaceutical carrier suitable for enteral or parenteral administration. Suitable carriers are substances that do not react with the polypeptides, for example lactose, glucose, sodium chloride, starches, magnesium stearate, talcum, vegetable oils, benzyl alcohols, gums, polyalkylene-

glycols, white petroleum jelly, cholesterol or other known medicinal carriers. The pharmaceutical preparations may be, for example, in the form of lyophilizates or in liquid form as solutions, suspensions or emulsions. They may be sterilized and/or contain assistants such as preserving, stabilizing, wetting or emulsifying agents. They may also contain further therapeutically valuable substances.

*Inter alia*, they may be combined primarily with the additives conventionally used in ACTH-therapy to prolong their action, for example oxypolygelatin, polyphlorethin phosphate, carboxymethyl cellulose or the aforementioned sparingly soluble metal compounds, especially the phosphates, pyrophosphates or hydroxides of zinc.

Furthermore, the activity of the new peptides can be prolonged by converting the peptides into their complexes with polymers or copolymers of aminoacids, especially those that have a preponderance of acid  $\alpha$ -aminoacids, such as glutamic acid or aspartic acid of the L-, D-, or D,L-configuration. The polymers and copolymers mentioned have free carboxyl groups in their side chains, whereas the terminal carboxyl group may be free or functionally converted, for example an ester group or an unsubstituted amide group or an amide group substituted by hydrocarbon groups primarily lower alkyl groups. The molecular weight of the polymers may be between 1000 and 100,000, preferably 2000—15,000. It is advantageous to use for the manufacture of the polyaminoacid preparations a water-soluble, physiologically tolerable salt, for example the sodium or ammonium salt or a salt with an organic base, such as triethylamine, procain, dibenzylamine, or another tertiary nitrogen base.

The aminoacid polymers are known or can be prepared by a known process, for example by that described by M. Idelson et al, J. A. Chem. Soc. 80, 4631 et seq. (1958). Thus, for example, glutamic acid- $\alpha$ -carboxylic anhydride- $\gamma$ -benzyl ester or -tertiary butyl ester may be caused to react in dioxan with ammonia or an amine in a certain molecular ratio, for example 100:1 (depending on the desired degree of polymerization), and the protective groups split off when polymerization is complete, for example the butyloxy group with hydrogen bromide in glacial acetic acid, and the tertiary butyloxy group with trifluoroacetic acid. To obtain polymers with a uniform, defined chain length, the polymers can be synthesized by the methods known in peptide chemistry (for example carbodiimide method, azide method or activated ester method).

The concentrations of the polymers in the pharmaceutical preparations depends on the solubility of the salt concerned and on the viscosity of the polymer. The polymer should

be present in the preparations in the dissolved state and it should be injectable.

The concentration of the adrenocorticotropically active peptide is so chosen that the preparation contains, for example, 10—50 international units.

The present invention further provides veterinary preparations, which comprise the peptides of the present invention or their

physiologically tolerable acid addition salts, derivatives or complexes, in admixture or conjunction with a carrier suitable for veterinary use.

The following Examples illustrate the invention.

In the thin-layer chromatography the following systems were used:

|    |            |                                                                             |
|----|------------|-----------------------------------------------------------------------------|
|    | System 43A | tertiary amyl alcohol+isopropanol+water (100:40:10)                         |
| 20 | " 43C      | tertiary amyl alcohol+isopropanol+water (100:40:55)                         |
|    | " 52       | n-butanol+glacial acetic acid+water (100:10:30)                             |
| 25 | " 100      | ethyl acetate+pyridine+glacial acetic acid+water (62:21:6:11)               |
|    | " 101      | n-butanol+pyridine+glacial acetic acid+water (30:20:6:24)                   |
|    | " 102      | ethyl acetate+methyl ethyl ketone+formic acid+water (50:30:10:10)           |
| 30 | " 110      | ethyl acetate+n-butanol+pyridine+glacial acetic acid+water (80:40:40:12:19) |

The abbreviations have the following meanings:

Z= carbobenzoxy  
BOC= tertiary butyloxycarbonyl  
tBu= tertiary butyl

#### EXAMPLE 1

##### 1. tertiary Butyloxycarbonyl - D - serine hydrate

A solution of 10.5 g (0.1 mol) of D-serine in 50 ml of 2N-sodium hydroxide solution is mixed with a solution of 15.7 g (0.11 mol) of tertiary butyloxycarbonyl azide in 50 ml of methanol, whereupon 11.1 g (0.11 mol) of triethylamine in 100 ml of methanol are stirred in dropwise at 40°C during the course of 4 hours. The batch is stirred for another 2 hours at 40°C and then overnight at room temperature, then adjusted to pH 6 to 7 by adding 2N-hydrochloric acid and freed from methanol under vacuum at 30°C. The aqueous solution is covered with ethyl acetate and at 0°C concentrated hydrochloric acid is stirred in to establish a pH of 1 to 2. The ethyl acetate phase is quickly separated, repeatedly washed with saturated sodium chloride solution, dried and evaporated under vacuum. The resulting oil (19.8 g) crystallizes as the monohydrate on being pasted with a small amount of water at 0°C. Yield: 15.95 g (=71% of theory). Melting at 50 to 52°C. After recrystallization from a small amount of water the melting point remains unchanged.  $[\alpha]_D^{20} = +9.1^\circ \pm 1^\circ$  (c=1.1 in water). According to its thin-layer chromatogram the product is unitary:  $R_{f_{43}} = 0.31$ ;  $R_{f_{100}} = 0.65$ .

##### 2. tertiary Butyloxycarbonyl - D - seryl - L - tyrosyl - L - seryl - L - methionine methyl ester

8.26 g (20 Millimols) of L-tyrosyl-L-seryl-L-methionine methyl ester are dissolved at 70°C in a mixture of 150 ml of acetonitrile and 10 ml of dimethylformamide, cooled to room temperature, and mixed with 4.46 g (20 millimols) of tertiary butyloxycarbonyl - D - serine. The solution is cooled to -5°C, a solution of 4.53 g (22 millimols) of dicyclohexyl carbodiimide in 10 ml of acetonitrile is stirred in and the whole is stirred for one hour at -5°C and then overnight at 0°C. 0.15 ml of Glacial acetic acid are added and the reaction mixture is stirred for 30 minutes at 0°C, the precipitated dicyclohexylurea filtered off and the filtrate evaporated under 0.1 mm Hg pressure. The residue is taken up in ethyl acetate, the solution washed at 0°C with N-hydrochloric acid, N-sodium bicarbonate solution and water, dried and evaporated under vacuum. On trituration with ether the residue turns solid. Yield: after recrystallization from methanol+ether: 9.1 g (=76% of theory). Melting at 152 to 155°C.  $[\alpha]_D^{20} = -7.0 \pm 1^\circ$  (c=0.9 in methanol). The thin-layer chromatogram of the substance contains the following Rf values:  $R_f = 0.29$  in chloroform+methanol (9:1);  $R_{f_{43}} = 0.78$ ;  $R_{f_{100}} = 0.85$ .

3. tertiary Butyloxycarbonyl - D - seryl - L - tyrosyl - L - seryl - L - methionine hydrazide.

5 A solution of 12 g (20 millimols) of tertiary butyloxycarbonyl - D - seryl - L - tyrosyl - L - seryl - L - methionine methyl ester in 50 ml of methanol is mixed with 5 ml (0.1 mol) of hydrazine hydrate and left to stand at room temperature. After 3 hours  
10 the precipitated crystalline hydrazide is filtered off and washed with water and ethanol; to yield 9.82 g (=82% of theory), melting at 148-152°C, after recrystallization from methanol at 150 to 153°C.  $[\alpha]_D^{20} = -6.3^\circ \pm 1^\circ$  (c=1 in methanol). According to its thin-layer chromatogram the substance is unitary:  $Rf_{45} = 0.65$ ;  $Rf_{52} = 0.57$ ;  $Rf_{100} = 0.51$ .

4. tertiary Butyloxycarbonyl - D - seryl - L - tyrosyl - L - seryl - L - methionyl - (γ - tertiary - butyl) - L - glutamyl - L - histidyl - L - phenylalanyl - L - arginyl - L - tryptophyl - glycine

1.43 g (2.4 Millimols) of tertiary butyloxycarbonyl - D - seryl - L - tyrosyl - L - seryl - L - methionine hydrazide are suspended at -10°C in 15 ml of dimethylformamide and mixed at this temperature with 1.58 ml of ice-cold 6N-hydrochloric acid. 0.5 ml of Ice-cold 5N-sodium nitrite solution are dropped in at -8°C and the batch is allowed to react for 25 minutes at -5 to -8°C. At the same time a solution of 1.65 g of γ - tertiary butyl - L - glutamyl - L - histidyl - L - phenylalanyl - L - arginyl - L - tryptophyl - glycine (1.9 millimols) [see British Patent Specification No. 992,957] in 5 ml of warm dimethylformamide is cooled to -10°C and mixed with 1.85 ml. of triethylamine. At -15°C the ice-cold solution of the BOC-tetrapeptide azide is introduced, the whole stirred for one hour at -10°C and then allowed to react further overnight at 0°C. A small amount of an inorganic salt settles out from the reaction solution; it is  
40 filtered off and the resulting solution concentrated to a small volume in a high vacuum. The crude, amorphous BOC-decapeptide is precipitated with 100 ml of 2N-ammonium hydroxide solution, suctioned off and washed neutral with water. After drying, there are obtained 1.88 g of crude decapeptide derivative which on crystallization from 50 ml of 90% methanol yields 1.2 g of pure BOC-decapeptide melting at 208°C with decomposition. The thin-layer chromatograms on silica gel in the systems 52 and 101 reveal only one spot each with Pauly-Ehrlich and Reindel-Hoppe reagents.  $Rf_{52} = 0.2$  and  $Rf_{101} = 0.6$ .  $[\alpha]_D^{20} = -16^\circ \pm 1^\circ$  (c=0.9 in 50%  
60 pyridine).

5. tertiary Butyloxycarbonyl - D - seryl - L - tyrosyl - L - seryl - L - methionyl - (γ - tertiary butyl) - L - glutamyl - L -

histidyl - L - phenylalanyl - L - arginyl - L - tryptophyl - glycyl - tertiary butyl - oxycarbonyl - L - lysyl - L - prolyl - L - valyl - glycyl - tertiary butyloxycarbonyl - L - lysyl - tertiary butyloxycarbonyl - L - lysyl - L - arginyl - L - arginyl - L - prolyl - L - vlyl - tertiary butyloxycarbonyl - L - lysyl - L - valyl - L - tyrosyl - L - proline tertiary butyl ester

0.725 g of tertiary Butyloxycarbonyl - D - seryl - L - tyrosyl - L - seryl - L - methionyl - (γ - tertiary butyl) - L - glutamyl - L - histidyl - L - phenylalanyl - L - arginyl - L - tryptophyl - glycine (0.5 millimol) and 1.11 g (0.5 millimol) of ε-tertiary butyloxycarbonyl - L - lysyl - L - prolyl - L - valyl - glycyl - tertiary butyloxycarbonyl - L - lysyl - tertiary butyloxycarbonyl - L - lysyl - L - arginyl - L - arginyl - L - prolyl - L - valyl - tertiary butyloxycarbonyl - L - lysyl - L - valyl - L - tyrosyl - L - proline - tertiary butyl ester trihydrochloride are stirred in 5 ml of 90% pyridine for one hour at 50°C under nitrogen, whereupon 175 mg (0.85 millimol) of dicyclohexyl carbodiimide are added and five hours later the identical amount of dicyclohexyl carbodiimide is again added. The batch is allowed to react for 24 hours at 50°C, the precipitated dicyclohexylurea is filtered off (161 mg) and the crude reaction product is precipitated with a large amount of ethyl acetate (1.77 g). The hydrochloride is converted into the triacetate on an ion exchange resin, "Amberlite" (Registered Trade Mark) IR-4B (acetate form) (diameter 16 mm, height 27 cm). For purification the totality of the protected cosapeptide in 50% tertiary butanol is chromatographed on a carboxymethyl cellulose column (diameter 16 mm, height 17 cm) by means of 50% tertiary butanol containing increasing proportions of 2N-acetic acid. Apart from fringe fractions, the pure protected tetra-cosapeptide ester can be eluted with 50% tertiary butanol+2N-acetic acid (96:4). Yield: 740 mg.

The thin-layer chromatogram of this fraction in the system 101 displays only one spot with Pauly, Reindel-Hoppe or ninhydrin reagent,  $Rf = 0.6$ .  $[\alpha]_D^{20} = 49^\circ \pm 2^\circ$  (c=0.62 in methanol). Ultraviolet spectrum in 0.1N-sodium hydroxide solution in methanol:  $\lambda_{max}$  283 mμ,  $\epsilon = 8600$  and  $\lambda_{max}$  289 mμ,  $\epsilon = 8950$ . Ratio Tyr:Try=1.9.

