

PATENT SPECIFICATION

1003.683



NO DRAWINGS

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Int Cl.:—C 07 c, d

COMPLETE SPECIFICATION

New Polypeptides 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:—

The present invention is concerned with new polypeptides and with their manufacture and use.

The present invention provides a tetrakosapeptide of the formula L - 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 and the corresponding tetrakosapeptide which contains the radical of glutamine instead of the glutamyl radical and their derivatives, acid addition salts, and heavy metal complexes. Heavy metal complexes are especially those of zinc, copper and cobalt.

The present invention provides more especially L - 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 itself, its sparingly soluble zinc complex, and the corresponding glutaminyl compound.

Derivatives are primarily functional derivatives such as esters, for example lower alkyl esters, for example methyl, ethyl, propyl, isopropyl, isobutyl and tertiary butyl esters, amides and hydrazides, as well as N-substitution products such as N-acyl, for example N-lower alkanoyl, more especially N-acetyl derivatives, and compounds containing the usual amino protecting groups.

The new compounds display a considerably adrenocorticotropic activity and are intended to be used in human and veterinary medicine as chemically pure and uniform compounds in place of ACTH. Suitable for the preparation of synthetic corticotropine derivatives having a prolonged activity is in particular the aforementioned sparingly soluble zinc complex. The compounds may also be used as intermediates for the manufacture of medicaments containing a longer chain of amino acids, such as the adrenocorticotropic hormones themselves.

The new tetrakosapeptides are obtained by the methods known for the manufacture of peptides, for which purpose the amino acids may be linked together in the order of succession specified above singly or in the form of preformed small peptide units.

Inter alia, one of the amino acid molecules or peptide molecules in the form of an ester may be linked with a further molecule of an amino acid or peptide containing a protected amino group in the presence of a condensing agent such as a carbodiimide or a phosphorus acid ester halide, or the amino acid ester or peptide ester containing a free amino group may be reacted with an amino acid or a peptide containing an activated carboxyl group (and a protected amino group), for example an acid halide, azide, anhydride, imidazolide, isoxazolide (for example from N-ethyl-

5-phenyl-isoxazolium-3¹-sulphonate; see Woodward et al., J. Am. Chem. Soc., 89 page 1011 [1961]) or an activated ester such as cyanomethyl ester or carboxymethyl thiol ester. Conversely an amino acid or a peptide containing a free carboxyl group (and a protected amino group) can be reacted with an amino acid or a peptide containing an activated amino group (and a protected carboxyl group), for example with a phosphite amide. All the aforementioned methods can be used for forming peptide bonds according to the present invention, but the processes used in the Examples below are particularly advantageous.

As mentioned above, there are various possibilities available for synthesising the tetrakosapeptides from the individual amino acids or small peptide units. According to one process, for example, the decapeptide L - seryl - L - tyrosyl - L - seryl - L - methionyl - L - glutamyl- (or glutamyl) - L - histidyl - L - phenylalanyl - L - arginyl - L - tryptophyl - glycine is condensed with the tetradeka peptide 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 as represented, for example, in Table 1 for the compound containing as the fifth amino acid L-glutamic acid. The decapeptide used as starting material can be prepared by the process described in British Specification No. 992,957. The tetradecapeptide is obtained, for example, by the reaction scheme shown in Table 2.

Throughout this Specification the symbol BOC represents a tertiary butyloxycarbonyl group, the symbol tBu a tertiary butyl group, the symbol iBu an isobutyl group, the symbol Z the benzyloxycarbonyl group, the symbol PZ the paraphenylazobenzyloxycarbonyl group and the symbol T the trityl 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 α,ω -diaminocarboxylic acid radicals, especially lysine radicals, of which the ω -amino group is protected by the tertiary butyloxycarbonyl group are described and claimed in our British Specification No. 957,892.

