

PATENT SPECIFICATION

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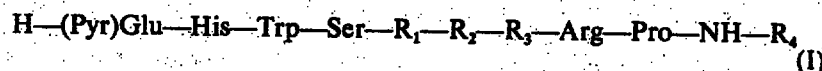
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(54) NONAPEPTIDE AMIDES

(71) We, TAKEDA YAKUHHIN KOGYO KABUSHIKI KAISHA also known as Takeda Chemical Industries Ltd., a joint-stock company organised under the laws of Japan, of 27, Doshomachi 2-chome, Higashi-ku, Osaka, Japan, 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 relates to novel nonapeptide amide derivatives having a strong ovulation inducing activity, which are represented by the formula:



wherein R_1 is Tyr or Phe; R_2 is D-Leu, D-Ile, D-Nle, D-Val, D-Nva, D-Abu, α -Aibu, D-Phe, D-Phe, D-Ser, D-Thr or D-Met; R_3 is Leu, Ile or Nle; and R_4 is alkyl of 1 to 3 carbon atoms which may be substituted with one or more hydroxyl groups.

The present invention relates also to a method for producing the nonapeptide amide derivatives (I).

In the present specification and the claims, amino acids and peptides are designated by abbreviations which are in common usage in the particular field of art or which have been approved by the Committee on Biochemical Nomenclature of IUPAC—IUB. The amino acid is in the L-configuration unless otherwise designated.

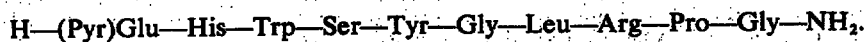
The following abbreviations are used, for instance.

Abu:	α -Aminobutyric acid
α -Aibu:	α -Aminoisobutyric acid
Arg:	Arginine
BOC:	t-Butoxycarbonyl
Bzl:	Benzyl
DCC:	N,N'-Dicyclohexylcarbodiimide
Gly:	Glycine
His:	Histidine
HONB:	N-Hydroxy-5-norbornene-2,3-dicarboximide
HOSu:	N-Hydroxysuccinimide
IBOC:	Isobornyloxycarbonyl
Ile:	Isoleucine
Leu:	Leucine
Nle:	Norleucine
Nva:	Norvaline
Met:	Methionine
OMe:	Methyl ester
OBzl:	Benzyl ester
ONB:	N-Hydroxy-5-norbornene-2,3-dicarboximide ester

	OSu:	N-Hydroxysuccinimide ester
	Phe:	Phenylalanine
	Phg:	α -Phenylglycine
	Pro:	Proline
5	(Pyr)Glu:	Pyroglutamic acid
	Ser:	Serine
	Thr:	Threonine
	Tos:	Tosyl
	Trp:	Tryptophan
10	Tyr:	Tyrosine
	Val:	Valine
	Z:	Benzyloxycarbonyl

Referring to the above substituent R₄, the straight chain or branched chain alkyl group of 1 to 3 carbon atoms which may be substituted with a hydroxyl group is exemplified by methyl, ethyl, *n*-propyl, *i*-propyl, hydroxymethyl, 1-hydroxyethyl, 2-hydroxyethyl, 2-hydroxy-*n*-propyl, 3-hydroxy-*n*-propyl and di-(hydroxymethyl)methyl.

It was known for many years that the hypothalamus contains factors which, at a higher level, control the secretion of tropic hormones from the pituitary. Recently, subsequent to the isolation of a thyrotropin-releasing hormone (TRH), a hormone which promotes the secretion of luteinizing hormone has been extracted in pure form from pigs and sheep and shown to be a decapeptide of the structure:



(A. V. Schally *et al.*, Biochem. Biophys. Res. Commun., 43, 1334 (1971); R. Guillemin *et al.*, Proc. Nat. Acad. Sci., U.S.A., 69, 278 (1972)). This finding has been followed by the synthesis of a number of similar peptides and biological tests have also been performed on these analogous peptides. However, even a minor modification in the above amino acid composition seriously diminishes the physiological activity of the peptide and the above chemical structure has been considered to be essential to the genesis of maximal physiological activity [A. V. Schally *et al.*, Biochem. Biophys. Res. Commun., 4, 366 (1972)].

With the above background, we have succeeded in synthesizing nonapeptide amide derivatives (I) and have surprisingly found that these compounds have more potent ovulation inducing activity than the naturally-occurring decapeptide. We have also found that those compounds act upon the pituitary to promote the secretion of both the luteinizing hormone and the follicle-stimulating hormone. We have further found that these compounds are useful not only as drugs for human beings, e.g. drugs for the diagnosis of the pituitary function or the gonadotropin deficiency and the therapy of amenorrhea, but also as veterinary drugs particularly for the purpose of animal breeding. The present invention is the culmination of those unexpected findings.

Thus, the present invention provides novel nonapeptide amide derivatives (I) which have a strong ovulation inducing activity; and a method for the production of the nonapeptide amide derivatives (I).

Further features of the present invention will be apparent from the following description.

The nonapeptide amide derivative (I) is produced by a method in which a reagent (A) L-Pyroglutamic acid, a protected L-glutamic acid (as hereinafter defined) or a peptide fragment which has an L-pyroglutamic acid unit or a protected L-glutamic acid unit at its N-terminal end is condensed with a reagent (B) an amine or peptide component wherein the reagent (A) and (B) taken together correspond to the amino acid sequence of (I), the active groups of the reagents (A) and (B) which are not required for the condensation reaction being optionally protected by a protecting group or groups, and wherein the protected L-glutamyl group, if any, is reacted with an acid or base to form the pyroglutamyl group and other protective groups, if any, are removed.

Thus, the reagent (A) is L-pyroglutamic acid or a peptide fragment which has an L-pyroglutamic acid unit at its N-terminal end and at the same time which form that point comprises a partial amino acid sequence of formula (I), and the reagent (B) to be condensed with the reagent (A) is an amine component which

corresponds to the balance of the nonapeptide amide derivative (I), the reagents (A) and (B) being optionally protected.

Basic combinations of the reagent (A) and the reagent (B) are exemplified in the following Table 1.

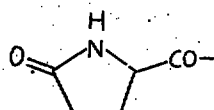
TABLE 1

Reagent	(A)	(B)
Combination		
1	H-(Pyr)Glu-OH	H-His-Trp-Ser-R ₁ -R ₂ -R ₃ -Arg-Pro-NH-R ₄
2	H-(Pyr)Glu-His-OH	H-Trp-Ser-R ₁ -R ₂ -R ₃ -Arg-Pro-NH-R ₄
3	H-(Pyr)Glu-His-Trp-OH	H-Ser-R ₁ -R ₂ -R ₃ -Arg-Pro-NH-R ₄
4	H-(Pyr)Glu-His-Trp-Ser-OH	H-R ₁ -R ₂ -R ₃ -Arg-Pro-NH-R ₄
5	H-(Pyr)Glu-His-Trp-Ser-R ₁ -OH	H-R ₂ -R ₃ -Arg-Pro-NH-R ₄
6	H-(Pyr)Glu-His-Trp-Ser-R ₁ -R ₂ -OH	H-R ₃ -Arg-Pro-NH-R ₄
7	H-(Pyr)Glu-His-Trp-Ser-R ₁ -R ₂ -R ₃ -OH	H-Arg-Pro-NH-R ₄
8	H-(Pyr)Glu-His-Trp-Ser-R ₁ -R ₂ -R ₃ -Arg-OH	H-Pro-NH-R ₄
9	H-(Pyr)Glu-His-Trp-Ser-R ₁ -R ₂ -R ₃ -Arg-Pro-OH	NH ₂ -R ₄

It has also been known that a protected L-glutamyl group shown by the general formula (II):



[wherein R₃ is an alkoxy group (e.g. methoxy, ethoxy, *n*-propoxy, *i*-propoxy, *n*-butoxy), an aralkyloxy group (e.g. benzyloxy) or amino] is easily converted to the L-pyroglutamyl group:



by contact with a base (e.g. ammonia) or an acid (e.g. acetic acid) and that the group (II) is equivalent to the L-pyroglutamyl group itself in this respect. In the method of the present invention, it is to be understood that the L-pyroglutamyl

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(i.e. H—(Pyr)Glu—) of the reagent (A) includes not only the L-pyroglutamyl group itself but also the protected L-glutamyl group of the formula (II). In the case in which (Pyr)Glu— of the reagent (A) is in the form of the group (II), the group (II) is easily converted to the L-pyroglutamyl group itself in accordance with *per se* known means.

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The condensation reaction according to this invention can be carried out by condensing means known for the formation of peptide linkages. Among such means of condensation are the DCC/HONB [Belgian Patent No. 796,399], the azide process, the chloride process, the acid anhydride process, the mixed acid anhydride process, the DCC process, the active ester process, the Woodward reagent K process, the carbodiimidazole process and the oxidation-reduction process [The Peptides, Vol. 1 (1966), Schröder and Lubke, Academic Press, New York, U.S.A.].

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Prior to the condensation reaction, one may protect the carboxyl and amino groups which are not involved in the contemplated reaction or activate the carboxyl or/and amino groups which will take part in the reaction, by means which are known *per se*. The carboxyl groups in the starting material may be protected in the form of metal salts (e.g. sodium and potassium salts) or esters (e.g. methyl, ethyl, benzyl, *p*-nitrobenzyl, *t*-butyl or *t*-amyl esters).

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Protective groups for the amino groups in the starting materials may be any of the conventional protecting groups of amino groups in peptide synthesis, e.g. benzyloxycarbonyl, *t*-butoxycarbonyl, isobornyloxycarbonyl.

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The hydroxyl group of serine may be protected with a conventional protective group such as benzyl, *t*-butyl or other ether-forming groups. The hydroxyl group of tyrosine may be protected with benzyl, *t*-butyl or other ether-forming groups; the guanidino group of arginine may be protected with such groups as nitro, tosyl, carbobenzoxy, isobornyloxycarbonyl or adamantyloxycarbonyl. As examples of activated carboxyl groups in starting materials, there may be mentioned the corresponding acid anhydride, azide, active esters (esters with alcohols or phenols (e.g. pentachlorophenol, 2,4,5-trichlorophenol, 2,4-dinitrophenol, cyanomethyl alcohol, *p*-nitrophenol, N-hydroxy-5-norbornene-2,3-dicarboximide, N-hydroxysuccinimide, N-hydroxyphthalimide or N-hydroxybenzotriazole). The activated amino groups in starting materials may be for example the corresponding phosphoric acid amides.

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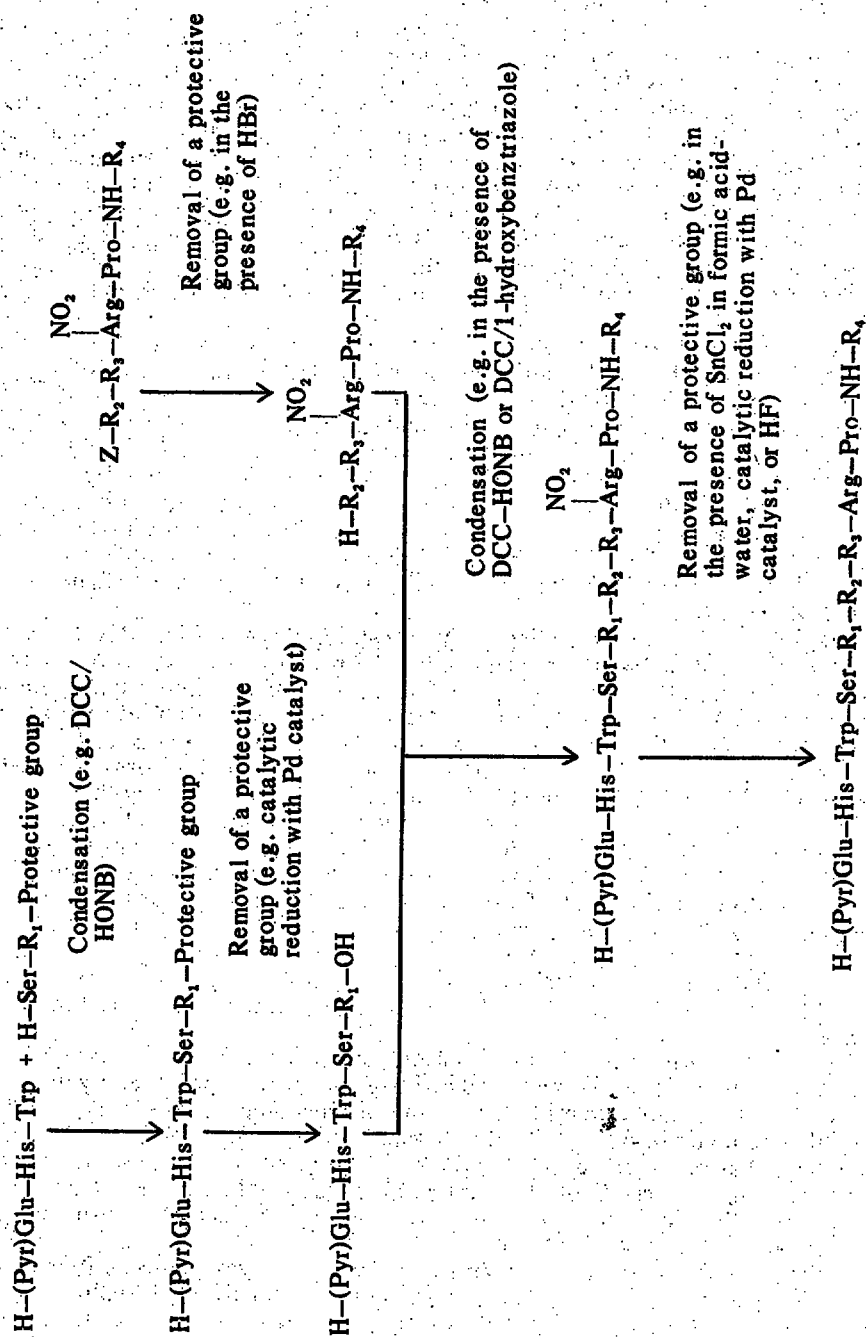
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The following table shows examples of combinations of such forms of carboxyl and amino groups in materials (A) and (B).

