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(54) Title: CHROMOGENIC SUBSTRATE

(57) Abstract

The invention relates to novel peptide derivatives with the formula: R_1-A_1-A_2-A_3-A_4-R_2 or its salt, wherein R_1 = H or a protective group; A_1 = H, Ile, Leu or Val; A_2 = Glu, Asp, Ser, Thr; A_3 = Gly or Glyc; A_4 = Arg or Lys; R_2 = 4-nitro aniline with the proviso that A_3 is Gly when A_4 is Lys and that A_3 is Glyc when A_4 is Arg. The invention also discloses the process for the preparation of the peptide derivatives, the method for the determination of the bacterial endotoxins by the use of the invented peptide derivatives and the use of these derivatives for determination of bacterial endotoxins.

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Chromogenic substrate

Field of the invention

The present invention relates to a new chromogenic synthetic substrate used for quantitative determination of bacterial endotoxins in physiological fluids, food, pharmaceuticals etc.

The background of the invention

Bacterial endotoxins are produced by Gram negative bacteria and are considered by most investigators to be a very important factor in the development of septecemia. Several methods for determination of endotoxin have been described based on the observation by Levin and Bang (1956) that endotoxins specifically activate the clotting system of Limulus Polyphemus. In the beginning a test, in which Limulus Amebocyte Lysate (LAL) in contact with endotoxins containing sources, produce a specific gelation, had been developed. More recently chromogenic and fluorogenic methods based on the above observation have been developed and can rapidly detect small amounts of endotoxins (H.C. Hemker: Handbook of Synthetic Substrates, 1983, Martinus Nijhoff Publisher, Boston).

Prior art

A.E. Torano et al have disclosed that an enzyme from Limulus amebocyte lysate shows similar specificity to mammalian blood coagulation factor X_a (Thrombosis Research 34, 407-417, 1984), which recognize the sequence -Ile-Glu-Gly-Arg- in its natural substrate prothrombin.

Other investigations have also shown the important role the COOH-terminal Gly-Arg sequence plays when analysing endotoxin. T. Harada et al, Biomedical Applications of the Horseshoe Crab (Limulidal), E. Cohen (ed), Alan R. Liss Inc. New York, 1979, pages 209-220, disclose in a table on page 213 different substrates which are used, but only those having the carboxy termial sequence Gly-Arg give interesting results. On page 212, lines 7-10 from the bottom, the authors point out: "These results clearly indicate that Limulus clotting enzyme displays a high specificity towards the peptide pNA having COOH-terminal Gly-Arg sequence".

US 4 188 264 and US 4 576 745 also give Gly-Arg as carboxy-terminal sequence in the substrate used for determination of endotoxin. Other substrates have been investigated, all of them showing Arg as carboxy terminal.

According to US 4 406 832 the carboxy terminal should be -Ala-Arg- or -Cys-Arg- and this substrate has given relative activity which is as good as or a little better than standard -Gly-Arg-. Due to the complicated mechanism in these reactions it is not possible to know which peptide sequence could give an acceptable result.

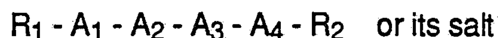
Surprisingly and against the prior art within the field, we have now found that a substrate which has the carboxy terminal sequence -Gly-Lys- gives a relative activity which is at least 20% better compared with known substrates.

The use of Glycolic acid (Glyc) in a substrate for determination of endotoxin has never been disclosed before, and it is very surprising that the effect is as good when using -Glyc-Arg- as when using Gly-Lys, both of them giving a better effect than the normally used Gly-Arg.

Description of the invention

The chromogenic synthetic peptide or peptide isostere derivative in the present invention shows high sensitivity in the method used for the determination of endotoxins.

The new substrates are characterized by the following formula:



$$R_1 - A_1 - A_2 - A_3 - A_4 - R_2 \quad \text{or its salt}$$

where

R_1 = H or a protective group

A_1 = H, Ile, Leu or Val

A_2 = Glu, Asp, Ser, Thr

A_3 = Gly or Glyc

A_4 = Arg or Lys

R_2 = 4-nitro aniline

with the proviso that A_3 is Gly when A_4 is Lys and that A_3 is Glyc when A_4 is Arg.

The present invention also discloses the process for the preparation of the peptid derivatives.

