

PATENT SPECIFICATION

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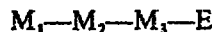
(54) TRIPEPTIDES AND THEIR PRODUCTION

(71) We, MERCK & CO. INC., a corporation duly organised and existing under the laws of the State of New Jersey, United States of America, of Rahway, New Jersey, United States of America, 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:—

This invention is directed to certain tripeptides and use as anti-depressant and/or thyroid hormone release stimulants and processes for their preparation.

The naturally occurring thyrotropin releasing hormone (TRH) is the tripeptide *L*-pyroglutamyl-*L*-histidyl-*L*-proline amide. Methods of synthesizing this peptide are known. Specific processes are described in U.S. 3,753,569; U.S. 3,746,697; U.S. 3,757,003, and U.S. 3,752,800. Analogs, derivatives and isomers of TRH are also disclosed in "Vitamins and Hormones, Advances in Research and Applications" volume 29 pg. 1—39, Academic Press, N.Y. and London (1971); "Chemistry and Biology of Peptides, Proceedings of the Third American Peptide Symposium" pg. 601—608, Ann Arbor Science Publishers Inc., Ann Arbor, Michigan (1972); Zh. Obshch. Khim 42 No. 2, pg. 483, February (1972); J. Med. Chem. 15, pg. 8, 219, 479 (1972); J. Med. Chem. 16, pg. 1137—1140 (1973); C.A. 75, 49547 w, 49548 x, 77268 z, 88942 r (1971); C.A. 74, 13401 m, (1971); and C.A. 73, 21767 c, 95001 v (1970). Besides its thyroid hormone release stimulating activity, TRH has also been found to have anti-depressant effect of substantially immediate onset. [Prage Jr., et al., LANCET pg. 999, Nov. 11, 1972; Plotnikoff, et al., SCIENCE, 178,417 (1972)].

The present invention provides tripeptides having the formula



(I)

and acid salts thereof in which M_1 is H-kic, H-kpc or H-pca, M_2 is his, his[N^{3im}—(C₁₋₆ alkyl)] or his[N^{3im}—(CH₂)_b—COOH] where b is 1, 2, 3 or 4, M_3 is *L*-pip, *L*-pro or *L*-tca, and E is —NH₂ or —OR where R is C₁₋₁₀ alkyl, including substituted alkyl and cycloalkyl, such that (i) when E is —NH₂, pca and *L*-pro do not occur together in the tripeptide, and (ii) when E is —OR, his and *L*-pro do not occur together in the tripeptide, and where the abbreviations for the amino acids are as defined in the specification and their configurations are *DL* or *L* or a mixture thereof.

The amino acids are abbreviated herein as follows:

	Amino Acids	Abbreviation	
	histidine	his	
	pyroglutamic acid	pca	
	proline	pro	
5	2-ketoimidazolidine-5-carboxylic acid	kic	5
	2-ketopiperidine-6-carboxylic acid	kpc	
	thiazolidine-4-carboxylic acid	tca	
	L-2-piperidinecarboxylic acid	L-pip	

10 The prefix *L*-, or *DL*- is used to designate an individual amino acid stereoisomer, or stereoisomer mixtures, respectively. Where no prefix is used, the amino acid includes both the *L*-stereoisomer and *DL*- mixtures. Thus, e.g. *pca* includes *L-pca* and *DL-pca* mixtures, including racemic mixtures. In general, the preferred tripeptides of Formula I are those in which M_1 , M_2 and M_3 all have the *L*- configuration.

15 In addition to the amino acid abbreviations, following is a list of other abbreviations used herein: 15

	FULL WORD(S)	ABBREVIATION	
	benzyloxycarbonyl	Z	
	2,4-dinitrophenyl	DNP	
20	dicyclohexylcarbodiimide	DCCI	20
	dimethylformamide	DMF	
	1-hydroxybenzotriazole	HBT	
	<i>tert.</i> -butyloxycarbonyl	BOC	
	trifluoroacetic acid	TFA	
25	resins	⊙	25
	azide	N ₃	

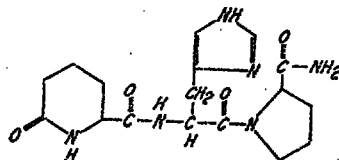
30 The present tripeptides encompass two general groups of compounds—the amides, where terminal group E in Formula I is —NH₂, and the esters, where the terminal group E is —OR, where R is a C₁₋₁₀ alkyl group, including unsubstituted as well as substituted alkyls and linear, branched and cyclic alkyls. Examples of suitable preferred R groups are *t*-butyl, *n*-decyl, cyclohexyl, *n*-nonyl and 2,4-dimethyl-*n*-heptyl; unsubstituted alkyl groups, especially methyl, are preferred.

One group of preferred tripeptides comprises those having the Formula I in which M_1 is *kic* or *kpc*. Examples of these tripeptides are:

35	H-kpc-his-L-pro—NH ₂ H-kpc-his-L-tca—OC ₆ H ₅ H-kic-his(N ^{3im} —(C ₁₋₆ alkyl))—L-pro—OC ₁₀ H ₂₁ H-kic-his(N ^{3im} —CH ₂ —COOH)—L-pro—OC ₁₀ H ₂₁ H- <i>DL</i> -kpc-his-tca—NH ₂	35
40	H-L-kic-his-L-pro—NH ₂ H-L-kpc-his(N ^{3im} —(C ₁₋₆ alkyl))—L-pro—OC(CH ₃) ₃ H-L-kpc-his(N ^{3im} —CH ₂ COOH)—L-pro—OC(CH ₃) ₃	40

H-DL-kic-L-his-L-tca-OC₂H₅,
H-DL-kpc-DL-his-L-pip-O-cyclohexyl and
H-L-kic-DL-his-L-pip-OC₂H₅,

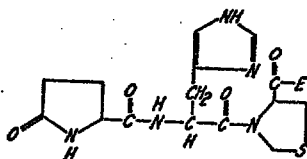
5 Especially preferred tripeptides are those in which M₁ in the Formula I is a six-membered heterocyclic ring containing amino acid residue, particularly H-L-kpc-L-his-L-pro-NH₂, which has the formula:



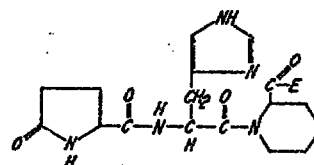
Another group of preferred tripeptides comprises those having Formula I in which M₁ is tca or L-pip. Examples of these preferred tripeptides are:

- 10 H-pca-his[N^{3im}-(C₁₋₆ alkyl)]-L-tca-NH₂
H-pca-his[N^{3im}-(CH₂)_b-COOH]-L-tca-NH₂
H-kic-his-L-pip-OCH(CH₃)₂
H-kpc-L-his-L-pip-NH₂
15 H-kic-DL-his-L-tca-OC₂H₅
H-DL-pca-his-L-tca-OC₂H₅, and
H-kpc-his-L-pip-NH₂

Especially preferred tripeptides are H-pca-his-tca-E and H-pca-his-L-pip-E and particularly (1) H-L-pca-L-his-L-tca-E and (2) H-L-pca-L-his-L-pip-E, which have the formulae:

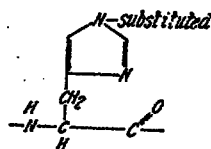


and



respectively.