TABLE 2

Examples of combinations	(A)		(B)	
	COOH	NH ₂	COOH	NH ₂
1*	Free	Protected	Protected	Free
2	Activated	Protected	Free	Free
3	Free	Protected	Protected	Activated

(Note) In the case designated by an asterisk*, a dehydrating agent (e.g. a carbodiimide reagent such as dicyclohexyl-carbodiimide) is preferably present in the reaction system. A reaction scheme according to this invention may be written as follows.



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This reaction may be conducted in the presence of a solvent. The solvent is generally chosen from those known to be useful for peptide condensation reactions. Thus, anhydrous or aqueous dimethylformamide, dimethylsulfoxide, pyridine, chloroform, dioxane, dichloromethane, tetrahydrofuran and suitable mixtures of such solvents may be mentioned by way of example.

The reaction temperature is generally chosen from within the range known to be employable for reactions leading to the formation of peptide bonds, i.e. preferably within the range of from $-20^{\circ}\text{C}.$ to $30^{\circ}\text{C}.$ Further, the precursor materials (protected peptides) of the contemplated compounds according to this invention may also be easily prepared by solid-phase synthetic processes.

After the condensation reaction has been completed, if the product carries protective groups, they can be removed by conventional procedures. Among such procedures are catalytic reduction in the presence of a catalyst such as palladium black, palladium-on-carbon or platinum, solvolysis by means of hydrogen fluoride or trifluoroacetic acid, and reduction with metallic sodium in liquid ammonia.

The peptide (I) thus produced can be recovered from the reaction product mixture by procedures known for the recovery of peptides, e.g. by extraction, distribution, column chromatography, etc.

The peptide (I) may also be recovered in the form of a salt or metal complex compound.

As acids which are able to form salts with the peptide (I), there may be mentioned inorganic acids such as hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, phosphoric acid, and organic acids such as formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic acid, fumaric acid, anthranilic acid, cinnamic acid, naphthalenesulfonic acid or sulfanylic acid.

The metals which are able to form metal complex compound with the peptide (I) include, among others, Zn^{2+} , Ni^{2+} , Co^{2+} , Cu^{2+} and Fe^{3+} . Such a metal complex compound can be produced by conventional procedures, for example, by reacting the peptide (I) with the hydroxide or oxide of one of the above mentioned metals at a pH of from 6 to 8.

The polypeptides (I) according to this invention have an LH—RH (luteinizing hormone releasing hormone) activity and, accordingly, are able to promote the secretion of LH (luteinizing hormone) and FSH (follicle stimulating hormone). Therefore, the polypeptides (I) are of use as drugs for promoting ovulation in women and in animals (e.g. rats, ewes, sows, cows, mares, quails or hens). The peptides can also be used for other pharmaceutical purposes for which conventional LH—RH, LH and FSH preparations have been employed.

Since the LH—RH activity of polypeptides (I) is about 5 to 60 times that of known naturally-occurring LH—RH, their dosage may be determined for each application on the basis of the above multiple whilst other factors (e.g. the subject of administration or the kind of disease) are also taken into consideration. For example, a suitable dosage may be selected from within the range of 2 ng. to 2 $\mu\text{g}.$ daily per kilogram of body weight.

Polypeptides (I) are primarily administered non-orally (e.g. by injection or by the rectal or vaginal route), although they may be orally administered in certain instances.

The dosage forms which may be employed include, for example, injections, suppositories, pessaries and powders. The injections can be prepared by dissolving from 10 γ to 100 γ of a polypeptide (I) in 1 ml. of physiological saline. Polypeptides (I) can also be made into lyophilized ampoule products with mannitol added as an excipient so that one may administer them as injections for extemporaneous use.

The starting material peptides employable in the method of this invention can be prepared either by known processes for peptide synthesis or by utilizing such processes as are found necessary.

For further illustration of the invention, examples are given as follows:

In those examples, the following abbreviations refer to the R_f value of a thin layer chromatography on silica gel with the following solvent system (ratios specified are by weight):

R _f ¹ :	chloroform-methanol-acetic acid, 9:1:0.5
R _f ² :	ethyl acetate-pyridine-acetic acid-water, 30:10:3:5
R _f ³ :	n-butanol-ethyl acetate-acetic acid-water, 1:1:1:1

The following trade marks are referred to in the Examples:

Biogel P—2: Materials for gel filtration manufactured by BIO-RAD, U.S.A.
 Sephadex LH—20: Esterified dextran gel manufactured by Pharmacia Fine Chemicals, Sweden
 Amberlite XAD—2: Polystyrene resin
 Amberlite IR—45: Weakly basic anion-exchange resin
 Amberlite IRA—410: Strongly basic anion-exchange resin
 Amberlite CG—410: Strongly basic anion-exchange resin
 Amberlite CG—45: Weakly basic anion-exchange resin
 All these resins are manufactured by Rohm & Haas Co. Ltd., U.S.A.

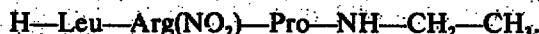
Example 1.

Production of



a) Preparation of $\text{Z}-\text{D}-\text{Leu}-\text{Leu}-\text{Arg}(\text{NO}_2)-\text{Pro}-\text{NH}\cdot\text{CH}_2\text{CH}_3$

In 50 ml. of dichloromethane are dissolved 1.58 g. of $\text{Z}-\text{D}-\text{Leu}-\text{OH}(\text{oil})$ and 1.2 g. of HONB, followed by the addition of 2.8 g. of



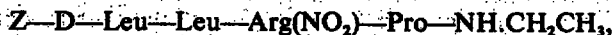
The mixture is chilled to 0°C and, following the addition of 1.4 g. of DCC, the mixture is stirred at room temperature for 8 hours. The by-product dicyclohexylurea is removed by filtration and the filtrate is evaporated to dryness under reduced pressure. The residue is dissolved in 150 ml. of ethyl acetate, washed twice with a 4% aqueous solution of sodium bicarbonate and twice with water, dried over sodium sulfate and finally evaporated to dryness under reduced pressure. The residue is reprecipitated from ethyl acetate-petroleum benzine. The procedure described gives 3.6 g. of the desired compound. Melting point: 134°C (decomp.). $[\alpha]_D^{25} -47.3^\circ$ ($c=0.54$ in methanol); $R_f=0.57$. Elemental analysis, $\text{C}_{33}\text{H}_{53}\text{O}_8\text{N}_9$.

Calcd: C, 56.31; H, 7.59; N, 17.91
 Found: C, 56.08; H, 7.82; N, 17.69

b) Preparation of



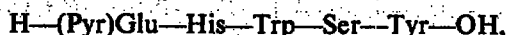
In 30 ml. of 25% hydrogen bromide-glacial acetic acid are dissolved 3.6 g. of



and the solution is swirled at room temperature for 50 minutes. To the solution are added 300 ml. of ether and the resulting precipitate is recovered by filtration. After being dried, the precipitate is dissolved in 50 ml. of water and run onto a column (2.5 x 20 cm) of Amberlite CG—410 (free base). The column is thoroughly washed with 30% aqueous methanol. The effluent and washings are pooled and concentrated under reduced pressure to remove the methanol, followed by lyophilization. The procedure described yields 3.1 g. of the free base of



$R_f=0.24$; $R_f=0.68$. In 4 ml. of dimethylformamide are dissolved 228 mg. of the above product of 290 mg. of



followed by the addition of 144 mg. of HONB. The mixture is chilled to -10°C and 90 mg. of DCC are added. The mixture is stirred at -10°C for 2 hours, at 0°C for 4 hours and at room temperature for 4 hours. The reaction mixture is filtered to remove the dicyclohexylurea. After the addition of ethyl acetate to the filtrate, the resulting precipitate is recovered by filtration. The precipitate is dried and dissolved in 20% aqueous ethanol. The solution is run onto a column of Amberlite XAD—2 (200 mesh (Japan Standards), 3 x 20 cm) and gradient elution is carried out with 20% ethanol and absolute ethanol (300 ml. each).

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The contemplated product emerges in the 260 ml.—360 ml. fractions. These fractions are concentrated under reduced pressure and lyophilized. The procedure gives 346 mg. of the desired product. $[\alpha]_D^{25}$ —38.28° (c=0.1 in 0.5% methanol); R_F^2 =0.235, R_F^3 =0.765.

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c) Preparation of



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In 20 ml. of 60% aqueous formic acid are dissolved 300 mg. of the NO₂-compound obtained in (b) above, followed by the addition of 600 mg. of SnCl₂·H₂O. The mixture is heated at 80—85°C for 2 hours, after which it is evaporated to dryness under reduced pressure. The residue is extracted with 50 ml. of warm water (80°C). The extract is filtered and the filtrate is run onto a column (3 × 20 cm) of Amberlite XAD—2 (200 mesh, Japan Standards). After washing with water, gradient elution is carried out with water and absolute ethanol (400 ml. each). The desired product emerges in the 150 ml. — 260 ml. fractions. These fractions are pooled and concentrated under reduced pressure to remove the ethanol. The concentrate is run onto a column (2 × 33 cm) of carboxy-methyl-cellulose and gradient elution is carried out with 0.005M to 0.2M ammonium acetate (pH 6.8). The desired peptide emerges in the 330 ml.—520 ml. fractions. These fractions are pooled and lyophilized to yield 282 mg. of white fluffy powder. $[\alpha]_D^{24}$ —32.66° (c=0.545 in 5% aqueous acetic acid); R_F^2 =0.10, R_F^3 =0.76. Aminoacid analysis (hydrolysis with 6N HCl in the presence of thioglycolic acid): His, 1.00; Arg, 0.96; Trp, 0.96; Ser, 1.00; Glu, 1.00; Pro, 1.04; Leu, 2.00; Tyr, 1.00.

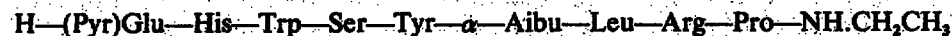
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Example 2.

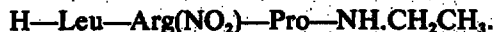
Production of

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a) Preparation of Z—α—Aibu—Leu—Arg(NO₂)—Pro—NH.CH₂CH₃

In 7 ml. of dimethylformamide are dissolved 0.6 g. of Z—α—Aibu—OH and 0.9 g. of



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The solution is chilled to 0°C and 0.71 g. of 1-ethyl-3-(3-dimethylamino)propylcarbodiimide, a water-soluble carbodiimide, is added, followed by stirring for 12 hours. The solvent is distilled off and the residue is dissolved in 100 ml. of chloroform. The solution is washed with 4% sodium bicarbonate and 1N hydrochloric acid, washed with water and dried over magnesium sulfate. The solvent is distilled off under reduced pressure and the residue is treated with ether to obtain a powder which is recovered by filtration.

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The powder is reprecipitated from ethyl acetate-ether to obtain 473 mg. of contemplated compound. Melting point: 104—107°C.

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$[\alpha]_D^{24}$ —45.8° (c=0.55 in DMF). R_F^1 =0.50
Elemental analysis, C₃₁H₅₀O₈N₆·0.5H₂O

Calcd.: C, 54.28; H, 7.50; N, 18.38
Found: C, 54.32; H, 7.32; N, 17.97

b) Preparation of



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In 3 ml. of 25% hydrogen bromide-glacial acetic acid are dissolved 432 mg. of



and the mixture is swirled at room temperature for 40 minutes, after which time 50 ml. of dry ether are added.