It also discloses the method for determination of bacterial endotoxins by the use of the derivative with the following formula:

$$R_1 - A_1 - A_2 - A_3 - A_4 - R_2 \quad \text{or its salt}$$

where

R_1 = H or a protective group

A_1 = H, Ile, Leu or Val

A_2 = Glu, Asp, Ser, Thr

A_3 = Gly or Glyc

A_4 = Arg or Lys

R_2 = an aromatic or heterocyclic group which gives the compound R_2-NH_2 by enzymatic hydrolysis

R_2-NH_2 are prior known compounds with chromogenic properties which permit quantifying of endotoxins by determination of splitted marker directly or after derivatization (H.C. Hemker: Loc. cit.) with the proviso that A_3 is Gly when A_4 is Lys and that A_3 is Glyc when A_4 is Arg.

The use of these derivatives for determination of bacterial endotoxins is also disclosed.

Example of compounds which could be R_2-NH-$_2$ are: p-nitroaniline, 3-carboxy-4-hydroxyaniline, 3-sulfo-4-nitroaniline, 3-alkoxy-4-nitroaniline, 3-carboxy-4-nitroaniline, 4-methyloxy-naphtylamine, 4-(N-ethyl-N-hydroxyethyl) aminoaniline, 5-amino-isophtalic acid-dimethyl ester, 5-amino-8-nitroquinoline, 7-amino-4-trifluormethyl coumarine, 7-amino-4-methyl coumarine, 4-amino-diphenylamine. The invention also discloses the use of these derivatives for determination of bacterial endotoxins.

The new peptide or peptide isostere derivatives contain as carboxyterminal Lys when combined with Gly and Arg when combined with Glyc. The combination Gly-Lys was considered unfavourable until present in substrates used for Limulus amebocyte lysate and the combination Glyc-Arg has never been used in this type of substrates before in methods for determination of bacterial endotoxins.

Description of synthesis

Conventional techniques for coupling and conventional protecting groups (Z, Boc etc) used within the peptide chemistry (M. Bodanzsky: Principles of Peptide Synthesis, Springer Verlag 1984) e.g. addition step-by-step of the amino acids at the C-terminal amino acid provided with a marker or synthesis of the N-terminal peptide fragment per se, which then is coupled to the C-terminal amino acid provided with a marker, have been used.

The synthesis of different substrates according to the invention will be discribed more in detail in the following not limited working examples.

Purification of the intermediates and end products were performed by precipitation, crystallization or gel filtration chromatography. The purified end products were lyophilized. Prefabricated glass plates of silicagel F_{254} were used for TLC analyses. After terminated chromatography the plates were inspected in U.V. light (254 nm) and were developed thereafter with ninhydrine and chlorine/dicarboxidine reagent. The Rf value given are results from single chromatographies.

Used solvent system for TLC have been indicated according to the following table:

Indication	Solvent system	Volume ratio
A	n-butanol: AcOH:water	3:2:1
Pa_6	chloroform: MeOH: AcOH:water	34:4:9:2
P1	chloroform: MeOH	9:1
Pa	chloroform: MeOH:AcOH	17:2:2

HPLC analysis were performed on Merck R.P. column (Hibar Lichracart) with 40% MeOH in 5% triethylaminophosphate pH 2.35 as eluent (1 ml/min). The optical activity of the end products were determined at 589 nm in 50% AcOH at a concentration of 0.4-1.0 g/100 ml at 25°C. The below mentioned abbreviations have the following meaning: (I.U.P.A.C. indication has been used were such exists).

Amino acids:

Arg =	arginine		Val =	valine
Gly =	glycine		Glu =	glutamic acid
Ile =	isoleucine		Asp =	aspartic acid
Leu =	leucine		Ser =	serine
			Thr =	threonine

All amino acids in the substrates have L-configurattion if not else is indicated.

The free amino acid or peptide is indicated by H- at the N-terminal amino group and -OH at the carboxy terminal group. The amino group is always given to the left and the carboxy to the right.

Abbreviations:

Ac =	acetyl	Et_3N =	triethylamine
AcOH =	acetic acid	Glyc =	glycolic acid
AMC =	7-amino-4-methylcoumarine	HOBT =	1-hydroxybenzotriazol
Boc =	t-butyloxycarbonyl	HPLC =	high performance liquid chromatography
Bz =	benzoyl		
Bzl =	benzyl	MeOH =	methanol
DCCI =	dicyclohexylcarbodiimide	ONp =	nitrophenyl ester
DCU =	dicyclohexylurea	OSu =	hydroxysuccinimide ester
DMAP =	dimethylaminopyridine	pNA =	p-nitroaniline
DMF =	dimethylformamide	TFA =	trifluoroacetic acid
EtOAc =	ethylacetate	Z =	benzyloxycarbonyl
EtOH =	ethanol		

Example 1

α-Ac-Ile-Glu-Gly-Lys-pNA·HCl   molecular weight = 644.15

1a) ε-Z-Lys-pNA·TFA   Molecular weight = 514.5

9 ml TFA is added to 10 mmol α-Boc-ε-Z-Lys-pNA dissolved in 25 ml methylenchloride. The solution is stirred for 30 minutes at room temperature and is precipitated with a mixture 2:1 of t-butylmethylether-petroleumether.
Yield 95%.
TLC: Rf = 0.60 (A).

