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(54) D-ALA², D-LYS⁴-SOMATOSTATIN

(71) We, ELI LILLY AND COMPANY, a corporation of the State of Indiana, United States of America, having a principal place of business at 307 East McCarty Street, City of Indianapolis, State of Indiana, 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 the tetradecapeptide

H-L-Ala-D-Ala-L-Cys-D-Lys-L-Asn-L-Phe-L-L-Phe-Trp-L-Lys-L-
Thr-L-Phe-L-Thr-L-Ser-L-Cys-OH, formula I

as well as to its pharmaceutically acceptable acid addition salts and to intermediates produced during the synthesis of the tetradecapeptide.

Somatostatin (also known as somatotropin release inhibiting factor) is a tetradecapeptide of the formula

H-L-Ala-Gly-L-Cys-L-Lys-L-Asn-L-Phe-L-Phe-L-Trp-L-Lys-
L-Thr-L-Phe-L-Thr-L-Ser-L-Cys-Oh.

This tetradecapeptide was isolated from ovine hypothalmic extracts and was found to be active in inhibiting the secretion of growth hormone (GH), also known as somatotropin. In this regard, see P. Brazeau, W. Vale, R. Burgus, N. Ling, M. Butcher, J. Rivier, and R. Guillemin, *Science*, *179*, 77 (1973).

D-Ala²-somatostatin is published in *Endocrinology, 98* (2), 336—343 (1976).

D-Lys⁴-somatostatin is disclosed in L. Farland *et al., Molecular and Cellular Endocrinology, 4*, 79—86 (1976).

The tetradecapeptide of formula I includes the non-toxic acid addition salts thereof. Its structure differs from that of somatostatin by the presence of a D-lysine residue in position 4 in place of an L-lysine residue, and by the presence of a D-alanine residue in position 2 in place of a glycine residue. For convenience, the tetradecapeptide of formula I is referred to as D-Ala², D-lys⁴-somatostatin.

Thus, this invention provides to a compound selected from those of the formula

H-L-Ala-D-Ala-L-Cys-D-Lys-L-Asn-L-Phe-L-Phe-L-Trp-L-Lys-L-
Thr-L-Phe-L-Thr-L-Ser-L-Cys-OH, formula I

and pharmaceutically acceptable non-toxic acid addition salts, and, as an intermediate, R - L - Ala - D - Ala - L - Cys(R_1) - D - Lys(R_2) - L - Asn - L - Phe - L - Phe - L - Trp(R_5) - L - Lys(R_2) - L - Thr(R_3) - L - Phe - L - Thr(R_3) - L - Ser(R_4) - L - Cys(R_1) - X, formula II; in which

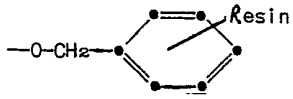
R is hydrogen or an α-amino protecting group;

R_1 is hydrogen or a thio protecting group;

R_2 is hydrogen or an ε-amino protecting group;

R_3 and R_4 each are hydrogen or a hydroxy protecting group;

R_5 is hydrogen or formyl; and
X is hydroxy or



in which resin is polystyrene; with the proviso that, when X is hydroxy, each of R, R_1, R_2, R_3, R_4, and R_5 is hydrogen, and, when X is

each of R, R_1, R_2, R_3, and R_4 is other than hydrogen.

The novel tetradecapeptide of formula I above is prepared by reacting the corresponding straight-chain tetradecapeptide of formula III, H - L - Ala - D - Ala - L - Cys - D - Lys - L - Asn - L - Phe - L - Phe - L - Trp - L - Lys - L - Thr - L - Phe - L - Thr - L - Ser - L - Cys - OH, with an oxidizing agent. This reaction converts the two sulfhydryl groups to a disulfide bridge.

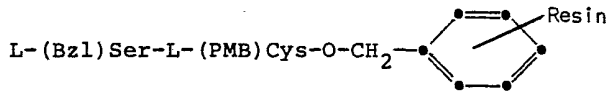
Pharmaceutically acceptable non-toxic acid addition salts include the organic and inorganic acid addition salts, for example, those prepared from acids such as hydrochloric, sulfuric, sulfonic, tartaric, fumaric, hydrobromic, glycolic, citric, maleic, phosphoric, succinic, acetic, nitric, benzoic, ascorbic, *p*-toluenesulfonic, benzenesulfonic, naphthalenesulfonic and propionic. Preferably, the acid addition salts are those prepared from acetic acid. Any of the above salts are prepared by conventional methods.

Also contemplated as being within the scope of this invention are intermediates of the formula II, R - L - Ala - D - Ala - L - Cys(R_1) - D - Lys(R_2) - L - Asn - L - Phe - L - Phe - L - Trp(R_5) - L - Lys(R_2) - L - Thr(R_3) - L - Phe - L - Thr(R_3) - L - Ser(R_4) - L - Cys(R_1) - X. Preferred intermediates include the following:

H - L - Ala - D - Ala - L - Cys - D - Lys - L - Asn - L - Phe - L - Phe - L - Trp - L - Lys - L - Thr - L - Phe - L - Thr - L - Ser - L - Cys - OH, formula III: and