6. D - Seryl - L - tyrosyl - L - seryl - L - methionyl - L - glutamyl - L - histidyl - L - phenylalanyl - L - arginyl - L - tryptophyl - glycyl - L - lysyl - L - prolyl - L - valyl - glycyl - L - lysyl - L - lysyl - L - arginyl - L - arginyl - L - prolyl - L - valyl - L - lysyl - L - valyl - L - tyrosyl - L - proline hexa-acetate

500 mg of Protected tetracosapeptide tertiary butyl ester are split with 5.5 ml of 90% trifluoroacetic acid during the course of 30 minutes at room temperature. The trifluoroacetate is precipitated with a large amount of peroxide-free ether, filtered off and washed with a large amount of ether and finally with petroleum ether. The amorphous residue is dissolved in water and the trifluoroacetate converted into the acetate on an ion exchange resin column of "Amberlite" IR-45. Yield: 450 mg of thin-layer chromatographically pure tetracosapeptide;  $R_{f_{101}}=0.5$  (on alumina).  $[\alpha]_D^{25}=-85^\circ \pm 1.5^\circ$  ( $c=0.62$  in 1% acetic acid).

Ultraviolet spectrum in 0.1N-sodium hydroxide solution:

$\lambda_{max}$  283  $m\mu$ ,  $\epsilon=8300$  and 288  $m\mu$ ,  $\epsilon=8600$ .

The peptide cannot be split with leucine amino peptidase. In the Saffran and Sayer tests it displays a substantial adrenocorticotropical action. As compared with the L-seryl analogue, its action is prolonged.

#### EXAMPLE 2

##### 25 1. BOC-D-Alanine

5 g (56.2 Millimols) of D-alanine in 30 ml of water are mixed with 3.2 g (39 millimols) of magnesium oxide and a solution of 12.4 ml (90 millimols) of tertiary butyloxycarbonyl azide in 30 ml of dioxan and the batch is allowed to react for 16 hours at 45°C while being well stirred. The reaction mixture is mixed with 30 ml of water, the insoluble product filtered off and the filtrate is concentrated to a small volume under vacuum. The concentrated solution is then extracted twice with ethyl acetate, the organic phases are washed with sodium bicarbonate and water, and the combined aqueous solutions are cooled to 0°C. The solution is adjusted to pH=4 with 10% citric acid solution, and the BOC-alanine is extracted with a large amount of ethyl acetate. The ethyl acetate phases are washed with saturated sodium chloride solution, dried over sodium sulphate and evaporated under vacuum. Yield: 4.5 g; melting at 83 to 84°C.  $[\alpha]_D^{25}=+25^\circ \pm 0.3^\circ$  ( $c=2.65$  in methanol). The compound displays the following  $R_f$  values in the thin-layer chromatogram on silica gel:  $R_{f_{430}}=0.35$ ,  $R_{f_{101}}=0.75$ .

##### 2. BOC-D-Ala-Tyr-Ser-Met-OCH<sub>3</sub>

3 g (7.2 Millimols) of H - Tyr - Ser - Met - OCH<sub>3</sub> are dissolved in a mixture of 2.7 ml of dimethylformamide and 50 ml of acetonitrile; 1.37 g (7.2 millimols) of BOC-D - alanine and then at -5°C 1.65 g (7.9 millimols) of dicyclohexyl carbodiimide are added. The whole is stirred for 2 hours at -5°C and then kept for 13 hours at 0°C. In addition to dicyclohexylurea, the BOC-tetrapeptide ester precipitates from the reaction solution. The precipitate is filtered off

and while still moist digester with 3×7.5 ml of warm dimethylformamide, the solvent is evaporated under a high vacuum and the residue crystallized from methanol. Yield: 2.01 g of protected tetrapeptide methyl ester melting at 186 to 187°C.  $[\alpha]_D^{25}=-2^\circ \pm 1^\circ$  ( $c=1.05$  in methanol). The thin-layer chromatogram of the compound on silica gel contains the following  $R_f$  values:

|                                     |       |
|-------------------------------------|-------|
| $R_{f_{430}}$                       | =0.7  |
| $R_t$ (chloroform+<br>methanol 9:1) | =0.4. |

It gives a positive reaction with Javell-Reindel-Hoppe reagent, Pauly reagent and potassium iodoplatinate.

##### 3. BOC - D - Ala - Tyr - Ser - Met - NH - NH<sub>2</sub>

1.7 g (2.9 Millimols) of BOC tetrapeptide ester are dissolved with heating in 12 ml of methanol, the solution is cooled to room temperature and stirred with 0.73 ml of hydrazine hydrate for 16 hours at room temperature, whereupon the hydrazide settles out as a thick, white precipitate. The batch is diluted with 12 ml of water, further stirred for one hour, cooled in an ice bath, and the partially crystalline precipitate is filtered off, thoroughly washed with water and dried over phosphorus pentoxide and sulphuric acid in a high vacuum. Yield: 1.42 g of protected tetrapeptide hydrazide; it melts at 176 to 177°C.  $[\alpha]_D^{25}=+16^\circ \pm 1^\circ$  ( $c=1.03$  in glacial acetic acid). In the thin-layer chromatogram on silica gel the following  $R_f$  values are found:

|                                     |       |
|-------------------------------------|-------|
| $R_f$ (100)                         | =0.65 |
| $R_f$ (chloroform+<br>methanol 9:1) | =0.2. |

The substance gives a positive reaction with Javell-Reindel-Hoppe reagent, Pauly reagent and potassium iodoplatinate.

##### 4. BOC - D - Ala - Tyr - Ser - Met - Glu(OtBu) - His - Phe - Arg - Try - Gly - OH

The decapeptide derivative is manufactured from 1.32 g of BOC - D - Ala - Tyr - Ser - Met - hydrazide and 1.61 g of H - Glu(OtBu) - His - Phe - Arg - Try - Gly - OH as described in Example 1(4). Yield: 1.9 g of crude BOC-decapeptide. Crystallization from 90% methanol furnishes 700 mg of crystalline peptide derivative melting at 206°C with decomposition, accompanied by 720 mg of undissolved amorphous product melting at 210°C. According to their thin-layer chromatograms on silica gel (systems 52 and 101) the two fractions are unitary and identical.  $R_{f_{101}}=0.75$ ;  $R_{f_{52}}=0.25$ .  $[\alpha]_D^{25}=-8^\circ \pm 1^\circ$  ( $c=1.06$  in 50% pyridine).

5. BOC - D - Ala - Tyr - Ser - Met - Glu(OtBu) - His - Phe - Arg - Try - Gly - Lys(BOC) - Pro - Val - Gly - Lys(BOC) - Lys(BOC) - Arg - Arg - Pro - Val - Lys(BOC) - Val - Tyr - Pro - OtBu
- 370 mg (0.25 Millimol) of BOC - D - Ala - Tyr - Ser - Met - Glu(OtBu) - His - Phe - Arg - Try - Gly - OH and 55 mg of H - Lys(BOC) - Pro - Val - Gly - Lys(BOC) - Lys(BOC) - Arg - Arg - Pro - Val - Lys(BOC) - Val - Tyr - Pro - OtBu are stirred under nitrogen in 5 ml of 80% pyridine for one hour at 45°C, then mixed with 80 mg of dicyclohexyl carbodiimide and after 5 hours another 80 mg of dicyclohexyl carbodiimide are added. The batch is allowed to react for 20 hours at 45°C, cooled to room temperature, the precipitated dicyclohexylurea is filtered off and the crude reaction product is precipitated with much ethyl acetate. Yield: 880 mg of crude protected tetracosapeptide ester which is still contaminated with starting material; it is purified by counter-current distribution according to Craig in the system chloroform+carbon tetrachloride+methanol+buffer (5:2:8:4) over 550 stages. (Buffer = 28.5 ml of glacial acetic acid+19.25 g of sodium acetate per litre of water).
- From fractions 69-90 there are obtained 400 mg of pure protected D - Ala<sup>1</sup> - β<sup>1-24</sup> - corticotropin tertiary butyl ester. [α]<sub>D</sub><sup>25</sup> = -48° ± 2° (c=0.52 in methanol). The ultraviolet spectrum of the compound in 0.1N-methanolic sodium hydroxide solution contains maxima at λ283 (ε=9300) and 289 mμ (=9600).
6. D - Ala - Tyr - Ser - Met - Glu - His - Phe - Arg - Try - Gly - Lys - Pro - Val - Gly - Lys - Lys - Arg - Arg - Pro - Val - Lys - Val - Tyr - Pro - OH, 6 CH<sub>3</sub> - COOH (D - Ala<sup>1</sup> - β<sup>1-24</sup> - corticotropin
- The pure protected 24-peptide derivative is converted into the pure tetracosapeptide hexaacetate with 90% trifluoroacetic acid as described in Example 1; its thin-layer chromatogram on alumina displays the R<sub>f,01</sub> value=0.6. In the Saffran test the peptide displays a high ACTH-activity.
- with 100 ml of boiling water, the insoluble urea derivative is suctioned off and the filtrate evaporated to dryness under vacuum. On crystallization from 30 ml of isopropanol, the residue yields 7.2 g of protected dipeptide ester hydrochloride melting at 178 to 180°C; its thin-layer chromatogram on silica gel plates in the system 102 displays an R<sub>f</sub> value of 0.62 (indicator: Sakaguchi reagent).
2. Z-Arg-Arg-Pro-OtBu
- A solution of 7.1 g of Z-Arg-Pro-OtBu in 70 ml of methanol and 6.8 ml of 2.18N-hydrochloric acid is hydrogenated during the course of 90 minutes in the presence of 700 mg of palladium charcoal (10% Pd). The catalyst is then filtered off and the filtrate evaporated to dryness under vacuum. The residue, together with 5.4 g of Z-Arg-OH, is dissolved in 17 ml of dimethylformamide, mixed with 17 ml of chloroform and cooled to 0°C. 4.5 g of Dicyclohexyl carbodiimide are stirred in and then 52 ml of chloroform are added. The mixture is stirred overnight at 0°C and then evaporated to dryness under vacuum. The residue is triturated with 100 ml of water, and the insoluble urea derivative suctioned off and washed with water and chloroform. The aqueous phase is separated, washed three times with chloroform and concentrated under vacuum. The concentrated solution is filtered through 300 ml of "Amberlite" IRA-400 (acetate form) and the diacetate of the peptide derivative is eluted with water. The eluate is collected fractionwise and tested in the thin-layer chromatogram in the system 102. The pure fractions (R<sub>f</sub>=0.35) are combined and evaporated to dryness under vacuum. Yield: 10.2 g (=97% of theory).
3. H-Arg-Arg-Pro-OtBu
- 4.5 g of Z-Arg-Arg-Pro-OtBu diacetate are hydrogenated in 25 ml of methanol in the presence of 500 mg of 10% palladium charcoal, the catalyst filtered off, the filtrate evaporated to dryness and the residue, together with 1 ml of triethylamine, dissolved in 28 ml of dimethylformamide. The solution is kept at 0°C and then used as it is for the synthesis of the nonapeptide derivative.
4. Z - Lys(BOC) - Pro - Val - Gly - Lys(BOC) - Lys(BOC) - Arg - Arg - Pro - OtBu
- 4.4 g of Z - Lys(BOC) - Pro - Val - Gly - Lys(BOC) - Lys(BOC) - NHNH<sub>2</sub> [prepared, for example, as described in British Patent No. 1,038,677] are dissolved in 44 ml of absolute dimethylformamide; 10.8 ml of N-hydrochloric acid are dropped in at -20°C, and then 4.4 ml of N-sodium nitrite solution are added, and the batch is further stirred for 20 minutes at -10°C. The above solution of H-Arg-Arg-Pro-OtBu is then added and the

## EXAMPLE 3

1. Z-Arg-Pro-OtBu
- 5.35 g of Z-Arg-OH and 5 g of proline tertiary butyl ester hydrochloride are suspended at room temperature in 70 ml of absolute chloroform, 3.1 g of dicyclohexyl carbodiimide are added and the mixture is heated to 50°C; the starting materials dissolve and at the same time a new crystalline precipitate forms. After 5 hours the batch is cooled to 0°C, suctioned and the residue washed with chloroform. The mixture of dicyclohexylurea and dipeptide is dried, extracted
- 115
- 120
- 125