1b) α-Boc-γ-O-Bzl-Glu-Gly-OH Molecular weight = 394.4

To 0.1 mol H-Gly-OH dissolved in 200 ml 1 molar $NaHCO_3$ a solution of 0.1 mol of α-Boc-γ-O-Bzl-Glu-O-Su in 200 ml dioxan is added. The mixture is stirred overnight at room temperature. Next day the solution is evaporated in vacuo to an oily residue which is dissolved in 100 ml water and washed with diethylether. The water phase is brought to pH 2 with $KHSO_4$ solution and extracted with EtOAc. After drying with Na_2SO_4, the EtOAc solution is evaporated and the substance precipitated with a mixture (1:1) of diethylether-petroleumether.
Yield 85%.
TLC: Rf = 0.5 (Pa).

1c) H-γ-O-Bzl-Glu-Gly-OH·TFA Molecular weight = 408.4

9 ml TFA is added to 10 mmol α-Boc-γ-O-Bzl-Glu-Gly-OH (1b) dissolved in 20 ml methylenchloride. The mixture is stirred for 30 minutes at room temperature, evaporated in vacuo to an oil and precipitated with diethylether.
Yield: 90%
TLC: Rf = 0.44 (A).

1d) α-Boc-Ile-γ-O-Bzl-Glu-Gly-OH Molecular weight = 507.6

To 0.1 mol H-γ-O-Bzl-Glu-Gly-OH·TFA dissolved in 250 ml 1 molar $NaHCO_3$ a solution of 0.1 mol α-Boc-Ile-O-Su in 250 ml dioxan is added. The mixture is stirred overnight at room temperature. Next day the solution is evaporated in vacuo, the residue dissolved in 100 ml water and washed with EtOAc. The water phase is brought to pH 3 with $KHSO_4$ solution and extracted with EtOAc. After drying with Na_2SO_4 the EtOAc solution is evaporated and the substance precipitated with petroleumether.
Yield: 92%
TLC: Rf = 0.6 (Pa)

1e) α-Boc-Ile-γ-O-Bzl-Glu-Gly-ε-Z-Lys-pNA Molecular weight = 890.0

3 mmol ε-Z-Lys-pNA·TFA (1a) dissolved in 25 ml DMF is neutralized in cold (-10°C) with Et_3N. To the solution 3 mmol α-Boc-Ile-γ-O-Bzl-Glu-Gly-OH (1d), 3 mmol HOBT and 3.2 mmol DCCI are added. The mixture is stirred for 1 hour in cold and overnight at room temperature. The formed DCU is filtered off and the solution is evaporated in vacuo to an oil which is dissolved in EtOAc and is washed with 2% $NaHCO_3$, 2% $KHSO_4$ and H_2O. After drying with with Na_2SO_4, the EtOAc phase is evaporated and the substance is precipitated with diethylether.
Yield: 61%
TLC: Rf = 0.67 (P_1)

1f) α-Ac-Ile-γ-O-Bzl-Glu-Gly-ε-Z-Lys-pNA Molecular weight = 831.9

3.5 ml TFA is added to 1.2 mmol α-Boc-Ile-γ-O-Bzl-Glu-Gly-ε-Z-Lys-pNA (1e) in 6 ml methylenchloride. The mixture is stirred for 30 minutes at room temperature and is precipitated with diethyleter. The dry substance is dissolved in 10 ml DMF and neutralized in cold (-10°C) with 160 µl Et_3N. 140 µl acetic anhydride is added and the mixture is stirred for 1 hour in cold and 2 hours at room temperature. The solution is evaporated in vacuo to an oil and the substance is precipitated with water.
Yield: 86%
TLC: Rf = 0.45 (P1).

