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⑦③ Proprietor: **MERCK & CO. INC.**
126, East Lincoln Avenue P.O. Box 2000
Rahway, New Jersey 07065 (US)

⑦② Inventor: **Veber, Daniel Frank**
290 Batleson Road
US-19002 Ambler Pennsylvania (US)

⑦④ Representative: **Blum, Rudolf E. et al,**
c/o E. Blum & Co Patentanwälte Vorderberg 11
CH-8044 Zürich (CH)

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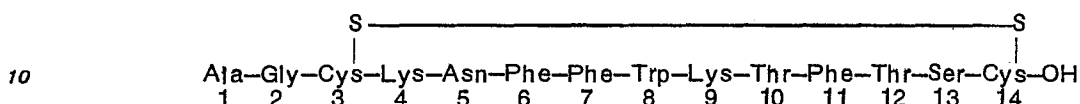
Somatostatin analogs, process for their preparation and their use in pharmaceuticals

SUMMARY OF THE INVENTION

The present invention concerns novel somatostatin analogs having a more potent biological activity and a longer duration of action than naturally occurring somatostatin.

5 BACKGROUND OF THE INVENTION

Somatostatin is a tetradecapeptide having the structure:



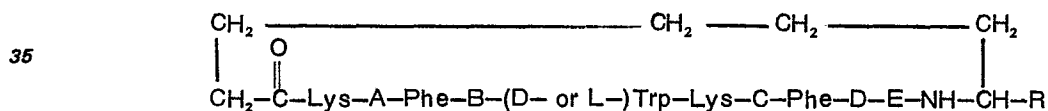
Somatostatin has the properties of inhibiting the release of growth hormone, inhibiting the release of insulin and glucagon and reducing gastric secretion. Somatostatin itself has a short duration of action because it is inactivated, *inter alia*, by aminopeptidases and carboxypeptidases present *in vivo*. This problem of the short duration of action has been partially solved in the prior art by preparing derivatives of somatostatin which have low solubility, thus attaining a slow release on subcutaneous injection. Once dissolved, however, the derivatives are no more stable to inactivation by aminopeptidases and carboxypeptidases than somatostatin itself.

In the French Patent No. 2,304,352 (American Home Products) are disclosed cyclic undecapeptides having a bridging group comprising 1 to 5 —CH₂—groups. Said cyclic peptides differ from the inventive somatostatin analogs in that they have the conventional 5-Asn and 13-Ser groups.

In the Netherlands Patent No. 76 07987 (Merck) there are also disclosed cyclic undecapeptides, in which the bridging group can comprise —CH₂—groups. Said peptides however have a 5-Asn, 5-Ala or 5-Abu group and 13-Ser group.

The present invention provides somatostatin analogs having higher biological activities and a longer duration of action than somatostatin and a novel method for preparing said analogs.

This invention is concerned with novel somatostatin analogs having a more potent biological activity and a longer duration of action than naturally occurring somatostatin having the structural formula:

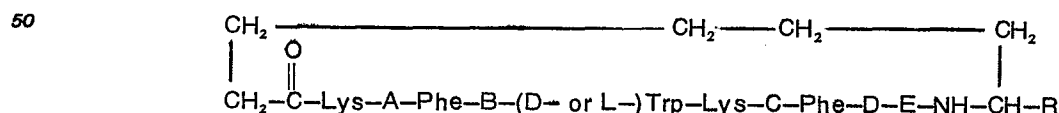


wherein

- 40
- R is H, COOH,
 - A is (Asn)_n, α-Abu, Pro, Ala wherein n = 0 or 1,
 - B is Phe, Tyr,
 - C and D are independently Thr, Val,
 - E is Ser, Pro, Ala or Gly,

45 wherein at least one of A and E is Pro and the ring formed by the peptide backbone contains 35 or 38 atoms and pharmaceutically acceptable non-toxic acid addition salts thereof.

The preferred somatostatin analogs of the present invention are illustrated by the following structural formula:



55 wherein

- R is H,
- A is Asn, Pro,
- B is Phe,
- C and D are Thr,
- E is Ser, Pro

60 and wherein at least one of A and E is Pro and the pharmaceutically acceptable non-toxic acid addition salts thereof.

Still further preferred somatostatin analogs are those having the structural formulas:



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Table I

<u>Abbreviated Designation</u>	<u>Amino acid</u>
Lys	L-lysine
Phe	L-phenylalanine
Trp	L-tryptophan
D-Trp	D-tryptophan
Thr	L-Threonine
Aha	7-aminoheptanoic acid
Tyr	L-tyrosine
Val	L-valine
Abu	L- α -aminobutyric acid
Ser	L-serine
Asn	L-asparagine
Pro	L-proline
Asu	D- or L- α -aminosuberic acid
<u>Abbreviated Designation</u>	<u>Protecting Groups</u>
INOC	isonicotinyloxycarbonyl
BOC	<i>tert</i> -butyloxycarbonyl
OMe	methyl ester
<i>t</i> Bu	<i>tert</i> -butyl
CBZ	benzyloxycarbonyl
Bzl	benzyl
2-Cl-CBZ	2-chlorobenzyloxycarbonyl
<u>Abbreviated Designation</u>	<u>Activating Groups</u>
ON _p	<i>p</i> -nitrophenyl ester
HSE	N-hydroxysuccinimide ester
HBT	1-hydroxybenzotriazole
<u>Abbreviated Designation</u>	<u>Condensing Agents</u>
DCCI	dicyclohexylcarbodiimide

	Abbreviated Designation	Reagents
5	TFA	trifluoroacetic acid
	TEA	triethylamine
	DIPEA	diisopropylethylamine
10	Abbreviated Designation	Solvents
	EPAW	ethyl acetate-pyridine- acetic acid-water
15	BAW	butanol-acetic acid-water
	CMW	chloroform-methanol-water
20	DMF	dimethylformamide
	THF	tetrahydrofuran

25 In accordance with the present invention, the novel somatostatin analogs are prepared by
cyclizing corresponding linear peptides. The linear peptides are prepared by using the solid phase
sequential synthesis technique. Accordingly, the process for preparing the somatostatin analogs of the
present invention comprises a) preparing a corresponding blocked linear peptide attached to a solid
30 the resin; d) treating the linear peptide with a cyclizing agent to obtain the cyclic peptide; and e)
removing the remaining blocking groups.