- whole is kept overnight at 0°C. The dimethylformamide is distilled off under a high vacuum and the residue triturated with ether to form a powder which is dissolved in warm 1% acetic acid saturated with ethyl acetate and mixed with "Amberlite" IRA-400 (acetate form) until chlorine ions can no longer be identified. The ion exchange resin is then suctioned off, the filtrate extracted with 2× 100 ml of ethyl acetate, and the organic phase is extracted twice more with 1% acetic acid. The combined aqueous solutions are evaporated to dryness under vacuum, the residue dissolved in 7:3 chloroform+methanol, chromatographed on 200 g of silica gel, and eluted with 600 ml of the above chloroform+methanol mixture and with 400 ml of methanol, to yield 4.56 g of the protected nonapeptide derivative (as the diacetate) which is unitary according to its thin-layer chromatogram ( $R_f=0.35$  in the system 102).
5. H - Lys(BOC) - Pro - Val - Gly - Lys(BOC) - Lys(BOC) - Arg - Arg - Pro - (a) Acetate:
- 25 A solution of 600 mg of the above nonapeptide derivative in 12 ml of methanol is hydrogenated in the presence of 60 mg of 10% palladium charcoal. When the absorption of hydrogen has ceased, the catalyst is suctioned off and the filtrate evaporated to dryness under vacuum. Yield: 530 mg (= 96% of theory). The compound is unitary according to its thin-layer chromatogram ( $R_f=0.08$  in the system 102).
- 30 (b) Hydrochloride:
- 35 300 mg of the nonapeptide derivative acetate obtained under (a) are mixed with 5 ml of pyridine and dissolved by adding 200 mg of pyridine hydrochloride. The solution is evaporated to dryness and the residue freed from pyridine acetate by being dried for several hours under a high vacuum at 40°C. The remove the excess pyridine hydrochloride the product is then triturated with acetone, whereupon the nonapeptide hydrochloride is obtained as a power which is insoluble in acetone.
- 40 6. BOC - D - Ser - Tyr - Ser - Met - Glu(OtBu) - His - Phe - Arg - Try - Gly - Lys(BOC) - Pro - Val - Gly - Lys(BOC) - Lys(BOC) - Arg - Arg - Pro - OtBu
- 45 100 mg of the above nonapeptide ester hydrochloride are dissolved in 2.2 ml of absolute pyridine and mixed with 120 mg of BOC - D - Ser - Tyr - Ser - Met - Glu(OtBu) - His - Phe - Arg - Try - Gly - OH (see Example 1). The mixture is stirred under nitrogen until all has dissolved. 100 mg of Dicyclohexyl carbodiimide are then added and the whole is stirred under nitrogen for 18 hours at 24°C. The precipitate formed is filtered off and the filtrate evaporated to dryness. The evaporation residue is triturated with ether and the resulting firm powder is subjected to a counter-current distribution according to Craig over 120 stages in the system chloroform+carbon tetrachloride (5:2)+methanol+0.5 molar aqueous ammonium acetate solution (2:1)=1.1; phase volume 3 ml. The crude product is dissolved in 15 ml of upper and lower phase and the solution distributed over the first 5 tubes of the distribution apparatus. Fractions 46 to 55 contain the pure protected nonadecapeptide ester (distribution number  $K=0.7$ ); according to its thin-layer chromatogram on silica gel it is unitary ( $R_{f10}=0.42$ ).
7. H - D - Ser - Tyr - Ser - Met - Glu His - Phe - Arg - Try - Gly - Lys - Pro - Val - Gly - Lys - Lys - Arg - Pro - OH, hexaacetate
- 80 A solution of 50 mg of the protected nonadecapeptide ester in 3 ml of 95% trifluoroacetic acid is kept for one hour at 22°C, then evaporated to dryness and the residue dissolved in 2N-acetic acid and the trifluoroacetate ions are removed on a column of a weakly basic ion exchange resin (Merck II) in the acetate form. The eluate is lyophilized and furnishes 40 mg of the free nonadecapeptide in the form of a colourless, amorphous powder. Paper-electrophoresis (pH 6.3; 1000 Volts, 2 hours' migration) reveals the product to be unitary: travelling distance of 8.5 cm (under identical conditions  $\beta^{1-24}$ -corticotropin travels 10.5 cm); in thin-layer chromatography on an alumina plate ( $R_{f101}=0.50$ ) the product is likewise found to be unitary. The product has a strong corticotropical activity; in the Sayer test the activity of D-Ser<sup>1</sup>- $\beta^{1-19}$ -corticotropin is found to be significantly superior to that of  $\beta^{1-19}$ -corticotropin (all are L-aminoacids).
- 85
- 90
- 95
- 100
- EXAMPLE 4
1. Z - Pro - Val - Lys(BOC) - Val - Tyr - Pro - OtBu
- (a) 5.14 g (6.75 millimols) of the pentapeptide ester H - Val - Lys(BOC) - Val - Tyr - Pro - OtBu [prepared, for example, according to the afore-mentioned British Patent No. 1,038,677] are dissolved in 10 ml of dimethylformamide, and 3.01 g (8.15 millimols) of benzyloxycarbonyl - L - proline - para - nitrophenyl ester are added. After 20 hours at room temperature the reaction mixture is worked up by dilution with 100 ml of ethyl acetate and the solution is washed with 50 ml of water, then with 4×25 ml of molar potassium carbonate solution and with 2×25 ml of water. The ethyl acetate layer is then cooled to 0°C and extracted with 2×25 ml of 1% aqueous citric acid solution, washed neutral and dried with sodium sulphate.
- 105
- 110
- 115
- 120
- 125

The protected hexapeptide ester is obtained in powder form by diluting the concentrated solution with dry ethyl ether. Yield: 97% of theory. According to its thin-layer chromatogram on silica gel the product is unitary in the following solvent systems:

Methanol  $R_f=0.97$   
 chloroform+methanol (95:5)  $R_f=0.26$   
 benzene+acetone (1:1)  $R_f=0.47$

The product is not crystalline; it melts at about 156 to 180°C.  $[\alpha]_D^{21}=-94.5$  ( $c=1.09$  in methanol);  $[\alpha]_D^{28}=-46.4$  ( $c=1.01$  in dimethylformamide).

(b) 7.61 g (10 Millimols) of the free pentapeptide ester H - Val - Lys(BOC) - Val - Tyr - Pro - OtBu are dissolved in 15 ml of dimethylformamide, and 1.4 ml of triethylamine are added. The mixture is cooled to -10°C and mixed with 4.73 g (12 millimols) of carbobenzoxy - L - proline - N - hydroxy - phthalimide ester [prepared according to Rec. Chim. Trav. Pays-Bas 81, page 683 (1962)]. The reaction mixture soon turns dark red and is worked up when all has passed into solution. The dissolution of the ester and the coupling take only 15 minutes. Working up consists in dilution with ethyl acetate, extraction with 60 ml of water to remove the dimethylformamide, extraction with 4×25 ml of saturated bicarbonate solution, one extraction with a 1% citric acid solution and washing until neutral. The solution is then dried with sodium sulphate, filtered, and the filtrate vigorously stirred into 1/2 litre of petroleum ether. The resulting suspension is diluted with 1/2 litre of petroleum ether, filtered and dried, to yield 9.83 g (=99% of theory) of a chromatographically pure product which is identical with the product obtained as described under (a).

2. H - Pro - Val - Lys(BOC) - Val - Tyr - Pro - OtBu

(a) Free hexapeptide ester

6.5 g (6.55 Millimols) of protected hexapeptide ester Z - Pro - Val - Lys(BOC) - Val - Tyr - Pro - OtBu are dissolved in 150 ml of methanol and hydrogenated for 23.5 hours in the presence of 1.3 g of palladium charcoal (10% Pd).

The methanolic solution is then filtered and the filtrate concentrated under vacuum.

A sample is kept at room temperature in an open vessel, whereupon crystals form which are used for seeding the main product. After 20 hours in an ice box the needles (1.41 g) are filtered off and washed first with methanol+ether and then with ether. With ether another 3.25 g of the peptide ester are obtained from the mother liquor; thus the total yield is 4.66 g (=83% of theory). Melting point 174 to 176°C.  $[\alpha]_D^{20}=-91^\circ \pm 0.5$  ( $c=1.87$  in methanol).

The peptide ester is soluble in methanol,

acetone, dimethylformamide and insoluble in ethyl acetate, ether and petroleum ether.

(b) Monoacetate:

2.5 g (2.52 Millimols) of the above protected hexapeptide ester are hydrogenated in glacial acetic acid+water (4:1) in the presence of 1.3 g of palladium charcoal. The absorption of hydrogen ceases after 2½ hours. The catalyst is filtered off, the filtrate evaporated under vacuum at a bath temperature of 40°C and the residue dried over sodium hydroxide under a high vacuum, to yield 2.34 g (=100% of theory) of a colourless resin which is unitary according to its thin-layer chromatogram.

The compound reveals the following  $R_f$  values (developed with Reindel-Hoppe reagent) on silica gel plates: System chloroform+methanol (8:2): 0.61; system 43A: 0.50

3. N<sup>α</sup> - Benzyloxycarbonyl - N<sup>δ</sup> - tert.butyl - oxycarbonyl - L - ornithine

26 g (112 millimols) of N<sup>δ</sup>-tertiary butyloxycarbonyl-L-ornithine (prepared according to British Patent Specification No. 957,892) are carbobenzoxyated in the usual manner. To acidify the alkaline reaction solution extracted with ether the pH is adjusted at 0°C to pH=3 with citric acid, once more extracted with ether, washed with water and dried, and the solvent distilled off, to yield 37.8 g (=92% of theory) of a colourless oil.

1 g of the above oil is dissolved in 5 ml of ether and caused to crystallize by adding 600 mg of dicyclohexylamine. The resulting salt is washed with ether. Yield: 1.28 g (=85% of theory). Melting at 133°C.

The bulk of the product crystallizes on dissolution in 100 ml of warm diisopropyl ether and cooling. There are obtained white crystals melting at 99°C, and after recrystallization from 100 ml of hot diisopropyl ether with the addition of 20 ml of ethyl acetate, at 101°C.  $[\alpha]_D^{21}=-3.2^\circ$  ( $c=2.45$  in absolute methanol).

4. N<sup>α</sup> - Benzyloxycarbonyl - N<sup>δ</sup> - tert.butyl - oxycarbonyl - L - ornithine - para - nitrophenyl ester

A solution of 14.6 g (40 millimols) of N<sup>α</sup> - Z - N<sup>δ</sup> - BOC - Orn - OH and 6.72 g of para-nitrophenol (48 millimols) in 75 ml of ethyl acetate is cooled to -20°C and mixed with 8.24 g (40 millimols) of dicyclohexylcarbodiimide. The batch is kept for 2 hours at 0°C, then filtered, the filtrate evaporated to dryness and the crystalline residue diluted with 75 ml of 10% acetonitrile in diisopropyl ether. After 24 hours at 0°C, the batch is filtered and washed with diisopropyl ether. The still moist material is dissolved in 50 ml of ethanol and 25 ml of diisopropyl ether are added. When the reaction mixture is kept for several hours in an ice box, small needles

of the above para-nitrophenyl ester form (11.9 g=61.5% of the theoretical yield), melting at 102°C. When the mother liquor is concentrated, it yields another 2.5 g (=19% of theory) of this ester.

After recrystallization from a small amount of ethanol the ester melts at 103°C.  $[\alpha]_D^{24} = -26.7^\circ$  ( $c=5.01$  in methanol).

The ester is soluble in dimethylformamide, acetone methylene chloride, ether, ethyl acetate, ethanol and acetonitrile, and sparingly soluble in diisopropyl ether and petroleum ether.

5. N<sup>ε</sup>-Benzyloxycarbonyl - N<sup>δ</sup>-tert.butyl - oxycarbonyl - L - ornithine hydroxy - phthalimide ester

A solution of 3.66 g (10 millimols) of N<sup>ε</sup>-Z - N<sup>δ</sup>-BOC - L - Orn - OH in 5 ml of anhydrous pyridine is cooled to -10°C and mixed with a solution of 2.06 g (10 millimols) of dicyclohexyl carbodiimide in 5 ml of pyridine. After 5 minutes 1.96 g of N - hydroxy - phthalimide are added and the mixture is kept for 16 hours at 0°C, then filtered and rinsed with pyridine. The filtrate is freed from pyridine under vacuum at 40°C, mixed with secondary butanol, once more evaporated to dryness and then taken up in a small amount of ether. After 48 hours at -8°C the resulting crystals (4.5 g=88% of the theoretical yield) are isolated; they melt at 125 to 127°C.

The crude crystallize is dissolved with heating in acetonitrile, the residual dicyclohexylurea and hydroxyphthalimide are filtered off and the filtrate is caused to crystallize by adding diisopropyl ether, to yield 3.12 g (=61% of theory) of pure product. After recrystallization from isopropyl alcohol it melts at 129°C.  $[\alpha]_D^{27} = -25.7^\circ$  ( $c=1$  in methanol).

6. Z - Orn(BOC) - Pro - Lys(BOC) - Val - Tyr - Pro - OtBu

(a) A solution of 2.3 g (2.5 millimols) of H - Pro - Val - Lys(BOC) - Val - Tyr - Pro - OtBu acetate in 5 ml of dimethylformamide is mixed with 1.71 g (3.5 millimols) of Z - Orn(BOC) - ONP. After 24 hours the reaction mixture is diluted by vigorously stirring in 200 ml of ether. During the course of 15 minutes a white precipitate forms; it is filtered off, washed with ether and dried. According to its thin-layer chromatogram the resulting heptapeptide derivative is unitary in the following solvent systems:

Benzene+acetone (1:1)  $R_f=0.37$   
chloroform+methanol (9:1)  $R_f=0.47$ .

After drying for 48 hours under vacuum there are obtained 2.73 g (=90% of theory) of a white powder melting at 142 to 145°C.  $[\alpha]_D^{20} = -38^\circ \pm 0.5$  ( $c=2.061$  in dimethylformamide);  $[\alpha]_D^{20} = -90^\circ \pm 1^\circ$  ( $c=0.970$  in methanol).

The product is soluble in dimethylformamide, acetic acid, alcohols and acetone, more sparingly soluble in acetonitrile and insoluble in ethyl acetate, ether, benzene and petroleum ether.

(b) A solution of 429 mg (0.5 millimol) of H - Pro - Val - Lys - (BOC) - Val - Tyr - Pro - OtBu in 2 ml of dimethylformamide is mixed with 305 mg (0.60 millimol) of N<sup>ε</sup>-benzyloxycarbonyl - N<sup>δ</sup>-tertiary butyloxy - carbonyl - L - ornithine hydroxyphthalimide ester. 0.1 ml of Triethylamine are added, whereupon the reaction mixture turns red and is worked up 15 minutes later. About 50 ml of ether are stirred into the solution which is filtered half an hour later, to yield 536 mg of the protected heptapeptide ester (=89% of theory) which is found to be analytically pure and identical with the product obtained as described under (a) above.