1) α-Ac-Ile-Glu-Gly-Lys-pNA·HCl Molecular weight = 644.15

10 ml triflic acid is added to a cold (-10°C) suspension of 1 mmol α-Ac-Ile-γ-O-Bzl-Glu-Gly-Z-Lys-pNA- (1f) in 10 ml methylenchloride. The mixture is stirred for 50 minutes at room temperature and precipitated with diethylether. The dried substance is ion exchanged on a Sephadex® QAE-25 column, in chloride form with 50% ETOH as eluent and is purified on a Merck Lobar® prepacked column (Lichroprep.® RP-8-B) with 50% MeOH as eluent (2 ml/minute). The purified product is lyophilized.

Yield: 42%
TLC: Rf = 0.2 (Pa_6)
HPLC: 98% purity
$[\alpha]^{25}_{D}$ = -63.1° (C= 0.5%)

Example 2

α-Ac-Ile-Ser-Gly-Lys-pNA·HCl Molecular weight = 602.11

2a) α-Boc-Gly-ε-Z-Lys-pNA Molecular weight = 557.6

5 mmol ε-Z-Lys-pNA·TFA (prepared as described in example 1a) dissolved in 25 ml DMF is neutralized in cold (-10°C) with Et_3N. To the solution 5 mmol α-Boc-Gly-OH, 5 mmol HOBT and 5 mmol DCCI are added. The mixture is stirred for 1 hour in cold and overnight at room temperature. The formed DCU is filtered off and the solution is evaporated in vacuo to an oil which is dissolved in EtOAc and washed with 2% $NaHCO_3$, 2% $KHSO_4$ and H_2O. After drying with Na_2SO_4 the EtOAc phase is evaporated in vacuo and the substance is precipitated with diethylether as an oil which solidifies in vacuo.
Yield: 73%
TLC: Rf = 0.55 (P_1).

2b) α-Boc-O-Bzl-Ser-Gly-ε-Z-Lys-pNA Molecular weight = 734.2

10 ml TFA is added to 5 mmol α-Boc-Gly-ε-Z-Lys-pNA (2a) dissolved in 20 ml methylenchloride. The mixture is stirred for 30 minutes at room temperature and the substance is precipitated with diethyleter. The dry substance is dissolved in 25 ml DMF and neutralized in cold (-10°C) with Et_3N. To the solution 5 mmol α-Boc-O-Bzl-Ser-OH, 5 mmol HOBT and 5.1 mmol DCCI are added. The mixture is stirred for 1 hour in cold and overnight at room temperature. The formed DCU is filtered off and the solution is evaporated in vacuo to an oil which is dissolved in EtOAc and washed with 2% $NaHCO_3$, 2% $KHSO_4$ and H_2O. After drying with Na_2SO_4 the EtOAc phase is evaporated and the substance is precipitated with diethylether as an oil which solidifies in vacuo.
Yield: 86%
TLC: Rf = 0.62 (P_1).

2c) α-Boc-Ile-O-Bzl-Ser-Gly-ε-Z-Lys-pNA Molecular weight: = 848.0

10 ml TFA is added to 2 mmol α-Boc-O-Bzl-Ser-Gly-ε-Z-Lys-pNA (2b) dissolved in 20 ml methylenchloride. The mixture is stirred for 30 minutes at room temperature and the substance is precipitated with diethylether. The dry substance is dissolved in 25 ml DMF and neutralized in cold (-10°C) with Et_3N. To the solution 2 mmol α-Boc-Ile-ONp is added. The mixture is stirred for 1 hour in cold and 48 hours at room temperature. The solution is evaporated in vacuo to an oil which is dissolved in EtOAc and washed with 2% $NaHCO_3$, 2% $KHSO_4$ and H_2O. After drying with Na_2SO_4 the EtOAc phase is evaporated and the substance is precipitated with diethylether.
Yield: 72%
TLC: Rf = 0.62 (P_1).

2d) α-Ac-Ile-O-Bzl-Ser-Gly-ε-Z-Lys-pNA Molecular weight = 789.9

5 ml TFA is added to 1 mmol α-Boc-Ile-O-Bzl-Ser-Gly-ε-Z-Lys-pNA (2c) dissolved in 10 ml methylenchloride. The mixture is stirred for 30 minutes at room temperature and the substance is precipitated with diethylether. The dry substance is dissolved in 10 ml DMF and neutralized in cold (-10°C) with 80 µl Et_3N. 70 µl acetic anhydride is added ad the mixture is stirred for 1 hour in cold and 2 hours at room temperature. The solution is evaporated in vacuo to an oil and the substance is precipitated with water.
Yield: 85%
TLC: Rf = 0.55 (P_1).