N-(BOC)-L-Ala-D-Ala-L-(PMB)Cys-D-(CPOC)Lys-L-Asn-L-Phe-

L-Phe-L-Trp-L-(CPOC)Lys-L-(Bzl)Thr-L-Phe-L-(Bzl)Thr-



In the above formulae defining the intermdiates R is either hydrogen or an α-amino protecting group. The α-amino protecting groups contemplated for R are well recognized by those of ordinary skill in the peptide art. Many of these are detailed in the treatise *Protective Groups in Organic Chemistry*, J. F. W. McOmie, Editor, Plenum Press, New York, 1973, in Chapter 2, authored by J. W. Barton. Illustrative of such protecting groups are benzyloxycarbonyl, *p*-chlorobenzyloxycarbonyl, *p*-bromobenzyloxycarbonyl, *o*-chlorobenzyloxycarbonyl, 2,6-dichlorobenzyloxycarbonyl, 2,4-dichlorobenzyloxycarbonyl, *o*-bromobenzyloxycarbonyl, *p*-methoxybenzyloxycarbonyl, *p*-nitrobenzyloxycarbonyl, *t*-butyloxycarbonyl (BOC), *t*-amyloxycarbonyl, 2-(*p*-biphenylylisopropyloxycarbonyl (BpOC), adamantyloxycarbonyl, isopropyloxycarbonyl, cyclopentyloxycarbonyl, (CPOC), cyclohexyloxycarbonyl, cycloheptyloxycarbonyl, triphenylmethyl (trityl), and *p*-toluenesulfonyl. Preferably, the α-amino protecting group defined by R is *t*-butyloxycarbonyl.

R_1 represents either the hydrogen of the sulfhydryl group of the cysteine or a protecting group for the sulfhydryl substituent. Illustrative suitable protecting groups are *p*-methoxybenzyl, benzyl, *p*-tolyl, benzhydryl, acetamidomethyl, trityl, *p*-nitrobenzyl, *t*-butyl, isobutyloxymethyl, as well as any of a number of trityl derivatives. For addition groups, see, for example, Houben-Weyl, *Methodes der Organischen Chemie*, "Synthese von Peptiden", Vols. 15/1 and 15/2, (1974),

Stuttgart, Germany. Preferably, the sulfhydryl protecting group defined by R_1 is *p*-methoxybenzyl.

R_2 represents either hydrogen on the ε-amino function of the lysine residue or a suitable ε-amino protecting group. Illustrative of such groups are the many of those mentioned hereinabove as being suitable for use as an α-amino protecting group. Included as typical such groups are benzyloxycarbonyl, *t*-butyloxycarbonyl, *t*-amyloxycarbonyl, cyclopentyloxycarbonyl, adamantyloxycarbonyl, *p*-methoxybenzyloxycarbonyl, *p*-chlorobenzyloxycarbonyl, *p*-bromobenzyloxycarbonyl, *o*-chlorobenzyloxycarbonyl, 2,6-dichlorobenzyloxycarbonyl, 2,4-dichlorobenzyloxycarbonyl, *o*-bromobenzyloxycarbonyl, *p*-nitrobenzyloxycarbonyl, isopropyloxycarbonyl, cyclohexyloxycarbonyl, cycloheptyloxycarbonyl, and *p*-toluenesulfonyl.

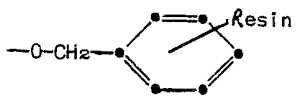
As will become apparent hereinafter, the process of preparing the tetradecapeptide of formula I involves periodic cleavage of the α-amino protecting group from the terminal amino acid present on the peptide chain. Thus, it will be appreciated that the identity of the ε-amino protecting group on the lysine residue is preferably such that it will not be cleaved under the conditions employed in selectively cleaving the α-amino protecting group. Appropriate selection of the α-amino and the ε-amino protecting groups is a matter well within the knowledge of a peptide chemist of ordinary skill in the art and depends upon the relative ease with which a particular protecting group can be cleaved. Thus, groups such as 2 - (*p* - biphenylyl)isopropyloxycarbonyl (BpOC) and trityl are very labile and can be cleaved even in the presence of mild acid. A moderately strong acid, such as hydrochloric acid, trifluoroacetic acid, or boron trifluoride in acetic acid, is required to cleave other groups such as *t*-butyloxycarbonyl, *t*-amyloxycarbonyl, adamantyloxycarbonyl, and *p*-methoxybenzyloxycarbonyl. Even stronger acid conditions are required to effect cleavage of other protecting groups such as benzyloxycarbonyl, halobenzyloxycarbonyl, *p*-nitrobenzyloxycarbonyl, cycloalkyloxycarbonyl, and isopropyloxycarbonyl. Cleavage of these latter groups requires drastic acid conditions such as the use of hydrogen bromide, hydrogen fluoride, or boron trifluoroacetate in trifluoroacetic acid. Of course, any of the more labile groups will also be cleaved under the stronger acid conditions. Appropriate selection of the amino protecting groups thus will include the use of a group at the α-amino function which is more labile than that employed as the ε-amino protecting group coupled with cleavage conditions designed to selectively remove only the α-amino function. In this context, R_2 preferably is cyclopentyloxycarbonyl, and, in conjunction therewith, the α-amino protecting group of choice for use in each of the amino acids which is added to the peptide chain preferably is *t*-butyloxycarbonyl.

The groups R_3 and R_4 both represent hydrogen or, separately, a protecting group for the alcoholic hydroxyl of threonine and serine, respectively. Typical such protecting groups are, for example, C_1—C_4 alkyl, such as methyl, ethyl, and *t*-butyl; benzyl; substituted benzyl, such as *p*-methoxybenzyl, *p*-nitrobenzyl, *o*-chlorobenzyl, and *p*-chlorobenzyl; C_1—C_3 alkanoyl, such as formyl, acetyl, and propionyl; and triphenylmethyl (trityl). Preferably, when R_3 and R_4 are protecting groups, the protecting group of choice in both instances is benzyl.

The group R_5 represents either hydrogen or formyl, the latter being a protecting group for the

of the tryptophan residue. The use of such a protecting group is optional and therefore R_5 may be hydrogen (N-unprotected) or formyl (N-protected).