7. H - Orn(BOC) - Pro - Val - Lys(BOC) - Val - Tyr - Pro - OtBu

(a) Acetate:

A solution of 1 g (0.83 millimol) of the protected heptapeptide ester described under (b) in 25 ml of 80% acetic acid is hydrogenated as described under 2. On completion of the hydrogenation the catalyst is filtered off, thoroughly washed, and the filtrate is evaporated to dryness, to leave 956 mg (=100% of theory) of pure material, which cannot be distinguished from the free base (see below) by thin-layer chromatography.  $[\alpha]_D^{20} = -87^\circ \pm 1$  ( $c=1.028$  in methanol);  $[\alpha]_D^{20} = -27^\circ \pm 1$  ( $c=1.095$  in 80% acetic acid).

(b) Free heptapeptide ester

A solution of 1 g (0.83 millimol) of the protected heptapeptide ester in methanol is hydrogenated as described under 2. The product is not obtained in crystalline form, but it no longer contains any starting material and is unitary according to its thin-layer chromatogram on silica gel plates; it reveals the following  $R_f$  values:

System 52  $=68$   
chloroform+methanol (90:10)  $=0.13$ .

After total hydrolysis and amino acid analysis according to Stein and Moore an amino acid ratio Pro:Val:Tyr:Orn:Lys=204:2.00:0.98:1.06:1.08 is found.

8. Z - Orn(BOC) - Orn(BOC) - Pro - Val - Lys(BOC) - Val - Tyr - Pro - OtBu

A solution of 2.69 g (2.38 millimols) of the heptapeptide ester acetate obtained according to 7(a) in 5 ml of dimethylformamide is mixed with 1.4 g (2.86 millimols) of Z-Orn(BOC)-ONP. The mixture is kept overnight at room temperature and then concentrated under a high vacuum, to leave a brown resin which on the addition of 100 ml

of warm acetonitrile yields a white precipitate. After 3 hours at 0°C the latter is filtered off, and the resulting powder washed with acetonitrile and with ether. After drying, the resulting octapeptide ester weighs 3.15 g (=93% of theory); it melts at 152 to 154°C.  $[\alpha]_D^{21} = -83.3^\circ \pm 0.5$  ( $c=1.046$  in methanol). The product is unitary according to its thin-layer chromatogram on silica gel and reveals the following Rf values:

Chloroform+methanol (95:5) =0.54  
benzene+acetone (1:1) =0.56.

The octapeptide ester is soluble in dimethylformamide, acetic acid, methanol and ethanol, more sparingly soluble in hot acetonitrile and insoluble in ethyl acetate and ether.

9. H - Orn(BOC) - Orn(BOC) - Pro - Val - Lys(BOC) - Val - Tyr - Pro - OtBu

(a) Acetate:

The protected octapeptide ester according to 7 is dissolved in 80% acetic acid and hydrogenated as described under 2. The catalyst is then filtered off and the filtrate evaporated at a maximum bath temperature of 30°C. According to its thin-layer chromatogram on silica gel plates the resulting product is unitary and reveals the following Rf values:

Chloroform+methanol (95:5) =0.06  
chloroform+methanol (9:1) =0.22  
chloroform+methanol (85:15) =0.26  
benzene+acetone (1:1) =0.03.

$[\alpha]_D^{25} = -49.4^\circ \pm 1$  ( $c=0.942$  in dimethylformamide);

$[\alpha]_D^{26} = -85.6^\circ \pm 1$  ( $c=1.266$  in methanol).

(b) Free octapeptide ester:

A solution of 500 mg of the protected octapeptide ester in methanol is hydrogenated as described under 2, to yield 441 mg of free base (=96% of theory). The Rf values do not differ from those of the acetate described above.  $[\alpha]_D^{25} = -51.8^\circ \pm 1$  ( $c=1.247$  in dimethylformamide);  $[\alpha]_D^{26} = -87.7^\circ \pm 1$  ( $c=1.079$  in methanol).

10. Z - Lys(BOC) - Pro - Val - Gly - Lys(BOC) - Lys(BOC) - Orn(BOC) - Orn(BOC) - Pro - Val - Lys(BOC) - Val - Tyr-Pro-OtBu

900 mg (0.80 Millimol) of the hexapeptide hydrazide Z - Lys(BOC) - Pro - Val - Gly - Lys(BOC) - Lys(BOC) - NH - NH<sub>2</sub> (prepared for example, according to British Patent Specification No. 1,038,677 are dissolved in 9 ml of pure dimethylformamide and the solution is cooled to -10°C. 1.6 ml of 2N-hydrochloric acid and 0.18 ml of 5N-sodium nitrite solution are added, the whole is stirred

for 10 minutes at -10°C, and a solution of 900 mg (0.61 millimol) of the octapeptide acetate described in 9(a) in 9 ml of dimethylformamide is added. 0.53 ml (4.0 millimol) of triethylamine are then added and the batch is kept for 18 hours in an ice box. The solution is then evaporated to dryness under a high vacuum and the residue triturated with acetonitrile. The tetradecapeptide derivative is insoluble in acetonitrile; it is filtered off and washed with 3×25 ml of acetonitrile.

There are obtained 1.554 g (=98% of theory) of a faintly yellowish product which reveals in the thin layer chromatogram only a trace of an impurity. None of the two starting materials can be identified. The Rf values (on silica gel) are:

Chloroform+methanol (95:5) =0.31  
dioxan+water (95:5) =0.76.

The product is amorphous but has a sharp melting point at 192 to 194°C.  $[\alpha]_D^{25} = -37.8^\circ \pm 1$  ( $c=1.050$  in dimethylformamide). As revealed by elementary analysis, the peptide derivative is obtained in the form of the dihydrate.

11. H - Lys(BOC) - Pro - Val - Gly - Lys(BOC) - Lys(BOC) - Orn(BOC) - Orn(BOC) - Pro - Val - Lys(BOC) - Val - Tyr - Pro - OtBu - tosylate

1 g (0.42 Millimol) of the protected tetradecapeptide ester are suspended in 150 ml of methanol and agitated at 40°C until all has dissolved. 400 mg of 5% palladium charcoal are then added and the solution is agitated for 18 hours at 40°C under hydrogen, filtered, and evaporated, to yield 793 mg (=84% of theory) of the ester.

For conversion into the tosylate the product is dissolved in 5 ml of pyridine, a solution of 70 mg of paratoluenesulphonic acid in 1 ml of pyridine is added, the pyridine evaporated to dryness under vacuum and the residue repeatedly washed with ether while triturating and decanting. After drying, there are obtained 721 mg of the tetradecapeptide ester tosylate as an amorphous powder which is substantially unitary as revealed by its thin-layer chromatogram and displays the following Rf values:

System 43C =0.49  
system 52 =0.61.

12. BOC - D-Ser-Tyr-Ser-Met-Glu(OtBu)-His - Phe - Arg - Try - Gly - Lys(BOC) - Pro - Val - Gly - Lys(BOC) - Lys(BOC) - Orn(BOC) - Orn(BOC) - Pro - Val - Lys(BOC) - Val - Tyr-Pro - OtBu

A suspension of 400 mg of the tetradecapeptide ester tosylate and 350 mg of BOC - D - Ser - Tyr - Ser - Met - Glu-

(OtBu) - His - Phe - Arg - Tyr - Gly - OH (cf. Example 1) in 5 ml of pyridine is stirred under nitrogen at 50°C until both components have dissolved (2 hours). The batch is then cooled to 40°C, 500 mg of dicyclohexyl carbodiimide are added and the whole is stirred for 18 hours at 40°C under nitrogen, then further for 20 hours at 0°C, and the precipitated dicyclohexylurea is filtered off. The filtrate is evaporated to dryness, the residue freed from excess dicyclohexyl carbodiimide by being repeatedly triturated and washed with petroleum ether, and the powder, which is insoluble in petroleum ether, is purified by being washed with ethyl acetate while triturating and decanting.

The resulting crude product melts unsharply at about 165 to 195°C and is further processed without first having been purified.

13. H - D - Ser - Tyr - Ser - Met - Glu - His - Phe - Arg - Try - Gly - Lys - Pro - Val - Gly - Lys - Lys - Orn - Orn - Pro - Val - Lys - Val - Tyr - Pro - OH - hexaacetate

100 mg of the crude protected tetracosapeptide derivative are dissolved in 5 ml of trifluoroacetic acid (containing 5% of water) and the solution is kept for 1 hour at 25°C, then evaporated to dryness under vacuum and the residue is immediately washed several times with anhydrous, peroxide-free ether with trituration and decantation. The resulting powder is mixed with 10 ml of N-acetic acid, with some dicyclohexylurea remaining undissolved. The solution is filtered and the filtrate freed from trifluoroacetate and paratoluenesulphonate ions by being run through a column of a weakly basic ion exchange resin (Merck II; acetate form). The column is rinsed with 10 ml of N-acetic acid, and the eluates are combined and lyophilized. According to its paper-electrophoresis (pH 6.2; 1000 Volts; 2 hours) the lyophilizate is not yet unitary; apart from a spot corresponding to free D-Ser<sup>1</sup>, Orn<sup>17</sup>, Orn<sup>18</sup>- $\beta^{1-24}$ -corticotropin (travelling distance 14 cm) two further, weaker side-spots are visible (travelling distance 2.5 and 18 cm).

The crude product is purified by chromatography on carboxymethyl cellulose (makers Messrs. Serva, Heidelberg; size of column: 0.9×14 cm). The solvent used—in which the carboxymethyl cellulose is suspended for preparing the column—is 0.05 ml of ammonium acetate solution, pH=5.2. The above lyophilizate is dissolved in 2 ml of this buffer solution, poured over the column and allowed to penetrate it. Elution is then performed with a linear buffer gradient prepared from 50 ml of 0.05-molar ammonium acetate solution, pH 5.2, and 50 ml of 1.0-molar ammonium acetate solution, pH 5.2. The elution is observed by means of an ultraviolet percolation absorption recorder. The main

fractions are combined, lyophilized to remove the ammonium acetate and the dry residue is dried at 40°C under 0.01 mm Hg pressure.

The free D-Ser<sup>1</sup>-Orn<sup>17</sup>, Orn<sup>18</sup>- $\beta^{1-24}$ -corticotropin displays an extremely strong and prolonged corticotropical activity.

#### EXAMPLE 5

1. H - Lys(BOC) - Pro - Val - Gly - Lys(BOC - Lys(BOC) - NH<sub>2</sub>  
5.0 g of Z - Lys(BOC) - Pro - Val - Gly - Lys(BOC) - Lys(BOC) - OCH<sub>3</sub> (prepared for example, according to the afore-mentioned British Patent No. 1,038,677) are dissolved with heating in 30 ml of methanol, and 400 ml of 6N-ammonia in methanol are added to the solution. After 3 days at room temperature the batch is evaporated and the residue recrystallized from aqueous tertiary butanol, to yield 4.8 g of hexapeptide amide melting at 197 to 198°C.  $[\alpha]_D^{25} = -48^\circ \pm 1^\circ$  (c=1.05 in methanol).

2.04 g of the above hexapeptide derivative are hydrogenated in 60 ml of methanol in the presence of 200 mg of 10% palladium carbon. The absorption of hydrogen is complete after 10 hours. Suctioning off the catalyst and evaporation furnishes H-Lys(BOC)-Pro - Val - Gly - Lys(BOC) - Lys(BOC)-NH<sub>2</sub> as a colourless resin which in thin-layer chromatography on silica gel is found to be free from unhydrogenated starting material; Rf<sub>110</sub>=0.15.

2. BOC - D - Ser - Tyr - Ser - Met - Glu(OtBu) - His - Phe - Arg - Try - Gly - Lys(BOC) - Pro - Val - Gly - Lys(BOC) - Lys(BOC) - NH<sub>2</sub>

A mixture of 150 mg of BOC-D-Ser-Tyr-Ser - Met - Glu(OtBu) - His - Phe - Arg - Try - Gly - OH (see Example 1), 200 mg of the above hexapeptide amide, 4 ml of pyridine and 12 mg of pyridine hydrochloride is stirred for 2 hours at 24°C under nitrogen. 120 mg of Dicyclohexyl carbodiimide are then added and the batch is further stirred for 24 hours at 24°C under nitrogen. After this time, the fine, gelatinous precipitate is removed on the centrifuge, and the supernatant pyridine solution is decanted and evaporated. The residue is triturated with ether and the ether-insoluble powder dried and freed from the hexapeptide amide by being triturated and washed with dilute acetic acid. Yield: 135 mg of crude protected hexadecapeptide derivative in the form of a powder that is insoluble in acetic acid. For purification it is suspended with stirring in 10 ml of aqueous methanol; a trace of by-product (acylurea, formed from the decapeptide and dicyclohexylurea) remains undissolved and is filtered off. The solution in aqueous methanol is evaporated to dryness and the residue taken up in dimethylform-

amide; a second portion of acylurea remains undissolved and is filtered off. Evaporation of the dimethylformamide solution furnishes the substantially pure protected hexadecapeptide derivative.

Thin-layer chromatogram on silica gel plates:  $R_{f_{101}}=0.80$ .