When the linear peptide is prepared on the resin, it is not critical which amino acid is selected to
be at the C-terminal position provided only that the sequence of amino acids in the linear peptide
corresponds to that in the desired somatostatin analog. Once a linear peptide has been cyclized one can
35 no longer determine which amino acid was at the C-terminus of the linear peptide. As an example to
illustrate this, either of the two following linear peptides, when cyclized, will give the identical
somatostatin analog:

40 D-Trp-(ϵ -2-Cl-CBZ)Lys-(O-Bzl)Thr-Phe-(O-Bzl)Thr-Pro-Aha-(ϵ -2-Cl-CBZ)Lys-Asn-Phe-
Phe-N₃

or

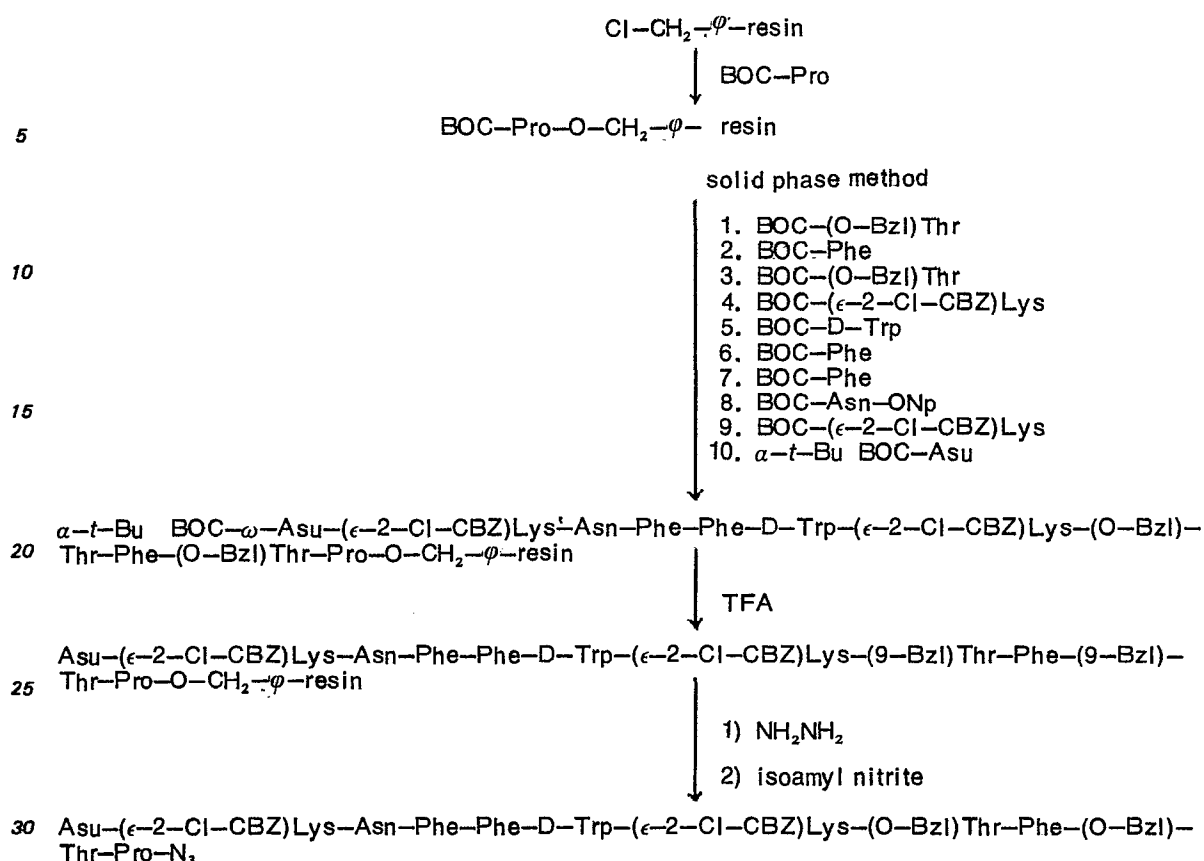
45 Aha-(ϵ -2-Cl-CBZ)Lys-Asn-Phe-Phe-D-Trp-(ϵ -2-Cl-CBZ)Lys-(O-
Bzl)Thr-Phe-(O-Bzl)Thr-Pro-N₃

↓ cyclization

cyclo[Aha-(ϵ -2-Cl-CBZ)Lys-Asn-Phe-Phe-D-Trp-(ϵ -2-Cl-CBZ)Lys-O-Bzl)Thr-Phe-
(O-Bzl)Thr-Pro].

50 It is evident that since the linear peptide is going to be cyclized, it does not matter which amino
acid is used to start the chain. Starting with Phe at the carboxyl end, as illustrated in the first of the two
examples above, has an advantage over the second example. In the first example, D-Trp, which can
react with *t*-butyl carbonium ions formed when BOC groups are removed, is the N-terminal amino acid
55 and thus will be added last and hence will be subjected to the least amount of exposure to *t*-butyl
carbonium ion.

In the case wherein R is COOH, it is preferable to employ the peptide having the amino acid
sequence in the second example with the exception that Aha is replaced by ω -Asu. The process for
preparing the required linear peptide may be illustrated by the following scheme:



The synthesis of the linear peptides by the solid phase technique is conducted in a stepwise manner on chloromethylated resin. The resin is composed of fine beads (20—70 microns in diameter) of a synthetic resin prepared by copolymerization of styrene with 1 to 2 percent divinyl-benzene. The benzene rings in the resin are chloromethylated in a Friedel-Crafts reaction with chloromethyl methyl ether and stannic chloride. The Friedel-Crafts reaction is continued until the resin contains 0.5 to 5 mmoles of chlorine per gram of resin.

The amino acid selected to be the C-terminal amino acid of the linear peptide is converted to its amino protected derivative. The carboxyl group of the selected C-terminal amino acid is bound covalently to the insoluble polymeric resin support, as for example, as the carboxylic ester of the resin-bonded benzyl chloride present in chloromethyl-substituted polystyrene-divinyl-benzene resin. After the amino protecting group is removed, the amino protected derivative of the next amino acid in the sequence is added along with a coupling agent, such as dicyclohexylcarbodiimide. The amino acid reactant may be employed in the form of a carboxyl-activated amino acid such as the ONp ester, an amino acid azide, and the like. Deprotection and addition of successive amino acids is performed until the desired linear peptide is formed.

The selection of protecting groups is, in part, dictated by particular coupling conditions, in part by the amino acid and peptide components involved in the reaction.

Amino-protecting groups ordinarily employed include those which are well known in the art, for example, urethane protecting substituents such as benzyloxycarbonyl (carbobenzoyl), p-methoxycarbonyl, p-nitrocarbonyl, t-butyloxycarbonyl, and the like. It is preferred to utilize t-butyloxycarbonyl (BOC) for protecting the α-amino group in the amino acids undergoing reaction at the carboxyl end of said amino acid. The BOC protecting group is readily removed following such coupling reaction and prior to the subsequent step by the relatively mild action of acids (i.e., trifluoro acetic acid, or hydrogen chloride in ethyl acetate).