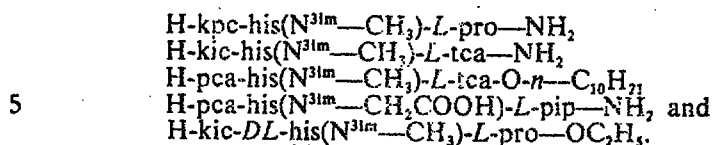
Another group of preferred tripeptides comprises those having Formula I in which M₂ is N^{3im}-substituted his. N^{3im}-substituted indicates substitution on the histidine imidazoline N in the 3 position, as illustrated in the following formula:



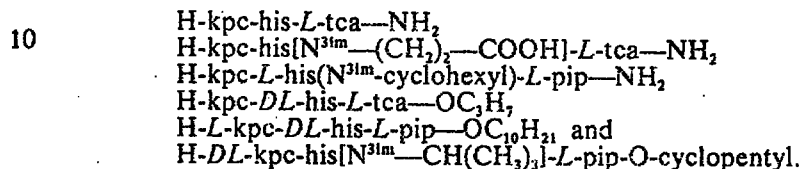
The substituting groups are C₁-C₆ alkyl and -(CH₂)_bCOOH, where b is 1, 2, 3 or 4. Methyl, *t*-butyl, cyclohexyl and carboxymethyl are preferred substituting groups. Examples of preferred tripeptides having, as M₂, N^{3im}-substituted-his are:

- 30 H-pca-his[N^{3im}-C₂H₅]-L-tca-O-2-ethyl-*n*-hexyl
H-DL-kpc-DL-his[N^{3im}-C₂H₅]-L-tca-NH₂
H-DL-kic-L-his[N^{3im}-(CH₂)_bCOOH]-L-pro-OC₁₀H₂₁
H-L-pca-L-his[N^{3im}-CH(CH₃)₃]-L-pro-NH₂
H-kpc-his[N^{3im}-*n*-C₄H₉]-L-pip-NH₂
35 H-L-kic-DL-his[N^{3im}-cyclohexyl]-L-tca-OCH(CH₃)₂ and
H-kic-his[N^{3im}-(CH₂)₃-COOH]-L-pip-O-cyclopentyl.

The preferred tripeptides of the group are exemplified by:

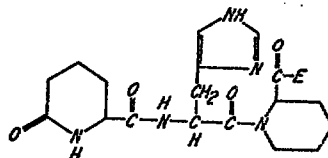


Another group of preferred tripeptides having Formula I comprises those in which M_1 is kpc and M_3 is L-tca or L-pip. Examples of these tripeptides are:



15 Particularly preferred peptides of this group are L-kpc-L-his-L-pip-E, which has the formula:

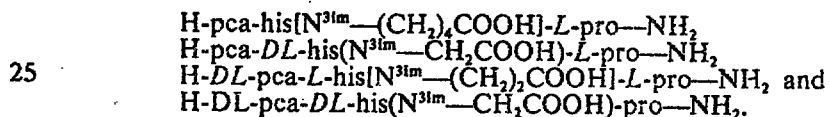
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Another preferred embodiment of this invention comprises tripeptides having the formula:



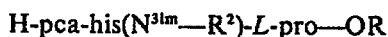
where R^1 is $-(\text{CH}_2)_b\text{-COOH}$, b being an integer from 1-4. Preferred R^1 is $-\text{CH}_2\text{-COOH}$. Examples of these tripeptides are



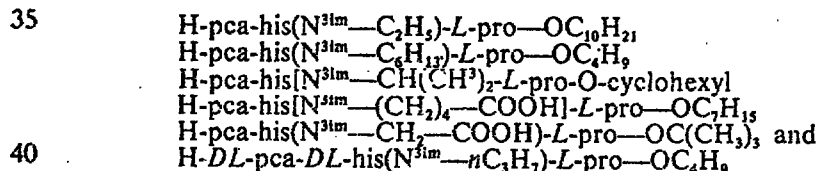
A preferred tripeptide of Formula II is H-L-pca-L-his($\text{N}^{31\text{m}}-\text{CH}_2\text{COOH}$)-L-pro-NH₂.

30 Another embodiment of this invention comprises tripeptides having the formula:

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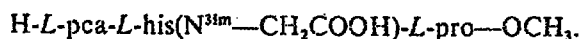
where R^2 is $\text{C}_1\text{-C}_6$ alkyl, e.g. $-\text{C}_2\text{H}_5$, cyclohexyl or $-\text{C}(\text{CH}_3)_3$, or $-(\text{CH}_2)_b\text{COOH}$ and where b and R are as defined above for formula I. Preferably R^2 is $-\text{CH}_3$ or $-\text{CH}_2\text{-COOH}$. Examples of tripeptides of formula II are:



and preferred tripeptides are

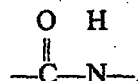


and



In addition to the tripeptides described, salts of these tripeptides are also an embodiment of the present invention. These salts include pharmaceutically acceptable salts of inorganic acids, e.g. HCl, H₂SO₄, HBr and H₃PO₄, and of organic acids, e.g. cyclohexylcarboxylic acid, tartaric acid, oxalic acid, fumaric acid, citric acid, malic acid, ascorbic acid, acetic acid, lactic acid and fatty acids, e.g. oleic, pantoic and palmitic acids. By pharmaceutically acceptable is meant that the salts are substantially non-toxic and have pharmacological activity like that of the tripeptide.

The tripeptides of the present invention can be prepared using various processes and techniques. The preparation involves coupling of appropriate amino acid components via the peptide linkage,

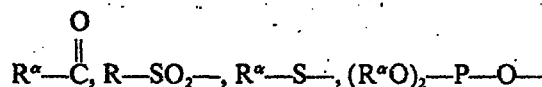


For example, the tripeptides of the present invention may be prepared by coupling M₁-M₂ and M₃ together through peptide linkages to form M₁-M₂-M₃, and subsequently converting the latter to M₁-M₂-M₃-E.