The group X represents the carboxyl terminal of the tetradecapeptide chain and can be hydroxyl in which case a free carboxyl group thereby is defined. In addition, X represents the solid resin support to which the carboxyl terminal moiety of the peptide is linked during its synthesis. This solid resin is represented by the formula



In any of the above, when X represents hydroxyl, each of R, R_1, R_2, R_3, R_4,

and R_5 is hydrogen. When X represents the solid resin support, each of R, R_1, R_2, R_3, and R_4 is a protecting group.

The following abbreviations, most of which are well known and commonly used in the art, are employed herein:

Ala — Alanyl
Asn — Asparaginyl
Cys — Cysteinyl
Gly — Glycinyl
Lys — Lysyl
Phe — Phenylalanyl
Ser — Seryl
Thr — Threonyl
Trp — Tryptophyl
DCC — N,N'-Dicyclohexylcarbodiimide
DMF — N,N-Dimethylformamide
BOC — *t*-Butyloxycarbonyl
PMB — *p*-Methoxybenzyl
CPOC — Cyclopentyloxycarbonyl
Bzl — Benzyl
BpOC — 2-(*p*-biphenylyl)isopropyloxycarbonyl

Although the selection of the particular protecting groups to be employed in preparing the compound of formula I remains a matter well within the oridinary skill of a synthetic peptide chemist, it is well to recognize that the proper selection of the protecting groups is dependent upon the particular succeeding reactions which must be carried out. Thus, the protecting group of choice must be one which is stable both to the reagents and under the conditions employed in the succeeding steps of the reaction sequence. For example, as already discussed to some degree hereinabove, the particular protecting group which is employed must be one which remains intact under the conditions which are employed for cleaving the α-amino protecting group of the terminal amino acid residue of the peptide fragment in preparation for the coupling of the next succeeding amino acid fragment to the peptide chain. It is also important to select, as protecting group, one which will remain intact during the building of the peptide chain and which will be readily removable upon completion of the synthesis of the desired tetradecapeptide product. All of these matters are well within the knowledge and understanding of a peptide chemist of ordinary skill in the art.

As is evident from the above discussion, the tetradecapeptide of formula I can be prepared by solid phase synthesis. This synthesis involves a sequential building of the peptide chain beginning at the C-terminal end of the peptide. Specifically, cysteine first is linked at its carboxyl function to the resin by reaction of an amino-protected, S-protected cysteine with a chloromethylated resin or a hydroxymethyl resin. Preparation of a hydroxymethyl resin is described by Bodanszky *et al., Chem. Ind.* (London), *38* 1597—98 (1966). The chloromethylated resin is commerically available from Lab Systems, Inc., San Mateo, California.

In accomplishing linkage of the C-terminal cysteine to the resin, the protected cysteine first is converted to its cesium salt. This salt then is reacted with the resin in accordance with the method described by B. F. Gisin, *Helv. Chim. Acta, 56,* 1476 (1973). Alternatively, the cysteine can be linked to the resin by activation of the carboxyl function of the cysteine molecule by application of readily recognized techniques. For example, the cysteine can be reacted with the resin in the presence of a carboxyl group activating compound such as N,N'-dicyclohexylcarbodiimide (DCC).

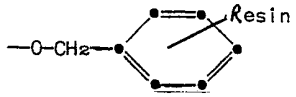
Once the free carboxyl cysteine has been appropriately linked to the resin support, the remainder of the peptide building sequence involves the step-wise addition of each amino acid to the N-terminal portion of the peptide chain. Necessarily, therefore, the particular sequence which is involved comprises a cleavage of the α-amino protecting group from the amino acid which represents the N-terminal portion of the peptide fragment followed by coupling of the next succeeding amino acid residue to the now free and reactive N-terminal amino acid. Cleavage of the α-amino protecting group can be effected in the presence of an acid such as hydrobromic acid, hydrochloric acid, trifluoroacetic acid, *p*-toluenesulfonic acid, benzenesulfonic acid, naphthalenesulfonic acid, and acetic acid, with formation of the respective acid addition salt product. Another method

which is available for accomplishing cleavage of the amino protecting group involves the use of boron trifluoride. For example, boron trifluoride diethyl etherate in glacial acetic acid will convert the amino-protected peptide fragment to a BF_3 complex which then can be converted to the deblocked peptide fragment by treatment with a base such as aqueous potassium bicarbonate. Any of these methods can be employed as long as it is recognized that the method of choice must be one which accomplishes cleavage of the N-terminal α-amino protecting group without disruption of any other protecting groups present on the peptide chain. In this regard, it is preferred that the cleavage of the N-terminal protecting group be accomplished using trifluoroacetic acid. Generally, the cleavage will be carried out at a temperature from 0°C. to room temperature.

Once the N-terminal cleavage has been effected, the product which results normally will be in the form of the acid addition salt of the acid which has been employed to accomplish the cleavage of the protecting group. The product then can be converted to the free terminal amino compound by treatment with a mild base, typically a tertiary amine such as pyridine, or triethylamine.

The peptide chain then is ready for reaction with the next succeeding amino acid. This can be accomplished by employing any of several recognized techniques. In order to achieve coupling of the next-succeeding amino acid to the N-terminal peptide chain, an amino acid which has a free carboxyl but which is suitably protected at the α-amino function as well as at any other active moiety is employed. The amino acid then is subjected to conditions which will render the carboxyl function active to the coupling reaction. One such activation technique which can be employed in the synthesis involves the conversion of the amino acid to a mixed anhydride. Thereby, the free carboxyl function of the amino acid is activated by reaction with another acid, typically a carbonic acid in the form of its acid chloride. Examples of such acid chlorides which can be used to form the appropriate mixed anhydrides are ethyl chloroformate, phenyl chloroformate, *sec*-butyl chloroformate, isobutyl chloroformate, and pivaloyl chloride.