The —OH group of Thr and Ser can be protected by the Bzl group and the ε-amino group of Lys can be protected by the INOC group or the 2-chlorobenzyloxycarbonyl (2-Cl-CBZ) group. In the case of Lys, it is preferred to protect the ε-amino group with 2-Cl-CBZ group as this group is removed simultaneously with the Bzl groups by treatment with HF after the linear peptide has been cyclized. The INOC group is not removed by HF and requires an additional treatment with Zn or catalytic hydrogenation. Neither group is affected by TFA, used for removing BOC protecting groups.

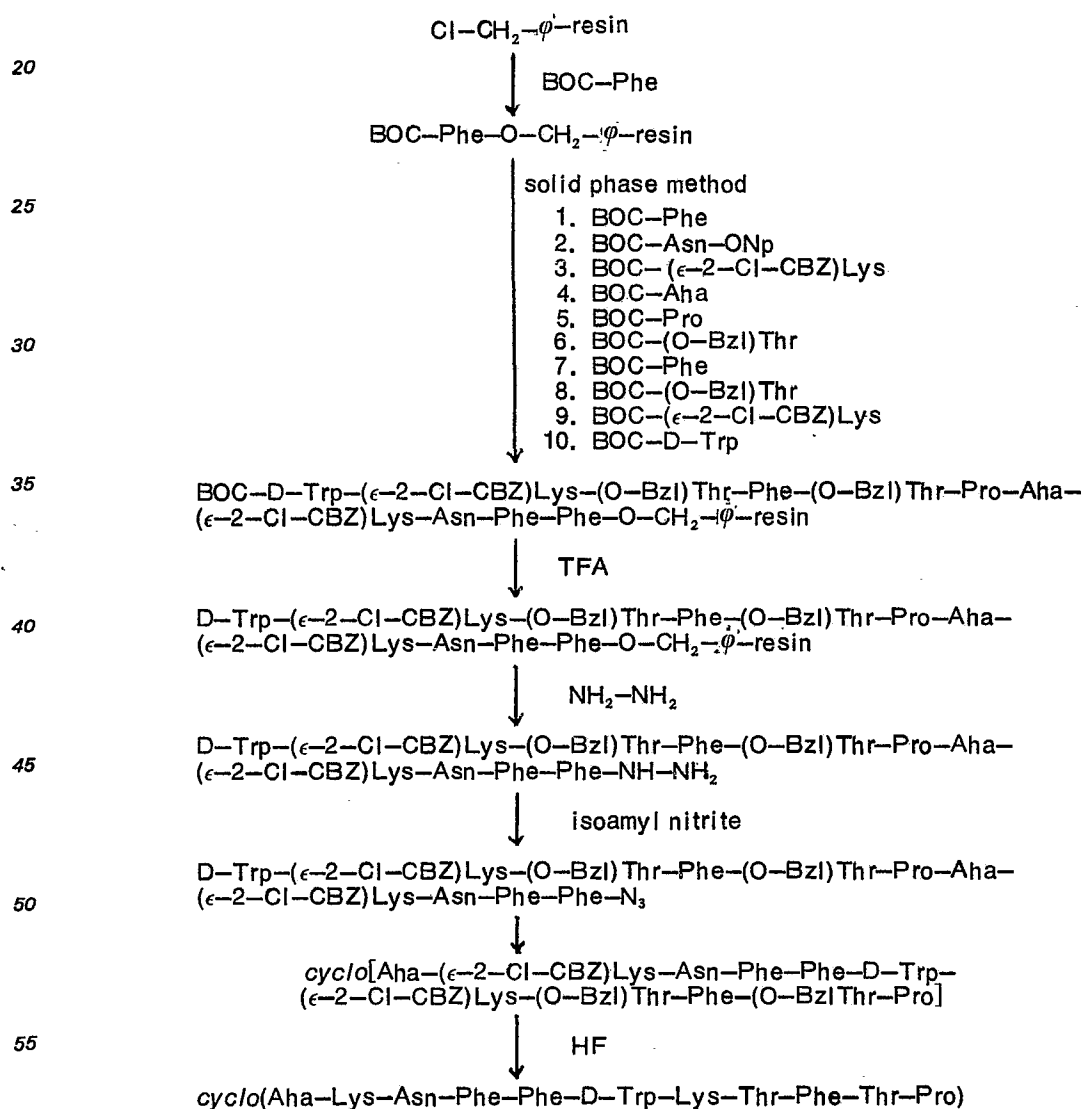
After the linear peptide has been formed on the solid phase resin, it may be removed from the resin by a variety of methods which are well known in the art. For example, the peptide may be cleaved from the resin with hydrazine and thus directly form the peptide hydrazide which may be subsequently

cyclized, *via* the azide, to the desired cyclic peptide. The hydrazide is converted to the corresponding azide by reaction with a reagent which furnishes nitrous acid *in situ*. Suitable reagents for this purpose include a lower alkyl nitrite (e.g. *t*-butyl nitrite, isoamyl nitrite) or an alkali metal nitrite salt (e.g., sodium nitrite, potassium nitrite) in the presence of a strong acid such as hydrochloric, phosphoric, sulfonic etc.

- 5 This reaction is carried out in the presence of either water and/or a non-aqueous solvent such as dimethylformamide, tetrahydrofuran, dioxane, chloroform, methylene chloride, etc., at a temperature between about -40°C . and $+20^{\circ}\text{C}$. Alternatively, the peptide may be removed from the resin by treatment with a lower alcohol such as methanol in the presence of an organic base such as triethylamine, thus resulting in the formation of the corresponding lower alcohol ester of the linear undecapeptide. The resulting ester may be converted to the hydrazide which may then be cyclized, *via* the azide, to the desired cyclic peptide. The preferred method for cleaving the peptide from the resin in the present invention is the use of hydrazine.