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The resulting precipitate is recovered by filtration and dried under reduced pressure over sodium hydroxide in a desiccator. The powder obtained is dissolved in 3 ml. of DMF and the solution is neutralized with 0.24 ml. of N-ethylmorpholine and chilled to 0°C. Then, 557 mg. of BOC—Tyr(Bzl)—OH and 298 mg. of HONB are added to the solution, followed by the addition of 280 mg. of DCC. The mixture is stirred at room temperature for 6 days. The dicyclohexylurea is filtered off and the DMF is distilled off under reduced pressure. The residue is dissolved in

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100 ml. of chloroform, washed with 1N hydrochloric acid and 4% sodium bicarbonate, washed with water and dried over magnesium sulfate, followed by evaporation to dryness under reduced pressure. The residue is crystallized from ethyl acetate.

Yield: 275 mg; melting point: 127–219°C.

$[\alpha]_D^{25}$ —27.6° (c=0.54 in DMF), R_f =0.60

Elemental analysis, $C_{44}H_{67}O_{10}N_{10} \cdot H_2O$

Calcd.: C, 57.82; H, 7.61; N, 15.32
Found: C, 58.32; H, 7.51; N, 15.10

c) Preparation of

BOC—Tyr(Bzl)— α —Aibu—Leu—Arg(NO₂)—Pro—NH.CH₂CH₃,

In 3 ml. of trifluoroacetic acid containing 0.1 ml. of anisole are dissolved 250 mg. of

BOC—Tyr(Bzl)— α —Aibu—Leu—Arg(NO₂)—Pro—NH.CH₂CH₃,

and the solution is swirled at room temperature for 30 minutes. To this are added 30 ml. of dry ether and the resulting precipitate is recovered by filtration and dried. The powder obtained is dissolved in 5 ml. of DMF and the solution is neutralized with 0.08 ml. of N-ethylmorpholine. Then, 150 mg. of BOC—Ser trichlorophenyl ester are added and the solution is stirred for 2 days. DMF is distilled off and the residue is triturated with ether to obtain a powder. The powder is recovered by filtration and reprecipitated twice from ethyl acetate.

Yield: 100 mg.; melting point: 140–142°C (decomp.);

$[\alpha]_D^{25}$ —34.8° (c=0.44 in DMF); R_f =0.74

Elemental analysis: $C_{47}H_{72}O_{12}N_{11} \cdot 3H_2O$

Calcd.: C, 54.43; H, 7.58; N, 14.84
Found: C, 54.66; H, 7.45; N, 14.91

d) Preparation of

H—(Pyr)Glu—His—Trp—Ser—Tyr— α —Aibu—Leu—Arg—Pro—NH.CH₂CH₃,

In 3 ml. of trifluoroacetic acid containing 0.1 ml. of anisole are dissolved 77 mg. of

BOC—Ser—Tyr(Bzl)— α —Aibu—Leu—Arg(NO₂)—Pro—NH.CH₂CH₃,

and the solution is swirled at room temperature for 30 minutes. To this are added 30 ml. of dry ether and the resulting precipitate is recovered by filtration and dried. The resulting powder is dissolved in 3 ml. of DMF and the solution is neutralized with 0.21 ml. of a 10% solution of N-ethylmorpholine in DMF. To this are added 37 mg. of crystals of

H—(Pyr)Glu—His—Trp—OH,

and the mixture is cooled to –10°C, followed by the addition of 21 mg. of HONB and 30 mg. of DCC. The mixture is stirred at 0°C for 12 hours. The dicyclohexylurea is filtered off and the filtrate is evaporated to dryness under reduced pressure. The residue is dissolved in 20% ethanol and adsorbed on a column (1 × 10 cm) of Amberlite XAD—2. Elution is carried out by the gradient method using 20% aqueous through absolute ethanol (200 ml. each). The fractions rich in the desired compound corresponding to the concentration range of aqueous ethanol of 40% to 70% are pooled, concentrated under reduced pressure and lyophilized. The described procedure gives 30 mg. of protected nonapeptide (R_f =0.31). With the addition of 0.1 ml. of anisole and 0.1 ml. of mercaptoethanol, this protected nonapeptide is treated with 3 ml. of anhydrous hydrogen fluoride and the mixture is stirred at 0°C for 60 minutes. The hydrogen fluoride is distilled off under reduced pressure and the residue is dissolved in 10 ml. of water. The solution is extracted three times with 2 ml. of ether and the water layers are pooled and run onto a column (2 × 4 cm) of Amberlite CG—410 (acetate form), which is washed with water. The eluant and aqueous washings are pooled and passed over a column of carboxymethyl-cellulose and a column of Amberlite XAD—2, as described in Example 1.

After lyophilization, there are obtained 14 mg. of a white fluffy powder. $[\alpha]_D^{25} = 54.1^\circ$ ($c = 0.31$ in 5% aqueous acetic acid); $R_f = 0.099$; $R_f = 0.715$. Amino acid analysis (hydrolysis with 6N HCl in the presence of thioglycolic acid): His 0.94; Arg 0.97; Trp 0.81; Ser 1.06; Glu 0.97; Pro 0.97; Leu 1.03; Tyr 1.03; α -Aibu 1.00.

Example 3.

Production of

H—(Pyr)Glu—His—Trp—Ser—Phe—D—Leu—Leu—Arg—Pro—NH.CH₂CH₃,

a) Preparation of H—(Pyr)Glu—His—Trp—Ser—Phe—OH

In 100 ml. of methanol are dissolved 4.0 g. of Z—Ser—Phe—OMe which are reduced at atmospheric pressure using 500 mg. of palladium black as the catalyst for 1.5 hours. The catalyst is removed by filtration and the methanol is distilled off under reduced pressure. The residue is dissolved in 25 ml. of DMF and 4.1 g. of crystals of H—(Pyr)Glu—His—Trp—OH are added. The mixture is chilled to -5°C , followed by the addition of 3.8 g. of HONB and 3 g. of DCC. The mixture is stirred at -5°C for 2 hours, at 0°C for 2 hours and, finally, at room temperature for 8 hours. The by-product dicyclohexylurea is filtered off and the filtrate is evaporated to dryness under reduced pressure. The residue is treated with ethyl acetate and the powder obtained is recovered by filtration (6.7 g.). This powder is dissolved in 30 ml. of 15% methanol-chloroform and the solution is run onto a column (6 x 12 cm) of silica gel prepared with 15% methanol-chloroform.

Elution is carried out using 600 ml. of 15% methanol-chloroform, 1 l. of 25% methanol-chloroform and 1 l. of 30% methanol-chloroform. The fractions between 400 ml. of 25% methanol-chloroform and 700 ml. of 30% methanol-chloroform are pooled and evaporated to dryness under reduced pressure. The residue is treated with ethyl acetate and the resulting powder is recovered by filtration. The procedure gives 4.2 g. of the methyl ester. $R_f = 0.29$.

In 20 ml. of methanol are suspended 3.5 g. of this methyl ester and, at 0°C , 8 ml. of 1N sodium hydroxide are added. The mixture is stirred for 1 hour, and is thereafter neutralized with 8 ml. of 1N hydrochloric acid. The methanol is distilled off under reduced pressure and 20 ml. of cold water are added. The resulting precipitate is recovered by filtration, washed with cooled water and, then, with ethanol-ether (4:6) and dried. The procedure gives 2.32 g. of the desired compound. $[\alpha]_D^{25} = 3.3^\circ$ ($c = 0.55$ in glacial acetic acid); $R_f = 0.61$.

b) Preparation of

H—(Pyr)Glu—His—Trp—Ser—D—Leu—Leu—Arg—Pro—NH.CH₂CH₃,

In 4 ml. of DMF are dissolved 290 mg. of

H—(Pyr)Glu—His—Trp—Ser—Phe—OH

and 228 mg. of

H—D—Leu—Leu—Arg(NO₂)—Pro—NH.CH₂CH₃,

followed by the addition of 130 mg. of HONB. The mixture is chilled to -10°C , after which 90 mg. of DCC are added. The mixture is stirred at -10°C for 2 hours, at 0°C for 3 hours and finally at room temperature for 5 hours.

In exactly the same manner as in Example 1, this reaction mixture is subjected to column chromatography on Amberlite XAD—2. The described procedure gives 342 mg. of protected nonapeptide.

$[\alpha]_D^{25} = 41.74^\circ$ ($c = 0.115$ in methanol); $R_f = 0.265$, $R_f = 0.78$.

In 20 ml. of a 60% aqueous solution of formic acid are dissolved 300 mg. of the above product, followed by the addition of 600 mg. of SnCl₂·H₂O. The mixture is allowed to stand at $80-85^\circ\text{C}$ for 2 hours, after which it is concentrated to dryness under reduced pressure. Then, in exactly the same manner as in Example 1, the concentrate is purified by means of a column of Amberlite XAD—2 and a column of carboxymethyl-cellulose. The procedure gives 211 mg. of a white fluffy powder.

$[\alpha]_D^{25} = 38.49^\circ$ ($c = 0.53$, in 5% aqueous acetic acid); $R_f = 0.12$; $R_f = 0.74$. Amino acid analysis (hydrolysis with 6N HCl in the presence of thioglycolic acid): His 1.00; Arg 1.04; Trp 0.98; Ser 0.92; Glu 1.00; Pro 1.00; Leu 1.04; Phe 1.00; Tyr 0.98.

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Example 4.

Production of

H—(Pyr)Glu—His—Trp—Ser—Tyr—D—Phg—Leu—Arg—Pro—NH.CH₂CH₃,

a) Preparation of Z—D—Phg—Leu—Arg(NO₂)—Pro—NH.CH₂CH₃,

In a mixture of 10 ml. of dimethylformamide and 20 ml. of dichloromethane are dissolved 580 mg. of Z—D—Phg—OH and 1.14 g. of

H—Leu—Arg(NO₂)—Pro—NH.CH₂CH₃,

To this mixture are added 400 mg. of HONB and, under cooling with ice, 500 mg. of DCC are added. The mixture is stirred at room temperature for 4 hours. The by-product dicyclohexylurea is filtered off and the filtrate is evaporated to dryness. The residue is dissolved in 100 ml. of ethyl acetate. The solution is washed with 4% sodium bicarbonate and water, dried over sodium sulfate and distilled to remove ethyl acetate. The residue is reprecipitated from ethyl acetate-petroleum

ether to obtain 1.4 g. of a powder.
R_f¹=0.65; R_f²=0.83
[α]_D²⁵—80.2° (c=0.5 in methanol).

b) Preparation of

H—(Pyr)Glu—His—Trp—Ser—Tyr—D—Phg—Leu—Arg—Pro—NH.CH₂CH₃,

In 20 ml. of acetic acid are dissolved 150 mg. of

Z—D—Phg—Leu—Arg(NO₂)—Pro—NH.CH₂CH₃,

With the addition of 100 mg. of palladium black, gaseous hydrogen is bubbled through the solution at atmospheric pressure to effect reduction.

After 4 hours, the catalyst is filtered off and 0.4 ml. of 1N hydrochloric acid is added. The solution is concentrated to dryness under reduced pressure and the concentrate is dissolved in 20 ml. of water and lyophilized. The resulting powder is dissolved in 8 ml. of dimethylformamide, and 0.25 ml. of a 10% solution of N-ethylmorpholine in dimethylformamide is added dropwise. 141 mg. of

H—(Pyr)Glu—His—Trp—Ser—Tyr—OH

are then added, followed by the addition of 50 mg. of 1-hydroxybenzotriazole and of 40 mg. of DCC at -5°C.

The mixture is stirred at -5°C for 2 hours, at 0°C for 2 hours and at room temperature for 8 hours. Ether is added to the reaction mixture and the resulting precipitate is recovered by filtration and dissolved in 20 ml. of water. The insolubles are filtered off and the filtrate is passed through a column of Amberlite IRA-410 (acetate form) which is then washed with water. The eluant and aqueous washings are pooled and run onto a column (1.5 × 25 cm) of carboxymethyl-cellulose and gradient elution is carried out using 0.005M ammonium acetate and 0.175M ammonium acetate (pH 6.8) (300 ml. each). The contemplated product emerges in the 170 ml. to 240 ml. fractions. These fractions are pooled and run onto a column (2 × 20 cm) of Amberlite XAD-2. The adsorbed compound is eluted by the gradient method using a mixture of water (250 ml.) and ethanol (280 ml.), whereupon the pure compound emerges in the 190 ml. to 240 ml. fractions. The eluate is concentrated under reduced pressure and lyophilized. The described procedure gives 110 mg. of the required product.

R_f¹=0.098, R_f²=0.73.

[α]_D²⁴—57.0° (c=0.56 in 5% aqueous acetic acid).

Aminoacid analysis (hydrolysis with 6N HCl in the presence of thioglycolic acid): His 0.96; Arg 1.04; Trp 0.92; Ser 0.93; Glu 1.00; Pro 1.00; Leu 1.01; Phg 0.98; Tyr 1.01.

Example 5.