3. H - D - Ser - Tyr - Ser - Met - Glu - His - Phe - Arg - Try - Gly - Lys - Pro - Val - Gly - Lys - Lys - NH<sub>2</sub> acetate

A solution of 20 mg of the purified hexadecapeptide derivative in 1 ml of 95% trifluoroacetic acid is kept for one hour at 24°C, then evaporated to dryness, and the residue is dissolved in dilute acetic acid. By elution on a column of a weakly basic ion exchange resin (Merck II) in the acetate form the trifluoroacetate ions are exchanged for acetate ions. The eluate yields on lyophilization 15 mg of the free hexadecapeptide amide as a colourless powder whose thin-layer chromatogram on alumina plates reveals an  $R_f$  value of 0.6 in system 101. In the Sayer test D-Ser<sup>1-16</sup>-corticotropin amide displays a significant corticotropical activity, whereas the corresponding L-Ser<sup>1-16</sup>-corticotropin is almost completely inactive.

#### EXAMPLE 6

1. BOC - D - Ser - Tyr - Ser - Met - Glu(OtBu) - His - Phe - Arg - Try - Gly - Lys(BOC) - Pro - Val - Gly - Lys(BOC) - Lys(BOC) - Arg - Arg - Pro - Val - Lys(BOC) - Val - Tyr - Pro - Asp(OtBu) - Gly - Ala - Glu(OtBu) - Asp(OtBu) - Glu(NH<sub>2</sub>) - Leu - Ala - Glu(OtBu) - Ala - Phe - Pro - Leu - Glu(OtBu) - Phe - OtBu

102 mg of BOC - D - Ser - Tyr - Ser - Met - Glu(OtBu) - His - Phe - Arg - Try - Gly-OH (see Example 1) are dissolved in 1.2 ml of 90% pyridine at 50°C and mixed successively with 0.035 ml of N-hydrochloric acid and a solution of 150 mg of H-Lys(BOC) - Pro - Val - Gly - Lys(BOC) - Lys(BOC) - Arg - Arg - Pro - Val - Lys(BOC) - Val - Tyr - Pro - Asp - (OtBu) - Gly - Ala - Glu(OtBu) - Asp(OtBu) - Glu(NH<sub>2</sub>) - Leu - Ala - Glu(OtBu) - Ala - Phe - Pro - Leu - Glu(OtBu) - Phe - OtBu. 1½ H<sub>2</sub>SO<sub>4</sub> [R. Schwyer and P. Sieber, Nature 199, page 172 (1963)] in 1.2 ml of 90% pyridine. While stirring at 50°C, an addition each of 0.15 ml of a 10% solution of dicyclohexyl carbodiimide in pyridine is made after 30 minutes, 1½ hours and 6 hours, the whole is stirred for 25 hours and then precipitated with 100 ml of peroxide-free ether. The resulting crude product is subjected to a 140-stage Craig distribution in a 8:8:17:7-mixture of chloroform+carbon tetrachloride+methanol and 0.1-molar ammonium acetate (pH 6.5). The fractions 50

to 80 contain according to the thin-layer chromatogram the protected  $\beta$ -corticotropin; they are combined and evaporated to dryness. Another 280-stage distribution in the identical solvent system furnishes on evaporation of fractions 125 to 154 ( $\lambda_{max}=140$ ;  $K=1.0$ ) 38 mg of pure product which reveals in the thin-layer chromatogram on alumina the  $R_{f_{100}}$  value of 0.5.

2. H - D - Ser - Tyr - Ser - Met - Glu - His - Phe - Arg - Try - Gly - Lys - Pro - Val - Gly - Lys - Lys - Arg - Arg - Pro - Val - Lys - Val - Tyr - Pro - Asp - Gly - Ala - Glu - Asp - Glu(NH<sub>2</sub>) - Leu - Ala - Glu - Ala - Phe - Pro - Leu - Glu - Phe - OH (D - Ser<sup>1-39</sup>- $\beta$ -corticotropin)

30 mg of the Protected D-Ser<sup>1-39</sup>-corticotropin derivative are dissolved in 3 ml of 95% trifluoroacetic acid and kept for one hour at 24°C, then evaporated to dryness and the residue is freed from trifluoroacetic acid ions by elution on a column of a weakly basic ion exchange resin (Merck II) in the acetate form. The eluate is lyophilized and yields the acetate of free D-Ser<sup>1-39</sup>-corticotropin as a colourless powder. In the thin-layer chromatography on silica gel and in the paper-electrophoresis the product displays the same behaviour as synthetic  $\beta$ <sup>1-39</sup>-corticotropin.  $R_{f_{101}}=0.4$ . Paper-electrophoresis (pH 6.3; 5 Volts/cm, 24 hours): distance travelled 7 cm.

The corticotropin action of D-Ser<sup>1-39</sup>-corticotropin is longer and substantially greater than that of the natural hormone, for example in the Sayer test.

#### EXAMPLE 7

1. BOC-Tyr-Gly-OCH<sub>3</sub>

10.12 g of BOC-tyrosine are dissolved in 45 ml of hot ethyl acetate and the solution after being cooled to 20°C, treated with a solution of 3.84 g of glycine methyl ester in 10 ml of ethyl acetate. 10.4 g of solid dicyclohexyl carbodiimide are added and the whole stirred for 18 hours at 25°C. The batch is then cooled to 0°C, the precipitated dicyclohexylurea filtered off after 30 minutes, and the crude dipeptide derivative isolated from the filtrate by concentration and precipitation with petroleum ether. The amorphous viscous mass is further purified by another precipitation from a mixture of ethyl acetate and petroleum ether. It can be crystallized from the same mixture of solvents. Melting point, 124—125°C.

The thin-layer chromatogram on silica gel gives the following  $R_f$  values:

|                                         |      |
|-----------------------------------------|------|
| $R_f$ (43 A)                            | 0.65 |
| $R_f$ (CHCl <sub>3</sub> -methanol=8:2) | 0.68 |
| $R_f$ (101)                             | 0.77 |

2. H-Tyr-Gly-OCH<sub>3</sub> hydrochloride

2 g of BOC-Tyr-Gly-OCH<sub>3</sub> are dissolved in 20 ml of absolute ethyl acetate while being heated, and treated with 20 ml of 4N-hydrochloric acid in absolute ethyl acetate at 20°C. After a few minutes a precipitate begins to form. After 30 minutes, the reaction mixture is concentrated to about 10 ml under reduced pressure and precipitation of the dipeptide-ester hydrochloride completed by the addition of 50 ml of petroleum ether. The batch is homogenized, and the amorphous product filtered off, washed with petroleum ether, and dried under a high vacuum at 30°C. The dipeptide ester hydrochloride is obtained as a hygroscopic powder melting at about 110–115°C (with decomposition). In the thin layer chromatogram it displays the following Rf values:

|    |                                      |      |
|----|--------------------------------------|------|
| 20 | Rf (43A)                             | 0.33 |
|    | Rf (CHCl <sub>3</sub> -methanol=8:2) | 0.36 |
|    | Rf (101)                             | 0.60 |

3. BOC-D-Ser-Tyr-Gly-OCH<sub>3</sub>

With slight heating, 1.69 g of H-Tyr-Gly-OCH<sub>3</sub> hydrochloride are dissolved in 10 ml of absolute dimethylformamide, then cooled to 0°C. There are added successively 0.84 ml of triethylamine, 1.23 g of BOC-D-Ser-OH, and 1.65 g of dicyclohexyl carbodiimide, and the mixture stirred for 20 hours at 25°C, and finally for 1 hour at 0°C. The mixture of triethylammonium chloride and dicyclohexylurea which has precipitated is filtered off, and the filtrate concentrated to obtain an oily residue. The addition of 30 ml of ethyl acetate and 100 ml of petroleum ether precipitates the crude product as a greasy substance. For preliminary purification the latter is dissolved with heating in a small amount of ethyl acetate, and precipitated by the addition of much petroleum ether (1.8 g). The product, which according to the thin-layer chromatogram is still impure, is for purification subjected to multiplicative distribution according to Craig in the solvent system ethyl acetate:benzene:0.05 molar aqueous ammonium acetate solution (2:1:2), the phase volumes being about 10 ml each. After 250 stages, the chromatographically pure tripeptide derivative is isolated from stages 45–74 ( $r_{\max}=60$ ;  $K=0.32$ ) by evaporating the solvent and sublimating off the ammonium acetate at 45°C under a high vacuum. Crystals from aqueous methanol, melting point 180–182°C. The thin-layer chromatogram on silica gel reveals the following Rf values:

|    |                                      |      |
|----|--------------------------------------|------|
|    | Rf (52)                              | 0.76 |
|    | Rf (101)                             | 0.78 |
| 60 | Rf (CHCl <sub>3</sub> -methanol—8:2) | 0.58 |

## 4. BOC-D-Ser-Tyr-Gly-OH

685 mg of BOC-D-Ser-Tyr-Gly-OCH<sub>3</sub> are dissolved in 5 ml of N-sodium hydroxide solution, the solution allowed to stand at 20°C for 10 minutes, then neutralized by the addition of 5 ml of N-hydrochloric acid. The protected tripeptide is extracted with 50 ml of N-butanol saturated with water, the butanol layer washed 4 times with 4 ml of water each time and concentrated to about 3 ml. Addition of 30 ml of petroleum ether yields a greasy precipitate which can be crystallized from ethyl acetate (melting point 168°C (with decomposition)). The substance is unitary according to the thin layer chromatogram. On silica gel it displays the following Rf values:

|          |      |
|----------|------|
| Rf (52)  | 0.60 |
| Rf (101) | 0.61 |

5. BOC-D-Ser-Tyr-Gly-Met-OCH<sub>3</sub>

425 mg of BOC-D-Ser-Tyr-Gly-OH and 240 mg of H-Met-OCH<sub>3</sub> hydrochloride are dissolved in 3 ml of absolute dimethylformamide, after which 0.168 ml of triethylamine is added and a solution of 288 mg of dicyclohexyl carbodiimide (in 5 ml of absolute ethyl acetate) added dropwise while stirring. Stirring is continued for 15 hours at 20°C and, after another hour at 0°C, the precipitated dicyclohexylurea is filtered off. From the filtrate the crude, greasy condensation product is precipitated by adding 50 ml of petroleum ether, and dried. It is purified by Craig distribution in the system methanol:buffer:chloroform:carbon tetrachloride (5:3:5:2) (buffer=28.5 ml of glacial acetic acid+19.25 g of ammonium acetate in 960 ml of water) over 400 stages the phase volumes being about 3 ml each. The chromatographically pure tetrapeptide derivative is isolated as a white amorphous powder from stages 103–134 ( $r_{\max}=120$ ;  $K=0.43$ ) by concentration to dryness and sublimating off the ammonium acetate under a high vacuum at 45°C. In the thin layer chromatogram on silica gel it displays the following Rf values:

|                                |      |
|--------------------------------|------|
| Rf (benzene+acetone 1:1)       | 0.21 |
| Rf (chloroform+methanol 85:15) | 0.62 |
| Rf (100)                       | 0.84 |

6. BOC-D-Ser-Tyr-Gly-Met-NH-NH<sub>2</sub>

160 mg of BOC-D-Ser-Tyr-Gly-Met-OCH<sub>3</sub> are dissolved in 1 ml of methanol, treated with 0.1 ml of hydrazine hydrate, and allowed to stand at 20°C overnight. The reaction mixture which has solidified to form a crystal cake is diluted with 8 ml of benzene and homogenized, filtered, and the filter residue washed with benzene+methanol (10:1). Drying in a high vacuum at 50°C yields pure tetrapeptide hydrazide melting at 190°C (with decomposition). In the thin-layer chrom-

atogram on silica gel it displays the following Rf values:

Rf (CHCl<sub>3</sub>-methanol 85:15) 0.21  
Rf (100) 0.65

- 5 7. Z - Glu(OtBu) - His - Phe - Arg - Try-Lys(BOC) - Pro - Val - Gly - Lys(BOC) - Lys(BOC) - Arg - Arg - Pro-Val - Lys(BOC) - Val - Tyr - Pro-OtBu - triacetate
- 10 625 mg (0.273 mmol) of H-Lys(BOC)-Pro - Val - Gly - Lys(BOC) - Lys(BOC)-Arg - Arg - Pro - Val - Lys(BOC) - Val-Tyr - Pro - OtBu 3 CH<sub>3</sub>COOH, prepared as described in British Patent No. 1,003,683
- 15 in 10 ml of dry pyridine are mixed with 0.17 g of para-toluene sulphonic acid. The solution is evaporated to dryness, the residue freed from pyridine acetate under a pressure of 0.01 mm of Hg at 35°C, and the pyridine tosylate eliminated by trituration with acetone and filtering with suction. There are obtained 726 mg of protected tetradecapeptide tritosylate as a colourless powder of melting point 155—162°C.
- 20 525 mg (0.2 mmol) of the tritosylate and 245 mg (0.24 mmol) of Z-Glu(OtBu)-His-Phe - Arg - Try - Gly - OH (obtained by condensation of Z-Arg-OH with H-Try-Gly-OCH<sub>3</sub>, splitting off the carbobenzoxy group from the tripeptide derivative, condensation of the free tripeptide ester with Z-Phe-OH, splitting off the carbobenzoxy group from the tetrapeptide ester, condensation of the free tetrapeptide ester with Z-Glu(OtBu)-His-N<sub>3</sub> and saponification of the methyl ester group with N-sodium hydroxide solution in 75% dioxan) are dissolved in 4 ml of 80% pyridine with stirring at 50°C. To the clear solution are added 52 mg (0.25 mmol) of
- 40 dicyclohexyl carbodiimide. After being allowed to stand for 20 hours at 50°C, the slightly turbid solution is cooled to 10°C, and the precipitated dicyclohexylurea filtered off. The solution is treated with much ether whereupon the tritosylate of the eicosapeptide precipitates. It is separated, dissolved in 10 ml of 60% methanol, and chromatographed through a column of 4 ml of "Amberlite" IRA-400 (acetate form). The eluate is evaporated under reduced pressure and triturated with ether. There are obtained 650 mg of crude, protected eicosapeptide triacetate. In the thin-layer chromatogram it displays the following Rf values:

55 On alumina, Rf (100) 0.34  
on silica gel, Rf (101) 0.76;  
Rf (110) 0.45;

- 60 Development with Reindel-Hoppe, Pauly, and Ehrlich reagents. There are also found unchanged hexapeptide and tetradecapeptide

derivatives and some faster running by-products.