Another method of activating the carboxyl function of the amino acid to achieve coupling is by conversion of the amino acid to its active ester derivative. Examples of such active esters are, for example, a 2,4,5-trichlorophenyl ester, a pentachlorophenyl ester, a *p*-nitrophenyl ester, an ester formed from 1-hydroxybenzotriazole, and an ester formed from N-hydroxysuccinimide. Another method for effecting coupling of the C-terminal amino acid to the peptide fragment involves carrying out the coupling reaction in the presence of at least an equimolar quantity of N,N'-dicyclohexylcarbodiimide (DCC). This latter method is preferred for preparing the tetradecapeptide of formula II where X is



Once the desired amino acid sequence has been prepared, the resulting peptide can be removed from the resin support. This is accomplished by treatment of the protected resin-supported tetradecapeptide with hydrogen fluoride. Treatment with hydrogen fluoride cleaves the peptide from the resin; in addition, however, it cleaves all remaining protecting groups present on the reactive moieties located on the peptide chain as well as the α-amino protecting group present at N-terminal amino acid. When hydrogen fluoride is employed to effect the cleavage of the peptide from the resin as well as removal of the protecting groups, it is preferred that the reaction be carried out in the presence of anisole. The presence of anisole has been found to inhibit the potential alkylation of certain amino acid residues present in the peptide chain. In addition, it is preferred that the cleavage be carried out in the presence of ethyl mercaptan. The ethyl mercaptan serves to protect the indole ring of the tryptophan residue and, furthermore, facilitates conversion of the blocked cysteines to their thiol forms. Also, when R_5 is formyl, the presence of ethyl mercaptan facilitates hydrogen fluoride cleavage of the formyl group.

Once the cleavage reaction has been accomplished, the product which is obtained is a straight-chain peptide containing 14 amino acid residues, formula III. In order to obtain the final product of formula I, it is necessary to treat the straight-chain tetradecapeptide under conditions which will effect its oxidation by converting the two sulfhydryl groups present in the molecule, one at each cysteinyl moiety, to a disulfide bridge. This can be accomplished by treating a dilute solution

of the linear tetradecapeptide with any of a variety of oxidizing agents including, for example, iodine, and potassium ferricyanide. Air also may be employed as oxidizing agent, the pH of the mixture preferably being from 2.5 to 9.0 and more preferably from 7.0 to 7.6. When air is used as oxidizing agent, the concentration of the peptide solution preferably is not greater than 0.4 mg. of the peptide per milliliter of solution, and more preferably is about 50 μg./ml.

The compounds of formula I may be administered to warm-blooded mammals, including humans, by any of several methods, including orally, sublingually, subcutaneously, intramuscularly or intravenously. Administration of the compound of formula I stimulates *in vivo* the release of growth hormone. This stimulatory effect is beneficial in those instances in which the host being treated requires a therapeutic treatment for insufficient secretion of somatotropin, such insufficient secretion being associated with adverse conditions such as primordial dwarfism. Preferably, the dose range for sublingual or oral administration is 1 mg. to 100 mg./kg. of body weight per day. Preferably, the dose range for intravenous, subcutaneous, or intramuscular adminstration is from 10 μg. to 1 mg./kg. of body weight per day, and more preferably, is from 50 μg. to 100 μg./kg. of body weight per day. It is evident that the dose range will vary widely depending upon the particular condition which is being treated as well as the severity of the condition.

It is also possible to administer the compound of formula I in the form of tablets containing other inocuous ingredients. Inert diluents or carriers, for example, magnesium carbonate or lactose, can be used together with conventional disintegrating agents, for example, maize starch and alginic acid, and lubricating agents, for example, magnesium stearate. Preferably, the amount of carrier or diluent will range from 5 to 95 percent of the final composition, and more preferably from 50 to 85 percent of the final composition. Suitable flavoring agents also can be employed in the final preparation rendering the composition more palatable for administration.

When the compound of formula I is to be administered intravenously, suitable carriers may be employed, such as, for example, isotonic saline, and phosphate buffer solutions.

The invention therefore also provides a pharmaceutical formulation comprising a compound of formula I, or a pharmaceutically acceptable non-toxic acid additional salt thereof, associated with a pharmaceutically acceptable carrier therefor.

The following examples are illustrative of the preparation of compound of formula I. All percentages and ratios are by volume, unless otherwise indicated.

Example 1

N-*t*-Butyloxycarbonyl-L-cysteinyl(S-*p*-methoxybenzyl) Methylated Polystyrene Resin

To 20.0 g. of chloromethylated polystyrene resin (Lab Systems, Inc., 0.75 mmoles/gram) suspended in 150 ml. of N,N-dimethylformamide (DMF) were added 3.7 grams (7.8 mmoles) of the cesium salt of N - *t* - butyloxycarbonyl - (S - *p* - methoxybenzyl)cysteine. The mixture was stirred at room temperature for three days. The resin then was filtered and washed successively with DMF, a mixture of 90 percent DMF and 10 percent water, and DMF. To the resin suspended in DMF was added a solution of 5.5 grams of cesium acetate in hot DMF. The mixture was stirred 16 hours at room temperature, for eight hours at 50°C., 16 hours at room temperature, for eight hours at 50°C., and for three days at room temperature. The resin then was filtered and was washed successively with DMF, a mixture of 90 percent DMF and 10 percent water, DMF, a mixture of 90 percent DMF and 10 percent water, DMF, and 95 percent ethanol. The resin then was dried *in vacuo* at 50°C. to obtain the title product containing 0.45 percent nitrogen (0.32 mmole/gram) and 0.80 percent sulfur (0.25 mmole/gram).