TABLE II

General Scheme for Preparing *Cyclo*(Aha-Lys-Asn-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Pro)



As reference to Table II will show, one preferred overall procedure for preparing the desired cyclic peptides of the present invention involves the stepwise synthesis of the linear peptide on a solid phase resin. More specifically, in the process for preparing *cyclo*(Aha-Lys-Asn-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Pro), the carboxyl end of the N-blocked amino acid phenylalanine is bound covalently to an insoluble polymeric resin support as the carboxylic acid ester of the resin-bonded benzyl chloride. The amino group of Phe is protected by the BOC group. After the attachment of the Phe is completed on the resin, the protecting group BOC is removed by treatment with TFA in CH_2Cl_2 . The subsequent amino acids are

attached, in the form of BOC-amino acid, using DCCI as the condensing agent or an active ester such as ONp. After the desired linear peptide has been prepared, the N-terminal amino group is selectively deblocked and the peptide is removed from the resin by treatment with hydrazine. The resulting linear peptide hydrazide with the N-terminal amino group deblocked having the amino acid sequence: D-Trp-(ϵ -2-Cl-CBZ)Lys-(O-Bzl)Thr-Phe-(O-Bzl)-Thr-Pro-Aha-(ϵ -2-Cl-CBZ)Lys-Asn-Phe-Phe-NH-NH₂ is treated with isoamyl nitrite in acid pH to form the corresponding azide. The azide solution is diluted with solvent and neutralized with an organic base. The linear peptide cyclizes to form *cyclo*[Aha-(ϵ -2-Cl-CBZ)Lys-Asn-Phe-Phe-D-Trp-(ϵ -2-Cl-CBZ)Lys-(O-Bzl)Thr-Phe-(O-Bzl)Thr-Pro]. During the cyclization the "pH" is checked and maintained at neutral by the addition of organic base. The "pH" in organic solvent is determined by the application of an aliquot of the solution to moistened narrow range pH paper.

After the linear peptide is cyclized, the remaining protective groups, 2-Cl-CBZ and Bzl, are removed in one step by treatment with HF in the presence of anisole. The crude cyclic peptides obtained by the process of Table II are purified by chromatography, preferably on "SEPHADEX" eluted with 50% aqueous acetic acid. "SEPHADEX" is a registered Trade Mark in the United Kingdom.

The following Examples illustrate methods of carrying out the present invention, but it is to be understood that these Examples are given for purposes of illustration and not of limitation. It is to be understood that changing the amino acid sequence of the polypeptide in accordance with the instructions provided by this disclosure, affords each of the compounds embraced by the description presented herein and embraced by the claims of this application.

Example 1

Preparation of *Cyclo*(Aha-Lys-Asn-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Pro)

Step a) — Preparation of BOC-D-Trp-(ϵ -2-Cl-CBZ)Lys-(O-Bzl)Thr-Phe-(O-Bzl)Thr-Pro-Aha-(ϵ -2-Cl-CBZ)Lys-Asn-Phe-Phe-O-CH₂- ϕ -resin

Chloromethyl resin (2% cross-linked Merrifield resin), 862.0 g. (2.37 moles), having 2.75 meq. chlorine/g., and 607.0 g. (2.37 moles, 1 equivalent) of BOC-Phe were added to 4320 ml. of peroxide-free tetrahydrofuran. The mixture was stirred in an oil bath at 80°C. bath temperature for 45 minutes. Triethylamine, 310.0 ml., was added and the reaction mixture stirred at 80°C. bath temperature for 70 hours, cooled to 25°C. and transferred to a stirred solid phase reaction column with 2000 ml of tetrahydrofuran. After removal of the solvent, the resin was washed using the stirred column with:

- 3 x 2000 ml. of tetrahydrofuran
- 4 x 5170 ml. of ethanol
- 1 x 5170 ml. of acetic acid
- 3 x 5170 ml. of water
- 3 x 5170 ml. of methanol
- 3 x 5170 ml. of chloroform.

The BOC-Phe-O-CH₂- ϕ -resin was dried *in vacuo* at 25°C. for 16 hours, giving 1203 g. of BOC-Phe-O-CH₂- ϕ -resin containing 0.937 mmole of phenylalanine/g. of resin.

BOC-Phe-O-CH₂- ϕ -resin (2.13 g.; 2.0 mmole) was carried through the procedures in Tables III and IV using 2 deblockings (2 minutes and 25 minutes) with 25% TFA in methylene chloride, and 2.5 equivalents of BOC-amino acid in the required sequence until the desired BOC-undecapeptide-O-CH₂- ϕ -resin was obtained.

In the coupling of BOC-Asn to Phe-Phe-O-CH₂- ϕ -resin, in place of BOC-AA, the *p*-nitrophenyl ester of BOC-Asn, (BOC-Asn-ONp), was used and the DCCI was omitted.

DCCI was used as the sole coupling agent in every remaining step except the coupling of BOC-Pro to Aha-(ϵ -2-Cl-CBZ)Lys-Asn-Phe-Phe-O-CH₂- ϕ -resin in which case the coupling was carried out in the presence of DCCI and 1-hydroxybenzotriazole monohydrate (HBT.H₂O).

The coupling of each amino acid proceeded smoothly. Best yields were obtained when the coupling was repeated in each step. When the coupling was repeated, the initial two chloroform washes, the deblocking step and the succeeding three chloroform washes were all omitted and replaced by a single chloroform wash.

The coupling reactions were carried out in methylene chloride, freshly degassed DMF or a mixture of these two solvents. The N-terminal amino group was blocked with a BOC group in each case; the hydroxy group of Thr was blocked with Bzl and the ϵ -amino group of Lys with 2-Cl-CBZ.

When the desired BOC-undecapeptide-O-CH₂- ϕ -resin was obtained, the N-terminal BOC group was removed by the terminal deblocking procedure set forth in Table V.

TABLE III

Solvent or reagent (number of treatments or washes)	CHCl ₃ (2)	25% TFA in CH ₂ Cl ₂ (2)	CHCl ₃ (3)	NEt ₃ -CH ₂ Cl ₂ (1:9) (2)	CHCl ₃ (3) CH ₂ Cl ₂ *	BOC AA in CH ₂ Cl ₂ , DMF or a mixture of both	0.5M DCCI in CH ₂ Cl ₂ **	DMF (1) MeOH (1) DMF (1) MeOH (1) CHCl ₃ (2)
Volume in ml.	40	40	40	40	40	25 ml.	10	40
Time in min.	5	2 and 25	2	5 and 5	2	5	5 min. coupling 30 min.	2

*When BOC Active Ester Coupling is employed: DMF (3) washes substituted for CH₂Cl₂.

**When BOC Active Ester Coupling is employed: No DCCI is added and coupling time is 600 min.

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TABLE IV

Protected Amino Acid	Solvent ml.
BOC-Phe (1.33 g.) recouple	CH ₂ Cl ₂ , 25 ml.
BOC-Asn-ONp (1.77 g.) recouple	DMF, 25 ml.
BOC-(ϵ -2-Cl-CBZ)Lys (2.08 g.) recouple	CH ₂ Cl ₂ , 25 ml.
BOC-Aha (1.23 g.) recouple	CH ₂ Cl ₂ , 25 ml.
BOC-Pro (1.08 g.) + HBT.H ₂ O (1.53 g.) recouple	DMF, 25 ml.
BOC-(O-Bzl)Thr (1.55 g.) recouple	CH ₂ Cl ₂ , 25 ml.
BOC-Phe (1.33 g.) recouple	CH ₂ Cl ₂ , 25 ml.
BOC-(O-Bzl)Thr (1.55 g.) recouple	CH ₂ Cl ₂ , 25 ml.
BOC-(ϵ -2-Cl-CBZ)Lys (2.08 g.) recouple	CH ₂ Cl ₂ , 25 ml.
BOC-D-Trp (1.52 g.) recouple	DMF, 5.5 ml. CH ₂ Cl ₂ , 19.5 ml.