2) α-Ac-Ile-Ser-Gly-Lys-pNA·HCl Molecular weight = 602.11

10 ml triflic acid is added to a cold (-10°C) suspension of 1 mmol α-Ac-Ile-O-Bzl-Ser-Gly-ε-Z-Lys-pNA (2d) in 10 ml methylenchloride. The mixture is stirred for 50 minutes at room temperature and precipitated with diethylether. The product is

ion exchanged and purified in the same way as in example 1.
Yield: 35%
TLC: Rf = 0.2 (Pa_6)
HPLC: 97% purity.
$[\alpha]^{25}_{D} = -50.1°$ (c= 0.5%)

Example 3

α-Ac-Ile-Thr-Gly-Lys-pNA·HCl Molecular weight = 616.13

3a) α-Boc-O-Bzl-Thr-Gly-ε-Lys-pNA Molecular weight = 748.2

10 ml TFA is added to 5 mmol α-Boc-Gly-ε-Z-Lys-pNA (2a) dissolved in 20 ml methylenchloride. The mixture is stirred for 30 minutes at room temperature and the substance is precipitated with diethylether. The dry substance is dissolved in 25 ml DMF and neutralized in cold (-10°C) with Et_3N. To the solution 5 mmol α-Boc-O-Bzl-Thr-OH, 5 mmol HOBT and 5.1 mmol DCCI are added. The mixture is stirred for 1 hour in cold and overnight at room temperature. The formed DCU is filtered off and the solution is evaporated in vacuo to an oil, which is dissolved in EtOAc and washed with 2% $NaHCO_3$, 2% $KHSO_4$ and H_2O. After drying with Na_2SO_4 the EtOAc phase is evaporated and the substance is precipitated with diethylether as an
oil which solidifies in vacuo.
Yield: 51%
TLC: Rf = 0.65 (P_1).

3b) α-Boc-Ile-O-Bzl-Thr-Gly-ε-Z-Lys-pNA Molecular weight = 862.0

10 ml TFA is added to 2 mmol α-Boc-O-Bzl-Thr-Gly-ε-Z-Lys-pNA (3a) dissolved in 20 ml methylenchloride. The mixture is stirred for 30 minutes at room temperature and the substance is precipitated with diethylether. The dry substance is dissolved in 25 ml DMF and neutralized in cold (-10°C) with Et_3N. To the solution 2 mmol α-Boc-Ile-ONp is added. The mixture is stirred for 1 hour in cold and 48 hours at room temperature. The solution is evaporated in vacuo to an oil which is dissolved in EtOAc and washed with 2% $NaHCO_3$, 2% $KHSO_4$ and H_2O. After drying with Na_2SO_4 the EtOAc phase is evaporated and the

substance is precipitated with diethylether.
Yield: 50%
TLC: Rf = 0.7 (P_1)

3c) α-Ac-Ile-O-Bzl-Thr-Gly-ε-Z-Lys-pNA Molecular weight = 803.9

5 ml TFA is added to 1 mmol α-Boc-Ile-O-Bzl-Thr-Gly-ε-Z-Lys-pNA (3b) dissolved in 10 ml methylenchloride. The mixture is stirred for 30 minutes at room temperature and the substance is precipitated with diethylether. The dry substance is dissolved in 10 ml DMF and neutralized in cold (-10°C) with 80 µl Et_3N. 70 µl acetic anhydride is added and the mixture is stirred for 1 hour in cold (-10°C) and
2 hours at room temperature. The solution is evaporated in vacuo to an oil and the substance is precipitated in water.
Yield: 86%
TLC: Rf = 0.55 (P_1)

3) Ac-Ile-Thr-Gly-Lys-pNA·HCl Molecular weight = 616.13

10 ml triflic acid is added to a cold (-10°C) suspension of 1 mmol α-Ac-Ile-O-Bzl-Thr-Gly-ε-Z-Lys-pNA (3c) in 10 ml methylenchloride. The mixture is stirred for 50 minutes at room temperature and precipitated with diethylether. The product is ion exchanged and purified in the same way as in example 1.
Yield: 36%
TLC: Rf = 0.21 (Pa_6)
HPLC: 97% purity
$[\alpha]^{25}_{D} = -53.2°$ (c= 0.3%)

Example 4

α-Ac-Ile-Glu-Glyc-Arg-pNA·HCl Molecular weight = 673.18
4a) Glyc-Arg-pNA·HCl Molecular weight: = 388.8

5 mmol Arg-pNA·2 HBr dissolved in 30 ml DMF is neutralized in cold (-10°C) with Et_3N. To the solution 5 mmol glycolic acid, 5 mmol HOBT and 5.1 mmol DCCI are added. The mixture is stirred for 1 hour in cold and 72 hours at room

temperature. The formed DCU is filtered off and the solution is evaporated in vacuo to an oil, which is purified on a Sephadex® QAE-25 column in chloride form with 90% ETOH as eluent.
Yield: 74%
TLC: Rf = 0.35 (A).