Production of

H—(Pyr)Glu—His—Trp—Ser—Tyr—D—Ser—Leu—Arg—Pro—NH.CH₂CH₃,

a) Preparation of Z—D—Ser—Leu—Arg(NO₂)—Pro—NH.CH₂CH₃,

In a mixture of 10 ml. of dioxane and 10 ml. of dimethylformamide are dissolved 955 mg. of Z—D—Ser—OH, 1.82 g. of

H—Leu—Arg(NO₂)—Pro—NH.CH₂CH₃,

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and 788 mg. of HONB. The solution is cooled to 0°C and 907 mg. of DCC are added. The mixture is stirred at 0°C for 2 hours and at room temperature for 5 hours. The by-product dicyclohexylurea is filtered off and the filtrate is evaporated to dryness under reduced pressure. The residue is dissolved in 100 ml. of chloroform. The solution is washed with 0.5N hydrochloric acid and 4% sodium bicarbonate in the order mentioned, dried over magnesium sulfate, and evaporated to dryness under reduced pressure. The residue is triturated with petroleum ether and the resulting powder is recovered by filtration and reprecipitated from ethanol-ether.

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Yield 2.4 g.; melting point: 123°—126° (decomp.)

$[\alpha]_D^{27}$ —49.8° (c=0.5 in methanol).

Elemental analysis, $C_{30}H_{47}O_9N_9 \cdot H_2O$

Calcd.:	C, 51.79;	H, 7.09;	N, 18.12
Found:	C, 52.01;	H, 6.96;	N, 18.04

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$R_f^1=0.40$

b) Preparation of

H—(Pyr)Glu—His—Trp—Ser—Tyr—D—Ser—Leu—Arg—Pro—NH.CH₂CH₃,

In a mixture of 20 ml. of methanol and 1 ml. of glacial acetic acid are dissolved 334 mg. of

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Z—D—Ser—Leu—Arg(NO₂)—Pro—NH.CH₂CH₃,

and, with palladium black as the catalyst, catalytic reduction is carried out at room temperature and at atmospheric pressure for 4 hours. The catalyst is filtered off and the filtrate is concentrated to dryness under reduced pressure. The residue is dissolved by the addition of 9.6 ml. of 0.1N hydrochloric acid and lyophilized.

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The dry powder and 280 mg. of

H—(Pyr)Glu—His—Trp—Ser—Tyr—OH

are dissolved in 3 ml. of DMF, and 100 mg. of 1-hydroxybenzotriazole and 0.615 ml. of a 10% solution of N-ethylmorpholine in DMF are added.

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The mixture is chilled to -5°C, followed by the addition of 165 mg. of DCC. The mixture is stirred at -5°C for 4 hours and at room temperature for 8 hours. The by-product urea derivative is filtered off and the filtrate is evaporated to dryness under reduced pressure. The residue is dissolved in 20 ml. of water and the insolubles are filtered off. The filtrate is run onto a column (10 ml.) of Amberlite IRA-410 (acetate form) and the effluent and washings are pooled and run onto a column (1.5 x 27 cm) of carboxymethyl-cellulose. Elution is carried out by the gradient elution method using 0.005 M ammonium acetate buffer (300 ml) and 0.15 M ammonium acetate buffer (300 ml). The desired compound emerges in the 280 ml.—322 ml. fractions. These fractions are pooled and lyophilized to obtain 135 mg. of crude product. This is dissolved in a small amount of water and placed on a column (1.5 x 17 cm) of Amberlite XAD-2. Elution is carried out by the gradient method using 5% aqueous ethanol (300 ml.) and 60% aqueous ethanol (330 ml.). The 196 ml. to 231 ml. fractions are pooled and, after removal of the ethanol by distillation, lyophilized. Yield: 62 mg.

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This product is dissolved on 0.1 N aqueous acetic acid and subjected to gel filtration on a column (1 x 54 cm) of Sephadex LH-20. After lyophilization, there are obtained 40 mg. of pure required product.

$[\alpha]_D^{27}$ —46.4° (c=0.5 in 5% aqueous acetic acid), $R_f^1=0.055$, $R_f^2=0.70$.

Amino acid analysis (hydrolysis with 6N HCl in the presence of thioglycolic acid): His 1.00; Arg 1.04; Trp 1.00; Ser 1.81; Glu 1.00; Pro 1.00; Leu 1.04; Tyr 0.96; ethylamine 1.04.

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Example 6.

Production of

H—(Pyr)Glu—His—Trp—Ser—Tyr—D—Abu—Leu—Arg—Pro—NH.CH₂CH₃,

a) Preparation of Z—D—Abu—Leu—Arg(NO₂)—Pro—NH.CH₂CH₃,

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In 5 ml. of dimethylformamide are dissolved 0.3 g. of Z—D—Abu—OH and 0.45 g. of H—Leu—Arg(NO₂)—Pro—NH.CH₂CH₃.

The solution is chilled to 0°C and 0.25 g. of HONB and 0.29 g. of DCC are

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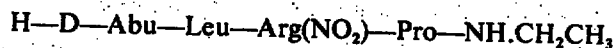
added. The mixture is stirred at 0°C for 1 hour and at room temperature for 5 hours. The urea derivative is filtered off and the filtrate is evaporated to dryness under reduced pressure. The residue is dissolved in 80 ml. of chloroform, washed with 1N hydrochloric acid and 4% sodium bicarbonate.

After washing with water, the residue is dried over magnesium sulfate and evaporated to dryness under reduced pressure. The residue is triturated with ether and the resulting powder is recovered by filtration and reprecipitated from ethyl acetate-petroleum benzene. Yield: 489 mg., melting point: 111–114°C. $[\alpha]_D^{25} -51.2^\circ$ (c=0.6 in methanol); $R_f = 0.66$.

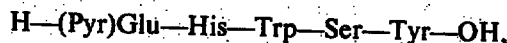
b) Preparation of

H-(Pyr)Glu-His-Trp-Ser-Tyr-D-Abu-Leu-Arg-Pro-NH.CH₂CH₃,
Z-D-Abu-Leu-Arg(NO₂)-Pro-NH.CH₂CH₃ is treated with 25% hydrogen bromide-glacial acetic acid and precipitated with ether.

The precipitate is run onto a column of Amberlite CG-410 (free base) to remove the hydrogen bromide. In 3 ml. of DMF are dissolved 220 mg. of the free base of



thus obtained and 290 mg. of



followed by the addition of 150 mg. of HONB. The mixture is chilled to -10°C and 85 mg. of DCC are added. The mixture is stirred at -10°C for 2 hours, at 0°C for 4 hours and at room temperature for 7 hours. Ethyl acetate is added to the reaction mixture and the resulting precipitate is recovered by filtration. The precipitate is dissolved in 20% aqueous methanol with heating and the solution is cooled, whereupon a precipitate separates out. This precipitate is recovered by filtration and dried. Yield: 284 mg. $R_f = 0.240$, $R_f^2 = 0.772$ (in both solvents, the product contains about 10% of impurity which gives a small R_f value).

In 15 ml. of 60% aqueous formic acid are dissolved 200 mg. of the above product, followed by the addition of 400 mg. of SnCl₂·H₂O. The mixture is heated to 80–85°C for 2 hours. The mixture is then evaporated to dryness under reduced pressure and the residue is extracted with 50 ml. of warm water.

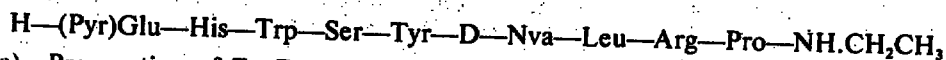
The insolubles are removed by filtration and the filtrate is passed through a column of Amberlite XAD-2, carboxymethyl-cellulose and Sephadex LH-20 in the order mentioned, followed by lyophilization. The described procedure gives 104 mg. of a white fluffy powder.

$[\alpha]_D^{24} -43.6^\circ$ (c=0.50 in 5% aqueous acetic acid), $R_f^2 = 0.08$.

Aminoacid analysis (hydrolysis with 6N HCl in the presence of thioglycolic acid): His 0.98; Arg 0.96; Trp 0.94; Ser 0.92; Glu 1.00; Pro 1.00; Leu 0.98; Abu 1.00; Tyr 1.00; ethylamine 1.02.

Example 7.

Production of



a) Preparation of Z-D-Nva-OH.

In the conventional manner, H-D-Nva-OH is reacted with carbobenzoxy chloride in 2N sodium hydroxide and the resulting compound is recrystallized from ethyl acetate-petroleum benzene. The procedure gives needles melting at 83–84°C.

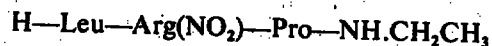
$[\alpha]_D^{25} +12.1^\circ$ (c=1.0 in methanol)

Elemental analysis, C₁₃H₁₇O₄N

Calcd.:	C, 62.14;	H, 6.82;	N, 5.57
Found:	C, 62.17;	H, 6.88;	N, 5.62

b) Preparation of Z-D-Nva-Leu-Arg(NO₂)-Pro-NH.CH₂CH₃,

In 5 ml. of DMF are dissolved 376 mg. of Z-D-Nva-OH, 685 mg. of



and 295 mg. of HONB and, then at 0°C, 340 mg. of DCC are added. The mixture is

stirred at 0°C for 2 hours and at room temperature for 8 hours. The by-product urea derivative is filtered off and 100 ml. of ethyl acetate are added to the filtrate. The mixture is washed with 0.5N hydrochloric acid, 4% sodium bicarbonate and water, and dried over sodium sulfate. The solution is evaporated to dryness under reduced pressure and triturated with ether. The resulting powder is recovered by filtration and purified by reprecipitation from ethyl acetate-ether. Yield: 900 mg., melting point: 105–107°C (decomp); $[\alpha]_D^{25}$ —51.2° (c=1 in methanol); R_f^1 =0.60
Elemental analysis: $C_{32}H_{51}O_8N_9$

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Calcd.: C, 55.71; H, 7.45; N, 18.27
Found: C, 55.54; H, 7.62; N, 18.09

c) Preparation of

H—(Pyr)Glu—His—Trp—Ser—Tyr—D—Nva—Leu—Arg—Pro—NH.CH₂CH₃,

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Starting with the Z—D—Nva—Leu—Arg(NO₂)—Pro—NH.CH₂CH₃, obtained in (b) and H—(Pyr)Glu—His—Trp—Ser—Tyr—OH, the same reaction and purification procedures as in Example 6b are followed to obtain the required compound.

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$[\alpha]_D^{25}$ —39.8° (c=0.51 in 5% aqueous acetic acid); R_f^1 =0.094; R_f^2 =0.74
Aminoacid analysis (hydrolysis with 6N HCl in the presence of thioglycolic acid): His 1.00; Arg 0.98; Trp 0.93; Ser 0.94; Glu 1.08; Pro 1.02; Nva 0.98; Leu 1.00; Tyr 0.98; ethylamine 1.04.

Example 8.

Production of

H—(Pyr)Glu—His—Trp—Ser—Phe—D—Nva—Leu—Arg—Pro—
NH.CH₂CH₂CH₃,

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a) Preparation of Z—D—Nva—Leu—Arg(NO₂)—Pro—NH.CH₂CH₂CH₃,
In 2 ml. of 25% hydrogen bromide-glacial acetic acid are dissolved 720 mg. of
Z—Leu—Arg(NO₂)—Pro—NH.CH₂CH₂CH₃,

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and the solution is shaken for 40 minutes. To this are added 20 ml. of dry ether and the resulting precipitate is recovered by filtration. The precipitate is thoroughly washed with dry ether. It is then dried over sodium hydroxide under reduced pressure in a desiccator.

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After drying, the product is dissolved in 5 ml. of DMF, and 250 mg. of Z—D—Nva—OH are added, followed by the addition of 0.15 ml. of triethylamine and 200 mg. of HONB. The mixture is cooled to 0°C and 230 mg. of DCC are added, followed by stirring at room temperature for 8 hours. To this are added 80 ml. of ethyl acetate and the insolubles are filtered off. The filtrate is washed with 1N hydrochloric acid, 4% sodium bicarbonate, and water, and dried over sodium sulfate.

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It is then evaporated to dryness under reduced pressure and the residue is triturated with petroleum ether. The resulting powder is purified by precipitation from ethyl acetate-petroleum ether. Yield: 718 mg.
 $[\alpha]_D^{25}$ —51.0° (c=1.0 in methanol); R_f^1 =0.64
Elemental analysis, $C_{33}H_{53}O_8N_9$,

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Calcd.: C, 56.23; H, 7.58; N, 17.89
Found: C, 56.14; H, 7.70; N, 17.58

b) Preparation of

H—(Pyr)Glu—His—Trp—Ser—Phe—D—Nva—Leu—Arg—Pro—
NH.CH₂CH₂CH₃,

In 3 ml. of 25% hydrogen bromide-glacial acetic acid are dissolved 500 mg. of

Z—D—Nva—Leu—Arg(NO₂)—Pro—NH.CH₂CH₂CH₃,

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and the solution is shaken for 50 minutes.