TABLE V
TERMINAL DEBLOCKING PROGRAM

5	Solvent or reagent (number of treatments or washes)	CHCl ₃ (1)	25% TFA in CH ₂ Cl ₂ + 1% Ethanedithiol (2)	CHCl ₃ (3)	MeOH (2) CH ₂ Cl ₂ (1) MeOH (2) CH ₂ Cl ₂ (2)
10	Vol. in ml.	40	40	40	40
15	Time in minutes	5	2 and 25	2	2

After the sequence of Tables III, IV and V were completed, the blocked undecapeptide-O-CH₂- ϕ -resin was dried overnight *in vacuo* and weighed 5.41 g.

Step b) — Preparation of D-Trp-(ϵ -2-Cl-CBZ)Lys-(O-Bzl)Thr-Phe-(O-Bzl)Thr-Pro-Aha-(ϵ -2-Cl-CBZ)Lys-Asn-Phe-Phe-NH-NH₂

To a mixture of 5.24 g. D-Trp-(ϵ -2-Cl-CBZ)Lys-(O-Bzl)Thr-Phe-(O-Bzl)Thr-Pro-Aha-(ϵ -2-Cl-CBZ)Lys-Asn-Phe-Phe-O-CH₂- ϕ -resin in 60 ml. freshly degassed DMF was added 5.0 ml. NH₂-NH₂. The mixture was magnetically stirred at room temperature for 1 hour. The mixture was filtered to remove the resin. The resin was washed with 4 x 30 ml. DMF. The filtrate and washings were concentrated *in vacuo* to near dryness. The semi-solid residue was triturated with 60 ml. ether to obtain a solid. The solid was collected by filtration, slurried with ether 3 x 20 ml. and dried *in vacuo* for 20 min. to yield 3.89 g. crude product. The solid was slurried with 4 x 30 ml. water to remove all traces of formylhydrazide and dried *in vacuo* overnight to give 3.02 g. of product.

Step c) — Preparation of D-Trp-(ϵ -2-Cl-CBZ)Lys-(O-Bzl)Thr-Phe-(O-Bzl)Thr-Pro-Aha-(ϵ -2-Cl-CBZ)Lys-Asn-Phe-Phe-N₃

D-Trp-(ϵ -2-Cl-CBZ)Lys-(O-Bzl)Thr-Phe-(O-Bzl)Thr-Pro-Aha-(ϵ -2-Cl-CBZ)Lys-Asn-Phe-Phe-NH-NH₂ (2.90 g., 1.47 mmole), prepared by the process set forth in step b), was suspended in 30 ml. freshly degassed DMF. The suspension was stirred magnetically at -40°C. under a nitrogen atmosphere. To the suspension was added 1.53 ml., 6.15N HCl in THF (9.41 mmole, 6.4 equivalents). The resulting clear acidic solution, "pH" 1.0 to 1.5, was warmed to -25°C. and 0.30 ml. isoamyl nitrite (0.135 ml./mmole, 1.5 equivalents) was added and stirring continued for 30 minutes. This solution of D-Trp-(ϵ -2-Cl-CBZ)Lys-(O-Bzl)Thr-Phe-(O-Bzl)Thr-Pro-Aha-(ϵ -2-Cl-CBZ)Lys-Asn-Phe-Phe-N₃ was used immediately in Step d).

Step d) — Preparation of Cyclo[Aha-(ϵ -2-Cl-CBZ)Lys-Asn-Phe-Phe-D-Trp-(ϵ -2-Cl-CBZ)Lys-(O-Bzl)Thr-Phe-(O-Bzl)Thr-Pro]

The solution of D-Trp-(ϵ -2-Cl-CBZ)Lys-(O-Bzl)Thr-Phe-(O-Bzl)Thr-Pro-Aha-(ϵ -2-Cl-CBZ)Lys-Asn-Phe-Phe-N₃ in DMF, obtained by the process set forth in Step c), was diluted in 1800 ml. freshly degassed DMF, precooled to -40°C. The solution was maintained under a nitrogen atmosphere and allowed to warm to -20°C. during which time the "pH" was maintained at 7.2 to 7.6 by the addition of 3.06 ml. N,N-diisopropylethylamine. The solution was maintained at -18°C. for 24 hours and then kept at 5°C. for an additional 3 days.

The solution was concentrated *in vacuo* to a thick oil, washed twice with ether and once with ethyl acetate and triturated with water to give a solid. The solid was collected by filtration and dried *in vacuo* overnight to give 2.06 g. of product.

Step e) — Preparation of Cyclo[Aha-Lys-Asn-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Pro]

Cyclo[Aha-(ϵ -2-Cl-CBZ)Lys-Asn-Phe-Phe-D-Trp-(ϵ -2-Cl-CBZ)Lys-(O-Bzl)Thr-Phe-(O-Bzl)Thr-Pro], 2.0 g., obtained by the process set forth in Step d), was dissolved in 3 ml. anisole and 30 ml. hydrogen fluoride at dry ice-acetone bath temperature. The solution was stirred magnetically at ice-bath temperature for 1 hour. The excess hydrogen fluoride was removed *in vacuo* at ice-bath temperature. The resulting oily residue was triturated with ethyl acetate to give a solid. The solid was collected by filtration, washed with ethyl acetate and dried *in vacuo* to give a 1.48 g. of product.

Step f) — Purification of Cyclo[Aha-Lys-Asn-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Pro]

The Cyclo[Aha-Lys-Asn-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Pro], 1.20 g., obtained by the process set forth in Step e), was dissolved in 25 ml. 50% aqueous acetic acid and charged to a column of

Sephadex G-50, (fine) (5 cm. x 115 cm., 2260 ml.) packed in 50% aqueous acetic acid. The column was eluted with 50% aqueous acetic acid at the rate of 17 ml./10 min./fraction. The effluent was monitored at 280 nm.