Example 2

t-Butyloxycarbonyl-L-alanyl-D-alanyl-L-S-*p*-Methoxybenzyl)cysteinyl-D-(cyclopentyloxycarbonyl)-lysyl-L-asparaginyl-L-phenylalanyl-L-phenylalanyl-L-tryptophyl-L-(N-cyclopentyloxycarbonyl)lysyl-L-(O-benzyl)threonyl-L-phenylalanyl-L-(O-benzyl)threonyl-L-(O-benzyl)seryl-L-(S-*p*-methoxy-benzyl)cysteinyl Methylated Polystyrene Resin

To a 300 ml. reaction vessel on a rocker were added 16.26 grams of the product

from Example 1. Sequences of deprotection, neutralization, coupling, and a recoupling were carried out for the addition of each amino acid to the peptide. A Beckman (Registered Trade Mark) 990 automatic peptide synthesizer was used for the entire sequence. The amino acids which were employed as well as the sequence of their employment is as follows: (1) N - *t* - butyloxycarbonyl - (O - benzyl) - L - serine; (2) N - *t* - butyloxycarbonyl - (O - benzyl) - L - threonine; (3) N - *t* - butyloxycarbonyl - L - phenylalanine; (4) N - *t* - butyloxycarbonyl - (O - benzyl) - L - threonine; (5) N^{α} - *t* - butyloxycarbonyl - N^{ϵ} - cyclopentyloxycarbonyl - L - lysine; (6) N^{α} - *t* - butyloxycarbonyl - L - tryptophan; (7) N - *t* - butyloxycarbonyl - L - phenylalanine; (8) N - *t* - butyloxycarbonyl - L - phenylalanine; (9) N - *t* - butyloxycarbonyl - L - asparagine, *p* - nitrophenyl ester; (10) N^{α} - *t* - butyloxycarbonyl - N^{ϵ} - cyclopentyloxycarbonyl - D - lysine; (11) N - *t* - butyloxycarbonyl - (S - *p* - methoxybenzyl) - L - cysteine; (12) N - *t* - butyloxycarbonyl - D - alanine; and (13) N - *t* - butyloxycarbonyl - L - alanine.

The sequence of deprotection, neutralization, coupling, and recoupling for the introduction of each amino acid into the peptide is as follows: (1) three washes (10 ml./gram resin) of three minutes each with chloroform; (2) removal of BOC group by treatment twice for twenty minutes each with 10 ml./gram resin of a mixture of 30 percent trifluoroacetic acid, 65 percent chloroform, and 5 percent triethylsilane; (3) two washes (10 ml./gram resin) of three minutes each with chloroform; (4) one wash (10 ml./gram resin) of three minutes with methylene chloride; (5) three washes (10 ml./gram resin) of three minutes each with a mixture of 90 percent *t*-butyl alcohol and 10 percent *t*-amyl alcohol; (6) three washes (10 ml./gram resin) of three minutes each with methylene chloride; (7) neutralization by three treatments of three minutes each with 10 ml./gram resin of 3 percent triethylamine in methylene chloride; (8) three washes (10 ml./gram resin) of three minutes each with methylene chloride; (9) three washes (10 ml./gram resin) of three minutes each with a mixture of 90 percent *t*-butyl alcohol and 10 percent *t*-amyl alcohol; (10) three washes (10 ml./gram resin) of three minutes each with methylene chloride; (11) addition of 0.8 mmole/gram resin of the protected amino acid and 0.8 mmole/gram resin of N,N'-dicyclohexylcarbodiimide (DCC) in 10 ml./gram resin of methylene chloride followed by mixing for 120 minutes; (12) three washes (10 ml./gram resin) of three minutes each with methylene chloride; (13) three washes (10 ml./gram resin) of three minutes each with a mixture of 90 percent *t*-butyl alcohol and 10 percent *t*-amyl alcohol; (14) three washes (10 ml./gram resin) of three minutes each with methylene chloride; (15) neutralization by three treatments of three minutes each with 10 ml./gram resin of 3 percent triethylamine in methylene chloride; (16) three washes (10 ml./gram resin) of three minutes each with methylene chloride; (17) three washes (10 ml./gram resin) of three minutes each with a mixture of 90 percent *t*-butyl alcohol and 10 percent *t*-amyl alcohol; (18) three washes (10 ml./gram resin) of three minutes each with methylene chloride; (19) three washes (10 ml./gram resin) of three minutes each with DMF; (20) addition of 0.8 mmole/gram resin of the protected amino acid and 0.8 mmole/gram resin of N,N'-dicyclohexylcarbodiimide (DCC) in 10 ml./gram resin of a 1:1 mixture of DMF and methylene chloride followed by mixing for 120 minutes; (21) three washes (10 ml./gram resin) of three minutes each with DMF; (22) three washes (10 ml./gram resin) of three minutes each with methylene chloride; (23) three washes (10 ml./gram resin) of three minutes each with a mixture of 90 percent *t*-butyl alcohol and 10 percent *t*-amyl alcohol (24) three washes (10 ml./gram resin) of three minutes each with methylene chloride; (25) neutralization by three treatments of three minutes each with 10 ml./gram resin of 3 percent triethylamine in methylene chloride; (26) three washes (10 ml./gram resin) of three minutes each with methylene chloride; (27) three washes (10 ml./gram resin) of three minutes each with a mixture of 90 percent *t*-butyl alcohol and 10 percent *t*-amyl alcohol; and (28) three washes (10 ml./gram resin) of three minutes each with methylene chloride.