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Table 1

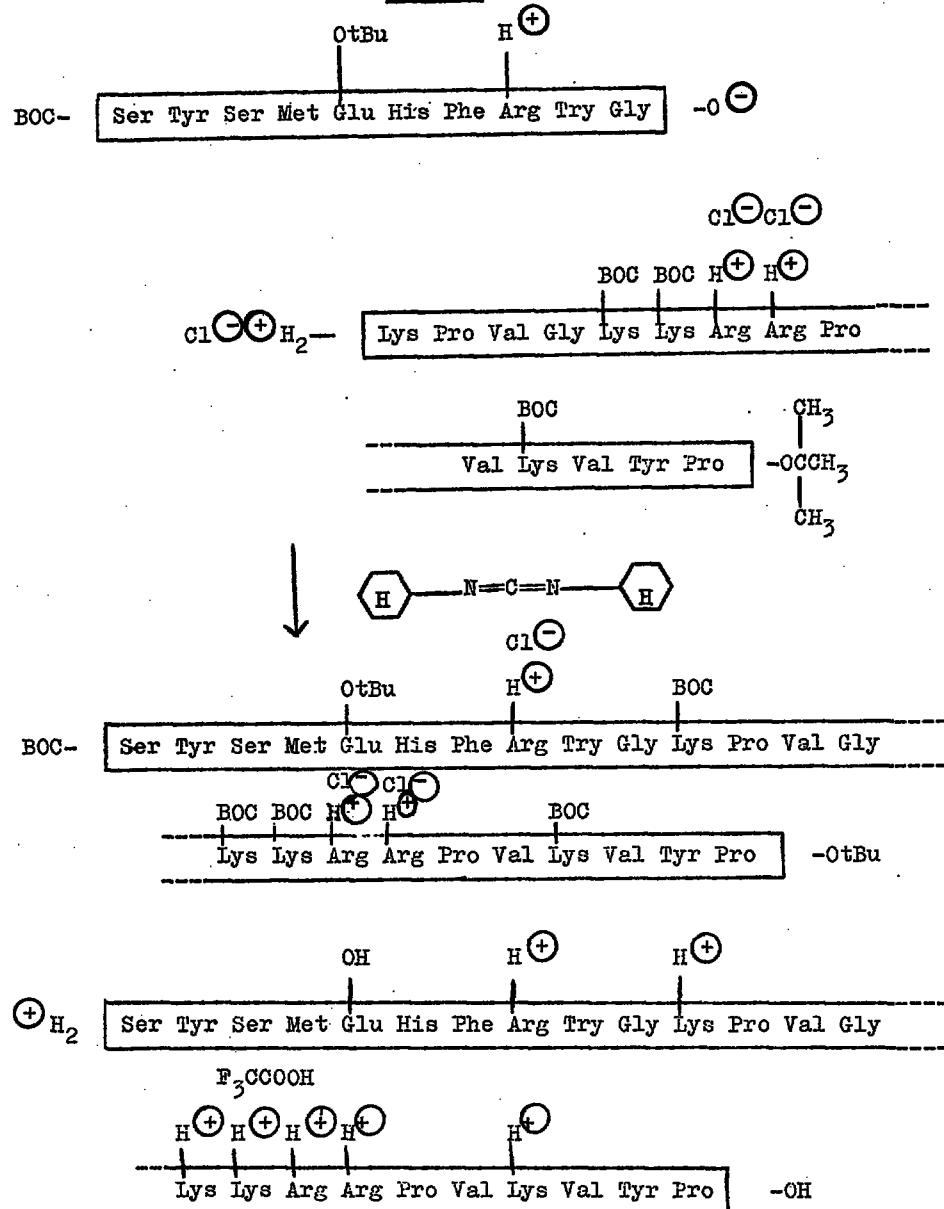
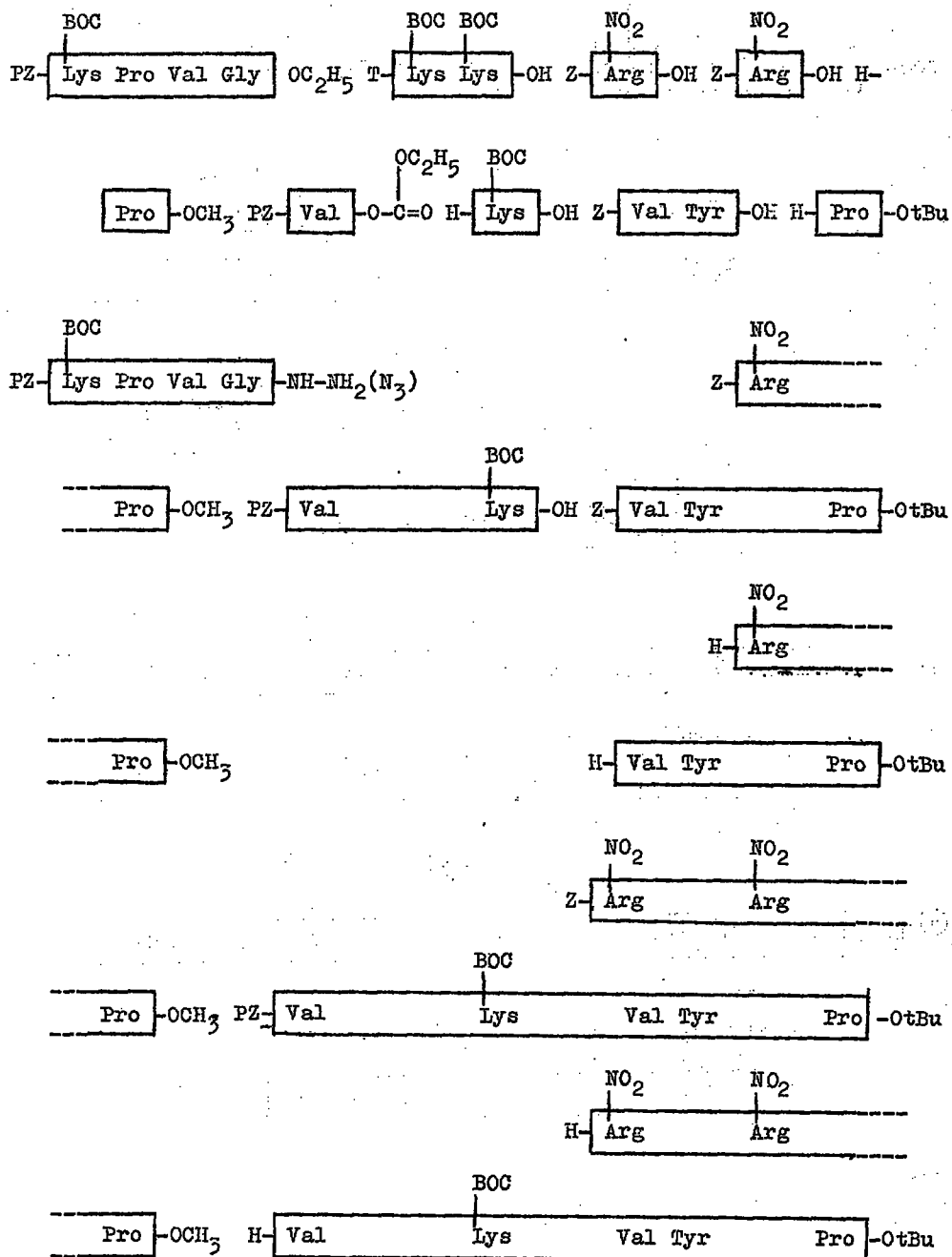
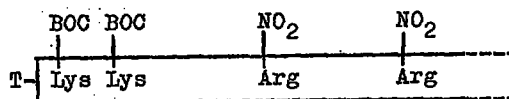
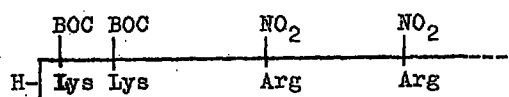
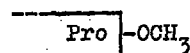
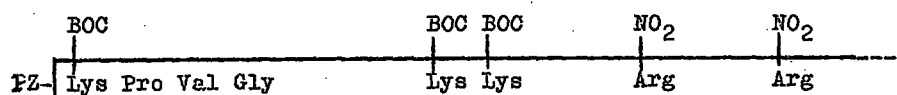
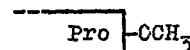
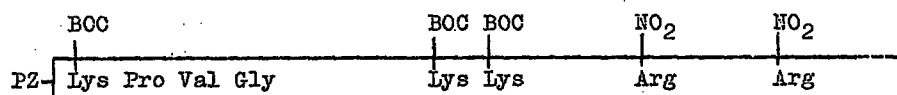
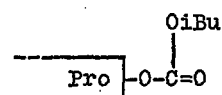
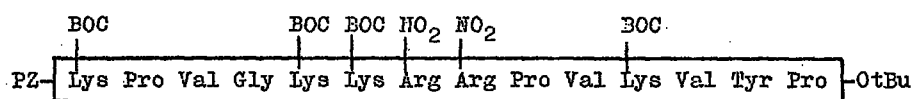
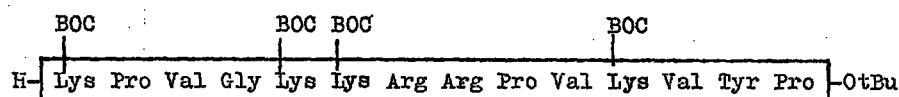