4b) α-Boc-γ-O-Bzl-Glu-Glyc-Arg-pNA·HCl Molecular weight = 708.2

To 5 mmol Glyc-Arg-pNA·HCl dissolved in 30 ml DMF and cooled to -10°C, 5 mmol α-Boc-γ-O-Bzl-Glu-OH, 5 mmol HOBT, 0.5 mmol DMAP and 5.1 mmol DCCI are added. The mixture is stirred for 1 hour in cold and 48 hours at room temperature. The formed DCU is filtered off and the solution is evaporated in vacuo to an oil which is dissolved in EtOAc and washed with 2% $NaHCO_3$, 2% $KHSO_4$ and H_2O. After drying with Na_2SO_4, the EtOAc is evaporated and the substance is precipitated with diethylether.
Yield: 66%
TLC: Rf = 0.35 (Pa_6).

4c) α-Boc-Ile-γ-O-Bzl-Glu-Glyc-Arg-pNA·HCl Molecular weight = 821.3

10 ml TFA is added to 2 mmol α-Boc-γ-O-Bzl-Glu-Glyc-Arg-pNA·HCl (4b) dissolved in 20 ml methylenchloride. The mixture is stirred for 30 minutes at room temperature and the substance is precipitated with diethyleter. The dry substance is dissolved in 25 ml DMF and neutralized in cold (-10°C) with Et_3N. To the solution 2 mmol α-Boc-Ile-ONp is added. The mixture is stirred for 1 hour in cold and 48 hours at room temperature. The solution is evaporated in vacuo to an oil which is dissolved in EtOAc and washed with 2% $NaHCO_3$, 2% $KHSO_4$ and H_2O. After drying with Na_2SO_4 the EtOAc phase is evaporated and the substance is precipitated with diethylether.
Yield: 69%
TLC: Rf = 0.43 (Pa_6)

4d) α-Ac-Ile-γ-O-Bzl-Glu-Glyc-Arg-pNA·HCl Molecular weight = 763.3

5 ml TFA is added to 1 mmol α-Boc-Ile-γ-O-Bzl-Glu-Glyc-Arg-pNA·HCl (4c) dissolved in 10 ml methylenchloride. The mixture is stirred for 30 minutes at room temperature and the substance is precipitated with diethylether. The dried

substance is dissolved in 10 ml DMF and neutralized in cold (-10°C) with 130 μl Et_3N. 120 μl acetic anhydride is added and the mixture is stirred for 1 hour in cold and 2 hours at room temperature. The solution is evaporated in vacuo to an oil and the substance is precipitated with H_2O.
Yield: 94%
TLC: Rf = 0.34 (Pa_6).

4 α-Ac-Ile-Glu-Glyc-Arg-pNA·HCl Molecular weight = 673.18

10 ml triflic acid is added to a cold (-10°C) suspension of 1 mmol α-Ac-Ile-γ-O-Bzl-Glu-Glyc-Arg-pNA·HCl (4d) in 10 ml methylenchloride. The mixture is stirred for 50 minutes at room temperature and precipitated with diethylether. The substance is ion exchanged and purified in the same way as in example 1.
Yield: 31%
TLC: Rf = 0.45 (A)
HPLC: 98% purity
$[\alpha]^{25}_{D} = -60.6°$ (c= 0.4%)

Comparison of the chromogenic substrates using a single-stage chromogenic LAL-test.*

A series of endotoxin containing solutions, making up a standard curve in the range 0.1-1.2 EU/ml, were assayed as described below using the different chromogenic substrates. The slopes of the resulting standard curves were compared and the slope for the substrate S-2423 was considered to be 100%.

A reagent (100 μl) consisting of a mixture of LAL (50% of the clotting concentration) and the chromogenic substrate (4.4 mM) in a Tris buffer of pH 7.9 is added to an equal volume of the sample. The resulting reaction (activation of the LAL and the subsequent hydrolyses of the chromogenic substrate) is carried out at 37°C. After 15 min. the reaction is terminated by the addition of 400 μl of acetic acid and the absorbance is read at 405 nm.