The above treatment sequence was employed for addition of each of the amino acids with the exception of the asparagine residue. The asparagine residue was incorporated via its *p*-nitrophenyl active ester. In doing so, Step (11) above was modified to the following 3-step sequence: (a) three washes (7.5—15 ml./gram resin) of three minutes each with DMF; (b) addition of 1.0 mmole/gram resin of the *p*-nitrophenyl ester of N - *t* - butyloxycarbonyl - L - asparagine in 7.5—15 ml./gram resin of a 1:1 mixture of DMF and methylene chloride followed by mixing for 720 minutes; and (c) three washes (7.5—15 ml./gram resin) of three minutes

each with DMF. Also, Step (20) was altered to duplicate the above Step (b) with the exception that a 3:1 mixture of DMF and methylene chloride was employed.

The finished peptide-resin was dried *in vacuo*. A sample of the product was hydrolyzed by refluxing it for 21 hours in a mixture of hydrochloric acid and dioxane. The amino acid analysis of the resulting product gave the following results, lysine being employed as standard: Asn, 1.03; 2Thr, 1.96; Ser, 0.98; 2Ala, 2.32; 3Phe, 2.91; 2Lys, 2.00; Trp, 0.80. The presence of cysteine was not determined since it is destroyed by the method of analysis.

Example 3

L-alanyl-D-alanyl-L-cysteinyl-D-lysyl-L-asparaginyl-L-phenylalanyl-L-phenylalanyl-L-tryptophyl-L-lysyl-L-threonyl-L-phenylalanyl-L-threonyl-L-seryl-L-cysteine

To a mixture of 10 ml. of anisole and 10 ml. of ethyl mercaptan were added 4.04 grams of the protected tetradecapeptide resin of Example 2. The mixture was cooled in liquid nitrogen, and 43 ml. of liquid hydrogen fluoride were added by distillation. The resulting mixture was allowed to warm to 0°C. and was stirred for 1.5 hours. The hydrogen fluoride then was distilled off, and ether was added to the remaining mixture. The resulting solid material was collected by filtration and washed with ether. The product was dried, and the deprotected tetradecapeptide was extracted from the resin mixture using 1M acetic acid. The acetic acid solution then was immediately lyophilized to dryness in the dark. The resulting slightly yellow solid was suspended in a mixture of 15 ml. of deoxygenated 1M acetic acid and 4 ml. of glacial acetic acid. The resulting suspension was filtered, and the filtrate was absorbed on a Sephadex (Registered Trade Mark) G-25 F column. The chromatographic conditions were: solvent, deoxygenated 0.2 M acetic acid; column size, 7.5×150 cm.; temperature, 26°C.; flow rate, 689 ml./hour; fraction volume, 24.1 ml.

Absorbance at 280 mμ of each fraction plotted versus fraction number indicated two main peaks with some small peaks. A collection of five sets of fractions was made. The fractions which were combined and their effluent volumes are as follows:

Fractions 111—195	(2651—4699 ml.)
Fractions 196—215	(4700—5181 ml.)
Fractions 216—246	(5182—5928 ml.)
Fractions 274—255	(5929—6145 ml.)
Fractions 256—310	(6146—7471 ml.)

The five samples were lyophilized to dryness in the dark and collected. UV spectroscopy indicated that the second sample (68.5 mg.) was the best product.

Example 4

Oxidation to ^{4}D-Ala^{2}, D-Lys^{4}-Somatostatin

The reduced D-Ala^{2}, D-Lys^{3}-somatostatin from Example 3 was diluted with distilled water to achieve a 50 μg./ml. concentration. Concentrated ammonium hydroxide was added to adjust the pH to 6.9. The solution was stirred at room temperature in the dark for 68 hours. An Ellman titration indicated that the oxidation was complete.

The reaction mixture was concentrated *in vacuo* to a volume of 10 ml., and 14 ml. of 50% acetic acid were added. The solution was added to a Sephadex G-25 F column. The chromatographic conditions were as follows: solvent, deoxygenated 50% acetic acid; column size, 5.0×90 cm.; temperature, 26°C.; flow rate, 321 ml./hour; fraction volume, 18.75 ml.

Absorbance at 280 mμ for each fraction plotted versus fraction number indicated two large peaks. The first peak represented aggregated forms of the product, and the second peak represented good monomeric product. The product represented by the second peak was collected, diluted with distilled water, and lyophilized to dryness. The resulting solid was dissolved in 7 ml. of degassed 0.2 M acetic acid, and the solution was applied to a Sephadex G-25 F column. The chromatographic conditions were: solvent, deoxygenated 0.2 M acetic acid; column size, 5.0×150 cm.; temperature, 26°C.; flow rate, 495 ml./hour; fraction volume, 16.5 ml.

Absorbance at 280 mμ of each fraction plotted versus fraction number indicated one large peak with a shoulder on each side. UV spectroscopy showed the large peak to be good product. Fractions 156—170 (effluent volumes 2558—2805 ml.) were combined and lyophilized to dryness in the dark to obtain 21.55 mg. of the desired product.

Optical rotation $[\alpha]_D^{26}$=—43.0° (1 percent acetic acid).

Amino acid analysis: Ala+D-Ala$_{1.98}$ 2Cys$_{1.90}$ 2Lys$_{2.0}$ Asn$_{1.0}$ 3Phe$_{2.88}$ Trp$_{0.89}$ Thr$_{1.92}$ Ser$_{0.88}$.