- 5 Fractions 105 to 115 were combined, concentrated to dryness in vacuo and the residue lyophilized from 20 ml. 10% aqueous acetic acid to give 648 mg. of substantially pure product. A 20 hour acid hydrolysate showed the following amino acid analysis:

	$\mu\text{mole/mg.}$	normalized to average
10		
	Lys	1.05
	Asp	0.571
	Thr	1.07
	Pro	0.533
15	Phe	1.54
	Trp (by U.V.)	0.530

Example 2

- 20 Preparation of *Cyclo*(Aha-Lys-Pro-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Ser)

Step a) — Preparation of D-Trp-(ϵ -2-Cl-CBZ)Lys-(O-Bzl)Thr-Phe-(O-Bzl)Thr-(O-Bzl)Ser-Aha-(ϵ -2-Cl-CBZ)Lys-Pro-Phe-Phe-O-CH₂- ϕ -resin

- 25 Chloromethyl resin (2% cross-linked Merrifield resin), 862.0 g. (2.37 moles), having 2.75 meq. chlorine/g., and 607.0 g. (2.37 moles, 1 equivalent) of BOC-Phe were added to 4320 ml. of peroxide-free tetrahydrofuran. The mixture was stirred in an oil bath at 80°C. bath temperature for 45 minutes. Triethylamine, 310.0 ml., was added and the reaction mixture stirred at 80°C. bath temperature for 70 hours, cooled to 25°C. and transferred to a stirred solid phase reaction column with 2000 ml. of tetrahydrofuran. After removal of the solvent, the resin was washed using the stirred column with:

- 30 3 x 2000 ml. of tetrahydrofuran
4 x 5170 ml. of ethanol
1 x 5170 ml. of acetic acid
3 x 5170 ml. of water
3 x 5170 ml. of methanol
35 3 x 5170 ml. of chloroform.

The BOC-Phe-O-CH₂- ϕ -resin was dried *in vacuo* at 25°C. for 16 hours, giving 1203 g. of BOC-Phe-O-CH₂- ϕ -resin containing 0.937 mmole of phenylalanine/g. of resin.

- 40 BOC-Phe-O-CH₂- ϕ -resin (2.31 g.; 2.0 mmoles) was carried through the procedures in Tables III of Example 1 and Table VI using 2 deblockings (2 minutes and 25 minutes) with 25% TFA in methylene chloride, and 2.5 equivalents of BOC-amino acid in the required sequence until the desired BOC-undecapeptide-O-CH₂- ϕ -resin was obtained.

- 45 DCCl was used as the coupling agent in every step. The coupling of each amino acid proceeded smoothly. Best yields were obtained when the coupling was repeated in each step. When the coupling was repeated, the initial two chloroform washes, the deblocking step and the succeeding three chloroform washes were all omitted and replaced by a single chloroform wash.

The coupling reactions were carried out in methylene chloride, or a mixture of freshly degassed DMF and methylene chloride. The N-terminal amino group was blocked with a BOC group in each case; the hydroxy group of Thr and Ser was blocked with Bzl and the ϵ -amino group of Lys with 2-Cl-CBZ.

- 50 When the desired BOC-undecapeptide-O-CH₂- ϕ -resin was obtained, the N-terminal BOC group was removed by the terminal deblocking procedure set forth in Table V of Example 1.

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TABLE VI

Protected Amino Acid	Solvent ml.
BOC-Phe (1.33 g.) recouple	CH ₂ Cl ₂ , 25 ml.
BOC-Pro (1.08 g.) recouple	CH ₂ Cl ₂ , 25 ml.
BOC-(ϵ -2-Cl-CBZ)Lys (2.08 g.) recouple	CH ₂ Cl ₂ , 25 ml.
BOC-Aha (1.23 g.) recouple	CH ₂ Cl ₂ , 25 ml.
BOC-(O-Bzl)Ser (1.48 g.) recouple	CH ₂ Cl ₂ , 25 ml.
BOC-(O-Bzl)Thr (1.55 g.) recouple	CH ₂ Cl ₂ , 25 ml.
BOC-Phe (1.33 g.) recouple	DMF, 25 ml.
BOC-(O-Bzl)Thr (1.55 g.) recouple	CH ₂ Cl ₂ , 25 ml.
BOC-(ϵ -2-Cl-CBZ)Lys (2.08 g.) recouple	CH ₂ Cl ₂ , 25 ml.
BOC-D-Trp (1.52 g.) recouple	DMF, 5.5 ml. CH ₂ Cl ₂ , 19.5 ml.

After the sequence of Tables III, V and VI were completed, the blocked undecapeptide-O-CH₂- ϕ -resin dried overnight *in vacuo* and weighed 5.30 g.

An acid hydrolysate showed the following amino acid composition:

	μ mole/mg.	normalized to average
Lys	0.627	1.84
Thr	0.511	1.5
Ser	0.207	0.61
Pro	0.337	0.99
Phe	1.02	3.00

Step b) — Preparation of D-Trp-(ϵ -2-Cl-CBZ)Lys-(O-Bzl)Thr-Phe(O-Bzl)Thr-(O-Bzl)Ser-Aha-(ϵ -2-CBZ)Lys-Pro-Phe-Phe-NH-NH₂

To a mixture of 5.0 g. D-Trp-(ϵ -2-Cl-CBZ)Lys-(O-Bzl)Thr-Phe(O-Bzl)Thr-(O-Bzl)Ser-Aha-(ϵ -2-Cl-CBZ)Lys-Pro-Phe-Phe-O-CH₂- ϕ -resin in 50 ml. freshly degassed DMF was added 5.0 ml. NH₂—NH₂. The mixture was magnetically stirred at room temperature for 55 minutes. The mixture was filtered to remove the resin. The filtrate and washings were concentrated *in vacuo* to an oily residue. The residue was triturated with ether and ethyl acetate to obtain a solid. The solid was collected by filtration and dried *in vacuo*. The solid was slurried, filtered and washed with water 8 $\times$ to remove formylhydrazide and dried *in vacuo* overnight to give 2.30 g. of product.

Step c) — Preparation of D-Trp-(ϵ -2-Cl-CBZ)Lys-(O-Bzl)Thr-Phe(O-Bzl)Thr-(O-Bzl)Ser-Aha-(ϵ -2-Cl-CBZ)Lys-Pro-Phe-Phe-N₃

D-Trp-(ϵ -2-Cl-CBZ)Lys-(O-Bzl)Thr-Phe(O-Bzl)Thr-(O-Bzl)Ser-Aha-(ϵ -2-Cl-CBZ)Lys-Pro-Phe-Phe-NH-NH₂ (2.2 g., 1.08 mmole), prepared by the process set forth in Step b), was dissolved in 40 ml. freshly degassed DMF. The solution was stirred magnetically at -25°C . To the solution was added 1.655 ml. of 5.72N HCl in THF (9.47 mmoles, 8.77 equivalents). To the resulting acidic solution, "pH" 1.0 to 1.5, was added 0.23 ml. (isoamyl nitrite (1.7 mmoles, 1.576 equivalents) and stirring continued for 30 minutes at -25°C . and then cooled to -40°C . This solution of D-Trp-(ϵ -2-Cl-CBZ)Lys-(O-Bzl)Thr-Phe(O-Bzl)Thr-(O-Bzl)Ser-Aha-(ϵ -2-Cl-CBZ)Lys-Pro-Phe-Phe-N₃ was used immediately in Step d).