Table 2



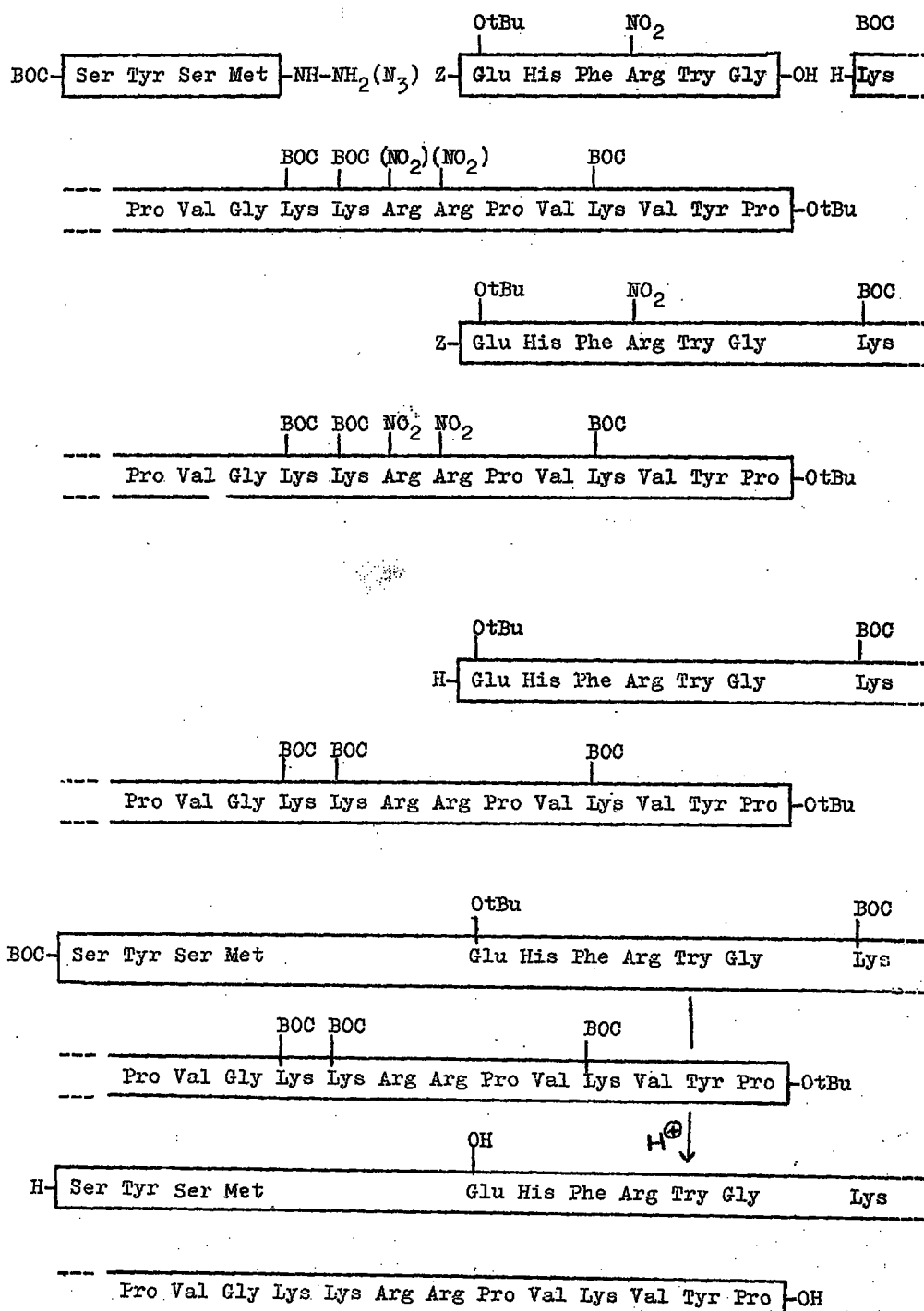


Th tetrakosapeptides are also obtained, for example, by condensing the tetrapeptide L - seryl - L - tyrosyl - L - seryl - L - methionine with the eikosa peptide L - glutamyl - (or glutaminy) - L - histidyl - L - phenylalanyl - L - arginyl - L - tryptophyl - glycyl - L - lysyl - L - prolyl - L - valyl - glycyl - L - lysyl - L - lysyl - L - arginyl - L - arginyl - L - prolyl - L - valyl - L - lysyl - L - valyl - L - tyrosyl - L - proline, for example as shown in Table 3.



methionine

Table 3





The tetrapeptide derivative used as starting material can be prepared by the process described in the aforementioned British Specification No. 992,957 and the hexapeptide by the process of British Specification No. 992,958.

Any free functional groups not participating in the reaction are advantageously protected, more especially by radicals that are easy to eliminate by hydrolysis or reduction, thus the carboxyl group preferably by esterification (for example with methanol, tertiary butanol, a benzyl alcohol or para-nitrobenzyl alcohol), the amino group, for example, by introducing the tosyl or trityl radical or the carbobenzoxy group or a coloured protective group, such as the para-phenylazo-benzyloxy-carbonyl group or the para-(para¹-methoxy-phenylazo)-benzyloxy-carbonyl group or more especially the tertiary butyloxy-carbonyl radical. For protecting the amino group in the guanido grouping of arginine the nitro group may be used, but it is not absolutely necessary to protect the aforementioned amino group of arginine during the reaction.

The conversion of a protected mercapto or amino group into a free group and the conversion of a functionally converted carboxyl group into a free carboxyl group in the course of the process used for the manufacture of the tetrakosapeptides and intermediates is carried out in the known manner by treatment with a hydrolysing or reducing agent respectively.

Depending on the reaction conditions used the new compounds are obtained in the form of bases or of their salts. From the salts the bases can be prepared in a known manner. When the bases are reacted with acids suitable for forming physiologically tolerable salts, there are obtained salts such, for example, as those of inorganic acids such as hydrohalic acids, for example hydrochloric or hydrobromic acid, perchloric, nitric or thiocyanic acid, sulphuric or phosphoric acids or organic acids such as formic, acetic, propionic, glycollic, lactic, pyruvic, oxalic, malonic, succinic, maleic, fumaric, malic, tartaric, citric, ascorbic, hydromaleic, dihydroxymaleic, benzoic, phenylacetic, 4-aminobenzoic 4-hydroxybenzoic, anthranilic, cinnamic, mandelic, salicylic, 4-amino-salicylic, 2-phenoxybenzoic, 2-acetoxy-benzoic, methanesulphonic, ethanesulphonic, hydroxyethanesulphonic, benzenesulphonic, para-toluenesulphonic, naphthalenesulphonic or sulphanilic acid.

The resulting tetrakosapeptide bases may, if desired, be converted into heavy metal complexes thereof.

The tetrakosapeptides and their derivatives, physiologically tolerable acid addition salts and heavy metal complexes of the present invention can 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 such, for example, as gelatine, lactose, glucose, sodium chloride, starches, magnesium stearate, talc, vegetable oils, benzyl alcohols, gums, poly-alkyleneglycols, white petroleum jelly, cholesterol or other known medicinal carriers. The pharmaceutical preparations may be in the form of, for example, tablets, dragees, ointments, powders, creams or suppositories, or in liquid form as solutions, suspensions or emulsions. There may be sterilized and/or may contain emulsifying agents. They may further contain other therapeutically useful substances.