D-Ala^2, D-Lys^4-somatostatin was tested for its *in vitro* activity in inhibiting gastric acid secretion. Large 5—6 inch bullfrogs were pithed. The gastric mucosa was freed from the muscle layers and was bisected longitudinally. The two halves were mounted in separate acrylic plastic chambers. The secretory area which was exposed was 2.85 square centimeters, and the volume of each half of the chamber was 5 ml. The solutions which were used to bathe the mucosa were the same as those used by Durbin et al., *Biochemica et Biophysics Acta, 321,* 553—560 (1973), with the exception that the serosal fluid contained sodium dihydrogen phosphate at a 1 millimolar concentration. Both sides of the chamber were aerated with a mixture of 95% oxygen and 5% carbon dioxide. The acid secretory rate was followed by maintaining the secretory solution at a pH of 4.5.

A concentration of 1×10^{-5} moles per liter of pentagastrin was used on the serosal side of the tissue to stimulate the acid secretory response. The serosal fluid was renewed every 40 minutes to prevent lowering of pentagastrin concentration by enzymatic hydrolysis of the peptide bonds. Addition of the compound to be tested was done by placing it in the serosal fluid each time the bathing solution was changed.

Spontaneous acid outputs for pentagastrin-stimulated secretion producing no less than 8 microequivalents/hour of acid served as controls. The effect of inhibition on gastric acid secretion was expressed as percent of inhibition from the control periods preceding the introduction of the test compound into the serosal buffer. Only one of the halves of the gastric mucosa was treated with the test compound, the other half serving as control to ensure continued viability of the tissue. After establishing steady state secretion, the test compound was added to the nutrient solution in an amount sufficient to attain an inhibitor concentration of 1×10^{-5} moles/liter. The acid was continually titrated to pH 4.5, and the volume of 12.5 mM sodium hydroxide utilized each 20 minutes was used to determine the acid secretory rate. The results were expressed as micro equivalents of acid secreted per hour.

Using this method of evaluation, somatostatin itself produced a percent inhibition of gastric acid secretion of 54.64 plus or minus 6.05 whereas D-Ala^2, D-Lys^4-somatostatin produced a percent inhibition of gastric acid secretion of 39.31 plus or minus 8.09.

D-Ala^2, D-Lys^4-somatostatin also was tested in dogs for its *in vivo* inhibition of gastric acid secretion. In dogs with chronic gastric fistula and Heidenhain pouch, gastric HCl secretion was induced by infusion of the C-terminal tetrapeptide of gastrin at 0.5 μg/kg hr. One dog served as control, receiving only the tetrapeptide. Another dog received the tetrapeptide and somatostatin while the test compound was adminstered to other dogs in place of somatostatin. After one hour of steady state secretion of HCl, somatostatin or the test compound was infused at 3 μg/kg hr. for one hour. Collection of gastric acid samples was continued for an additional 1.5 hours at 15 minute intervals. Relative to the control, somatostatin inhibited gastric acid secretion by 99.1%, whereas D-Ala^2, D-Lys^4-somatostatin provided an inhibition of 50.9%.

D-Ala^2, D-Lys^4-somatostatin was also shown to inhibit pancreatic secretion. In three dogs having both pancreatic and total gastric fistula, secretion from the pancreas was induced by infusion of secretin at 0.5 unit/kg hr. and gastric HCl by infusion of tetragastrin at 0.5 μg/kg hr. After a steady response was established, adminstration of the test compound was begun. One of the dogs was retained as control, and, of the remaining two dogs, one received somatostatin and the other D-Ala^2, D-Lys^4-somatostatin, each for one hour at 3 μg/kg hr. Peak inhibitory effects expressed as percent changes over control are as follows:

Pancreatic Secretion

	Volume	HCO_3^-	Protein
Somatostatin	−72	−85.1	−69.4
D-Ala^2, D-Lys^4-somatostatin	−61	−68	−55.4

D-Ala², D-Lys⁴-somatostatin also was tested for its activity with respect to the release of growth hormone. The procedure which was employed is carried out using mature male Spraque-Dawley rats (Laboratory Supply Company, Indianapolis, Indiana). The test is a modification of the method of P. Brazeau, W. Vale, and R. Guilleman, *Endocrinology, 94,* 184 (1974). In this assay one set comprising five groups of eight rats each was employed. First, sodium pentabarbital was administered to all of the rats of a particular set to stimulate growth hormone secretion. In the set, one group is the control group and received only saline. Two of the groups received somatostatin, one at 2 μg/rat, subcutaneously, and the other at 50 μg/rat, subcutaneously. The other two groups received D-Ala², D-Lys⁴-somatostatin, one at 2 μg/rat, subcutaneously and the other at 50 μg/rat, subcutaneously. The degree of inhibition of serum growth hormone concentration then was determined with respect to the control group, and the relative activities of D-Ala², D-Lys⁴-somatostatin and somatostatin itself were compared.

At a dose level of 2 μg/rat, D-Ala², D-Lys⁴-somatostatin stimulated growth hormone secretion by 114 percent over control whereas somatostatin had no effect whatever on growth hormone secretion. At a dose level of 50 μg/rat, D-Ala², D-Lys⁴-somatostatin stimulated growth hormone secretion by almost 200 percent over control, while somatostatin itself produced a 33 percent inhibition.

D-Ala², D-Lys⁴-somatostatin was tested for its *in vivo* activity in inhibiting glucagon and insulin secretion upon stimulation with L-alanine. Normal mongrel dogs of either sex were fasted overnight. Control blood samples were obtained, and then an intravenous infusion of saline, somatostatin, or D-Ala², D-Lys⁴-somatostatin was started. After 30 minutes, L-alanine additionally was adminstered intravenously for a period of 15 minutes. The infusion of saline, somatostatin, or D-Ala², D-Lys⁴-somatostatin was continued for 15 minutes after completion of the alanine infusion. The total dose of somatostatin or D-Ala², D-Lys⁴-somatostatin which was infused is 200—500 μg/dog (0.20—0.30 μg/kg/minute), and the total dose of L-alanine infused was 1 mmol/kg.