8. H - Glu(OtBu) - His - Phe - Arg - Try-Gly - Lys(BOC) - Pro - Val - Gly-Lys(BOC) - Lys(BOC) - Arg - Arg-Pro - Val - Lys(BOC) - Val - Tyr-Pro - OtBu - tetraacetate

600 mg of the crude eicosapeptide derivative are dissolved in 10 ml of methanol and hydrogenated overnight in the presence of 0.5 ml of glacial acetic acid and 1g of palladium carbon (10%). The catalyst is then filtered off, and the filtrate evaporated under reduced pressure. The resulting decarboxybenzoxylated product is dissolved in 10 ml of tertiary butanol+water (1:1) and chromatographed through a column of 6 g of carboxymethyl cellulose using a gradient between 100 ml of 50% tertiary butanol and 100 ml of 50% tertiary butanol+glacial acetic acid (9:1). The fractions which contain a thin-layer chromatographically unitary eicosapeptide derivative are combined and evaporated under reduced pressure. The Rf values of the protected eicosapeptide ester-tetraacetate on silica gel are: Rf (54)=0.50; Rf (101)=0.68; Rf (110)=0.12.

When all the protective groups have been split off with 90% trifluoroacetic acid, the free eicosapeptide displays the following Rf values:

Rf (101) on silica gel 0.25  
Rf (101) on alumina 0.30.

9. BOC - D - Ser - Tyr - Gly - Met-Glu(OtBu) - His - Phe - Arg - Try-Gly - Lys(BOC) - Pro - Val - Gly-Lys(BOC) - Lys(BOC) - Arg - Arg-Pro - Val - Lys(BOC) - Val - Tyr-Pro-OtBu

102 mg of BOC-D-Ser-Tyr-Gly-Met-NHNH<sub>2</sub> are dissolved in 0.7 ml of absolute dimethylformamide and cooled to 0°C. There is then added 0.165 ml of 4.12N-hydrochloric acid and the batch immediately cooled to -10°C. After the addition of 0.036 ml of 5N-sodium nitrite solution, the batch is allowed to react for 10 minutes at -10°C, after which a solution, cooled to 0°C, of 298 mg of H - Glu(OtBu) - His - Phe-Arg - Try - Gly - Lys(BOC) - Pro - Val-Gly - Lys(BOC) - Lys(BOC) - Arg - Arg-Pro - Val - Lys(BOC) - Val - Tyr - Pro-OtBu - tetraacetate and 0.12 ml of triethylamine in 1 ml of absolute dimethylformamide is added. The solution is allowed to stand for 24 hours at 0°C, concentrated to obtain an oily residue, and treated with 10 ml of water. The condensation product which precipitates is filtered off and dried in a high vacuum at 40°C. For purification, the product is subjected to multiplicative distribution in the solvent system methanol+

buffer+chloroform+carbon tetrachloride (32:16:21:9) (buffer=28.5 ml of glacial acetic acid+19.25 mg of ammonium acetate in 950 ml of water) over 350 stages, the phase volumes being 3 ml each. From tubes 85—116 ( $r_{\max}=100$ ;  $K=0.4$ ) there is obtained on evaporation to dryness and drying of the residue at 40°C in a high vacuum the chromatographically pure, protected tetracosapeptide acetate as a white, amorphous powder. In the thin-layer chromatogram it displays the following Rf values:

|    |               |          |      |
|----|---------------|----------|------|
| 15 | On silica gel | Rf (43C) | 0.27 |
|    |               | Rf (52)  | 0.18 |
| 15 | On alumina    | Rf (100) | 0.38 |

10. H-D-Ser-Tyr-Gly-Met-Glu-His-Phe-Arg-Try-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-OH

20 163 mg of protected tetracosapeptide acetate are dissolved in 3.2 ml of 90% trifluoroacetic acid and the solution allowed to stand at 20°C for 25 minutes. The solution is then 25 diluted with about 15 ml of water, concentrated to about 2 ml, and lyophilized. For conversion into the acetate, the trifluoroacetate of the free tetracosapeptide is dissolved in a small amount of water and filtered through 30 a column ( $\phi=7.5$  mm;  $l=16$  cm) of a slightly basic ion exchanger (for example Merck No. II) in the acetate form. The eluate is concentrated to a small volume (about 2 ml), lyophilized, and dried for 30 35 minutes at 40°C in a high vacuum. There is obtained chromatographically and electrophoretically pure D-Ser<sup>1</sup>-Gly<sup>3</sup>- $\beta^{1-24}$ -corticotropin acetate as a white, amorphous powder.

40 In the thin-layer chromatogram on alumina in system 101, its Rf value is 0.56 ( $\beta^{1-24}$ -corticotropin under identical conditions: Rf=0.51) and in electrophoresis (16 Volts/cm) at pH 6.1 (pyridine acetate buffer) it travels 45 in the course of 2 hours 8.6 cm towards the cathode.

#### EXAMPLE 8

A dry ampoule is prepared from the following ingredients:

|    |      |                                                                |
|----|------|----------------------------------------------------------------|
| 50 | mg   |                                                                |
|    | 0.5  | D-Ser <sup>1</sup> - $\beta^{1-24}$ -corticotropin hexaacetate |
|    | 1.23 | ZnSO <sub>4</sub> ·7H <sub>2</sub> O                           |
|    | 1.38 | Na <sub>2</sub> PO <sub>4</sub> ·12H <sub>2</sub> O            |
|    | 40.0 | mannitol                                                       |

55 Before use, the contents of the dry vial are mixed with 1 ml of distilled water in a solution ampoule, to form a suspension having a pH of 7.6.

#### EXAMPLE 9

A dry ampoule is prepared from the following ingredients:

|  |      |                                                                |    |
|--|------|----------------------------------------------------------------|----|
|  | mg   |                                                                |    |
|  | 0.5  | D-Ser <sup>1</sup> - $\beta^{1-24}$ -corticotropin hexaacetate | 60 |
|  | 1.23 | ZnSO <sub>4</sub> ·7H <sub>2</sub> O                           | 65 |
|  | 40.0 | mannitol                                                       |    |

and a solution ampoule containing a solution of

|  |      |                                         |    |
|--|------|-----------------------------------------|----|
|  | mg   |                                         |    |
|  | 1.38 | NaPO <sub>4</sub> ·12H <sub>2</sub> O   | 70 |
|  | 0.1  | "Versene" (Registered Trade Mark) —Fe-3 |    |
|  |      | in distilled water to make 1.0 ml.      |    |

Before use the contents of the two ampoules are mixed; the resulting suspension has a pH of 7.6.

#### EXAMPLE 10

A solution ampoule is prepared from the following ingredients:

|  |       |                                                                |    |
|--|-------|----------------------------------------------------------------|----|
|  | mg    |                                                                |    |
|  | 0.5   | D-Ser <sup>1</sup> - $\beta^{1-24}$ -corticotropin hexaacetate | 80 |
|  | 280.0 | gelatin                                                        |    |
|  | 5.0   | phenol                                                         |    |
|  |       | distilled water to make 1.0 ml.                                | 85 |

#### EXAMPLE 11

An aqueous solution of D-Ser<sup>1</sup>- $\beta^{1-24}$ -corticotropin hexaacetate is filtered sterile and mixed under aseptic conditions with sodium polyphloreitin phosphate and sodium chloride, filled into vials and lyophilized, so that a dry vial is obtained which contains

|  |       |                                                                |    |
|--|-------|----------------------------------------------------------------|----|
|  | mg    |                                                                |    |
|  | 0.5   | D-Ser <sup>1</sup> - $\beta^{1-24}$ -corticotropin hexaacetate | 95 |
|  | 23.20 | sodium polyphloreitin phosphate (86.5% strength)               |    |
|  | 12.28 | sodium chloride                                                |    |

Before use the content of the dry phial is mixed with 2ml of distilled water contained in a solution ampoule.

#### EXAMPLE 12

A suspension is prepared from the following components:

|  |                                                                |         |  |     |
|--|----------------------------------------------------------------|---------|--|-----|
|  |                                                                |         |  |     |
|  | D-Ser <sup>1</sup> - $\beta^{1-24}$ -corticotropin hexaacetate | 1.0-mg  |  | 105 |
|  | ZnCl <sub>2</sub>                                              | 10.5-mg |  |     |
|  | Na <sub>2</sub> HPO <sub>4</sub>                               | 1.7-mg  |  |     |
|  | benzyl alcohol                                                 | 17.0-mg |  |     |
|  | NaCl                                                           | 2.5-mg  |  | 110 |
|  | NaOH to attain a pH value of 8.0                               |         |  |     |
|  | Distilled water to make up                                     | 2 ml    |  |     |

## EXAMPLE 13

2.0 g of poly-L-glutamic acid having an average molecular weight of about 11,000 are dissolved in about 5.7 ml of 10% sodium hydroxide solution so that the pH of the solution amounts to 7.4. In this solution are dissolved 5.0 mg of D-Seryl-L-tyrosyl-L-seryl-L-methionyl-L-glutamyl-L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophyl-glycyl-L-lysyl-L-prolyl-L-valyl-glycyl-L-lysyl-L-lysyl-L-arginyl-L-arginyl-L-prolyl-L-valyl-L-lysyl-L-valyl-L-tryosyl-L-proline-hexaacetate (D-Ser<sup>1</sup>- $\beta^{1-24}$ -corticotropin hexaacetate) and 0.2 mg of merthiolate and the solution made up to 10 ml with distilled water. The solution is filtered under sterile conditions. It contains per ml:

|    |                                               |          |
|----|-----------------------------------------------|----------|
| 20 | D-Ser <sup>1</sup> - $\beta^{1-24}$ -cortico- |          |
|    | tropin                                        | 0.5 mg   |
|    | poly-L-glutamic acid                          | 200.0 mg |
|    | sodium hydroxide solution                     |          |
|    | up to pH 7.4                                  |          |
| 25 | merthiolate                                   | 0.02 mg  |
|    | distilled water to make up                    | 1.0 ml.  |

## EXAMPLE 14

2.0 g of poly-L-glutamic acid are dissolved in 5.7 ml of 10% sodium hydroxide solution so that the pH of the solution is 7.4. In the resulting solution are dissolved 5.0 mg of D-Ser<sup>1</sup>- $\beta^{1-24}$ -corticotropin hexaacetate and 0.2 mg of merthiolate, 1 ml of a hydrochloric acid-zinc chloride solution (pH 2.8) containing 5.2 mg of zinc chloride per ml is added. The pH is adjusted to 7.8 with sodium hydroxide solution and made up to a volume of 10 ml with distilled water.

## EXAMPLE 15

2.0 g of poly-L-glutamic acid are dissolved in about 5.7 ml of a 10% sodium hydroxide solution so that the pH of the solution is 7.4. In this solution are dissolved 5.0 mg of D-Ser<sup>1</sup>- $\beta^{1-24}$ -corticotropin hexaacetate and 0.2 mg of merthiolate. There are then added 1 ml of hydrochloric acid solution (pH 2.8) containing 5.2 mg of zinc chloride, and 0.85 mg of (anhydrous) disodium phosphate. The pH is adjusted to 7.8 with sodium hydroxide solution. The volume is made up to 10 ml with water.

## EXAMPLE 16

In the same manner as described in Examples 8-15, preparations can be made that contain as active peptide the D-Ala<sup>1</sup>- $\beta^{1-24}$ -corticotropin (described in Example 2), or the D-Ser<sup>1</sup>- $\beta^{1-19}$ -corticotropin (described in Example 3), or D-Ser<sup>1</sup>-Orn<sup>17,18</sup>- $\beta^{1-24}$ -corticotropin (described in Example 4), or D-Ser<sup>1</sup>- $\beta^{1-18}$ -corticotropin amide (described in Example 5), or D-Ser<sup>1</sup>- $\beta^{1-30}$ -

corticotropin (described in Example 6), or D-Ser<sup>1</sup>-Gly<sup>3</sup>- $\beta^{1-24}$ -corticotropin (described in Example 7).

## WHAT WE CLAIM IS:—

1. A peptide that differs from an ACTH-active peptide containing 16 to 39 aminoacid residues and having an N-terminal aminoacid residue with L-configuration, solely in that the N-terminal aminoacid residue has D-configuration.