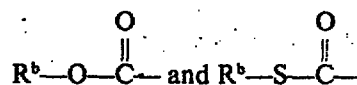
Alternatively, M₁, M₂ and M₃-E may be sequentially coupled in any order to produce M₁-M₂-M₃-E, for example, L-pca-L-his-M₃-E can be produced by first preparing L-pca-L-his-N₂ and then coupling it with M₃-E. In this procedure, M₂ and M₃-E may have been originally prepared with amino-protecting groups and it is necessary to remove such groups before coupling can take place. If that is the case, the protecting group on M₃-E will be removed during the coupling of M₃-E to M₂ or to M₁-M₂ and the group on M₂ will be removed during the coupling of M₂ or of M₂-M₃-E to M₁.

Where the peptide prepared is linear, coupling involves reaction between an α -amino group and a carboxyl group of different amino acid components. The general practice in controlling and directing this coupling is to protect or block, in the amino acid components, the functional groups that are not intended to take part in peptide bond formation. Important functional requirements of the blocking groups are that (1) they should not adversely affect the coupling reaction and (2) they should be conveniently removable as required during the peptide preparation.

Various types of blocking or protecting groups are available and may be used. Suitable amino-protecting groups include acyl type groups having the formulae



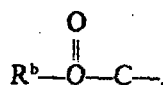
where R^a is alkyl, aryl, aralkyl, alkaryl, or substituted alkyl or aryl; urethane type groups having the formula



where R^b is a hydrocarbon group, e.g. alkyl, aryl, alkaryl, aralkyl-substituted alkyl or aryl, or arylidene having the formula R^c=C- where R^c is an aryl group; a substituted hydrocarbon group or an alkylamino or heterocyclic group.

Representative examples of the acyl-type groups are formyl, *p*-toluenesulfonyl, chloroacetyl, trifluoroacetyl, phthaloyl, phosphoryl, benzenesulfonyl, *o*-nitrophenoxyacetyl, *o*-nitrophenylsulfonyl and dibenzylphosphoryl. Examples of alkyl and aryl blocking groups are triphenylmethyl, benzyl, trialkylsilyl, benzylthiomethyl, dinitrophenyl and diphenyl. Useful arylidene groups are exemplified by benzylidene and 2-hydroxy-5-chlorobenzylidene.

Most useful of the amino blocking groups are those of the urethane type having the formula



when R^b is as defined above. Examples of the especially useful urethane-type

protecting groups are those in which R² is benzyl, substituted benzyl, e.g. *p*-methoxybenzyl, *p*-nitrobenzyl, *p*-phenylazobenzyl, *p*-bromobenzyl and 2-nitrobenzyl, cycloalkyl, e.g. cyclohexyl and cyclopentyl, substituted cycloalkyl e.g. methylcyclohexyl, dodecylcyclohexyl and isobutylcyclopentyl, adamantyl, piperidino, dimethylamino and alkyl groups e.g. *n*-propyl, *t*-butyl, *t*-amyl, *n*-octyl and *n*-dodecyl.

In addition to the blocking groups described above, the amino function can also be protected by salt formation, provided the amino group is sufficiently basic.

Carboxyl group protection of the amino acid component is generally effected by esterification, amide or hydrazide formation and salt formation.

Useful carboxyl protecting esters include alkyl esters such as methyl, ethyl, *t*-butyl and decyl, aryl esters such as benzyl, phenyl and benzhydryl, substituted alkyl esters, and substituted phenyl esters such as pentamethylbenzyl, phenylazo-phenyl and *o*-cyanobenzyl.

Hydrazides that are useful as carboxyl-protecting groups are, in general, substituted. Examples of such hydrazides are benzoyloxylhydrazide, *tert*-butyloxy-carbonylhydrazide, phenylhydrazide and tritylhydrazide.

Protection of the carboxyl group by forming an amide is of little general use since the amide group is difficult to remove without disrupting the peptide bond itself. However, where, as here, the tripeptide end group may be an amide, this means of carboxyl protection can be advantageously used.

In the case of some of the ester carboxyl protecting groups, these groups also have what is known as an activating effect. This activation refers to enhancement of the coupling reactivity. The extent of the activation is dependent on the particular ester chemical configuration, the general rule being that activation is determined by the extent to which the protecting group makes the carboxyl function more susceptible to nucleophilic attack. Examples of carboxyl activating ester groups are *p*-nitrophenyl, 2,4-dinitrophenyl, *N*-hydroxysuccinimidyl, perfluorophenyl, cyanomethyl, perchlorophenyl, 2,4,5-trichlorophenyl, 4-methylthio-phenyl, 8-hydroxyquinolyl, 1-hydroxybenzotriazole, and thioesters, e.g. *p*-nitrobenzylthio, *p*-nitrophenylthio and phenylthio.

Coupling of the amino acid components can be accomplished by various reactions. Among reactions are included:

(a) direct condensation of N^α-blocked amino acid component with carboxyl blocked amino acid component,

(b) conversion of N^α-blocked amino acid component to the azide followed by reaction with a carboxyl blocked amino acid component,

(c) reaction of N^α-blocked amino acid halide or mixed anhydride with carboxyl blocked amino acid component,

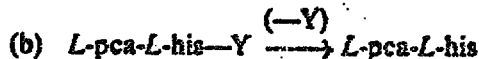
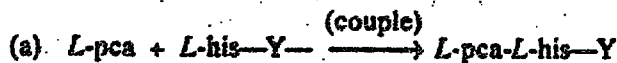
(d) reaction of N^α-blocked amino acid active ester, with carboxyl blocked amino acid,

(e) direct coupling of N^α-blocked amino acid component with carboxyl blocked amino acid component using a coupling agent, e.g. a carbodiimide; a carbodiimide plus a hydroxybenzotriazole; a carbodiimide plus *N*-hydroxysuccinimide; triphenylphosphite plus imidazole; triphenylphosphine plus dipyrindyl-2,2'-disulfide; or Woodward's reagent.

The coupling reactions especially useful are the azide method (b) and method (e) using a carbodiimide.

Various preparative schemes can be used for obtaining the present peptides. A convenient scheme utilizes sequential coupling of individual amino acids to prepare first a dipeptide intermediate and then the final tripeptide.

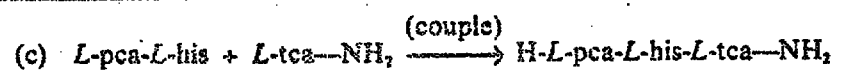
In carrying out the coupling steps, the component reacting at its carboxyl group is generally protected at its alpha amino group while the component reacting at its alpha amino group is generally protected at its carboxyl group. Where the component also has a secondary functional group, e.g. the imidazole group in histidine, it may also be protected. The following equations illustrate such a stepwise sequence. The sequence shows an amide preparation-analogous sequence is used to prepare the ester tripeptides. Y, in the equations, is a carboxyl protecting group.