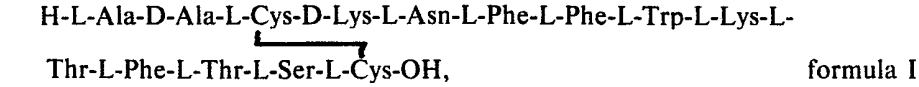
Somatostatin infusion caused a decrease in basal serum insulin concentration and inhibited the rise in concentration of both glucagon and insulin during the infusion of L-alanine. In comparison, the D-Ala², D-Lys⁴-somatostatin, infused at a rate of 0.253 μg/kg/min., caused a slight decrease in basal secretion of both insulin and glucagon and produced a partial inhibition of the increase in serum concentration of both insulin and glucagon normally produced by the infusion of L-alanine.

D-Ala², D-Lys⁴-somatostatin also was evaluated for *in vivo* activity in inhibiting glucagon secretion upon stimulation with insulin. Normal mongrel dogs of either sex were fasted overnight. After control blood samples had been obtained, an intravenous infusion of saline, somatostatin, or D-Ala², D-Lys⁴-somatostatin was commenced. After 15 minutes, insulin, 0.3 units/kg., was injected intravenously. The infusion of saline, somatostatin, or the test compound was continued for two hours, and blood samples were obtained at various intervals throughout the test. The total dose of the somatostatin or test compound ranged from 120—260 μg/dog (0.07—0.13 μg/kg/min.). Adminstration of insulin produced a reduction in the blood glucose concentration and increase in serum glucagon concentration. Infusion of somatostatin blocked the increase in serum glucagon concentration but had no affect on the reduction of the blood glucose concentration.

In comparison, when, instead of somatostatin, D-Ala², D-Lys⁴-somatostatin was infused at a rate of 0.114 μg/kg/min., it was found that it did not inhibit the increase in serum glucagon concentration produced by insulin administration.

WHAT WE CLAIM IS:—

1. A compound of the formula



formula I

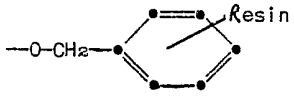
or a pharmaceutically acceptable non-toxic acid addition salt thereof.

2. A process for preparing the compound of formula I, as defined in claim 1, which comprises reacting the corresponding straight-chain tetradecapeptide of formula III, H - L - Ala - D - Ala - L - Cys - D - Lys - L - Asn - L - Phe - L -

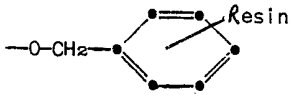
Phe - L - Trp - L - Lys - L - Thr - L - Phe - L - Thr - L - Ser - L - Cys - OH, with an oxidizing agent.

3. A compound of formula II, R - L - Ala - D - Ala - L - Cys(R_1) - D - Lys(R_2) - L - Asn - L - Phe - L - Phe - L - Trp(R_5) - L - Lys(R_2) - L - Thr(R_3) - L - Phe - L - Thr(R_3) - L - Ser(R_4) - L - Cys(R_1) - X; in which

R is hydrogen or an α-amino protecting group;
R_1 is hydrogen or a thio protecting group;
R_2 is hydrogen or an ε-amino protecting group;
R_3 and R_4 each are hydrogen or a hydroxy protecting group;
R_5 is hydrogen or formyl; and
X is hydroxy or



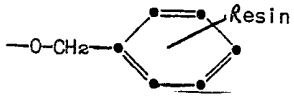
in which Resin is polystyrene; with the proviso that, when X is hydroxy, each of R, R_1, R_2, R_3, R_4, and R_5 is hydrogen, and, when X is



each of R, R_1, R_2, R_3, and R_4 is other than hydrogen.

4. Compound of claim 3, in which X is hydroxy.

5. Compound of claim 3, in which X is



6. Compound of claim 5, in which R is *t*-butyloxycarbonyl.

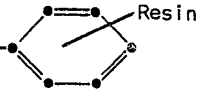
7. Compound of claim 5, in which R_1 is *p*-methoxybenzyl.

8. Compound of claim 5, in which R_2 is cyclopentyloxycarbonyl.

9. Compound of claim 5, in which R_3 and R_4 are benzyl.

10. Compound of claim 5, having the formula N - (BOC) - L - Ala - D - Ala - L - (PMB)Cys - D - (CPOC)Lys - L - Asn - L - Phe - L - Phe - L - Trp - L - (CPOC)Lys - L - (Bzl)Thr - L - Phe - L - (Bzl)Thr - L - (Bzl)Ser - L - (PMB)Cys - O - CH_2 -

N-(BOC)-L-Ala-D-Ala-L-(PMB)Cys-D-(CPOC)Lys-L-Asn-L-Phe-
L-Phe-L-Trp-L-(CPOC)Lys-L-(Bzl)Thr-L-Phe-L-(Bzl)Thr-
L-(Bzl)Ser-L-(PMB)Cys-O-CH_2—



11. A pharmaceutical formulation comprising a compound as claimed in claim 1, or a pharmaceutically acceptable non-toxic acid addition salt thereof, associated with a pharmaceutically acceptable carrier therefor.

12. A compound as claimed in claim 1 substantially as hereinbefore described with particular reference to Example 4.

13. A process as claimed in claim 2 substantially as hereinbefore described with particular reference to Example 4.

14. A compound as claimed in claim 3 substantially as hereinbefore described with particular reference to either of Examples 2 and 3.

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