The new tetrakosapeptides and their derivatives, physiologically tolerable acid addition salts and heavy metal salts can be used in the form of veterinary preparations comprising these compounds in admixture or conjunction with a carrier suitable for veterinary use.

The following Examples illustrate the invention.

The following abbreviations are used for the paper chromatographic systems:

- 43 : tertiary amyl alcohol-isopropanol-water (100:40:55);
- 45 : secondary butanol-aqueous ammonia of 3% strength (100:44);
- 49 : secondary butanol-triethylamine-diethyl barbituric acid-water-isopropanol (100:0.8:1.8 grams:60:10);
- 50 : tert.amyl alcohol-isopropanol-triethylamine-sodium-5:5-diethylbarbiturate-water (100:40:0.8:1.8 grams:50);
- 54 : secondary butanol-isopropanol-monochloroacetic acid-water (70:10:3 grams:40).

EXAMPLE 1

1) PZ-Lys-(BOC)-Pro-Val-Gly-NH-NH₂

A mixture of 1.15 grams (1.5 millimols) of PZ - Lys - (BOC) - Pro - Val-Gly-OCH₃, (British Specification No. 957,892), 15 cc of absolute methanol and 0.6 cc of hydrazine hydrate is refluxed for one hour, evaporated to dryness and the hydrazide

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is precipitated with much ether. The initially gelatinous product ~~solidified~~ *solidifies* when scratched with a glass rod. It is filtered off and thoroughly washed with ether on the suction filter. After drying over sulphuric acid there are obtained 1.1 grams of PZ-tetrapeptide hydrazide melting at 130 to 131°C.

A specimen recrystallized from acetonitrile has the melting point $F=163-165^{\circ}\text{C}$. The hydrazide is readily soluble in cold acetic acid of 25% strength.

2) Z-Arg(NO₂)-Pro-OCH₃

3.06 grams (23.7 millimols) of proline methyl ester in 20 cc of acetonitrile are added to a solution of 7.18 grams (20.3 millimols) of carbobenzoxy-nitro-L-arginine in 20 cc of dimethylformamide. The mixture is cooled with ice and sodium chloride to about -5° to -10°C and then treated with a solution of 4.9 grams of dicyclohexyl carbodiimide in 12 cc of dimethylformamide:acetonitrile=1:1. The reaction solution is then diluted with 20 cc of ice-cold acetonitrile and kept for 17 hours at 0°C . The urea formed is filtered off, washed with acetonitrile and the solution is treated with 5 drops of glacial acetic acid. After 30 minutes the whole is evaporated to dryness, the residue dissolved in ethyl acetate, the precipitated urea is once more filtered off through cotton wool and the ethyl acetate solution is washed with 3×10 cc of N-hydrochloric acid, then twice with water and finally with N-sodium bicarbonate solution until, when the alkaline extracts are acidified, no turbidity appears; finally the whole is washed with water until neutral.

When the dried ethyl acetate solution is concentrated to a small volume, the carbobenzoxy dipeptide ester *precipitates* substantially quantitatively. Yield: 6.16 grams (65.5% of the theoretical). The product melts at 155 to 157°C after having sintered at 153°C .

3) H-Arg(NO₂)-Pro-OCH₃

8.4 grams of Z-Arg(NO₂)-Pro-OCH₃ are dissolved with heating in 27 cc of glacial acetic acid and then treated at room temperature with 27 cc of approximately 4N-hydrogen bromide solution in glacial acetic acid. After one hour the whole is concentrated in vacuum at 40°C to a small volume and the decarbenzoxy solution product is precipitated with much absolute ether. The initially tacky product is treated with absolute ether until it forms a fine powder. For purification the dihydrobromide of the dipeptide ester is once more precipitated from methanol+ether. The pale yellowish compound is dissolved in 4 cc of water, 150 cc of chloroform are added and the mixture is cooled to 0°C in an ice bath. While whirling the mixture vigorously, solid potassium carbonate is added in portions until all the water has been consumed and the potassium carbonate separates in solid form; the latter is once more extracted with fresh chloroform and the combined chloroform extracts are dried over anhydrous potassium carbonate and filtered through a little "Celite" (Registered Trade Mark) in a G₄ glass suction filter. After having evaporated the chloroform at 40°C there are obtained 5.5 grams (=90% of the theoretical yield) of nitro-L-arginyl-proline methyl ester. The compound is further worked up as it is.

4) Z-Arg(NO₂)-Arg(NO₂)-Pro-OCH₃

8.34 grams (25.2 millimols) of H-Arg(NO₂)-Pro-OCH₃ in 25 cc of freshly distilled dimethylformamide are combined with a solution of 8.92 grams (25.2 millimols) of Z-Arg(NO₂)-OH, and the mixture is diluted with 50 cc of acetonitrile and then cooled to -10°C . The whole is kept for 30 minutes at -10°C and then treated while being stirred with a solution of 5.71 grams (27.7 millimols) of dicyclohexyl-carbodiimide in 12.5 cc of ice-cold acetonitrile and the whole is allowed to react for 22 hours at 0°C . The urea is filtered off and the filtrate is concentrated in vacuum to a small volume. The syrupy residue is dissolved in chloroform, washed with 3×10 cc of N-hydrochloric acid, then with 2×10 cc of water and finally with N-sodium bicarbonate solution and N-sodium carbonate solution until, when the alkaline extracts are acidified, no more precipitate or turbidity appears. Finally, the chloroform extracts are washed neutral with water and dried over sodium sulphate and evaporated, to yield 10.4 grams (=62% of theory) of crude carbobenzoxy-tripeptide ester.

When a sample of the crude product is subjected to scission with 2N-hydrogen bromide solution in glacial acetic acid for one hour at room temperature there appears in the paper chromatogram in the systems 54 and 49 apart from the tripeptide also a further amount of nitro-arginine and another by-product. For purification 4.6 grams of the crude product are crystallized from 140 cc of butanol. Yield: 2.2 grams of

pure carbobenzoxy-tripeptide ester melting at 120°C with decomposition. Optical rotation $[\alpha]_D^{25} = -43.9^\circ \pm 1^\circ$ ($c = 1.032$ in methanol).

The ultra-violet spectrum of the carbobenzoxy-tripeptide ester displays at 271 m μ the maximum typical of nitroarginine ($\epsilon = 32200$).