To this are added 60 ml. of dry ether, and the resulting precipitate is recovered by filtration and dissolved in 40 ml. of water. The solution is passed through a column (1 × 14 cm) of Amberlite CG—410 (free base) which is then

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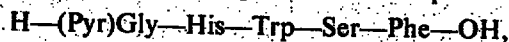
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washed with 30% aqueous methanol. The eluant and washings are pooled and the methanol is distilled off under reduced pressure. The residue is lyophilized to obtain 320 mg. of the free base



In 2 ml. of DMF are dissolved 225 mg. of the above product and 280 mg. of

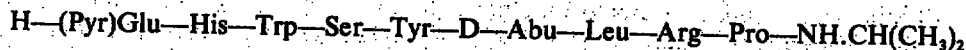


followed by the addition of 130 mg. of HONB. The mixture is chilled to -10°C and 87 mg. of DCC are added. The mixture is stirred at -10°C for 3 hours, at 0°C for 2 hours and at room temperature for 6 hours. To this reaction mixture are added 40 ml. of ethyl acetate and the resulting precipitate is recovered by filtration and dissolved in 30% aqueous ethanol under heating. The insolubles are filtered off and the filtrate is cooled, whereupon a precipitate is obtained. The precipitate is recovered by filtration, washed with cold water and dried. Yield: 340 mg. In 20 ml. of 60% aqueous formic acid are dissolved 300 mg. of the above product, followed by the addition of 580 mg. of $\text{SnCl}_2 \cdot \text{H}_2\text{O}$. The mixture is heated at 85°C for 2 hours. The reaction mixture is evaporated to dryness under reduced pressure and extracted with warm water.

The extract is purified by chromatography on Amberlite XAD-2, carboxymethyl-cellulose and Biogel P-2. The procedure gives 170 mg. of desired product. $[\alpha]_D^{25} -37.7^\circ$ ($c=0.505$ in 5% aqueous acetic acid); $\text{Rf}^1=0.13$, $\text{Rf}^2=0.76$. Aminoacid analysis (hydrolysis with 6N HCl in the presence of thioglycolic acid): His 1.02; Arg 0.98; Trp 0.97; Ser 0.98; Glu 1.02; Pro 1.00; Leu 1.00; Nva 1.02; Phe 0.98.

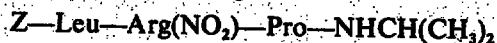
Example 9.

Production of



a) Preparation of $\text{Z-D-Abu-Leu-Arg(NO}_2\text{)-Pro-NHCH(CH}_3\text{)}_2$.

In 3 ml. of 25% hydrogen bromide-glacial acetic acid are dissolved 700 mg. of



and the solution is swirled for 60 minutes, after which 50 ml. of dry ether are added. The resulting precipitate is recovered by filtration and dried. The dried product is dissolved in 6 ml. of DMF. To this solution are added 240 mg. of Z-D-Abu-OH , and the solution is neutralized with 0.15 ml. of triethylamine.

Then, 200 mg. of HONB are added and the mixture is chilled to 0°C , followed by the addition of 230 mg. of DCC. The mixture is stirred at room temperature for 6 hours. To this are added 100 ml. of ethyl acetate and the insolubles are filtered off. The filtrate is washed with 0.5N hydrochloric acid and 4% sodium bicarbonate and then dried over sodium sulfate. It is then evaporated to dryness under reduced pressure and treated with ether. The resulting powder is reprecipitated from ethyl acetate-ether. Yield 725 mg.

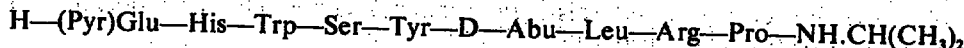
$[\alpha]_D^{25} -52.0^\circ$ ($c=0.5$ in methanol), $\text{Rf}^1=0.62$.

Elemental analysis, $\text{C}_{32}\text{H}_{51}\text{O}_8\text{N}_9$

Calcd.: C, 55.17; H, 7.45; N, 18.27

Found: C, 55.42; H, 7.59; N, 18.01

b) Preparation of



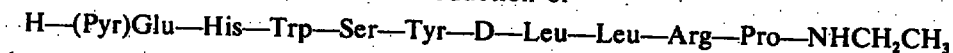
From the $\text{Z-D-Abu-Leu-Arg(NO}_2\text{)-Pro-NH.CH(CH}_3\text{)}_2$ obtained at a) above and $\text{H-(Pyr)Glu-His-Trp-Ser-Tyr-OH}$, the above compound is obtained by exactly the same procedure as that set forth in Example 5.

$[\alpha]_D^{25} -41.0^\circ$ ($c=0.62$ in 5% aqueous acetic acid); $\text{Rf}^1=0.11$; $\text{Rf}^2=0.74$.

Aminoacid analysis (hydrolysis with 6N HCl in the presence of thioglycolic acid): His 1.00; Arg 0.90; Trp 0.87; Ser 0.97; Glu 1.00; Pro 0.94; Abu 1.00; Leu 1.02; Tyr 0.96.

Example 10.

Production of



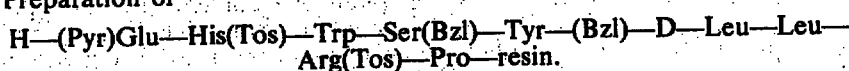
by a conventional method solid-phase method as described in "Solid Phase

Peptide Synthesis" by J. M. Stewart and J. D. Young, published by W. H. Freeman and Company, San Francisco (1969).

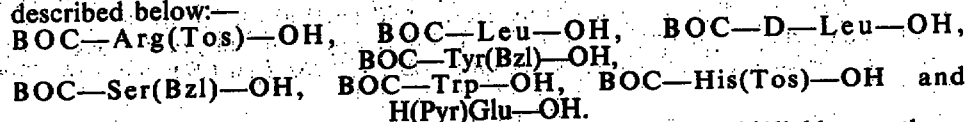
a) Preparation of BOC-Pro-resin.

In 50 ml. of DMF are placed 15 g. of chloromethylated resin (Chloromethylated poly-styrene for Peptide Synthesis, divinylbenzene 2%, 100—200 mesh (Japan Standard) and Cl content 9.45 millimoles), followed by the addition of 6.1 g of BOC-Pro—OH and 3.33 ml. of triethylamine. The mixture is shaken for 5 days. The resin is recovered by filtration and thoroughly washed with DMF, ethanol, water, ethanol and ether in that order, and dried. Yield: 17.55 g. Aminoacid analysis reveals that this resin contains 0.314 millimoles/g. of BOC—Pro.

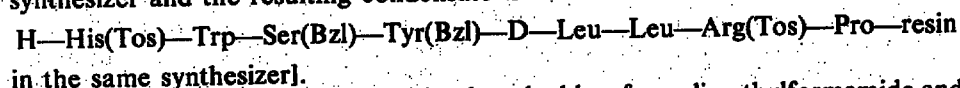
b) Preparation of



The reaction vessel of an automatic peptide synthesizer (Simadzu Seisakusho Model APS—800) is charged with 3.145 g of the BOC-Pro-resin obtained at (a) above. The resin is made swollen with dichloromethane for 12 hours, after which each aminoacid is fed on the following cycle and in the forms and in the order described below:—



Dichloromethane (3 minutes $\times$ 3) $\rightarrow$ 50% trifluoroacetic acid/dichloromethane (10 min. and 30 min.) $\rightarrow$ dichloromethane (3 min. $\times$ 3) $\rightarrow$ ethanol (3 min. $\times$ 3) $\rightarrow$ dichloromethane (3 min. $\times$ 3) $\rightarrow$ 10% triethylamine/chloroform (10 min.) $\rightarrow$ chloroform (3 min. $\times$ 3) $\rightarrow$ dichloromethane (3 min. $\times$ 2) $\rightarrow$ BOC-amino acid-anhydride (synthesized from BOC-amino acid and DCC in the conventional manner) (30 min. and 60 min.) $\rightarrow$ acylation (dichloromethane, triethylamine and acetic anhydride) (1 hour) $\rightarrow$ dichloromethane (3 min. $\times$ 3) [H—(Pyr)Glu—OH alone is directly condensed with DCC in DMF in the automatic peptide synthesizer and the resulting condensate is condensed with

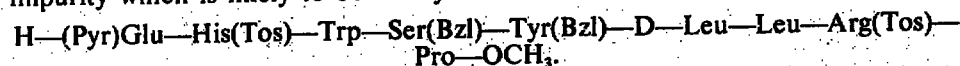


Finally, the resin is washed with ethanol, chloroform dimethylformamide and ether, and dried. Yield 3.89 g.

c) Preparation of



In 20 ml. of methanol are suspended 3.52 g. of the resin obtained at (b) and the suspension is cooled to 20°C. The ethylamine obtained by heating 80% aqueous ethylamine in a warm-water bath is bubbled into the suspension through sodium hydroxide. After sufficient saturation, the mixture is stirred at room temperature for 40 hours. The resin is recovered by filtration and the filtrate is evaporated to dryness under reduced pressure. The residue is treated with ether to yield 835 mg. of a crude powdery product. This crude product includes a large proportion of an impurity which is likely to be methyl ester i.e.



Therefore, 503 mg. of this product are dissolved in methanol-DMF (8 ml.—8 ml.) and, in the same manner above, ethylamine is bubbled into the solution. The solution is stirred for 34 hours, whereby the methyl ester is converted to the amide.

The solution is then evaporated to dryness under reduced pressure and the residue is treated with ether. The resultant powder is recovered by filtration. Yield: 465 mg.

d) Preparation of

H—(Pyr)Glu—His—Trp—Ser—Tyr—D—Leu—Leu—Arg—Pro—NH.CH₂CH₃

In the presence of 0.4 ml. of anisole and 0.4 ml. of mercaptoethanol, 327.8 mg. of the crude product obtained in c) are dissolved in 8 ml. of anhydrous hydrogen fluoride. The solution is stirred at 0°C for one hour, after which it is evaporated to dryness under reduced pressure. The residue is dissolved in 20 ml. of water and the insolubles are filtered off. The filtrate is passed through a column (1.5 x 20 cm) of Amberlite IRA—410 (acetate form) and then purified by chromatography on carboxymethyl-cellulose and Amberlite XAD—2. The product is subjected to gel filtration on a column of Sephadex LH—20. The described procedure gives 98 mg. of required product.

[α]_D²⁵—32.0° (c=0.52 in 5% aqueous acetic acid); R_F²=0.10; R_F³=0.75.
Aminoacid analysis (hydrolysis with 6N HCl in the presence of thioglycolic acid): His 0.98; Arg 1.00; Trp 0.87; Ser 0.90; Glu 1.02; Pro 1.02; Leu 2.01; Tyr 0.98; ethylamine 1.10.

Example 11.

Production of

H—(Pyr)Glu—His—Trp—Ser—Tyr—D—Nle—Leu—Arg—Pro—NH.CH₂CH₃

a) Preparation of BOC—D—Nle—Leu—Arg(NO₂)—Pro—NH.CH₂CH₃

In 10 ml. of DMF are dissolved 346 mg. of BOC—D—Nle—OH and 820 mg. of H—Leu—Arg(NO₂)—Pro—NH.CH₂CH₃. The mixture is chilled to 0°C, followed by the addition of 322 mg. of HONB and 371 mg. of DCC. The mixture is stirred at 0°C for 2 hours and at room temperature overnight. The urea compound, a by-product, is removed by filtration and the filtrate is extracted with 10 ml. of chloroform. The extract is washed with 0.1N hydrochloric acid, 5% sodium bicarbonate and water, and dried over anhydrous sodium sulfate.

The chloroform is evaporated and the residue is triturated with ether and reprecipitated from ethanol-ether to give the required compound. Yield: 811 mg. Melting point: 124—126°C (decomp.) [α]_D²⁵—46.2° (c=0.5 in methanol).
Elemental analysis, C₃₀H₃₅O₈N₅·½H₂O

Calcd.: C, 53.08; H, 8.31; N, 18.57
Found: C, 53.07; H, 8.27; N, 19.05;

b) Preparation of

H—(Pyr)Glu—His—Trp—Ser—Tyr—D—Nle—Leu—Arg—Pro—NH.CH₂CH₃,
BOC—D—Nle—Leu—Arg(NO₂)—Pro—NH.CH₂CH₃ which is prepared at a above is treated with trifluoroacetic acid, precipitated with ether and recovered by filtration. The product is passed through a column of Amberlite CG—45 (free base) to remove the trifluoroacetic acid. In 2 ml. of DMF are dissolved 190 mg. of the compound

H—D—Nle—Leu—Arg—(NO₂)—Pro—NH.CH₂CH₃,

and 168 mg. of

H—(Pyr)Glu—His—Trp—Ser—Tyr—OH.

The mixture is cooled to 0°C, followed by the addition of 65 mg. of HONB and 75 mg. of DCC. The mixture is stirred at 0°C for 2 hours and at room temperature overnight. The urea compound, a by-product, is removed by filtration and ethyl acetate is added to the filtrate. The resulting precipitate is recovered by filtration and treated with 4 ml. of anhydrous hydrogen fluoride in the presence of 0.1 ml. of anisole and 0.1 ml. of 2-mercaptoethanol at 0°C for one hour. The hydrogen fluoride is evaporated under reduced pressure and the residue is thoroughly dried and dissolved in 10 ml. of water. The solution is run onto a column of Amberlite IRA—410 (acetate form) and the eluant is adsorbed on a column of carboxymethyl-cellulose (1.2 x 22 cm) and gradient elution is carried out with 0.005 M ammonium acetate (200 ml.) and 0.15 M ammonium acetate (200 ml). The fractions from 147 ml. to 168 ml. are pooled and lyophilized to give the pure required product. Yield: 40 mg.