2. A peptide that differs from an (all-L)-ACTH-active peptide containing a 16 to 39 aminoacid residue sequence (beginning from the N-terminus) of a natural corticotropin, in which sequence at least one of the aminoacid residues in the positions 1 to 5, 17, 18 and 25 to 33 may be replaced by the residue of another natural  $\alpha$ -aminoacid or of a homologue thereof, solely in that the N-terminal aminoacid residue has D-configuration.

3. An acid addition salt of a peptide as claimed in claim 1 or 2.

4. A derivative of a peptide as claimed in claim 1 or 2.

5. A complex of a peptide as claimed in claim 1 or 2.

6. A peptide that differs from an (all-L)-ACTH-active peptide containing a 16 to 39 aminoacid residue sequence (beginning from the N-terminus) of a natural corticotropin, in which sequence at least one of the aminoacid residues in the positions 2 to 5, 17, 18 and 25 to 33 may be replaced by the residue of another natural  $\alpha$ -aminoacid or of a homologue thereof, solely in that the N-terminal aminoacid residue is the residue of D-serine.

7. A peptide that differs from an (all-L)-ACTH-active peptide containing a 16 to 39 aminoacid residue sequence (beginning from the N-terminus) of a natural corticotropin, in which sequence the first aminoacid residue may be replaced by the residue of alanine, proline or threonine, and/or the second aminoacid residue may be replaced by the residue of phenylalanine, and/or the third aminoacid residue may be replaced by the residue of glycine or alanine, and/or the fourth aminoacid residue may be replaced by the residue of norvaline, norleucine or  $\alpha$ -aminobutyric acid, and/or the fifth aminoacid residue may be replaced by the residue of glutamine, and/or each of the seventeenth and eighteenth aminoacid residues may be replaced by the residue of ornithine or lysine, and/or the twentyfifth aminoacid residue may be replaced by the residue of valine, solely in that the N-terminal aminoacid residue has D-configuration.

8. A peptide that differs from an (all-L)-ACTH-active peptide containing a 20 to 25 aminoacid residue sequence (beginning from the N-terminus) of a natural corticotropin, in which sequence at least one of the amino-



## EXAMPLE 13

2.0 g of poly-L-glutamic acid having an average molecular weight of about 11,000 are dissolved in about 5.7 ml of 10% sodium hydroxide solution so that the pH of the solution amounts to 7.4. In this solution are dissolved 5.0 mg of D - Seryl - L - tyrosyl - L - seryl - L - methionyl - L - glutamyl - L - histidyl - L - phenylalanyl - L - arginyl - L - tryptophyl - glycyl - L - lysyl - L - prolyl - L - valyl - glycyl - L - lysyl - L - lysyl - L - arginyl - L - arginyl - L - prolyl - L - valyl - L - lysyl - L - valyl - L - tryosyl - L - proline - hexaacetate (D - Ser<sup>1</sup> -  $\beta^{1-24}$ -corticotropin hexaacetate) and 0.2 mg of merthiolate and the solution made up to 10 ml with distilled water. The solution is filtered under sterile conditions. It contains per ml:

|    |                                                  |          |
|----|--------------------------------------------------|----------|
| 20 | D - Ser <sup>1</sup> - $\beta^{1-24}$ - cortico- |          |
|    | tropin                                           | 0.5 mg   |
|    | poly-L-glutamic acid                             | 200.0 mg |
|    | sodium hydroxide solution                        |          |
|    | up to pH 7.4                                     |          |
| 25 | merthiolate                                      | 0.02 mg  |
|    | distilled water to make up                       | 1.0 ml.  |

## EXAMPLE 14

2.0 g of poly-L-glutamic acid are dissolved in 5.7 ml of 10% sodium hydroxide solution so that the pH of the solution is 7.4. In the resulting solution are dissolved 5.0 mg of D-Ser<sup>1</sup>- $\beta^{1-24}$ -corticotropin hexaacetate and 0.2 mg of merthiolate, 1 ml of a hydrochloric acid-zinc chloride solution (pH 2.8) containing 5.2 mg of zinc chloride per ml is added. The pH is adjusted to 7.8 with sodium hydroxide solution and made up to a volume of 10 ml with distilled water.

## EXAMPLE 15

2.0 g of poly-L-glutamic acid are dissolved in about 5.7 ml of a 10% sodium hydroxide solution so that the pH of the solution is 7.4. In this solution are dissolved 5.0 mg of D-Ser<sup>1</sup>- $\beta^{1-24}$ -corticotropin hexaacetate and 0.2 mg of merthiolate. There are then added 1 ml of hydrochloric acid solution (pH 2.8) containing 5.2 mg of zinc chloride, and 0.85 mg of (anhydrous) disodium phosphate. The pH is adjusted to 7.8 with sodium hydroxide solution. The volume is made up to 10 ml with water.

## EXAMPLE 16

In the same manner as described in Examples 8—15, preparations can be made that contain as active peptide the D-Ala<sup>1</sup>- $\beta^{1-24}$ -corticotropin (described in Example 2), or the D - Ser<sup>1</sup> -  $\beta^{1-19}$  - corticotropin (described in Example 3), or D-Ser<sup>1</sup>-Orn<sup>17,18</sup>- $\beta^{1-24}$  - corticotropin (described in Example 4), or D - Ser<sup>1</sup> -  $\beta^{1-18}$  - corticotropin amide (described in Example 5), or D-Ser<sup>1</sup>- $\beta^{1-39}$ -

corticotropin (described in Example 6), or D-Ser<sup>1</sup>,Gly<sup>3</sup>- $\beta^{1-24}$ -corticotropin (described in Example 7).

## WHAT WE CLAIM IS:—

1. A peptide that differs from an ACTH-active peptide containing 16 to 39 aminoacid residues and having an N-terminal aminoacid residue with L-configuration, solely in that the N-terminal aminoacid residue has D-configuration.

2. A peptide that differs from an (all-L)-ACTH-active peptide containing a 16 to 39 aminoacid residue sequence (beginning from the N-terminus) of a natural corticotropin, in which sequence at least one of the aminoacid residues in the positions 1 to 5, 17, 18 and 25 to 33 may be replaced by the residue of another natural  $\alpha$ -aminoacid or of a homologue thereof, solely in that the N-terminal aminoacid residue has D-configuration.

3. An acid addition salt of a peptide as claimed in claim 1 or 2

4. A derivative of a peptide as claimed in claim 1 or 2.

5. A complex of a peptide as claimed in claim 1 or 2.

6. A peptide that differs from an (all-L)-ACTH-active peptide containing a 16 to 39 aminoacid residue sequence (beginning from the N-terminus) of a natural corticotropin, in which sequence at least one of the aminoacid residues in the positions 2 to 5, 17, 18 and 25 to 33 may be replaced by the residue of another natural  $\alpha$ -aminoacid or of a homologue thereof, solely in that the N-terminal aminoacid residue is the residue of D-serine.

7. A peptide that differs from an (all-L)-ACTH-active peptide containing a 16 to 39 aminoacid residue sequence (beginning from the N-terminus) of a natural corticotropin, in which sequence the first aminoacid residue may be replaced by the residue of alanine, proline or threonine, and/or the second aminoacid residue may be replaced by the residue of phenylalanine, and/or the third aminoacid residue may be replaced by the residue of glycine or alanine, and/or the fourth aminoacid residue may be replaced by the residue of norvaline, norleucine or  $\alpha$ -aminobutyric acid, and/or the fifth aminoacid residue may be replaced by the residue of glutamine, and/or each of the seventeenth and eighteenth aminoacid residues may be replaced by the residue of ornithine or lysine, and/or the twentyfifth aminoacid residue may be replaced by the residue of valine, solely in that the N-terminal aminoacid residue has D-configuration.

8. A peptide that differs from an (all-L)-ACTH-active peptide containing a 20 to 25 aminoacid residue sequence (beginning from the N-terminus) of a natural corticotropin, in which sequence at least one of the amino-



acid residues in the positions 2 to 5, 17, 18 and 25 may be replaced by the residue of another natural  $\alpha$ -aminoacid or of a homologue thereof, solely in that the N-terminal aminoacid residue is the residue of D-serine.

9. A peptide that differs from an (all-L)-ACTH-active peptide containing a 20 to 25 aminoacid residue sequence (beginning from the N-terminus) of a natural corticotropin, in which sequence the second aminoacid residue may be replaced by the residue of phenylalanine, and/or the third aminoacid residue may be replaced by the residue of glycine or alanine, and/or the fourth aminoacid residue may be replaced by the residue of norvaline, norleucine or  $\alpha$ -aminobutyric acid, and/or the fifth aminoacid residue may be replaced by the glutamine, and/or each of the seventeenth and eighteenth aminoacid residues may be replaced by the residue of ornithine or lysine, and/or the twentyfifth aminoacid residue may be replaced by the residue of valine, solely in that the N-terminal aminoacid residue is the residue of D-serine.

10. A peptide that differs from an (all-L)-ACTH-active peptide containing a 20 to 25 aminoacid residue sequence (beginning from the N-terminus) of a natural corticotropin, in which sequence each of the arginine<sup>17,18</sup> residues is replaced by the residue of ornithine and/or the serine<sup>9</sup> residue is replaced by the glycyl residue and/or the glutamyl<sup>15</sup> residue is replaced by the glutaminyl residue, solely in that the N-terminal aminoacid residue is the residue of D-serine.

11. The tetracosapeptide of the formula D - seryl - L - tyrosyl - L - seryl - L - methionyl - L - glutamyl - L - histidyl - L - phenyl - alanyl - L - arginyl - L - tryptophyl - glycyl - L - lysyl - L - prolyl - L - valyl - glycyl - L - lysyl - L - lysyl - L - arginyl - L - arginyl - L - prolyl - L - valyl - L - lysyl - L - valyl - L - tyrosyl - L - proline or the corresponding compound containing the glutamine radical instead of the glutamyl radical.

12. The tetracosapeptide of the formula D - alanyl - L - tyrosyl - L - seryl - L - methionyl - L - glutamyl - L - histidyl - L - phenyl - alanyl - L - arginyl - L - tryptophyl - glycyl - L - lysyl - L - prolyl - L - valyl - glycyl - L - lysyl - L - lysyl - L - arginyl - L - arginyl - L - prolyl - L - valyl - L - lysyl - L - valyl - L - tyrosyl - L - proline or the corresponding compound containing the glutamine radical instead of the glutamyl radical.

13. The tetracosapeptide of the formula D - seryl - L - tyrosyl - L - seryl - L - methionyl - L - glutamyl - L - histidyl - L - phenyl - alanyl - L - arginyl - L - tryptophyl - glycyl - L - lysyl - L - prolyl - L - valyl - glycyl - L - lysyl - L - lysyl - L - ornithyl - L - ornithyl - L - prolyl - L -

valyl - L - lysyl - valyl - L - tyrosyl - L - proline or the corresponding compound containing the glutamine radical instead of the glutamyl radical.

14. The tetracosapeptide of the formula D - seryl - L - tyrosyl - glycyl - L - methionyl - L - glutamyl - L - histidyl - L - phenyl - alanyl - L - arginyl - L - tryptophyl - glycyl - L - lysyl - L - prolyl - L - valyl - glycyl - L - lysyl - L - lysyl - L - arginyl - L - arginyl - L - prolyl - L - valyl - L - lysyl - L - valyl - L - tyrosyl - L - proline or the corresponding compound containing the glutamine radical instead of the glutamyl radical.

15. The hexadecapeptide of the formula D - seryl - L - tyrosyl - L - seryl - L - methionyl - L - glutamyl - L - histidyl - L - phenylalanyl - L - arginyl - L - tryptophyl - glycyl - L - lysyl - L - prolyl - L - valyl - glycyl - L - lysyl - L - lysine or the corresponding compound containing the glutamine radical instead of the glutamyl radical.

16. The nonadecapeptide of the formula D - seryl - L - tyrosyl - L - seryl - L - methionyl - L - glutamyl - L - histidyl - L - phenylalanyl - L - arginyl - L - tryptophyl - glycyl - L - lysyl - L - prolyl - L - valyl - glycyl - L - lysyl - L - lysyl - L - arginyl - L - arginyl - L - proline or the corresponding compound containing the glutamine radical instead of the glutamyl radical.

17. D-Ser<sup>1</sup>- $\beta^{1-39}$ -corticotropin or the corresponding compound containing the glutamine radical instead of the glutamyl<sup>15</sup> radical.

18. An acid addition salt of a peptide as claimed in any one of claims 6 to 17.

19. A derivative of a peptide as claimed in any one of claims 6 to 17.

20. A complex of a peptide as claimed in any one of claims 6 to 17.

21. The C-terminal amide of a peptide as claimed in any one of claims 1, 2 and 6 to 17.

22. A complex of a peptide as claimed in any one of claims 1, 2 and 6 to 17 with a complex-forming metal.

23. A complex of a peptide as claimed in any one of claims 1, 2 and 6 to 17 with zinc phosphate, zinc pyrophosphate and/or zinc hydroxide.