5) H-Arg(NO₂)-Arg(NO₂)-Pro-OCH₃

A mixture of 2.19 grams (3.3 millimols) of Z-Arg(NO₂)-Arg(NO₂)-Pro-OCH₃ and 13.2 cc of 2N-hydrobromic acid solution in glacial acetic acid is decarboxylated for one hour at room temperature. The excess acid is evaporated and the decarboxylation product is precipitated with much absolute ether. The crude product is dissolved in 2 cc of water, extracted twice with fresh ethyl acetate, the ethyl acetate phases are once again washed with water and the combined aqueous solutions are poured over a column of ion-exchanger Merck II. The free tripeptide ester is eluted with 200 cc of water and the water is then evaporated in vacuum at 40°C. Yield: 1.3 grams (= 74% of the theoretical).

The paper chromatogram in the systems 43, 49 and 54 of the free tripeptide ester produces with ninhydrin only one positive spot each. R_f values: 43/0.42, 49/0.67 and 54/0.49.

6) T-Lys(BOC)-Lys(BOC)-Arg(NO₂)-Arg(NO₂)-Pro-OCH₃

1.07 grams (2.01 millimols) of H-Arg(NO₂)-Arg(NO₂)-Pro-OCH₃ in 16.5 cc of a 1:1 mixture of dimethylformamide and acetonitrile are cooled to -10°C. While stirring the mixture vigorously there are rapidly added, 1.44 grams of T-Lys(BOC)-Lys(BOC)-OH (afore-mentioned British Specification No. 957,892), the whole is diluted with 10 cc of previously cooled acetonitrile and after 10 minutes 456 mg (2.2 millimols) of dicyclohexyl carbodiimide in 5 cc of ice-cold acetonitrile are added. The mixture is allowed to react for 20 hours at 0°C; the urea is then filtered off and the filtrate is evaporated in vacuum at 40°C. The unreacted tripeptide ester is precipitated with much ethyl acetate and the ethyl acetate solution is then evaporated to dryness and the residue is dissolved in a small amount of acetone. The mixture is filtered through cotton wool and the N-trityl-pentapeptide ester is precipitated with much ether.

A specimen of the trityl-pentapeptide ester split with anhydrous trifluoroacetic acid reveals in the paper chromatogram in the system 49, in addition to the pentapeptide methyl ester ($R_f = 0.29$), a very small amount of the two starting materials [dipeptide-H-lys-lys-OH ($R_f = 0.12$) and tripeptide ester nitro-arginyl-nitro-arginyl-proline methyl ester ($R_f = 0.53$)].

For analysis 200 mg of the product are subjected to a Craig distribution over 100 stages between methanol of 80% strength and a 1:1-mixture of chloroform and carbon tetrachloride. The bulk of the substance (180 mg) is found in stages 16-28 which are combined and once more precipitated from acetone + ether. Melting point: 134-136°C. Optical rotation $[\alpha]_D^{25} = -41.20 \pm 0.4^\circ$ ($c = 2.709$ in methanol). Ultra-violet spectrum: λ_{max} 271 m μ , $\epsilon = 32,500$, in absolute alcohol.

7) H-Lys(BOC)-Lys(BOC)-Arg(NO₂)-Arg(NO₂)-Pro-OCH₃

1.46 grams (1.19 millimols) of N^o-trityl-pentapeptide methyl ester (Example 6) in 50 cc of acetic acid of 75% strength are subjected to splitting for 45 minutes at 30°C; the acetic acid is then evaporated in a high vacuum at 30°C and the residue is distributed between acetic acid of 1% strength and ether. On evaporation of the ethereal solution a quantitative yield of triphenyl carbinol is obtained. The acetic acid solution is likewise evaporated in a high vacuum at 40°C and the residue distributed in a separating funnel between butanol and N-sodium carbonate solution. The pH value of the aqueous phase must be 8.5. The butanol extracts are washed with water until neutral and then dried over sodium sulphate. Yield: 1.03 grams (= 88% of the theoretical).

The compound is used for further working up without first having been purified.

8) PZ-Lys(BOC)-Pro-Val-Gly-Lys(BOC)-Lys(BOC)-Arg(NO₂)-Arg(NO₂)-Pro-OCH₃

A mixture of 900 mg (1.2 millimols) of PZ-Lys(BOC)-Pro-Val-Gly-hydrazide and 10 cc of dimethylformamide is cooled to -10°C. 4 cc of N-hydrochloric acid are then slowly run in, whereupon 1.4 cc of ice-cold N-sodium nitrite solution are vigorously stirred in dropwise at -10°C. After 30 seconds the azide begins to separate out as a sticky substance. The mixture is allowed to react for another 3 minutes at -10°C and then treated with 150 cc of ice water. The tacky azide, which is



seconds

difficult to filtrate, is extracted with ice-cold ethyl acetate and the ethyl acetate phases are washed 3 times with water until neutral, then dried in the cold over magnesium sulphate and filtered through a cold G4-glass suction filter into an ice cooled solution of 1.03 grams (about 1 millimol) of H-Lys(BOC)-Lys(BOC)-Arg(NO₂)-Arg(NO₂)-Pro-OCH₃. The mixture is allowed to react for 22 hours at 0°C and then for 3 hours at 30°C. The reaction solution is washed with 40 cc of water, then with 4 × 10 cc of acetic acid solution of 1% strength, then with 5 × 10 cc of N-sodium bicarbonate solution and finally with water and saturated sodium chloride solution. On evaporation of the dried solution the nonapeptide derivative settles out. Yield: 1.70 grams of crude product.

For purification 1.52 grams of the crude product are dissolved in a small amount of chloroform and then filtered through a column of 45 grams of silica gel. One reprecipitation of the eluate from 10 cc of chloroform with ether yields 1.06 grams of PZ-nonapeptide methyl ester, melting at 134 to 140°C with decomposition.

In the thin-layer chromatogram (silica gel G; product of Merck) in the system dioxan:water=9:1 only one substance of R_f value 0.75 can be identified. In the systems chloroform:acetone=7:3 and benzene:acetone=1:1 the compound remains at the starting point. The substance, crystallized from acetonitrile, melts at 136—138°C; it exhibits in the UV-spectrum in ethanol maxima at $\lambda=272\text{ m}\mu$ ($\epsilon=26600$) and $\lambda=320\text{ m}\mu$ ($\epsilon=22400$).

9) PZ - Lys(BOC) - Pro - Val - Gly - Lys(BOC) - Lys(BOC) - Arg(NO₂)-Arg(NO₂)-Pro-OH

A mixture of 1.06 grams (0.62 millimols) of PZ-nonapeptide ester (cf. 8 above), 6 cc of dioxan of 75% strength and 1.24 cc of 1.95N-sodium hydroxide solution is hydrolysed for 15 minutes at room temperature. The reaction solution is then poured into 110 cc of ice water containing 2.5 cc of N-hydrochloric acid and the floccular precipitate is filtered through a G3 glass suction filter. The precipitate is thoroughly washed with water and then dried over phosphorus pentoxide in a high vacuum to yield 970 mg of an amorphous product. R_f value in the thin-layer chromatogram=0.52 for dioxan:water=9:1.