[α]_D²⁵—42.2° (c=0.5 in 5% aqueous acetic acid) R_F²=0.11, R_F³=0.755
Aminoacid analysis (hydrolysis with 6N HCl in the presence of thioglycolic acid) His 10.5; Arg 1.00; Trp 0.93; Ser 0.89; Tyr 1.00; Gly 1.00; Pro 1.02; Leu 1.00; Nle 1.00; ethylamine 1.05

Example 12.

Production of

H—(Pyr)Glu—His—Trp—Ser—Phe—D—Phe—Leu—Arg—Pro—NH.CH₂CH₃,

a) Preparation of H—D—Phe—Leu—Arg(NO₂)—Pro—NH.CH₂CH₃,

To a mixture of 10 ml. of dichloromethane and 1 ml. of DMF are added 600 mg. of Z—D—Phe—OH, 500 mg. of HONB and 950 mg. of

H—Leu—Arg(NO₂)—Pro—NH.CH₂CH₃,

and the mixture is heated. The mixture is cooled to 0°C, and 500 mg. of DCC are added. The mixture is stirred for 4 hours at room temperature, and the dicyclohexyl urea, a by-product, is removed by filtration. The filtrate is evaporated to dryness under reduced pressure and the residue is dissolved in chloroform. The solution is washed twice with 4% sodium bicarbonate and twice with water, and dried over anhydrous magnesium sulfate and evaporated to dryness under reduced pressure. Immediately, the residue is dissolved in 2 ml. of glacial acetic acid. After the addition of 5 ml. of 25% hydrogen bromide-glacial acetic acid, the mixture is stirred at room temperature for one hour, followed by the addition of 50 ml. of dry ether. The precipitate which separates is recovered by filtration, thoroughly washed with ether and dried. The precipitate is dissolved in 10 ml. of 30% aqueous methanol. The solution is run onto a column of Amberlite IRA—410 (free form), which is eluted with 100 ml. of 30% aqueous methanol. The eluant is pooled and concentrated to remove methanol.

Lyophilization of the residue gives the required compound

H—D—Phe—Leu—Arg(NO₂)—Pro—NH.CH₂CH₃,

as a white powder. Yield: 1.67 g.

[α]_D²⁶—94.26° (c=1.01 in methanol)

Elemental analysis: C₂₃H₄₅O₆N₅·H₂O

Calcd.: C, 54.09; H, 7.62; N, 20.28

Found: C, 53.84; H, 7.79; N, 19.91

b) Preparation of

H—(Pyr)Glu—His—Trp—Ser—Phe—D—Phe—Leu—Arg—Pro—NH.CH₂CH₃,

In 6 ml. of DMF are dissolved 290 mg. of

H—(Pyr)Glu—His—Trp—Ser—Phe—OH

and 240 mg. of

H—D—Phe—Leu—Arg(NO₂)—Pro—NH.CH₂CH₃,

followed by the addition of 150 mg. of HONB. The mixture is cooled to -10°C, and 90 mg. of DCC are added. The mixture is stirred at -10°C for 2 hours, at 0°C for 2 hours and at room temperature for 6 hours. The urea compound, a by-product, is removed by filtration and 40 ml. of ether are added to the filtrate. The precipitate which separates is recovered by filtration. Reprecipitation from ethanol and ethyl acetate gives crude protected peptide. Yield: 510 mg. The crude peptide is dissolved in 40 ml. of 60% aqueous formic acid. After the addition of 1 g. of SnCl₄·H₂O to the solution, the mixture is left standing at 80—85°C for 2 hours. The mixture is evaporated to dryness under reduced pressure. After the addition of 50 ml. of water to the residue, the mixture is adjusted to pH 7.0 with an aqueous solution of ammonia. The precipitate obtained is removed by filtration. The filtrate is purified on a column of Amberlite XAD—2 and carboxymethyl-cellulose and lyophilized by the same procedure as in Example 1, whereupon the required compound is obtained as a white fluffy powder. Yield: 392 mg.

[α]_D²⁶—71.8° (c=0.195 in 5% aqueous acetic acid)

Rf²=0.14, Rf³=0.76

Amino acid analysis (hydrolysis with 6N HCl in the presence of thioglycolic acid)
His 0.98; Arg 1.00; Trp 0.97; Ser 0.93; Glu 1.00; Pro 0.98; Leu 1.00; Phe 1.96

Example 13.

Production of

H—(Pyr)Glu—His—Trp—Ser—Tyr—D—Thr—Leu—Arg—Pro—NH.CH₂CH₃,

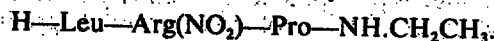
a) Preparation of BOC—D—Thr—Leu—Arg(NO₂)—Pro—NH.CH₂CH₃,

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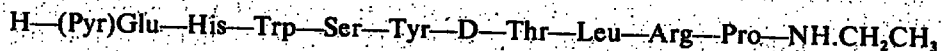
In 10 ml. of acetonitrile are dissolved 438 mg. of BOC-D-Thr-OH (an oily substance) and 418 mg. of HONB, followed by the addition of 912 mg. of



The mixture is cooled to 0°C and then 949 mg. of DCC are added. The mixture is stirred at room temperature for 10 hours and the insolubles are removed by filtration. The filtrate is evaporated to dryness under reduced pressure and the residue is extracted with 100 ml. of ethyl acetate. The extract is washed with 5% sodium bicarbonate, a 10% aqueous solution of citric acid and water in that order and dried over anhydrous sodium sulfate. It is then evaporated to dryness under reduced pressure and the residue is reprecipitated from ethanol-ether. The procedure gives 900 mg. of the required compound. Melting point: 119–121°C (decomp.), $[\alpha]_D^{25}$ –38.6° (c=0.5 in methanol), R_f = 0.45. Elemental analysis, $\text{C}_{28}\text{H}_{41}\text{O}_9\text{N}_3\cdot\text{H}_2\text{O}$.

Calcd.: C, 49.76; H, 7.90; N, 18.65
Found: C, 49.64; H, 7.70; N, 18.46

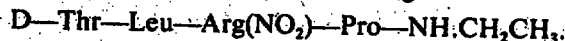
b) Preparation of



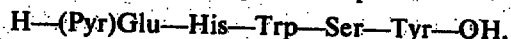
In 10 ml. of trifluoroacetic acid are dissolved 657 mg. of



and the solution is shaken for 40 minutes. To this are added 60 ml. of ether and the resulting precipitate is recovered by filtration and dissolved in 30 ml. of water. The aqueous solution is passed through a column (1 x 10 cm) of Amberlite IR-45 (free base) which is then washed with 30% aqueous methanol. The eluant and washings are pooled and concentrated to remove methanol under reduced pressure. The residue is lyophilized. The procedure gives 471 mg. of



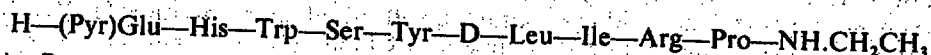
In 2 ml. of DMF are dissolved 167 mg. of this product and 175 mg. of



followed by the addition of 67 mg. of HONB. The mixture is chilled to –5°C and 77 mg. of DCC are added. The mixture is stirred at –5°C for 3 hours and then at room temperature for 10 hours. The insolubles are filtered off and 50 ml. of ethyl acetate are added to the filtrate. The resulting precipitate is recovered by filtration and reprecipitated from DMF-ethyl acetate. Yield: 290 mg. A 160 mg. portion of this product is dissolved in 10 ml. of 60% aqueous formic acid and, with the addition of 400 mg. of $\text{SnCl}_2\cdot\text{H}_2\text{O}$, the solution is treated at 80°C, for 2 hours. The reaction mixture is evaporated to dryness under reduced pressure. 10 ml. of water are added and the insolubles are filtered off. The filtrate is purified by chromatography on Amberlite XAD-2, carboxymethyl-cellulose and Bio-gel P-2. The procedure gives 65 mg. of the required compound; $[\alpha]_D^{25}$ –37.4° (c=0.5 in 5% aqueous acetic acid), R_f = 0.12, R_f = 0.68. Amino acid analysis (hydrolysis with 6N HCl in the presence of thioglycolic acid): His 1.00; Arg 0.97; Trp 0.94; Thr 1.03; Ser 0.90; Glu 1.00; Pro 1.06; Leu 1.06; Tyr 1.00; ethylamine 1.10.

Example 14.

Production of



a) Preparation of Z-Ile-Arg(NO₂)-Pro-NH.CH₂CH₃

In 12 ml. of 25% hydrogen bromide-glacial acetic acid are dissolved 1.43 g. of Z-Arg(NO₂)-Pro-NH.CH₂CH₃ and the mixture is shaken for 40 minutes at room temperature. To this solution are added 300 ml. of dry ether and the resulting precipitate is collected by filtration and dried in a desiccator over sodium hydroxide. The powder obtained is dissolved in 25 ml. of DMF and the solution is neutralized with 0.84 ml. of triethylamine and chilled to 0°C. 795 mg. of Z-Ile-OH, 590 mg. of HONB and 680 mg. of DCC are added, and the mixture is stirred at 0°C for 5 hours and for 8 hours at room temperature. The reaction mixture is filtered and the filtrate is evaporated to dryness. The residue is dissolved

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in 100 ml. of chloroform, washed with 0.1N hydrochloric acid, 5% sodium bicarbonate and water, and then dried over magnesium sulfate. After evaporation of the solvent, the residue is precipitated from ethyl acetate-ether. Reprecipitation from ethanol-ether gives 1.1 g. of the required product; melting point: 103–105°C.

$[\alpha]_D^{25}$ —58.1° (c=1.0 in methanol), R_f^1 =0.48

Elemental analysis, $C_{27}H_{42}O_7N_8 \cdot \frac{1}{2}H_2O$

Calcd.: C, 54.08; H, 7.23; N, 18.68

Found: C, 53.80; H, 7.15; N, 18.84

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b) Preparation of Z—D—Leu—Ile—Arg(NO₂)—Pro—NH.CH₂CH₃,

In 4 ml. of 25% hydrogen bromide-glacial acetic acid are dissolved 590 mg. of

Z—Ile—Arg(NO₂)—Pro—NH.CH₂CH₃,

and the mixture is swirled at room temperature for 40 minutes. To this solution are added 50 ml. of dry ether and the resulting precipitate is collected by filtration and dried in a desiccator over sodium hydroxide. The powder obtained is dissolved in 8 ml. of DMF and the solution is neutralized with 0.14 ml. of triethylamine and chilled to 0°C. 264 mg. of Z—D—Leu—OH, 216 mg. of HONB and 247 mg. of DCC are added, and the mixture is stirred at 0°C for 2 hours and at room temperature for 12 hours. The reaction mixture is then processed as described as a) above to yield 620 mg. of the required product; melting point: 112–113°C.

$[\alpha]_D^{25}$ —47.8° (c=0.5 in methanol), R_f^1 =0.53

Elemental analysis, $C_{33}H_{53}O_8N_9 \cdot H_2O$

Calcd.: C, 54.91; H, 7.68; N, 17.47

Found: C, 54.72; H, 7.52; N, 17.19

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c) Preparation of

H—(Pyr)Glu—His—Trp—Ser—Tyr—D—Leu—Ile—Arg—Pro—NH.CH₂CH₃,

In 3 ml. of 25% hydrogen bromide-glacial acetic acid are dissolved 220 mg. of

Z—D—Leu—Ile—Arg(NO₂)—Pro—NH.CH₂CH₃,

and the solution is swirled at room temperature for 50 minutes. To this solution are added 50 ml. of dry ether and the resultant precipitate is collected by filtration and dried in a desiccator over sodium hydroxide. This material is dissolved in a small quantity of water and passed through a column (1.2 × 7 cm) of Amberlite IR—45 (free form). The eluant and washings are combined and lyophilized. The material is dissolved in 5 ml. of DMF together with 230 mg. of

H—(Pyr)Glu—His—Trp—Ser—Tyr—OH

and 108 mg. of HONB. The mixture is chilled to –10°C and 124 mg. of DCC are added. The mixture is stirred at –10° for 2 hours, for 2 hours at 0°C and for 10 hours at room temperature, and then filtered. The filtrate is evaporated to dryness and the residue is purified on a column (1.8 × 20 cm) of Amberlite XAD—2 (200–300 mesh) with a gradient elution of 5% aqueous ethanol to 80% aqueous ethanol (250 ml. each). The required product is eluted in 150 ml.—300 ml. fractions. These fractions are concentrated under reduced pressure and lyophilized. The material thus obtained is dissolved in 7 ml. of 60% aqueous formic acid and 450 mg. of SnCl₄·H₂O are added. The mixture is heated at 80–85° for 2 hours. The insolubles are filtered off and the filtrate is evaporated to dryness under reduced pressure. The residue is extracted with 30 ml. of warm water (80°C). The extract is filtered, the filtrate is applied to a column (1.8 × 15 cm) of Amberlite XAD—2 (200–300 mesh, Japan Standards), and the column is thoroughly washed with water. For elution, a linear gradient of aqueous ethanol (20–80%, 250 ml.—250 ml.) is used. The desired compound emerges in 100 ml.—230 ml. fractions. These fractions are pooled and concentrated under reduced pressure to remove ethanol. The concentrate is applied to a column (1.2 × 40 cm) of carboxymethyl cellulose, and the product is eluted by linear gradient elution (0.005 M—0.2 M ammonium acetate, pH 6.8, 200 ml.—200 ml.). The desired peptide emerges in 180 ml.—290 ml. fractions. These fractions are pooled and lyophilized. Yield: 130 mg.