2-nitro-alkyl e.g. amantyl, n-OR
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If Y is step (a) is an activating group, steps (b) and (c) may be combined. Also, although *L*-pca is not shown to be protected at its amino group, it may be protected if desired. In that case, an additional step would be required to remove the protecting group to yield the *H-L-pca-L-his-L-tca-NH₂*.

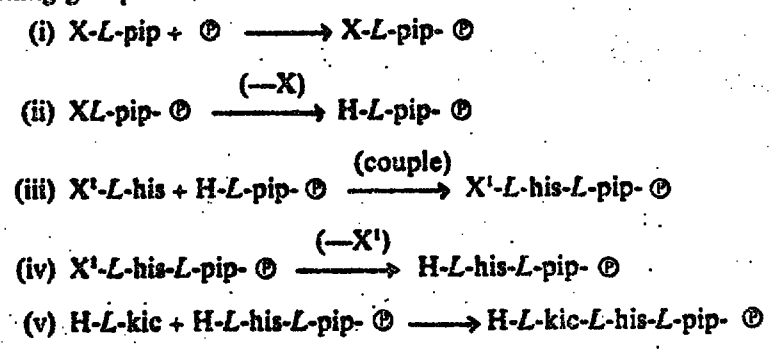
The reaction sequence shown in the equations (a) to (c) is illustrative and not meant to be limiting. Thus, the sequence used may involve coupling *M₁* amino acid with *M₂-NH₂* (or OR) amino acid component to produce the *M₁ M₂-NH₂* (or OR) dipeptide which in turn is coupled with the *M₁* amino acid to produce the desired *M₁-M₂-M₃-NH₂* (or OR) tripeptide.

In the case where *M₂* is to be an *N³*-substituted *L*-his, the tripeptide may be prepared from the corresponding peptide *M₁-L-his-M₃-E* with a *C₁₋₄* alkyl iodide or a compound of formula *I-(CH₂)_b-COOH*, where *b* is 1, 2, 3 or 4. For example, *H-L-pca-L-his-L-pro-NH₂* or *H-L-kpc-L-his-L-tca-NH₂* is reacted with *I-CH₂-COOH* to produce *H-L-pca-L-his(N³-CH₂COOH)-L-pro-NH₂* or *H-L-kpc-L-his(N³-CH₂COOH)-L-tca-NH₂*, respectively.

Another scheme for preparing the present tripeptides utilizes a resin matrix on which the peptide is sequentially built. This scheme may involve a soluble resin system or an insoluble system. The insoluble resin system is preferred and is conventionally referred to as solid phase (or Merrifield) synthesis. The insoluble resin is generally one that has sites at which carboxyl groups can be found. An example of such a resin is one commonly referred to as Merrifield resin. One form of this resin is a copolymer of styrene and divinylbenzene which is chloromethylated to provide carboxyl reactive sites. The peptide is built up one amino acid at a time until the desired tripeptide is formed. The tripeptide is then freed from the resin.

Thus, a tripeptide in accordance with the present invention, may be prepared by coupling by peptide linkage, *M₂* with *M₁-⊙* where ⊙ is a resin support having sites capable of bonding with the carboxylic acid of *M₁* and *M₂* and *M₁* are as defined above to produce *M₂-M₁-⊙*, coupling by peptide linkage, *M₃-M₂-⊙* with *M₁* (where *M₁* is as defined above) to produce *M₃-M₂-M₁-⊙*, trans-esterifying *M₃-M₂-M₁-⊙* with ROH where R is *C₁₋₄* alkyl to produce *M₃-M₂-M₁-OR* and ⊙-H, separating *M₃-M₂-M₁-OR* from ⊙-H and optionally replacing the -OR in *M₃-M₂-M₁-OR* with -NH₂. In this procedure, *M₂* is conveniently coupled by use of *X-M₂*, where X is an amino-blocking group, to produce *X-M₂-M₁-⊙*, which is deblocked to produce *M₂-M₁-⊙*.

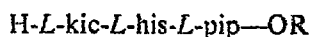
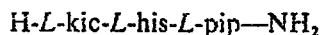
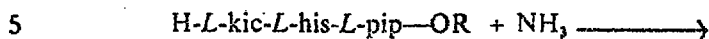
This procedure is illustrated in the reaction equation sequence set out below. In the equations, P is used to describe the resin and X and X' represent amino blocking groups.



The next step (or steps) in the sequence involves freeing the tripeptide from the resin ⊙. Depending on the reaction system used, the freed tripeptide may have an acid, ester or amide terminal group. Where, after the resin is cleaved and the terminal group is the acid, this tripeptide can be converted to the corresponding ester or amide by conventional procedures. Similarly, where the freed tripeptide has an ester terminal group, the amide group may, if desired, be substituted for it by conventional procedures.

A most convenient method for separating the tripeptide from the resin ⊙ is by transesterifying with a suitable alcohol. One advantage of this method is that ester is obtained directly and the ester can be readily converted to the amide if desired. These reactions are illustrated below:

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Tripeptide EsterTripeptide Amide

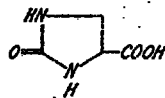
Although not shown in the above reaction equation, the nitrogens in the kic and the histidine imidazole group can be blocked during the reaction if desired, with the blocking groups being removed at an appropriate point in the peptide preparation. N^{3im} -substituted-his- containing tripeptides can also be prepared using solid phase synthesis, with the substituting group being added either before or during the course of the synthesis.

In both schemes described above and in peptide preparation generally, the blocking and/or activating groups are added and removed. Both removal and addition of blocking groups is accomplished using techniques and reaction systems known and available to those skilled in the peptide art. Examples of some reactions by which the protecting or blocking groups are removed are reduction with sodium in liquid NH_3 , hydrogenolysis (e.g. using Pd/C as a catalyst) treatment with hydrohalo acid (e.g. HCl or HBr) in acetic acid, or treatment with TFA. The particular reaction system used to deblock, that is to remove a blocking group, as well as to block a group in an amino acid component is chosen so that, (1) if necessary, removal of blocking groups is selective and (2) it does not adversely affect the peptide preparation process.

Following are examples illustrating the preparation of tripeptides of Formula I and some intermediates. All parts are by weight unless otherwise indicated.