200 mg of the product distributed over 121 stages in the system methanol:water:chloroform:carbon-tetrachloride=8:2:5:5 yield 162 mg of the pure peptide derivative of K-value=0.65.

Ultra-violet spectrum in absolute alcohol: λ_{max} 320 m μ ($\epsilon=21700$) and 271 m μ ($\epsilon=37200$).

The substance, crystallized from acetonitrile, melts at 140—145° with decomposition.

10) PZ-Val-Lys(BOC)-OH

A mixture of 4.75 grams (13.4 millimols) of PZ-valine and 65 cc of absolute dioxan is cooled in ice water in a manner such that part of the dioxan is solid. There are then added 3.45 cc (14.4 millimols) of N-tributylamine and after another 5 minutes 1.38 cc (13.4 millimols) of chloroformic acid ethyl ester and the whole is allowed to react for 15 minutes while being cooled.

4 grams (17.2 millimols) of N^ε-BOC-lysine are slowly stirred into 32 cc of water containing 2.45 cc (17.2 millimols) of N-triethylamine. The last portions of N^ε-BOC-lysine do not dissolve readily. On cooling with ice a small amount of solid matter separates again from the aqueous solution. This solution is rapidly added with vigorous stirring and cooling to the freshly prepared solution of the mixed anhydride and the mixture is allowed to react for 30 minutes at room temperature. The reaction solution is concentrated in vacuum at 40°C to a small volume and then treated, while being cooled with ice, with 100 cc of water and 40 cc of citric acid solution of 10% strength. When the smeary precipitate is rubbed with ether it turns solid. The crude PZ-valyl-N^ε-BOC-lysine is filtered off, washed copiously with water and ether and dried in a high vacuum at 50°C. Yield: 4.41 grams. Melting point: 167—169°C, (after sintering at 165°C).

The ethereal phase is separated and dried over sodium sulphate. When the ether is evaporated, another 1.07 grams of the PZ-dipeptide separate; melting point 167—169°C.

The total yield amounts to 5.48 grams (=70% of the theoretical). After having been crystallized once from ethyl acetate the analytical fraction melts at 167—169°C.

The ultra-violet spectrum in absolute alcohol displays maxima at 230 m μ ($\epsilon=13400$) and at 322 m μ ($\epsilon=23000$).



product.



11) Z-Val-Tyr-Pro-OtBu

11.25 grams (27.4 millimols) of carbobenzoxy-valyl-tyrosine (British Patent No. 876,571), in 100 cc of freshly distilled acetonitrile are mixed with a solution of 4.65 grams (27.4 millimols) of proline tertiary butyl ester in 25 cc of acetonitrile and cooled in an ice bath to 0°C. A solution of 6.21 grams of dicyclohexyl carbodiimide in 10 cc of cold acetonitrile is then added and the mixture is allowed to react for 15 hours at 0°C. The urea which crystallizes out is filtered off (yield: 90% of the theoretical) and the reaction solution is mixed with 1 cc of glacial acetic acid. After 15 minutes the acetonitrile is evaporated in vacuum, the residue is dissolved in ethyl acetate and the precipitated urea is once more filtered off. The ethyl acetate solution is extracted with 2 × 10 cc of ice-cold 2N-hydrochloric acid, and then with 2N-sodium carbonate solution until an acidified sample no longer produces a precipitate, and finally with water until neutral. The ethyl acetate extracts are dried over sodium sulphate and evaporated under diminished pressure. Yield: 14.1 grams (=88% of the theoretical) of amorphous carbobenzoxy-tripeptide ester.

This carbobenzoxy-tripeptide ester is readily soluble in most organic solvents except ether, petroleum ether and benzene. It is further worked up without first having been purified.

12) H-Val-Tyr-Pro-OtBu

14.13 grams (24.9 millimols) of Z-Val-Tyr-Pro-OtBu in 250 cc of methanol of 90% strength containing 4.5 cc of glacial acetic acid are subjected to hydrogenolytic splitting in the presence of 2 grams of palladium-carbon catalyst of 10% strength. The liberated carbon dioxide is absorbed with potassium hydroxide solution in a second interposed duck-shaped hydrogenation vessel. After 2 hours no more hydrogen is being taken up. The catalyst is filtered off and the filtrate is evaporated to dryness in vacuum at 40°C; the residue is distributed between 200 cc of ethyl acetate and 2 × 20 cc of ice-cold 2N-sodium carbonate solution. The sodium carbonate solutions are once more extracted with fresh ethyl acetate and the ethyl acetate extracts are washed with water until neutral and then dried over sodium sulphate. Evaporation in vacuum yields 7.69 grams (=71% of theory) of the free tripeptide ester.

In the paper chromatogram in the systems 43, 45 and 54 the tripeptide ester migrates with the solvent front and produces with ninhydrin a Pauly reagent one spot each.

13) PZ-Val-Lys(BOC)-Val-Tyr-Pro-OtBu

A solution of 10.36 grams (17.7 millimols) of PZ-Val-Lys(BOC)-OH and 7.69 grams (17.7 millimols) of H-Val-Tyr-Pro-OtBu in 140 cc of freshly distilled dimethylformamide is treated at 0°C with 4.2 grams of dicyclohexyl carbodiimide (15% excess) in 12 cc of dimethylformamide, and the whole is kept for 2 days at 0°C. The urea is filtered off and the filtrate mixed with 0.5 cc of glacial acetic acid and left to stand for another 30 minutes. The solvent is then evaporated in vacuum to leave a small volume and the syrupy residue is dissolved in much ethyl acetate. The ethyl acetate phase is washed with 4 × 25 cc of 0.2N-ammonium hydroxide solution, 2 × 30 cc of water, 2 × 30 cc of ice-cold citric acid solution of 10% strength and finally with water until it is neutral. After drying with sodium sulphate and evaporating the solvent there are obtained 17 grams of crude amorphous reaction product. For purification the crude product is dissolved in 100 cc of chloroform and the solution is poured over a silica gel column (570 grams; diameter 5.6 cc, 41 cm high) deactivated with 10% of water. On elution with chloroform the orange-red zone migrates slowly, whereas with 2:1 chloroform+ethyl acetate 2 fractions can be eluted.

The first fraction (4.8 grams) is still considerably contaminated with dicyclohexylurea and PZ-dipeptide and can be crystallized from acetonitrile only in a poor yield, whereas the second fraction (7.2 grams) is again obtained as a gelatinous precipitate from 200 cc of acetonitrile. Yield: 5.1 grams of PZ-pentapeptide ester melting at 154—158°C.

The analytically pure fraction, obtained by a further crystallization, melts at 157—159°C.