24. A complex of the tetracosapeptide of the formula D - seryl - L - tyrosyl - L - seryl - L - methionyl - L - glutamyl - L - histidyl - L - phenylalanyl - L - arginyl - L - tryptophyl - glycyl - L - lysyl - L - prolyl - L - valyl - glycyl - L - lysyl - L - lysyl - L - arginyl - L - arginyl - L - prolyl - L - valyl - L - lysyl - L - valyl - L - tyrosyl - L - proline or of the corresponding compound containing the glutamine radical instead of the glutamyl radical, with zinc



- phosphate, zinc pyrophosphate and/or zinc hydroxide.
25. A complex of a peptide as claimed in any one of claims 1, 2 and 6 to 17 with gelatine.
26. A complex of a peptide as claimed in any one of claims 1, 2 and 6 to 17 with polyphlorethine phosphate.
27. A complex of a peptide as claimed in any one of claims 1, 2 and 6 to 17 with a polymer or copolymer of (an) amino acid(s) containing a preponderance of (an) acid amino acid(s).
28. A complex of a peptide as claimed in any one of claims 1, 2 and 6 to 17 with poly-glutamic acid whose  $\gamma$ -carboxyl group is free or functionally converted.
29. A complex of the tetracosapeptide of the formula D - seryl - L - tyrosyl - L - seryl - L - methionyl - L - glutamyl - L - histidyl - L - phenylalanyl - L - arginyl - L - tryptophyl - glycyl - L - lysyl - L - prolyl - L - valyl - glycyl - L - lysyl - L - lysyl - L - arginyl - L - arginyl - L - prolyl - L - valyl - L - lysyl - L - valyl - L - tyrosyl - L - proline or of the corresponding compound containing the glutamine radical instead of the glutamyl radical, with polyphlorethine phosphate.
30. A complex of the tetracosapeptide of the formula D - seryl - L - tyrosyl - L - seryl - L - methionyl - L - glutamyl - L - histidyl - L - phenylalanyl - L - arginyl - L - tryptophyl - glycyl - L - lysyl - L - prolyl - L - valyl - glycyl - L - lysyl - L - lysyl - L - arginyl - L - arginyl - L - prolyl - L - valyl - L - lysyl - L - valyl - L - tyrosyl - L - proline or of the corresponding compound containing the glutamine radical instead of the glutamyl radical, with poly-glutamic acid.
31. A pharmaceutical preparation which comprises a peptide as claimed in claim 1, in admixture or conjunction with a pharmaceutically suitable carrier.
32. A pharmaceutical preparation which comprises a peptide as claimed in claim 2, in admixture or conjunction with a pharmaceutically suitable carrier.
33. A pharmaceutical preparation which comprises a physiologically tolerable acid addition salt of a peptide as claimed in claim 1 or 2, in admixture or conjunction with a pharmaceutically suitable carrier.
34. A pharmaceutical preparation which comprises a physiologically tolerable derivative of a peptide as claimed in claim 1 or 2, in admixture or conjunction with a pharmaceutically suitable carrier.
35. A pharmaceutical preparation which comprises a physiologically tolerable complex of a peptide as claimed in claim 1 or 2, in admixture or conjunction with a pharmaceutically suitable carrier.
36. A pharmaceutical preparation which comprises a peptide as claimed in claim 6, in admixture or conjunction with a pharmaceutically suitable carrier.
37. A pharmaceutical preparation which comprises a peptide as claimed in claim 7, in admixture or conjunction with a pharmaceutically suitable carrier.
38. A pharmaceutical preparation which comprises a peptide as claimed in claim 8, in admixture or conjunction with a pharmaceutically suitable carrier.
39. A pharmaceutical preparation which comprises a peptide as claimed in claim 9, in admixture or conjunction with a pharmaceutically suitable carrier.
40. A pharmaceutical preparation which comprises a peptide as claimed in claim 10, in admixture or conjunction with a pharmaceutically suitable carrier.
41. A pharmaceutical preparation which comprises a peptide as claimed in claim 11, in admixture or conjunction with a pharmaceutically suitable carrier.
42. A pharmaceutical preparation which comprises a peptide as claimed in claim 12, in admixture or conjunction with a pharmaceutically suitable carrier.
43. A pharmaceutical preparation which comprises a peptide as claimed in claim 13, in admixture or conjunction with a pharmaceutically suitable carrier.
44. A pharmaceutical preparation which comprises a peptide as claimed in claim 14, in admixture or conjunction with a pharmaceutically suitable carrier.
45. A pharmaceutical preparation which comprises a peptide as claimed in claim 15, in admixture or conjunction with a pharmaceutically suitable carrier.
46. A pharmaceutical preparation which comprises a peptide as claimed in claim 16, in admixture or conjunction with a pharmaceutically suitable carrier.
47. A pharmaceutical preparation which comprises a peptide as claimed in claim 17, in admixture or conjunction with a pharmaceutically suitable carrier.
48. A pharmaceutical preparation which comprises a physiologically tolerable acid addition salt of a peptide as claimed in any one of claims 6 to 17, in admixture or conjunction with a pharmaceutically suitable carrier.
49. A pharmaceutical preparation which comprises a physiologically tolerable derivative of a peptide as claimed in any one of claims 6 to 17, in admixture or conjunction with a pharmaceutically suitable carrier.
50. A pharmaceutical preparation which comprises a physiologically tolerable complex of a peptide as claimed in any one of claims 6 to 17, in admixture or conjunction with a pharmaceutically suitable carrier.
51. A pharmaceutical preparation which

comprises the C-terminal amide of a peptide as claimed in any one of claims 1, 2 and 6 to 17, in admixture or conjunction with a pharmaceutically suitable carrier.

5 52. A pharmaceutical preparation which comprises a physiologically tolerable complex of a peptide as claimed in any one of claims 1, 2 and 6 to 17 with a complex-forming metal, in admixture or conjunction with a pharmaceutically suitable carrier.

10 53. A pharmaceutical preparation which comprises a physiologically tolerable complex of a peptide as claimed in any one of claims 1, 2 and 6 to 17 with zinc phosphate, zinc pyrophosphate and/or zinc hydroxide, in admixture or conjunction with a pharmaceutically suitable carrier.

15 54. A pharmaceutical preparation which comprises a physiologically tolerable complex of the tetracosapeptide of the formula D-seryl - L - tyrosyl - L - seryl - L - methionyl - L - glutamyl - L - histidyl - L - phenylalanyl - L - arginyl - L - tryptophylglycyl - L - lysyl - L - prolyl - L - valylglycyl - L - lysyl - L - lysyl - L - arginyl - L - arginyl - L - prolyl - L - valyl - L - lysyl - L - valyl - L - tyrosyl - L - proline or of the corresponding compound containing the glutamine radical instead of the glutamyl radical, with zinc phosphate, zinc pyrophosphate and/or zinc hydroxide, in admixture or conjunction with a pharmaceutically suitable carrier.

30 55. A pharmaceutical preparation which comprises a physiologically tolerable complex of a peptide as claimed in any one of claims 1, 2 and 6 to 17 with gelatine, in admixture or conjunction with a pharmaceutically suitable carrier.

40 56. A pharmaceutical preparation which comprises a physiologically tolerable complex of a peptide as claimed in any one of claims 1, 2 and 6 to 17 with polyphlorethine phosphate, in admixture or conjunction with a pharmaceutically suitable carrier.

45 57. A pharmaceutical preparation which comprises a physiologically tolerable complex of a peptide as claimed in any one of claims 1, 2 and 6 to 17 with a polymer or copolymer or (an) aminoacid(s) containing a preponderance of (an) acid  $\alpha$ -aminoacid(s), in admixture or conjunction with a pharmaceutically suitable carrier.

50 58. A pharmaceutical preparation which comprises a physiologically tolerable complex of a peptide as claimed in any one of claims 1, 2 and 6 to 17 with poly-glutamic acid whose  $\gamma$ -carboxyl group is free and whose terminal carboxyl group is free or functionally converted, in admixture or conjunction with a pharmaceutically suitable carrier.

60 59. A pharmaceutical preparation which comprises a physiologically tolerable complex of the tetracosapeptide of the formula D-seryl - L - tyrosyl - L - seryl - L - meth-

ionyl - L - glutamyl - L - histidyl - L - phenylalanyl - L - arginyl - L - tryptophylglycyl - L - lysyl - L - prolyl - L - valylglycyl - L - lysyl - L - lysyl - L - arginyl - L - arginyl - L - prolyl - L - valyl - L - lysyl - L - valyl - L - tyrosyl - L - proline or of the corresponding compound containing the glutamine radical instead of the glutamyl radical, with polyphlorethine phosphate, in admixture or conjunction with a pharmaceutically suitable carrier.

70 60. A pharmaceutical preparation which comprises a physiologically tolerable complex of the tetracosapeptide of the formula D-seryl - L - tyrosyl - L - seryl - L - methionyl - L - glutamyl - L - histidyl - L - phenylalanyl - L - arginyl - L - tryptophylglycyl - L - lysyl - L - prolyl - L - valylglycyl - L - lysyl - L - lysyl - L - arginyl - L - arginyl - L - prolyl - L - valyl - L - lysyl - L - valyl - L - tyrosyl - L - proline or of the corresponding compound containing the glutamine radical instead of the glutamyl radical, with polyglutamic acid, in admixture or conjunction with a pharmaceutically suitable carrier.

80 61. A pharmaceutical preparation having a composition substantially as described in any one of Examples 8 to 16 herein.

90 62. A veterinary preparation which comprises a peptide as claimed in any one of claims 1, 2 and 6 to 17 or a physiologically tolerable acid addition salt, derivative or complex thereof, in admixture or conjunction with a carrier suitable for veterinary use.

100 63. Tertiary butyloxycarbonyl - D - seryl - L - tyrosyl - L - seryl - L - methionyl - ( $\gamma$  - tertiary butyl) - L - glutamyl - L - histidyl - L - phenylalanyl - L - arginyl - L - tryptophyl - glycyl - tertiary butyloxycarbonyl - L - lysyl - L - prolyl - L - valylglycyl - tertiary butyloxycarbonyl - L - lysyl - tertiary butyloxycarbonyl - L - lysyl - arginyl - L - arginyl - L - prolyl - L - valyl - tertiary butyloxycarbonyl - L - lysyl - L - valyl - L - tyrosyl - L - proline, or its tertiary butyl ester or amide, or a corresponding compound which contains the glutaminyl radical instead of the glutamyl radical.

110 64. Tertiary butyloxycarbonyl - D - seryl - L - tyrosyl - glycyl - L - methionyl - ( $\gamma$  - tertiary butyl) - L - glutamyl - L - histidyl - L - phenylalanyl - L - arginyl - L - tryptophyl - glycyl - tertiary butyloxycarbonyl - L - lysyl - L - prolyl - L - valylglycyl - tertiary butyloxycarbonyl - L - lysyl - tertiary butyloxycarbonyl - L - lysyl - arginyl - L - arginyl - L - prolyl - L - valyl - tertiary butyloxycarbonyl - L - lysyl - L - valyl - L - tyrosyl - L - proline, or its tertiary butyl ester or amide, or a corresponding compound which contains the glutaminyl radical instead of the glutamyl radical.



5 65. Tertiary butyloxycarbonyl - D - alanyl - L - tyrosyl - L - seryl - L - methionyl - (γ - tertiary butyl) - L - glutamyl - L - histidyl - L - phenylalanyl - L - arginyl - L - tryptophyl - glycyl - tertiary butyloxycarbonyl - L - lysyl - L - prolyl - L - valyl - glycyl - tertiary butyloxycarbonyl - L - lysyl - tertiary butyloxycarbonyl L - lysyl - arginyl - L - arginyl - L - prolyl - L - valyl - tertiary butyloxycarbonyl - L - lysyl - L - valyl - L - tyrosyl - L - proline, or its tertiary butyl ester or amide, or a corresponding compound which contains the glutaminyl radical instead of the glutamyl radical.

15 66. Tertiary butyloxycarbonyl - D - alanyl - L - tyrosyl - L - seryl - L - methionyl - (γ - tertiary butyl) - L - glutamyl - L - histidyl - L - phenylalanyl - L - arginyl - L - tryptophyl - glycyl - tertiary butyloxycarbonyl - L - lysyl - L - prolyl - L - valyl - glycyl - tertiary butyloxycarbonyl - L - lysyl - tertiary butyloxycarbonyl L - lysyl - tertiary butyloxycarbonyl - L - ornithyl - L - prolyl - L - valyl - tertiary butyloxycarbonyl - L - lysyl - L - valyl - L - tyrosyl - L - proline, or its tertiary butyl ester or amide, or a corresponding compound which contains the glutaminyl radical instead of the glutamyl radical.

67. Tertiary butyloxycarbonyl - D - seryl - L - tyrosyl - L - seryl - L - methionyl - (γ - tertiary butyl) - L - glutamyl - L - histidyl - L - phenylalanyl - L - arginyl - L - tryptophyl - glycyl - tertiary butyloxycarbonyl - L - lysyl - L - prolyl - L - valyl - glycyl - tertiary butyloxycarbonyl - L - lysyl - tertiary butyloxycarbonyl L - lysyl - L - arginyl - L - arginyl - L - proline, or its tertiary butyl ester or amide, or a corresponding compound which contains the glutaminyl radical instead of the glutamyl radical.

68. A peptide that differs from an ACTH-active peptide containing 16 to 39 aminoacid residues and having an N-terminal aminoacid residue with L-configuration, each of the α-amino group and the side-chain-amino groups being protected by a tertiary butyloxycarbonyl group and each of the terminal carboxyl group and the side-chain-carboxyl group being protected by a tertiary butyl ester group, solely in that the N-terminal aminoacid residue is the residue of D-serine.

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