EXAMPLE 1.(A) Preparation of L-2-ketoimidazolidine-5-carboxylic acid

A solution of 25 grams (g) of NaOH in 760 milliliters (ml) of water was prepared in a vessel and placed in a cooling bath. 5 ml of bromine was added to the solution with stirring. When the temperature of this mixture reached about 0°C , 26.6 g. of N^α -benzyloxycarbonyl asparagine was dissolved in the mixture. The solution was then warmed to 75°C . and kept at this temperature for 45 minutes. The solution was then cooled and extracted twice with 150 mls. of methylene chloride each time. The extracted solution was evaporated to dryness and the solid remaining was dried overnight in a vacuum oven. The dried solid was extracted with ethanol. The extract was evaporated to the point where the product L-2-ketoimidazolidine-5-carboxylic acid,



precipitated.

After filtration of the product, it was dried under vacuum. About 4.20 grams of product was obtained. The melting point was $166^\circ\text{--}170^\circ\text{C}$. Elemental analysis of the product showed C=34.87% (theo. C=36.93%), H=4.52% (theo. H=4.65%) and N=19.5% (theo. N=21.53%).

(B) Preparation of L-2-ketoimidazolidine-5-carboxyl-L-histidyl-L-proline-OCH₃

19.8 g (6.36 millimoles [mm]) N^α -*tert*-butyloxycarbonyl- N^{3im} 2,4-dinitrophenyl-L-histidyl-L-proline- $\textcircled{\text{O}}$, 2.0 equivalents of L-2-ketoimidazolidine-5-carboxylic acid, 2.0 equivalents of dicyclohexylcarbodiimide and 1.3 grams of 1-hydroxybenzotriazole were mixed in DMF and allowed to react at room temperature (R.T.) for about 5 hours. At the end of this time, reaction was substantially complete and the yield of H-L-kic- N^{3im} -DNP-L-his-L-pro- $\textcircled{\text{O}}$ was 98%. The dinitrophenyl blocking group was removed with $\text{HSC}_2\text{H}_4\text{OH/DMF}$ and the product, H-L-kic-L-his-L-pro- $\textcircled{\text{O}}$ was washed with acetic acid, methanol and CH_2Cl_2 .

The washed H-L-kic-L-his-L-pro- OCH_3 (4.7 g) was added to 188 ml. of methanol and 47.1 ml. triethylamine. This mixture was stirred at R.T. for 5 days and then allowed to stand at R.T. for about 6 days. The mixture was then filtered, evaporated to dryness and purified by elution through silica column (ratio of weight of silica to weight of material applied to it being 200:1). The eluent was a mixture of 70 parts by volume CHCl_3 , 30 parts by volume CH_3OH and 3 parts by volume H_2O . The product H-L-kic-L-his-L-pro- OCH_3 was isolated by lyophilization. Yield was 1.54 grams.

(C) Preparation of L-2-ketoimidazolidine-5-carbonyl-L-histidyl-L-proline-amide
0.8 grams of H-L-kic-L-his-L-pro- OCH_3 was added to about 10 ml. of liquid NH_3 and kept at R.T. for 7 days. This reaction mixture was dissolved in methanol. After evaporation *in vacuo* the residue was freeze dried. The primary product obtained was H-L-kic-L-his-L-pro- NH_2 .

EXAMPLE 2.

Preparation of DL-2-ketopiperidine-6-carbonyl-L-histidyl-L-proline- OCH_3 ; - NH_2

By substituting DL-2-ketopiperidine-6-carboxylic acid for L-2-ketoimidazolidine-5-carboxylic acid and using substantially the same procedures as in Examples 1B and 1C, the tripeptides, H-DL-kpc-L-his-L-pro- OCH_3 and H-DL-kpc-L-his-L-pro- NH_2 were prepared.

Examples of other tripeptides which are prepared using the general procedure of Example 1B are:

- H-kpc-his-L-tca- OC_2H_5
- H-DL-pca-L-his-L-tca- $\text{O}-n\text{-C}_6\text{H}_{13}$ and
- H-kic-his-L-pip- $\text{OC}(\text{CH}_3)_3$

and these esters are converted to the corresponding amides using the procedure of Example 1C.

EXAMPLE 3.

(A) Preparation of L-pyroglutamyl-L-histidine- OCH_3

A vessel equipped for agitation and cooling was charged with L-histidine methyl ester dihydrochloride (72.9 g.), L-pyroglutamic acid (39.1 g.) and acetonitrile (1200 ml). This mixture was cooled to 0°C . with stirring. Triethylamine (84 ml.) was added gradually over a 10-minute period, maintaining the temperature at 0°C . This mixture was then allowed to stand for 15 minutes. A solution of 76.5 g. of dicyclohexylcarbodiimide in 180 ml. of acetonitrile was then added gradually over a 5-10 minute period. The resulting mixture was allowed to stand at 0°C . for 30 minutes, then allowed to warm to room temperature, and then stirred for 24 hours at room temperature.

The mixture was then filtered and the filter cake was washed 3 times with acetonitrile (100 ml. each time). This filtrate was discarded.

The washed cake was then slurried in the funnel four (4) times with methanol (150 ml. each time). This filtrate was evaporated under vacuum to a volume of 300 ml. and then 1200 ml. of diethyl ether was added slowly with stirring. This mixture was allowed to stand overnight (although 30 minutes is adequate) and was then filtered. The filter cake was washed twice with a 9 volume:1 volume, diethyl ether:methanol mixture (75 ml. each time). This washed filter cake was then slurried with 500 ml. of chloroform for two hours. The slurry was filtered and the filter cake was washed four times with chloroform (45 ml. each time).

This washed cake (42.1 grams) was added to 168 ml. of methanol and heated rapidly to boiling. This hot mixture was filtered. The filtrate was allowed to cool to room temperature and was allowed to stand at room temperature for 3 hours. White crystalline product L-pyroglutamyl-L-histidine- OCH_3 was formed. This was filtered off and washed with 10 ml. of methanol. This washed material was dried. The yield of L-pyroglutamyl-L-histidine- OCH_3 was 24.1 g (28.7%). The product m.p. was $208^\circ\text{--}210^\circ\text{C}$. Thin layer chromatographic (TLC) analysis showed the product to be 99% by weight pure.

(B) Preparation of L-pyroglutamyl-L-histidine hydrazide

In a vessel fitted with thermometer and stirrer and placed in an ice bath, 33.6 g. of L-pyroglutamyl-L-histidine- OCH_3 was dissolved in 640 ml. of methanol.

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When the solution temperature reached 10°C, 400 ml. of anhydrous hydrazine of purity at least 97% by weight was added gradually over 5 minutes, maintaining the temperature below 20°C. The reaction mixture was stirred for an additional 6 minutes at 18–20°C., and then transferred to a second vessel and stripped under vacuum.

The resulting white solid was redispersed in 1040 ml. of ethanol-2BA (benzene denatured-absolute) and then the resulting mixture was stripped of ethanol. This redispersion/stripping step was repeated a second time.