The ultra-violet spectrum of the compound in absolute alcohol displays maxima at 227 mμ ($\epsilon = 20700$) and 322 mμ ($\epsilon = 21100$).

In the thin-layer chromatogram (silica gel G; product of Merck) the R_f -values revealed are:

0.23 (chloroform + acetone 95:5) and

0.60 (benzene + acetone 1:1).

14) H-Val-Lys(BOC)-Val-Tyr-Pro-OtBu

A mixture of 1.4 grams of PZ-Val-Lys(BOC)-Val-Tyr-Pro-OtBu, 100 cc of methanol and 400 mg of palladium-carbon catalyst of 10% strength is shaken for 6 hours in an autoclave with hydrogen under 5 atmospheres pressure. The catalyst is filtered off, repeatedly washed with methanol and the solvent is evaporated in vacuum. The residue is triturated with much ether and dried in a high vacuum. 980 mg of a fine, amorphous powder is obtained.

In the systems 54 and 49 the compound migrates with the solvent front.

The R_f value is 0.65 in the thin-layer chromatogram (silica gel G) in the system dioxane + water 9:1.

15) PZ-Lys(BOC)-Pro-Val-Gly-Lys(BOC)-Lys(BOC - Arg(NO₂) - Arg(NO₂) - Pro-Val-Lys(BOC)-Val-Tyr-Pro-OtBu

276 mg (0.16 millimol) of PZ-Lys-Pro-Val-Gly-Lys(BOC)-Lys(BOC)-Arg(NO₂)-Arg(NO₂)-Pro-OCH₃ (dried over phosphorus pentoxide in a high vacuum at 80°C) are dissolved in a mixture of 1 cc of absolute dimethylformamide and 2 cc of absolute tetrahydrofuran and the whole is cooled to -10°C in a cooling bath of ice and sodium chloride. 0.16 millimol of triethylamine in 1.6 cc of tetrahydrofuran is then added and after 5 minutes 0.16 millimol of chloroformic acid isobutyl ester in 1.6 cc of absolute tetrahydrofuran is introduced. The whole is allowed to react in the cooling bath for 15 minutes and then treated with a solution of 140 mg of H-Val-Lys(BOC)-Val-Tyr-Pro-OtBu in 2 cc of absolute tetrahydrofuran, stirred for 15 minutes in an ice bath and then for 1 hour at room temperature; the solvent is then evaporated in vacuum at 40°C and the reaction product is precipitated with much ether. The dried crude product (395 mg) in 4 cc of alcohol-free chloroform is poured over a column of alumina (activity III; 40 grams) and eluted with 100 cc of chloroform. The yellow zone containing the peptide derivative migrates slowly. The whole is then eluted with 80 cc of chloroform + methanol 95:5, to yield 290 mg of chromatographically unitary product. R_f value (dioxan + water 9:1) = 0.75 in the thin-layer chromatogram.

The substance melts, when crystallized from acetonitrile, at 160—165°C. The UV-spectrum in ethanol exhibits maxima at $\lambda = 272 \text{ m}\mu$ ($\epsilon = 36800$) and $319 \text{ m}\mu$ ($\epsilon = 20400$).

16) H-Lys(BOC)-Pro-Val-Gly-Lys(BOC)-Lys(BOC) - Arg - Arg - Pro - Val - Lys(BOC)-Val-Tyr-Pro-OtBu, 3 CH₃COOH.

A mixture of 320 mg of PZ-tetradeka-peptide tertiary butyl ester (cf. 14 above), 6 cc of acetic acid of 90% strength and 100 mg of palladium-carbon catalyst of 10% strength is shaken for 5 hours with hydrogen under a pressure of 5 atmospheres (gauge). The reaction solution is then poured into a duck-shaped hydrogenation vessel and shaken for another 17 hours with 100 mg of fresh palladium catalyst under normal conditions. To absorb the carbon dioxide formed, another duck-shaped hydrogenation vessel filled with potassium hydroxide solution is interposed. The catalyst is then filtered off and thoroughly washed with acetic acid of 90% strength and with methanol, and the whole is evaporated to dryness in vacuum to yield 220 mg of a white amorphous powder.

The ultra-violet spectrum in absolute alcohol displays at $278 \text{ m}\mu$ the maximum ($\epsilon = 1500$) typical of tyrosin.

In the paper-chromatogram in the systems 49, 50 and 54 the compound migrates with the solvent front and produces positive reactions with ninhydrin and Pauly and Sakaguchi reagents.

17) BOC - Ser - Tyr - Ser - Met - Glu(O - tBu) - His - Phe - Arg - Try - Gly-Lys(BOC) - Pro - Val - Gly - Lys(BOC) - Lys(BOC) - Arg - Arg - Pro - Val-Lys(BOC) - Val - Tyr - Pro - OtBu

210 mg of tetradeka-peptide tertiary butyl ester (cf. 16 above) are rapidly dissolved at 0°C in 10 cc of 0.1N-hydrochloric acid and the resulting solution is subjected to lyophilization in a high vacuum. The fine, white residue is then further dried for 2 hours at 50°C in a high vacuum over phosphorus pentoxide. At the same time,

160 mg (0.11 millimol) of BOC-Ser-Tyr-Ser-Met-Glu(OtBu)-His-Phe-Arg-Try-Gly-OH (British Specification No. 992,957), are dissolved with heating in 1 cc of freshly distilled dimethylformamide, cooled to room temperature and added to the trihydrochloride of the tetradeka peptide, then diluted with another 2.5 cc of dimethylformamide, and after 10—15 minutes a clear solution is obtained, which is kept for 2 hours in an ice bath. 42 mg (0.2 millimol) of dicyclohexyl-carbodiimide in 0.6 cc of ice-cold acetonitrile are then added, and the mixture is allowed to react for 13 hours at 0°C and then for 48 hours at room temperature. The crude reaction product is then precipitated with much ethyl acetate and dried in a high vacuum at 40°C. Yield of crude product: 330 mg.

EXAMPLE 2

H - Ser - Tyr - Ser - Met - Glu - His - Phe - Arg - Try - Gly - Lys - Pro - Val - Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-OH $\text{CH}_3 \cdot \text{COOH}$

100 mg of crude, protected tetrakosapeptide (Example 1; still contaminated with starting peptides) are treated for 1 hour at room temperature with 2 cc of anhydrous trifluoroacetic acid. The excess acid is evaporated in vacuum at room temperature and the resulting residue is triturated with much absolute ether. The amorphous scission product is subjected to continuous high voltage electrophoresis (700 Volts; 30 milliAmperes) in 4.5 cc of 0.5 N-acetic acid. Rate of application: 1 cc per hour. In all, 23 fractions are collected. The fractions 1—9, 10, 12—13, 14—15, 16 and 17—23 are evaporated. As revealed by electrophoretic analysis, the bulk of the desired tetrakosapeptide is contained in fractions 12—15. For purification the combined fractions 12—15 are dissolved in 2 cc of 0.01-molar ammonium acetate buffer and poured over a column of carboxymethylcellulose (diameter: 1 cm, height: 1.16 cm; 2.5 grams). The carboxymethylcellulose is introduced into the column with 100 cc of 0.01-molar ammonium acetate buffer and then eluted with another 50 cc of the same buffer. The peptide is then eluted from the column with ammonium acetate buffer (pH=5.4) of increasing molarity (0.1-m to 0.7-m).