$[\alpha]_D^{25}$ —39.2° (c=0.5 in 5% aqueous acetic acid); R_f^2 =0.11

Aminoacid analysis (hydrolysis with 6N HCl in the presence of thioglycolic acid): His 0.97; Arg 1.02; Trp 0.92; Ser 0.94; Glu 1.00; Pro 1.04; Ile 1.00; Leu 1.00; Tyr 0.97

Example 15:

Production of

H—(Pyr)Glu—His—Trp—Ser—Tyr—D—Leu—Nle—Arg—Pro—NH.CH₂CH₃

a) Preparation of Z—Nle—Arg(NO₂)—Pro—NH.CH₂CH₃

In 10 ml. of 25% hydrogen bromide-glacial acetic acid are dissolved 715 mg. of

Z—Arg(NO₂)—Pro—NH.CH₂CH₃

and the mixture is swirled at room temperature for 30 min. To this solution are added 250 ml. of dry ether and the resulting precipitate is collected by filtration and dried in a desiccator over sodium hydroxide. The powder thus obtained is dissolved in 15 ml. of DMF together with 400 mg. of Z—Nle—OH and the solution is chilled to 0°C. To this is added 0.45 ml. of triethylamine, and then 295 mg. of HONB and 340 mg. of DCC. The mixture is stirred at 0°C. for 3 hours and for 10 hours at room temperature. The reaction mixture is then processed as described in Example 14a) to give the desired product. Yield: 824 mg.; melting point: 109—110°C.

$[\alpha]_D^{22}$ —50.4° (c=0.5 in ethanol). R_f^2 =0.41

Elemental analysis, C₂₇H₄₂O₇N₈· $\frac{1}{2}$ H₂O

Calcd.: C, 54.08; H, 7.23; N, 18.68

Found: C, 53.79; H, 7.09; N, 18.34

b) Preparation of Z—D—Leu—Nle—Arg(NO₂)—Pro—NH.CH₂CH₃

In 5 ml. of 25% hydrogen bromide-glacial acetic acid are dissolved 590 mg. of

Z—Nle—Arg(NO₂)—Pro—NH.CH₂CH₃

and the mixture is shaken for 40 minutes at room temperature. To this are added 200 ml. of dry ether, and the resulting precipitate is collected by filtration, and dried in a desiccator over sodium hydroxide. This product is dissolved in 5 ml. of DMF and the solution is neutralized with 0.14 ml. of triethylamine at 0°C. To this are added 264 mg. of Z—D—Leu—OH, 216 mg. of HONB and 247 mg. of DCC at 0°C. The mixture is stirred for 2 hours at 0°C and for 10 hours at room temperature, and is then processed as described in Example 14b) to yield 594 mg. of the desired product: melting point: 101—103°C.

$[\alpha]_D^{23}$ —45.2° (c=0.5 in methanol), R_f^2 =0.55

Elemental analysis, C₃₃H₅₃O₈N₉·H₂O

Calcd.: C, 54.91; H, 7.68; N, 17.47

Found: C, 54.63; H, 7.54; N, 17.08

c) Preparation of

H—(Pyr)Glu—His—Trp—Ser—Tyr—D—Leu—Nle—Arg—Pro—NH.CH₂CH₃

In 4 ml. of 25% hydrogen bromide-glacial acetic acid are dissolved 350 mg. of

Z—D—Leu—Nle—Arg(NO₂)—Pro—NH.CH₂CH₃

and the mixture is swirled for 40 minutes at room temperature. To this mixture are added 300 ml. of dry ether and the resulting precipitate is collected by filtration and dried in a desiccator over sodium hydroxide.

The material thus obtained is dissolved in 2 ml. of water and the solution is passed through a column of (1.2 × 7 cm) of Amberlite IR—45 (free form). The column is washed well with water. Eluant and washings are combined and lyophilized. This product is coupled with 345 mg. of

H—(Pyr)Glu—His—Trp—Ser—Tyr—OH

in 6 ml. of DMF in the presence of 180 mg. of HONB and 206 mg. of DCC. The mixture is worked up and the product obtained is purified by chromatography on Amberlite XAD—2 as described in Example 14c). Removal of the nitro group and purification of the required peptide are also carried out in a similar manner to that described in Example 14c). Yield: 213 mg.

$[\alpha]_D^{25}$ —38.6° (c=0.5 in 5% aqueous acetic acid). $R_f^2=0.10$.
Aminoacid analysis: hydrolysis with 6N HCl in the presence of thioglycolic acid:
His 0.98; Arg 1.00; Trp 0.91; Ser 0.95; Glu 1.00; Pro 1.08; Leu 1.00; Nle 1.00; Tyr 0.96

Example 16.

Production of

H—(Pyr)Glu—His—Trp—Ser—Tyr—D—Val—Leu—Arg—Pro—NH.CH₂CH₃,

a) Preparation of BOC—D—Val—Leu—Arg(NO₂)—Pro—NH.CH₂CH₃,

To a solution of 540 mg. of BOC—D—Val—OH(oil) and 1.14 g. of

H—Leu—Arg(NO₂)—Pro—NH.CH₂CH₃,

in 10 ml. of DMF are added 537 mg. of HONB and 620 mg. of DCC at 0°C. The mixture is stirred for 3 hours at 0°C and for 10 hours at room temperature, and then filtered. The filtrate is evaporated to dryness under reduced pressure and the residue is dissolved in 100 ml. of ethyl acetate. The solution is washed with 0.1N hydrochloric acid and 5% sodium bicarbonate, dried over anhydrous sodium sulphate, and evaporated to dryness. The residue is triturated with petroleum ether to give the crude product, which is then reprecipitated from ethyl acetate-ether. Yield: 1.1 g.; melting point: 144—146°C (decomp.).

$[\alpha]_D^{25}$ —43.7° (c=1.0 in methanol). $R_f^1=0.55$

Elemental analysis: C₂₉H₃₃O₈N₇·½H₂O

Calcd.: C, 52.39; H, 8.18; N, 18.96
Found: C, 52.40; H, 8.32; N, 18.52

b) Preparation of

H—(Pyr)Glu—His—Trp—Ser—Tyr—D—Val—Leu—Arg—Pro—NH.CH₂CH₃,

In 5 ml. of trifluoroacetic acid are dissolved 300 mg. of

BOC—D—Val—Leu—Arg(NO₂)—Pro—NH.CH₂CH₃,

and the mixture is swirled for 30 minutes at room temperature. To this are added 50 ml. of dry ether and the resulting white solid is recovered and dried under reduced pressure in a desiccator over sodium hydroxide. This material is dissolved in 20 ml. of 20% aqueous methanol and the solution is applied to a column (1 × 5 cm) of Amberlite IR—45 (free form). The column is washed with the same solvent. Eluant and washings are combined, concentrated to a small volume, and lyophilized: 165 mg. This material (150 mg.) is dissolved in 2 ml. of DMF together with 168 mg. of

H—(Pyr)Glu—His—Trp—Ser—Tyr—OH

and 65 mg. of HONB, and the mixture is cooled to 0°C. To this are added 75 mg. of DCC and the mixture is stirred for 2 hours at 0°C and for 12 hours at room temperature. The reaction mixture is filtered and diluted with 50 ml. of ethyl acetate. The precipitate is recovered by filtration dried and purified by column chromatography on silica gel (4 g.). For elution, an R_f^2 solvent system is used. The fractions which contain the protected nonapeptide are combined and the solvent is evaporated to dryness: 140 mg. This protected nonapeptide (110 mg.) is treated with 4 ml. of anhydrous hydrogen fluoride in the presence of 0.1 ml. of anisole and 0.02 ml. of mercaptoethanol at 0°C for one hour. Hydrogen fluoride is removed under reduced pressure and the residue is extracted with 20 ml. of water. The extract is washed with ether and passed through a column (1.5 × 3 cm) of Amberlite IRA—400 (acetate form), and the column is washed with water. Eluant and washings are combined and lyophilized. The material is dissolved in a small quantity of water and the solution is applied to a column (1.2 × 30 cm) of carboxymethyl-cellulose. For elution, a linear gradient of ammonium acetate (0.005 M—0.2 M, 150 ml.—150 ml.) is used. The desired product emerges in the 110 ml.—130 ml. fractions. These fractions are combined and lyophilized. Yield: 87 mg.

$[\alpha]_D^{25}$ —40.8° (c=0.5 in 5% aqueous acetic acid), $R_f^2=0.12$, $R_f^3=0.66$

Aminoacid analysis (hydrolysis with 6N HCl in the presence of thioglycolic acid):
His 1.00; Arg 1.02; Trp 0.85; Ser 0.95; Glu 0.98; Pro 1.02; Val 0.91; Leu 0.95; Tyr 1.00; ethylamine 1.01

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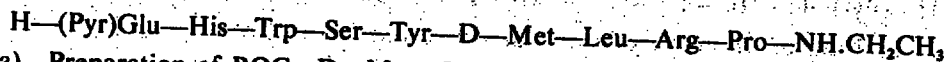
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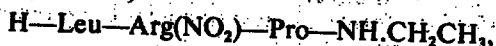
Example 17.

Production of



a) Preparation of BOC—D—Met—Leu—Arg(NO₂)—Pro—NH.CH₂CH₃

BOC—D—Met—Leu—Arg(NO₂)—Pro—NH.CH₂CH₃ is prepared in a similar manner to that described in Example 16a) from 623 mg. of BOC—D—Met—OH, 1.14 g. of



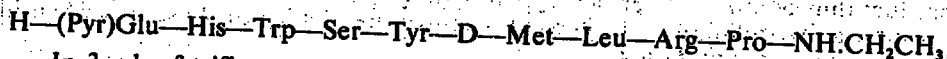
537 mg. of HONB, and 620 mg. of DCC. Yield: 1.1 g.; melting point: 125—130°C (decomp.).

[α]_D²⁵—51.5° (c=1.0 in methanol), R_f²=0.59

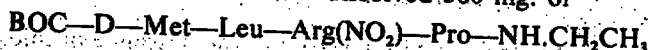
Elemental analysis, C₂₉H₃₃O₈N₅S

Calcd.: C, 50.64; H, 7.77; N, 18.32; S, 4.65
Found: C, 50.51; H, 7.52; N, 18.54; S, 4.30

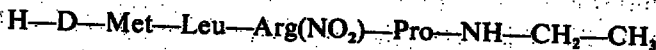
b) Preparation of



In 3 ml. of trifluoroacetic acid are dissolved 300 mg. of



and the mixture is swirled for 20 minutes at room temperature. To this are added 40 ml. of dry ether and the resulting white precipitate is recovered by filtration and dried. This material is converted to the free form by passing through a column (1 × 5 cm) of Amberlite IR—45 (free form) as described in Example 16b). The resulting partially protected tetrapeptide,



(147 mg.) is reacted with 171 mg. of



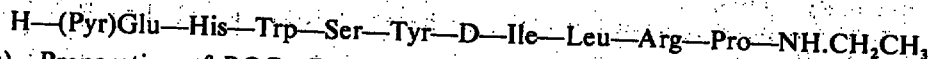
in the presence of 67 mg. of HONB and 77 mg. of DCC to give the partially protected nonapeptide. This compound is purified on a column of Amberlite XAD—2, and then treated with SnCl₂·H₂O in 60% aqueous formic acid for 2 hours at 80—85°C. The final product thus obtained is purified by chromatography on Amberlite XAD—2, and carboxymethyl-cellulose. All these procedures are similar to those described in Example 16b). Yield: 79 mg.

[α]_D²⁵=39.6° (c=0.5 in 5% aqueous acetic acid), R_f²=0.16

Aminoacid analysis (hydrolysis with 6N HCl in the presence of thioglycolic acid): His 1.02; Arg 1.00; Trp 0.90; Ser 0.89; Glu 1.00; Pro 1.00; Met 0.91; Leu 0.98; Tyr 1.02; ethylamine 1.00

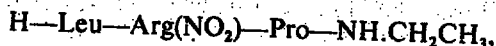
Example 18.