The resulting white solid was next dispersed in 800 ml. of DMF. This dispersion was then evaporated under vacuum to a weight of 230 g. This evaporated material was dispersed in 800 ml. of ethanol-2BA, allowed to stand for 45 minutes at room temperature and filtered. The filter cake was washed twice with ethanol-2BA (80 ml. each time) and dried. This dried product was dispersed in 3200 ml. of methanol and heated to boiling. The mixture was allowed to cool to room temperature, with stirring, and filtered, and the filter cake was washed twice with methanol (60 ml. each time). The product was dried under vacuum. Yield of *L*-pyroglutamyl-*L*-histidine-hydrazide was 25.69 g. (76.5%). TLC analysis showed this product to be substantially pure.

(C) Preparation of *L*-pyroglutamyl-*L*-histidyl-*L*-thiazolidine-4-carboxylic acid amide

800 mg. (3 mm) of *L*-pyroglutamyl-*L*-histidine hydrazide was suspended in 80 ml. DMF. The suspension was cooled to –20°C. and brought to a pH of 1.5 by addition of 5.9M HCl in THF.

4 ml. of a mixture of 1 volume of isoamylnitrite and 10 volumes of DMF was added. The mixture was stirred for 25 minutes while the temperature was kept at –20°C. Then, 500 mg. (3 mm) of *L*-thiazolidine-4-carboxylic acid was added and the pH was adjusted to 8.5 by addition of 4.1 ml. triethylamine. The mixture was stored at –15°C. for seven days.

DMF was then removed *in vacuo*. The residue was dissolved in 40 ml. of 0.4M sodium carbonate-bicarbonate buffer (pH=10) and the solution was extracted four times with a mixture of 30 ml. *n*-butanol/40 ml. chloroform.

The aqueous layer was evaporated and the residue was extracted with 50, 25 and 25 ml. of methanol. The methanol solutions were combined and evaporated *in vacuo*. The residue remaining is *L*-pyroglutamyl-*L*-histidyl-*L*-thiazolidine-4-carboxylic acid.

The *L*-pyroglutamyl-*L*-histidyl-*L*-thiazolidine-4-carboxylic acid (615 mg.; 1.5 mm) was dissolved in 30 ml. DMF. 260 mg. (1.7 mm) of HBT was added to the solution and the pH adjusted to 4 with triethylamine. 180 mg. (3.4 mm) of NH₄Cl was added followed by 385 mg. (1.7 mm + 10% excess) of DCCI. The reaction mixture was stirred overnight at R.T.

The reaction mixture was then filtered. The filtrate was evaporated, the residue was dissolved in 20 ml. of eluent (80:20:2) by volume chloroform:methanol:water) and charged to a 200-g. silica gel column.

The fractions containing the product *L*-pyroglutamyl-*L*-histidyl-*L*-thiazolidine-carboxylic acid amide were pooled and evaporated; yield was 370 mg. of the product—substantially pure by TLC.

Other tripeptides which can be prepared using the general procedure of Example 3 are:

H-*L*-kic-*L*-his-*L*-pro—NH₂,
H-*DL*-kpc-his-*L*-pip—NH₂,
H-pca-his-*L*-pip—NH₂,
H-*L*-kpc-his-*L*-pip—NH₂, and
H-kic-his-*L*-tca—NH₂.

EXAMPLE 4.

Preparation of *L*-pyroglutamyl-N^{31m}-carboxymethyl-*L*-histidyl-*L*-proline amide

H-*L*-pca-*L*-his-*L*-pro—NH₂ was added to a DMF solution containing 1.1 equivalents of monoiodoacetic acid. The reaction mixture was stirred for about 18 hours and the solution evaporated to dryness. The dried residue was chromatographed on a suitable dry silica gel column using a mixture of CHCl₃:CH₃OH:H₂O (50:40:10 by volume) as eluent, taking 5 ml. cuts. The fractions containing first major product eluted contained substantially pure H-*L*-

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pca-L-his(N^{31m}-CH₂COOH)-L-pro-NH₂. Other products in the reaction mixture were H-L-pca-L-his(N^{11m}, N^{31m}-di-CH₂COOH)-L-pro-NH₂ and H-L-pca-L-his(N^{11m}-CH₂COOH)-L-pro-NH₂.

In some cases, the N^{31m}-substituted L-his-containing product may be eluted as an iodide salt. This salt can be neutralized e.g. with the resin sold under the Trade Mark Dowex 1 in hydroxide form, to obtain the free tripeptide product.

Other N^{31m} substituted-his containing tripeptides which were prepared from the appropriate tripeptide using the general procedure of Example 4 are:

H-L-pca-DL-his(N^{31m}-n-hexyl)-L-pro-OCH₃,
H-DL-pca-DL-his[N^{31m}-(CH₂)₄-COOH]-L-pro-NH₂,
H-kic-his[N^{31m}-(CH₂)₂-COOH]-tca-NH₂,
H-DL-kpc-his(N^{31m}-C₆H₅)-L-pip-OC₂H₅,
H-L-kic-L-his(N^{31m}-CH₂COOH)-L-pro-OCH₃ and
H-pca-DL-his(N^{31m}-cyclohexyl)-L-tca-NH₂.

EXAMPLE 5.

Preparation of L-pyroglutamyl-histidyl-L-piperidine-2-carboxylic acid methyl ester

1.5 g. of H-L-pca-L-his-NH-NH₂ was dispersed in 40 ml. of DMF and cooled to -30°C. with stirring. To this dispersion was added 4.08 ml. of 5.25 M HCl in THF. Then, in a period of about 40 minutes, 0.77 ml. of isoamyl nitrite was added (0.65 ml. initial charge followed, by three increments of 0.051 ml, 0.035 ml, and 0.035 ml). To this mixture was added 965 mg (5.37 millimoles) of L-piperidine-2-carboxylic acid methyl ester hydrochloride. Next about 4.1 ml. of triethylamine was added. The reaction mixture was then maintained at +5C. for 96 hours and then at R.T. overnight. [During this period three additional increments (0.2 ml, 0.4 ml and 0.2 ml) of triethylamine were added to maintain pH at about 7.2].

At the end of this time, the triethylamine hydrochloride was filtered off. This residue was washed with DMF/diethyl ether (125 ml/50-75 ml.). The wash was combined with the filtrate. This filter/wash procedure was repeated two more times. The final filtrate and washings were then evaporated *in vacuo* to yield 2.87 g. of crude tripeptide product. This crude product was purified by elution through silica gel column with 90:10:1 by volume chloroform:methanol:water mixture. Products recovered were H-L-pca-L-his-L-pip-OCH₃ (787 mg) and H-L-pca-DL-his-L-pip-OCH₃ (671 mg).