Fractions of 10 cc volume each are collected in an automatic fraction collector. After 15 fractions, the peptide has been quantitatively removed from the column. A total of 15 fractions is obtained. The vessels 10 to 13 contain electrophoretically pure tetrakosapeptide. The path covered after 1 hour at 3000 Volts and pH=1.9 is 13 to 17 cm.

In the in-vitro test according to Saffran and Schally the peptide obtained reveals a considerable adrenocorticotrophic activity.

EXAMPLE 3

A solution of 5.1 mg (about 1.5×10^{-8} mol) of the above tetrakosapeptide in 2.0 ml of 0.1N-potassium chloride solution is treated with 0.020 ml of 0.1N-hydrochloric acid and 0.020 ml of 0.1-molar zinc sulphate solution. The solution thus contains: $\text{HCl}: 2 \times 10^{-3}$; $\text{Zn}^{+2}: 2 \times 10^{-6}$ mol; tetrakosapeptide: about 1.5×10^{-8} mol.

The solution is titrated with 0.1N-sodium hydroxide solution. The titration curve shows a zinc complex with $\text{pK}=8.35$. At and above the pH of 8.35, the sparingly soluble complex begins to precipitate in the form of a very finely divided gel. It is centrifuged off at a pH of 9.5 and washed with water. The complex has a strong ACTH-activity.

EXAMPLE 4

1 injection vial contains:

	a)	b)
30920*	1 mg	3 mg
Mannitol	10 mg	10 mg

Method

30920* and mannitol are dissolved in water for the purpose of injection, so that 1.0 ml of solution contains 1 mg and 3 mg of 30920* respectively and 10 mg of mannitol. The solution is filtered under sterile conditions and is put into sterilized injection vials of 1.0 ml each under aseptic conditions and lyophilized in the conventional manner. The vials are then sealed under aseptic conditions.

*30920 = H - Ser - Tyr - Ser - Met - Glu - His - Phe - Arg - Try - Gly - Lys - Pro - Val - Gly - Lys - Lys - Arg - Arg - Pro - Val - Lys - Val - Tyr - Pro - OH, $\text{CH}_3 \cdot \text{COOH}$.

WHAT WE CLAIM IS:—

1. A process for the manufacture of a tetrakosapeptide of the formula L-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 L-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 a derivative, physiologically tolerable acid addition salt or heavy metal complex thereof, wherein tertiary butyloxycarbonyl-L-seryl-L-tyrosyl-L-seryl-L-methionyl-L-glutamyl-(γ -tertiary butyl ester) (or L-glutamyl)-L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophyl-glycyl-L-lysyl-L-lysyl-L-arginyl-L-arginyl-L-prolyl-L-valyl-L-tyrosyl-L-proline is condensed with N^ε-tertiary butyloxycarbonyl-L-lysyl-N^ε-tertiary butyloxycarbonyl-L-lysyl-L-arginyl-L-arginyl-L-prolyl-L-valyl-N^ε-tertiary butyloxycarbonyl-L-lysyl-L-valyl-L-tyrosyl-L-proline tertiary butyl ester, and, if desired, the protected amino and carboxyl groups are liberated and, if desired, the resulting free tetrakosapeptide is converted into a physiologically tolerable acid addition salt or heavy metal complex thereof.

2. A process as claimed in claim 1, wherein the condensation is carried out in the presence of a carbodiimide.

3. A process for the manufacture of a tetrakosapeptide of the formula L-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-tyrosyl-L-proline, or L-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-tyrosyl-L-proline, or a derivative, physiologically tolerable acid addition salt or heavy metal complex thereof, wherein tertiary butyloxycarbonyl-L-seryl-L-tyrosyl-L-seryl-L-methionine azide is condensed with L-glutamyl-(γ -tertiary butyl ester)-L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophyl-glycyl-N^ε-tertiary butyloxycarbonyl-L-lysyl-L-prolyl-L-valyl-glycyl-N^ε-tertiary butyloxycarbonyl-L-lysyl-L-arginyl-L-arginyl-L-prolyl-L-valyl-N^ε-tertiary butyloxycarbonyl-L-lysyl-L-valyl-L-tyrosyl-L-proline tertiary butyl ester, and, if desired, the protected amino and carboxyl groups are liberated and, if desired, the resulting tetrakosapeptide is converted into a physiologically tolerable acid addition salt or heavy metal complex thereof.

4. A process as claimed in claim 1, and conducted substantially as described in Example 1 herein.

5. A process as claimed in claim 1, and conducted substantially as described in Example 2 herein.

6. A process as claimed in claim 1, and conducted substantially as described in Example 3 herein.

7. Tertiary butyloxycarbonyl-L-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-prolyl-L-valyl-tertiary butyloxycarbonyl-L-lysyl-L-valyl-L-tyrosyl-L-proline-tertiary butyl ester.

8. Tertiary butyloxycarbonyl-L-seryl-L-tyrosyl-L-seryl-L-methionyl-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-prolyl-L-valyl-tertiary butyloxycarbonyl-L-lysyl-L-valyl-L-tyrosyl-L-proline-tertiary butyl ester.

9. L-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 a derivative, physiologically tolerable acid addition salt or heavy metal complex thereof.



- 5 10. L - 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 a derivative or physiologically tolerable acid addition salt thereof. 5
- 10 11. A zinc complex of L - 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. 10
12. A pharmaceutical preparation which comprises a compound as claimed in claim 7 or 8 in admixture or conjunction with a pharmaceutically suitable carrier. 15
13. A pharmaceutical preparation which comprises a compound as claimed in claim 9 in admixture or conjunction with a pharmaceutically suitable carrier. 15
14. A pharmaceutical preparation which comprises a compound as claimed in claim 10 in admixture or conjunction with a pharmaceutically suitable carrier. 15
15. A pharmaceutical preparation which comprises a compound as claimed in claim 11 in admixture or conjunction with a pharmaceutically suitable carrier. 15
16. A pharmaceutical preparation having the composition substantially as described in Example 4 herein. 20
- 20 17. A veterinary preparation which comprises a compound as claimed in any one of claims 7 to 11 in admixture or conjunction with a carrier suitable for veterinary use. 20

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