Production of



a) Preparation of BOC—D—Ile—Leu—Arg(NO₂)—Pro—NH.CH₂CH₃

BOC—D—Ile—Leu—Arg(NO₂)—Pro—NH.CH₂CH₃ is prepared in a similar manner to that described in Example 16a) from 579 mg. of BOC—D—Ile—OH, 1.14 g. of



537 mg. of HONB and 620 mg. of DCC. Yield: 0.9 g.;

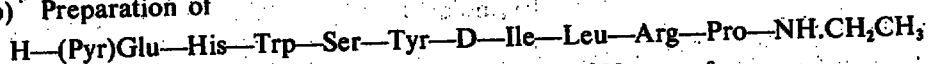
melting point: 127—131°C (decomp.)

[α]_D²⁵—48.3° (c=1.0 in methanol)

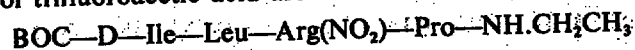
Elemental analysis, C₃₀H₃₅O₈N₅

Calcd.: C, 53.79; H, 8.27; N, 18.18
Found: C, 53.81; H, 8.31; N, 18.65

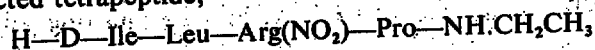
b) Preparation of



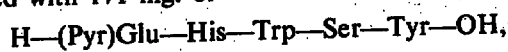
In 3 ml. of trifluoroacetic acid are dissolved 300 mg. of



and the mixture is shaken for 25 minutes at room temperature. To this are added 40 ml. of dry ether and the resulting precipitate is recovered by filtration and dried. This material is converted to the free form by passage through a column (1 x 5 cm) of Amberlite IR-45 (free form) as described in Example 16b). The resulting partially protected tetrapeptide,



(142 mg.) is reacted with 171 mg. of



67 mg. of HONB, and 77 mg. of DCC to give the partially protected nonapeptide. This compound is purified on a column of Amberlite XAD-2, and then treated with $\text{SnCl}_2 \cdot \text{H}_2\text{O}$ in 60% aqueous formic acid for 2 hours at 80-85°C. The desired product thus obtained is purified by chromatography on Amberlite XAD-2, and carboxymethyl-cellulose. All these procedures are similar to those described in Example 16b). Yield: 93 mg.

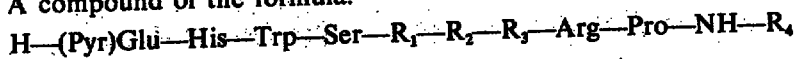
$[\alpha]_D^{25} -41.5^\circ$ ($c=0.5$ in 5% aqueous acetic acid).

$\text{Rf}^1=0.13$, $\text{Rf}^2=0.66$.

Amino acid analysis (hydrolysis with 6N HCl in the presence of thioglycolic acid): His 0.98; Arg 1.00; Trp 0.89; Ser 0.91; Glu 0.95; Pro 1.00; Ile 1.00; Leu 1.00; Tyr 1.00; ethylamine 1.01.

WHAT WE CLAIM IS:—

1. A compound of the formula:



wherein:

R_1 is Tyr or Phe; R_2 is D-Leu, D-Ile, D-Nle, D-Val, D-Nva, D-Abu, α -Aibu, D-Phe, D-Phg, D-Ser, D-Thr or D-Met; R_3 is Leu, Ile or Nle and R_4 is alkyl of 1 to 3 carbon atoms which may be substituted with one or more hydroxyl groups.

2. A compound as claimed in claim 1, wherein R_2 is D-Leu, D-Nle, D-Nva, D-Phg, D-Phe, α -Aibu or D-Ser, and R_1 , R_3 and R_4 have the meanings given in claim 1.

3. A compound as claimed in claim 1, wherein R_2 is D-Val, D-Ile, D-Thr or D-Met, and R_1 , R_3 and R_4 have the meanings given in claim 1.

4. A compound as claimed in claim 1, wherein R_4 is ethyl.

5. A compound as claimed in claim 1, wherein R_1 is Tyr, R_2 is D-Leu, R_3 is Leu and R_4 is ethyl.

6. A compound as claimed in claim 1, wherein R_1 is Tyr, R_2 is α -Aibu, R_3 is Leu and R_4 is ethyl.

7. A compound as claimed in claim 1, wherein R_1 is Phe, R_2 is D-Leu, R_3 is Leu and R_4 is ethyl.

8. A compound as claimed in claim 1, wherein R_1 is Tyr, R_2 is D-Phg, R_3 is Leu and R_4 is ethyl.

9. A compound as claimed in claim 1, wherein R_1 is Tyr, R_2 is D-Ser, R_3 is Leu and R_4 is ethyl.

10. A compound as claimed in claim 1, wherein R_1 is Tyr, R_2 is D-Abu, R_3 is Leu and R_4 is ethyl.

11. A compound as claimed in claim 1, wherein R_1 is Tyr, R_2 is D-Nva, R_3 is Leu and R_4 is ethyl.

12. A compound as claimed in claim 1, wherein R_1 is Phe, R_2 is D-Nva, R_3 is Leu and R_4 is *n*-propyl.

13. A compound as claimed in claim 1, wherein R_1 is Tyr, R_2 is D-Abu, R_3 is Leu and R_4 is *i*-propyl.

14. A compound as claimed in claim 1, wherein R_1 is Tyr, R_2 is D-Nle, R_3 is Leu and R_4 is ethyl.

15. A compound as claimed in claim 1, wherein R_1 is Phe, R_2 is D-Phe, R_3 is Leu and R_4 is ethyl.

16. A compound as claimed in claim 1, wherein R_1 is Tyr, R_2 is D-Thr, R_3 is Leu and R_4 is ethyl.



b) Preparation of

H-(Pyr)Glu-His-Trp-Ser-Tyr-D-Ile-Leu-Arg-Pro-NH.CH₂CH₃

In 3 ml. of trifluoroacetic acid are dissolved 300 mg. of

BOC-D-Ile-Leu-Arg(NO₂)-Pro-NH.CH₂CH₃

and the mixture is shaken for 25 minutes at room temperature. To this are added 40 ml. of dry ether and the resulting precipitate is recovered by filtration and dried. This material is converted to the free form by passage through a column (1 x 5 cm) of Amberlite IR-45 (free form) as described in Example 16b). The resulting partially protected tetrapeptide,

H-D-Ile-Leu-Arg(NO₂)-Pro-NH.CH₂CH₃

(142 mg.) is reacted with 171 mg. of

H-(Pyr)Glu-His-Trp-Ser-Tyr-OH,

67 mg. of HONB, and 77 mg. of DCC to give the partially protected nonapeptide. This compound is purified on a column of Amberlite XAD-2, and then treated with SnCl₂.H₂O in 60% aqueous formic acid for 2 hours at 80-85°C. The desired product thus obtained is purified by chromatography on Amberlite XAD-2, and carboxymethyl-cellulose. All these procedures are similar to those described in Example 16b). Yield: 93 mg.

[α]_D²⁵ -41.5° (c=0.5 in 5% aqueous acetic acid).

R_f²=0.13, R_f³=0.66.

Aminoacid analysis (hydrolysis with 6N HCl in the presence of thioglycolic acid): His 0.98; Arg 1.00; Trp 0.89; Ser 0.91; Glu 0.95; Pro 1.00; Ile 1.00; Leu 1.00; Tyr 1.00; ethylamine 1.01.

WHAT WE CLAIM IS:-

1. A compound of the formula:

H-(Pyr)Glu-His-Trp-Ser-R₁-R₂-R₃-Arg-Pro-NH-R₄

wherein:

R₁ is Tyr or Phe; R₂ is D-Leu, D-Ile, D-Nle, D-Val, D-Nva, D-Abu, α-Aibu, D-Phe, D-Phg, D-Ser, D-Thr or D-Met; R₃ is Leu, Ile or Nle and R₄ is alkyl of 1 to 3 carbon atoms which may be substituted with one or more hydroxyl groups.

2. A compound as claimed in claim 1, wherein R₁ is D-Leu, D-Nle, D-Nva, D-Phg, D-Phe, α-Aibu or D-Ser, and R₁, R₂ and R₄ have the meanings given in claim 1.

3. A compound as claimed in claim 1, wherein R₂ is D-Val, D-Ile, D-Thr or D-Met, and R₁, R₃ and R₄ have the meanings given in claim 1.

4. A compound as claimed in claim 1, wherein R₄ is ethyl.

5. A compound as claimed in claim 1, wherein R₁ is Tyr, R₂ is D-Leu, R₃ is Leu and R₄ is ethyl.

6. A compound as claimed in claim 1, wherein R₁ is Tyr, R₂ is α-Aibu, R₃ is Leu and R₄ is ethyl.

7. A compound as claimed in claim 1, wherein R₁ is Phe, R₂ is D-Leu, R₃ is Leu and R₄ is ethyl.

8. A compound as claimed in claim 1, wherein R₁ is Tyr, R₂ is D-Phg, R₃ is Leu and R₄ is ethyl.

9. A compound as claimed in claim 1, wherein R₁ is Tyr, R₂ is D-Ser, R₃ is Leu and R₄ is ethyl.

10. A compound as claimed in claim 1, wherein R₁ is Tyr, R₂ is D-Abu, R₃ is Leu and R₄ is ethyl.

11. A compound as claimed in claim 1, wherein R₁ is Tyr, R₂ is D-Nva, R₃ is Leu and R₄ is ethyl.

12. A compound as claimed in claim 1, wherein R₁ is Phe, R₂ is D-Nva, R₃ is Leu and R₄ is *n*-propyl.

13. A compound as claimed in claim 1, wherein R₁ is Tyr, R₂ is D-Abu, R₃ is Leu and R₄ is *i*-propyl.

14. A compound as claimed in claim 1, wherein R₁ is Tyr, R₂ is D-Nle, R₃ is Leu and R₄ is ethyl.

15. A compound as claimed in claim 1, wherein R₁ is Phe, R₂ is D-Phe, R₃ is Leu and R₄ is ethyl.

16. A compound as claimed in claim 1, wherein R₁ is Tyr, R₂ is D-Thr, R₃ is Leu and R₄ is ethyl.



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17. A compound as claimed in claim 1, wherein R_1 is Tyr, R_2 is D-Leu, R_3 is Ile and R_4 is ethyl.

18. A compound as claimed in claim 1, wherein R_1 is Tyr, R_2 is D-Leu, R_3 is Nle, and R_4 is ethyl.

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19. A compound as claimed in claim 1, wherein R_1 is Tyr, R_2 is D-Val, R_3 is Leu and R_4 is ethyl.

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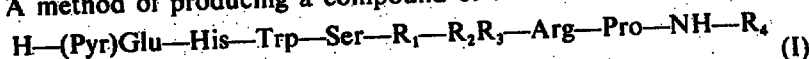
20. A compound as claimed in claim 1, wherein R_1 is Tyr, R_2 is D-Met, R_3 is Leu and R_4 is ethyl.

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21. A compound as claimed in claim 1, wherein R_1 is Tyr, R_2 is D-Ile, R_3 is Leu and R_4 is ethyl.

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22. A method of producing a compound of the formula (I):



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wherein R_1 is Tyr or Phe, R_2 is D-Leu, D-Ile, D-Nle, D-Val, D-Nva, D-Abu, α -Aibu, D-Phe, D-Phg, D-Ser, D-Thr, or D-Met; R_3 is Leu, Ile or Nle and R_4 is alkyl of 1 to 3 carbon atoms which may be substituted with one or more hydroxyl groups, which method comprises condensing a reagent (A) L-pyroglutamic acid, a protected L-glutamic acid (as hereinbefore defined) or a peptide fragment which has an L-pyroglutamic acid unit or a protected L-glutamic acid unit at its N-terminal end with a reagent (B) an amine or peptide amide component wherein the reagents (A) and (B) taken together correspond to the amino acid sequence of the compound of formula (I), the active groups of the reagents (A) and (B) not required for the condensation reaction being optionally protected; reacting the protected L-glutamyl group, if any, with an acid or base to form the pyroglutamyl group and removing other protective group(s) if any.

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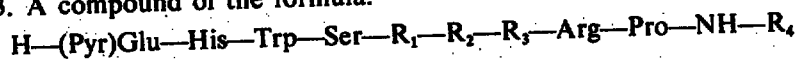
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23. A compound of the formula:



in which

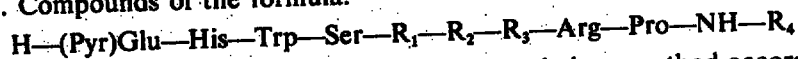
R_1-R_4 are as defined in claim 1 substantially as herein described with reference to any of the specific examples.

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24. A method according to claim 22 substantially as herein described with reference to any of the specific examples.

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25. Compounds of the formula:



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in which R_1-R_4 are as defined in claim 1 when made by a method according to claim 22 or 24.

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