Substituting H-L-pip-NH₂ for H-L-pip-OCH₃ in Example 5, the analogous H-L-pca-his-L-pip-NH₂ is prepared.

Other tripeptides which are prepared using the general procedure of Example 5 are:

H-L-pca-his-L-tca-OCH₃,
H-L-kpc-his-L-pip-OCH₃,
H-L-pca-his-L-tca-NH₂,
H-L-kic-his-L-pip-O-n-C₄H₉,
H-L-kic-his-L-pip-O-n-C₁₀H₂₁ and
H-L-kpc-his-L-tca-OCH(CH₃)₂.

In addition to the methods of preparation illustrated by the examples and disclosed above, the tripeptides of the present invention may also be prepared using methods for preparing tripeptides of the TRH type disclosed in the art cited above.

Tripeptides of the present invention have been found to have anti-depressant activity and thyrotropin-releasing hormone activity. Their activity as anti-depressants is as central nervous system (CNS) stimulants and was determined by testing representative tripeptides of the present invention using *in vivo* assay based on restoration of anti-convulsant action of methazolamide in picolinic-acid-treated mice. The thyrotropin-releasing hormone activity of the present tripeptides was determined using the *in vivo* assay substantially as described in "Vitamins and Hormone" R. S. Harris et al, 29, 3-4 (1971).

In some cases, unlike TRH, the present tripeptides showed a separation of activity e.g. they had greater anti-depressant activity than thyrotropin-releasing hormone activity. This type of separated activity would offer the advantage of making the tripeptide especially useful at proper dosages for treating depression

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without causing significant thyrotropin release when not desired or necessary.

5 The following table contains activity data for a number of representative peptides of the present invention. The procedures used to obtain the data were those described above. The results are expressed numerically as multiples of the TS (thyrotropin stimulation) and AD (anti-depressant) activity compared to the control TS and AD activities of TRH which have the value 1. The designation S.N. is used to indicate substantially no detectable activity at the dosage level tested.

TABLE I
THYROTROPIN RELEASING HORMONE AND ANTI-DEPRESSANT ACTIVITIES

No	Peptide	TS	AD
Control	TRH	1	1
1	<i>L</i> -2-ketopiperidine-6-carbonyl- <i>L</i> -histidyl- <i>L</i> -thiazolidine-4-carboxamide	1	8
2	<i>L</i> -2-ketopiperidine-6-carbonyl- <i>L</i> -histidyl- <i>L</i> -proline amide	1	4
3	<i>DL</i> -2-ketopiperidine-6-carbonyl- <i>L</i> -histidyl- <i>L</i> -proline amide	0.2	3
4	<i>L</i> -pyroglutamyl- <i>DL</i> -histidyl- <i>L</i> -thiazolidine-4-carboxamide	0.2	2
5	<i>L</i> -pyroglutamyl- <i>L</i> -histidyl- <i>L</i> -2-piperidine-carboxylic acid methyl ester	0.2	1
6	<i>L</i> -2-ketoimidazolidine-5-carbonyl- <i>L</i> -histidyl- <i>L</i> -proline amide	0.1	1
7	<i>L</i> -pyroglutamyl-(<i>N</i> ^{31m} -carboxymethyl- <i>L</i> -histidyl)- <i>L</i> -proline amide	0.1	1
8	<i>L</i> -2-ketopiperidine-6-carbonyl- <i>L</i> -histidyl- <i>L</i> -2-piperidinecarboxylic acid methyl ester	0.016	1
9	<i>L</i> -pyroglutamyl- <i>DL</i> -histidyl- <i>DL</i> -2-piperidinecarboxylic acid methyl ester	S.N.	0.5
10	<i>L</i> -pyroglutamyl- <i>DL</i> -histidyl- <i>L</i> -2-piperidinecarboxylic acid methyl ester	S.N.	1
11	<i>L</i> -pyroglutamyl- <i>L</i> -histidyl- <i>L</i> -2-piperidinecarboxamide	1	1
12	<i>DL</i> -2-ketopiperidine-6-carbonyl- <i>L</i> -histidyl- <i>L</i> -proline methyl ester	0.02	S.N.
13	<i>L</i> -2-ketoimidazolidine-5-carbonyl- <i>L</i> -histidyl- <i>L</i> -proline methyl ester	0.001	S.N.

10 The data in Table I clearly illustrate the pharmacological effectiveness and unexpected separation of TS and AD activities of peptides of this invention.

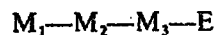
Based on the *in vivo* effectiveness of such peptides as thyrotropin-releasing activators and as central nervous system stimulants (anti-depressant activity), they may be effectively used for treating mammals suffering from central nervous system depression and/or in need of thyrotropin release activation.

15 The peptides can be administered by any convenient method, e.g. orally, parenterally, intravenously, sublingually, by insufflation or by suppository.

20 Dosage levels adequate to produce the desired effect are used. Preferably the dosage range will be from 0.05 to 100 mg. per dose depending on the method of administration. Dosage intervals will depend on the extent of relief desired. The tripeptides can be administered alone, in pharmaceutically acceptable carriers or in formulations with other pharmaceutically active substances, as desired.

WHAT WE CLAIM IS:—

1. A tripeptide having the formula:



25 or an acid salt thereof in which M_1 is H-kic, H-kpc or H-pca, M_2 is his, his[N^{31m}—(C₁₋₆ alkyl)] or his[N^{31m}—(CH₂)_b—COOH], where b is 1, 2, 3 or 4, M_3 is