Step d) — Preparation of Cyclo[(O-Bzl)Thr-(O-Bzl)Ser-Aha-(ϵ -2-Cl-CBZ)Lys-Pro-Phe-Phe-D-Trp-(ϵ -2-Cl-CBZ)Lys-(O-Bzl)Thr-Phe]

The solution of D-Trp-(ϵ -2-Cl-CBZ)Lys-(O-Bzl)Thr-Phe(O-Bzl)Thr-(O-Bzl)Ser-Aha-(ϵ -2-Cl-CBZ)Lys-Pro-Phe-Phe-N₃ in DMF, obtained by the process set forth in step c), was diluted in 2000 ml. freshly degassed DMF, precooled to -40°C . The solution was maintained under a nitrogen atmosphere and allowed to warm to -20°C . during which time the "pH" was maintained at 7.2 to 7.6 by the addition of 3.2 ml. N,N-diisopropylethylamine. The solution was maintained at -18°C . for 24 hours and then kept at 5°C . for an additional 24 hours.

The solution was concentrated *in vacuo* to a thick oil, triturated once with a mixture of ether and petroleum ether, twice with ether, and once with a mixture of ethyl acetate and ether. The residue was dried and triturated with a total of 150 ml. water to give a solid. The solid was collected by filtration and dried *in vacuo* overnight to give 2.25 g. of product.

An acid hydrolysate showed the following amino acid composition:

	$\mu\text{mole/mg.}$	normalized to average
Lys	0.858	1.95
Thr	0.871	1.98
Ser	0.446	1.02
Pro	0.450	1.03
Phe	0.450	1.03
Trp	0.089	0.20

Step e) — Preparation of Cyclo(Aha-Lys-Pro-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Ser)

Cyclo[(O-Bzl)Thr-(O-Bzl)Ser-Aha-(ϵ -2-Cl-CBZ)Lys-Pro-Phe-Phe-D-Trp-(ϵ -2-Cl-CBZ)Lys-(O-Bzl)Thr-Phe], 2.1 g., obtained by the process set forth in Step d), was dissolved in 3 ml. anisole and 30 ml. hydrogen fluoride at dry ice-acetone bath temperature. The solution was stirred magnetically at ice-bath temperature for 20 minutes. The excess hydrogen fluoride was removed *in vacuo* at ice-bath temperature. The resulting oily residue was maintained *in vacuo* for an additional 20 minutes at ice-bath temperature and triturated with ethyl acetate to give a solid. The solid was collected by filtration and dried *in vacuo* for 3 hours to give 1.44 g. of product.

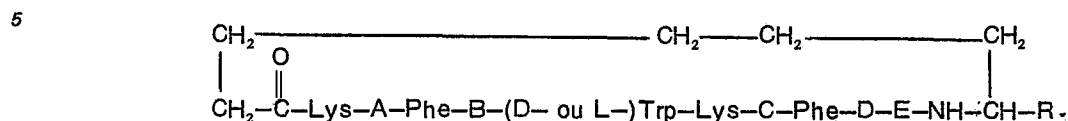
Step f) — Purification of Cyclo(Aha-Lys-Pro-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Ser)

The Cyclo(Aha-Lys-Pro-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Ser), 1.44 g., obtained by the process set forth in Step e), was divided into two equal portions. Each portion was dissolved in 10 ml. acetic acid to which was added 10 ml. water. The resulting cloudy solution was charged to a column of Sephadex G-50, (5 cm. $\times$ 115 cm., 2260 ml.) packed in 50% aqueous acetic acid. The column was eluted with 50% aqueous acetic acid at the rate of 17.2 ml./10 min./fraction. The effluent was monitored at 280 nm.

Fractions 96 to 107 from the first column and fractions 98 to 109 from the second column were combined, concentrated to dryness *in vacuo* and the residue lyophilized from 20 ml. 10% aqueous acetic acid to give 1.1155 g. of substantially pure product. A 20 hour acid hydrolysate showed the following amino acid analysis:

Revendications

1. Composés de formule



10 dans laquelle

- 15 R représente H, COOH,
 A représente (Asn)_n, alpha-Abu, Pro, Ala, et n = 0 ou 1,
 B représente Phe, Tyr,
 C et D représentent indépendamment Thr, Val,
 E représente Ser, Pro, Ala, Gly,

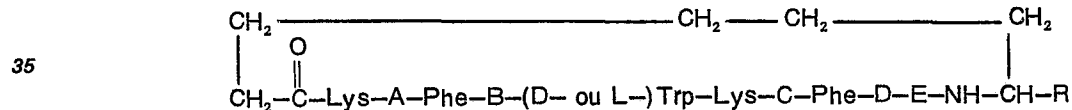
l'un au moins de A et E représentant Pro, et le cycle formé par le squelette de peptide contient 35 ou 38 atomes, et leurs sels formés par addition avec des acides non toxiques acceptables pour l'usage pharmaceutique.

2. Le composé selon la revendication 1 dans lequel

- 25 R représente H,
 A représente Asn, Pro,
 B représente Phe,
 C et D représentent Thr,
 E représente Ser, Pro,

l'un au moins des symboles A et E représentant Pro.