*) Limulus Ameboecyte Lysate (H.C. Hemker Loc. cit.)

Table I shows the results for the activated LAL with substrates as measured by the concentration of hydrolized pNA. Substrate S-2423 (Ac-Ile-Glu-Gly-Arg-pNA·HCl) is taken as standard.

Table I

Screening of substrate for single stage method - endotoxin with substrate concentration 2.2 mM (in the reaction solution).

So = 2.2 mM

Substrate	Peptide sequence	Relative activity
S-2423	Ac-Ile-Glu-Gly-Arg-pNA·HCl	100
S-2834	Ac-Ile-Glu-Gly-Lys-pNA·HCl	126
S-2854	Ac-Ile-Ser-Gly-Lys-pNA·HCl	131
S-2860	Ac-Ile-Glu-Glycolyl-Arg-pNA·HCl	142

This table clearly shows that when using -Gly-Lys or Glyc-Arg as carboxy terminal sequence in the substrate, the activity is surprisingly considerably higher than when the known substrate S-2423, which has Gly-Arg as carboxyterminal sequence, is used.

CLAIMS.

1. Peptide derivatives, characterized by the following formula:

$$R_1 -A_1 -A_2 -A_3 -A_4 -R_2 \text{ or its salt}$$

where

R_1 = H or a protective group
A_1 = H, Ile, Leu or Val
A_2 = Glu, Asp, Ser, Thr
A_3 = Gly or Glyc
A_4 = Arg or Lys
R_2 = 4-nitro aniline

with the proviso that A_3 is Gly when A_4 is Lys and that A_3 is Glyc when A_4 is Arg.

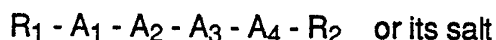
2. Peptide derivatives according to claim 1, characterized by that A_1 is Ile.

3. Peptide derivative according to claim 1, characterized by that it is chosen from:
alpha-Ac-Ile-Glu-Gly-Lys-pNA
alpha-Ac-Ile-Ser-Gly-Lys-pNA
alpha-Ac-Ile-Thr-Gly-Lys-pNA or
alpha-Ac-Ile-Glu-Glyc-Arg-pNA.

4. Process for the preparation of peptide derivatives according to any of claims 1-3, characterized in that the synthesis is performed by step-by-step addition of the amino acids at the C-terminal amino acid provided with a marker.

5. Process for the preparation of peptide derivatives according to any of claims 1-3, characterized in that the synthesis is performed by synthesis of the N-terminal peptide fragment per se, which then is coupled to the C-terminal amino acid provided with a marker.

6. Method for determination of bacterial endotoxins, characterized by the use of peptide derivates with the following formula:



$$R_1 - A_1 - A_2 - A_3 - A_4 - R_2 \quad \text{or its salt}$$

where

R_1 = H or a protective group

A_1 = H, Ile, Leu or Val
A_2 = Glu, Asp, Ser, Thr
A_3 = Gly or Glyc
A_4 = Arg or Lys
R_2 = an aromatic or heterocyclic group which gives the compound R_2-NH_2 by enzymatic hydrolysis, which can be determined quantitatively, with the proviso that when A_3 is Gly when A_4 is Lys and that A_3 is Glyc when A_4 is Arg.

7. Method for determination of bacterial endotoxins, characterized by the use of peptide derivatives according to claim 6 in which A_1 is Ile.