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THYROTROPIN RELEASING HORMONE AND ANTI-DEPRESSANT ACTIVITIES

No	Peptide	TS	AD
Control	TRH	1	1
1	<i>L</i> -2-ketopiperidine-6-carbonyl- <i>L</i> -histidyl- <i>L</i> -thiazolidine-4-carboxamide	1	8
2	<i>L</i> -2-ketopiperidine-6-carbonyl- <i>L</i> -histidyl- <i>L</i> -proline amide	1	4
3	<i>DL</i> -2-ketopiperidine-6-carbonyl- <i>L</i> -histidyl- <i>L</i> -proline amide	0.2	3
4	<i>L</i> -pyroglutamyl- <i>DL</i> -histidyl- <i>L</i> -thiazolidine-4-carboxamide	0.2	2
5	<i>L</i> -pyroglutamyl- <i>L</i> -histidyl- <i>L</i> -2-piperidine-carboxylic acid methyl ester	0.2	1
6	<i>L</i> -2-ketoimidazolidine-5-carbonyl- <i>L</i> -histidyl- <i>L</i> -proline amide	0.1	1
7	<i>L</i> -pyroglutamyl-(N ³ im-carboxymethyl- <i>L</i> -histidyl)- <i>L</i> -proline amide	0.1	1
8	<i>L</i> -2-ketopiperidine-6-carbonyl- <i>L</i> -histidyl- <i>L</i> -2-piperidinecarboxylic acid methyl ester	0.016	1
9	<i>L</i> -pyroglutamyl- <i>DL</i> -histidyl- <i>DL</i> -2-piperidinecarboxylic acid methyl ester	S.N.	0
10	<i>L</i> -pyroglutamyl- <i>DL</i> -histidyl- <i>L</i> -2-piperidinecarboxylic acid methyl ester	S.N.	1
11	<i>L</i> -pyroglutamyl- <i>L</i> -histidyl- <i>L</i> -2-piperidinecarboxamide	1	1
12	<i>DL</i> -2-ketopiperidine-6-carbonyl- <i>L</i> -histidyl- <i>L</i> -proline methyl ester	0.02	S
13	<i>L</i> -2-ketoimidazolidine-5-carbonyl- <i>L</i> -histidyl- <i>L</i> -proline methyl ester	0.001	S

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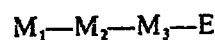
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1. A tripeptide having the formula:



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ACTIVITIES

	TS	AD
	1	1
	1	8
	1	4
	0.2	3
	0.2	2
	0.2	1
	0.1	1
	0.1	1
yl ester	0.016	1
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	0.02	S.N
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L-pip, L-pro or L-tca, and E is —NH₂ or —OR where R is C₁₋₁₀ alkyl, including substituted alkyl and cycloalkyl, such that (i) when E is —NH₂, pca and L-pro do not occur together in the tripeptide, and (ii) when E is —OR, his and L-pro do not occur together in the tripeptide, and where the abbreviations for the amino acids are as defined in the specification and their configurations are DL or L or a mixture thereof.

2. A tripeptide as claimed in Claim 1 in which M₂ is his.

3. A tripeptide as claimed in Claim 1 in which M₂ is N^{3im} C₁₋₆ alkyl-his or N^{3im}—(CH₂)_b—COOH-his.

4. A tripeptide as claimed in Claim 1 in which E is —NH₂.

5. A tripeptide as claimed in Claim 4 in which M₃ is L-pip.

6. A tripeptide as claimed in Claim 5 in which M₁ is H-kic.

7. A tripeptide as claimed in Claim 4 in which M₂ is L-his.

8. A tripeptide as claimed in Claim 7 in which M₁ is H-L-kpc.

9. A tripeptide as claimed in Claim 7 in which M₁ is H-L-pca and M₃ is L-pip or L-tca.

10. A tripeptide as claimed in Claim 1 in which E is —OR.

11. A tripeptide as claimed in Claim 10 in which M₃ is L-pip.

12. A tripeptide as claimed in Claim 10 in which M₂ is L-his.

13. A tripeptide as claimed in Claim 11 in which M₁ is H-L-pca, H-L-kic or H-L-kpc and M₃ is L-pip or L-tca.

14. A tripeptide as claimed in Claim 10 in which R is —CH₃.

15. A tripeptide as claimed in Claim 13 in which R is —CH₃.

16. A method of preparing a tripeptide as claimed in Claim 1, that comprises coupling through peptide linkages of M₁—M₂ and M₂ to produce M₁—M₂—M₃, which is subsequently converted to M₁—M₂—M₃—E, M₁, M₂, M₃ and E being as defined in Claim 1.

17. A method of preparing a tripeptide as claimed in Claim 1, that comprises sequential coupling of M₁, M₂ and M₃—E in any order to produce M₁—M₂—M₃—E, where M₁, M₂, M₃ and E are as defined in Claim 1.

18. A method as claimed in Claim 17 in which M₂ and M₃—E, as originally prepared, have amino-protecting groups and the group on M₃—E is removed during the coupling of M₃—E to M₂ or M₁—M₂ and the group on M₂ is removed during the coupling of M₂ or of M₂—M₃—E with M₁.

19. A method of preparing the tripeptide H-L-pca-L-his—M₃—E that comprises preparing L-pca-L-his—N₃ and coupling it with M₃—E, where M₃ and E are as defined in Claim 1.

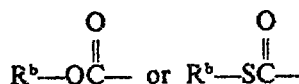
20. A method of preparing a tripeptide as claimed in Claim 1 in which M₂ is N^{3im}-substituted L-his, that comprises reacting a tripeptide having the formula M₁—L-his—M₃—E with a C₁₋₆ alkyl iodide or a compound of formula I—(CH₂)_b—COOH, where M₁, M₃ and b are as defined in Claim 1.

21. A method as claimed in Claim 20 that comprises reacting H-L-pca-L-his-L-pro—NH₂ or H-L-kpc-L-his-L-tca—NH₂ with I—CH₂—COOH to produce H-L-pca-L-his(N^{3im}—CH₂COOH)—L-pro—NH₂ or H-L-kpc-L-his(N^{3im}—CH₂COOH)—L-tca—NH₂, respectively.

22. A method of preparing a tripeptide as claimed in Claim 1 that comprises coupling by peptide linkage, M₂ with M₃—⊙, where ⊙ is a resin support having sites capable of bonding with the carboxylic acid of M₃ and M₂ and M₃ are as defined in Claim 1, to produce M₂—M₃—⊙, coupling by peptide linkage, M₁—M₂—⊙ with M₁ (where M₁ is as defined in Claim 1) to produce M₁—M₂—M₃—⊙, transesterifying M₁—M₂—M₃—⊙ with ROH where R is C₁₋₆ alkyl to produce M₁—M₂—M₃—OR and ⊙—H, separating M₁—M₂—M₃—OR from ⊙—H and optionally replacing the —OR in M₁—M₂—M₃—OR with —NH₂.

23. A method as claimed in Claim 22 in which M₂ is coupled by use of X—M₂ where X is an amino-blocking group, to produce X—M₂—M₃—⊙, which is de-blocked to produce M₂—M₃—⊙.

24. A method as claimed in Claim 23 in which the blocking group X is of the urethane type, i.e. has the formula



where R^b is a hydrocarbon, substituted hydrocarbon, alkylamino or heterocyclic group.



25. A method of producing a peptide as claimed in Claim 1, substantially as hereinbefore described in any one of Examples 1 to 5.

26. A peptide as claimed in Claim 1, when prepared by a method as claimed in any one of Claims 16 to 25.

5 27. A pharmaceutical composition containing a peptide as claimed in any one of Claims 1 to 15 and 26, together with a pharmaceutically acceptable carrier. 5

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