3. Procédé de préparation des peptides répondant à la formule



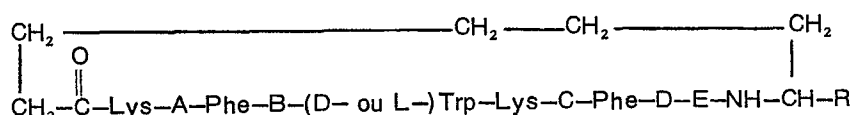
dans laquelle

- 40 R représente H, COOH,
 A représente (Asn)_n, alpha-Abu, Pro, Ala, et n = 0 ou 1,
 B représente Phe, Tyr,
 C et D représentent indépendamment Thr, Val,
 45 E représente Ser, Pro, Ala, Gly,

l'un au moins des symboles A et E représentant Pro et le cycle formé par le squelette de peptide contient 35 ou 38 atomes, et leurs sels formés par addition avec des acides non toxiques acceptables pour l'usage pharmaceutique, qui comprend:

- 50 a) la préparation d'un peptide linéaire correspondant bloqué fixé sur une résine en phase solide et dans lequel le groupe —OH de Thr et Ser est bloqué par Bzl; le groupe -amino de Lys est bloqué par 2-Cl-CBZ; le groupe amino à azote terminal est bloqué par BOC et dans le cas où R représente COOH, le groupe acide carboxylique à carbone terminal est bloqué par un groupe tert-butyle;
 b) l'élimination sélective du groupe BOC protégeant le groupe amino à azote terminal et, dans le cas où R représente COOH, l'élimination du groupe tert-butyle;
 55 c) le traitement du peptide linéaire fixé à la résine par l'hydrazine provoquant la séparation du peptide d'avec la résine et la formation de l'hydrazide du peptide linéaire avec le groupe de l'azote terminal débloquent;
 d) le traitement du peptide linéaire par le nitrite d'isoamyle à pH acide, donnant l'azide correspondant; la dilution et la neutralisation de la solution de l'azide conduisant au peptide cyclique correspondant, et
 60 e) le traitement du peptide cyclique par HF provoquant l'élimination simultanée des groupes bloquants Bzl et 2-Cl-CBZ.

4. Une composition comprenant une quantité efficace du point de vue thérapeutique d'un ou plusieurs peptides de structure



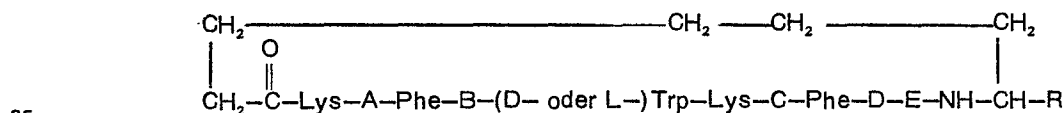
5 dans laquelle

- R représente H, COOH,
 A représente (Asn)_n, alpha-Abu, Pro, Ala, et n = 0 ou 1,
 10 B représente Phe, Tyr,
 C et D représentent indépendamment Thr, Val,
 E représente Ser, Pro, Ala, Gly,

l'un au moins des symboles A et E représentant Pro et le cycle formé par le squelette du peptide con-
 15 tient 35 ou 38 atomes, et leurs sels formés par addition avec des acides non toxiques acceptables pour
 l'usage pharmaceutique dans un excipient acceptable pour l'usage pharmaceutique.

Patentansprüche

20 1. Verbindungen mit der Formel:



25 worin

- R H, COOH ist
 A (Asn)_n, α-Abu, Pro, Ala, worin n = 0 oder 1, ist
 30 B Phe, Tyr ist
 C und D unabhängig Thr, Val sind,
 E Ser, Pro, Ala, Gly ist,

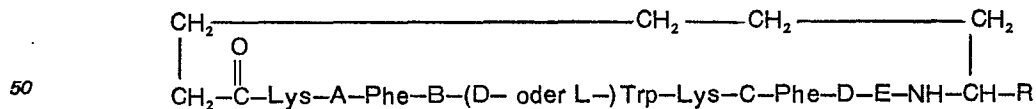
worin mindestens eines von A und E Pro ist und der Ring, der durch das Peptidgerüst gebildet wird, 35
 oder 38 Atome enthält, und pharmazeutisch brauchbare nicht-toxische Säureadditionssalze davon.

2. Verbindung nach Anspruch 1, worin

- R H ist,
 A Asn, Pro ist,
 40 B Phe ist,
 C und D Thr sind,
 E Ser, Pro ist,

worin mindestens eines von A und E Pro ist.

45 3. Verfahren zur Herstellung der Peptide mit der Formel:



worin

- R H, COOH ist,
 A (Asn)_n, α-Abu, Ala, worin n = 0 oder 1, ist,
 B Phe, Tyr ist,
 C und D unabhängig Thr, Val sind,
 60 E Ser, Pro, Ala, Gly ist,

worin mindestens eines von A und E Pro ist und der Ring, der durch das Gerüst gebildet wird, 35
 oder 38 Atome enthält, und pharmazeutisch brauchbarer nicht-toxischer Säureadditionssalze davon,
 das darin besteht:

a) ein entsprechendes blockiertes, lineares Peptid, das an ein festphasiges Harz gebunden ist, her-
 65 zustellen, worin die —OH-Gruppe von Thr und Ser durch Bzl blockiert ist; die ε-Aminogruppe von Lys

durch 2-Cl-CBZ blockiert ist; die N-endständige Aminogruppe durch BOC blockiert ist und für den Fall, daß R COOH ist, die C-endständige Carbonsäuregruppe durch t-Butyl blockiert ist;

b) die BOC-Gruppe von der N-endständigen Aminogruppe selektiv zu entfernen und für den Fall, daß R COOH ist, die t-Butylgruppe zu entfernen;

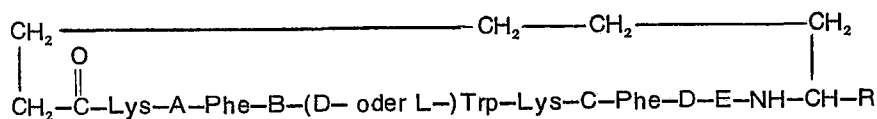
5 c) das an das Harz gebundene lineare Peptid mit Hydrazin zu behandeln, zur Entfernung des Peptids von dem Harz, unter Erzielung des linearen Peptidhydrazids mit entblockierter N-endständiger Gruppe;

d) das lineare Peptid mit Isoamylnitrit bei saurem pH-Wert zu behandeln unter Erzielung des entsprechenden Azids; die Azidlösung zur verdünnen und zu neutralisieren unter Erzielung des entsprechenden cyclischen Peptids und;

10 e) das cyclische Peptid mit HF zu behandeln zur gleichzeitigen Entfernung der Bzl- und 2-Cl-CBZ-Blockierungsgruppen.

4. Zusammensetzung enthaltend eine therapeutisch wirksame Menge von einem oder mehreren Peptiden mit der Struktur:

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worin

R H, COOH ist,
A (Asn)_n, α-Abu, Pro, Ala, worin n = 0 oder 1, ist,
25 B Phe, Tyr ist,
C und D unabhängig Thr, Val sind,
E Ser, Pro, Ala, Gly ist,

worin mindestens eines von A und E Pro ist und der Ring, der durch das Peptidgerüst gebildet wird, 35
30 oder 38 Atome enthält, und pharmazeutisch brauchbare, nicht-toxische Säureadditionssalze davon in einem pharmazeutisch brauchbaren Exciipienten.

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