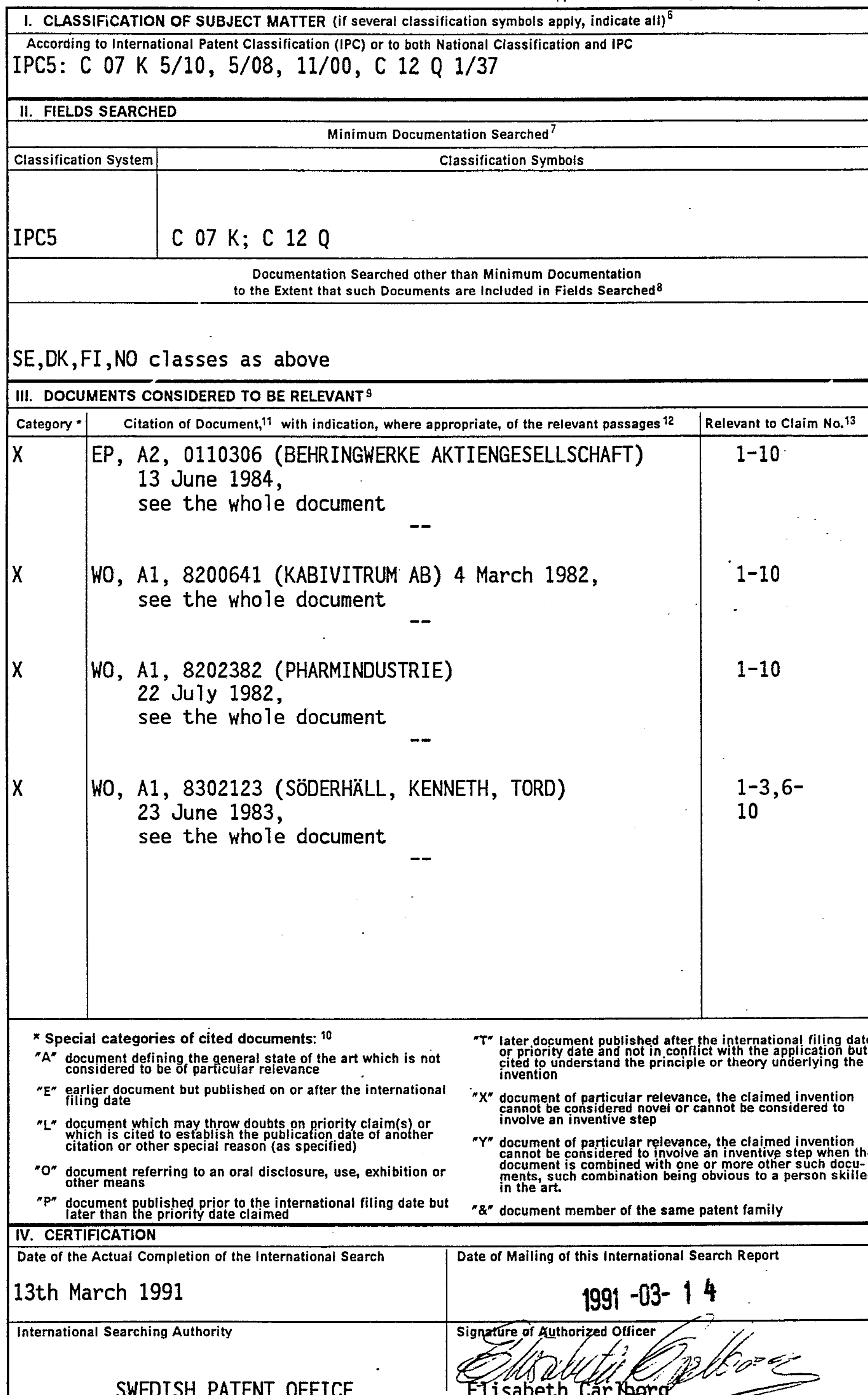
8. Method for determination of bacterial endotoxins, characterized by the use of peptide derivatives according to claim 6 chosen from:
alpha-Ac-Ile-Glu-Gly-Lys-pNA
alpha-Ac-Ile-Ser-Gly-Lys-pNA
alpha-Ac-Ile-Thr-Gly-Lys-pNA or
alpha-Ac-Ile-Glu-Glyc-Arg-pNA.

9. Method for determination of bacterial endotoxins, characterized by the use of peptide derivatives according to claim 6 in which NH_2-R_2 is a chromogenic group.

10. Use of any of the peptide derivatives according to any of claims 1-3 for determination of bacterial endotoxins.

INTERNATIONAL SEARCH REPORT

International Application No PCT/SE 90/00797



I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 07 K 5/10, 5/08, 11/00, C 12 Q 1/37

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 07 K; C 12 Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	EP, A2, 0110306 (BEHRINGWERKE AKTIENGESELLSCHAFT) 13 June 1984, see the whole document --	1-10
X	WO, A1, 8200641 (KABIVITRUM AB) 4 March 1982, see the whole document --	1-10
X	WO, A1, 8202382 (PHARMINDUSTRIE) 22 July 1982, see the whole document --	1-10
X	WO, A1, 8302123 (SÖDERHÄLL, KENNETH, TORD) 23 June 1983, see the whole document --	1-3,6-10

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
13th March 1991	1991 -03- 14
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Elisabeth Carlborg

Form PCT/ISA/210 (second sheet) (January 1985)

International Application No. PCT/SE 90/00797

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
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ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.PCT/SE 90/00797

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on 91-01-31
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

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