TITLE:
SDK PEPTIDES
DESCRIPTION

The present invention relates to SDK peptides, to a preparation process of the same and to therapeutic compositions containing them.

The abbreviations used in this Specification follow the 1983 Recommendations on Nomenclature on Symbolism for Amino Acids Peptides of the IUPAC-IUB Joint Commission on Biochemical Nomenclature (Eur. J. Biochem. 138, 9-37 (1984)). For ease of understanding, however, certain of these abbreviations are set out below :

Asp or D	aspartic acid
Ser or S	serine
Lys or K	lysine
Pro or P	proline
Thr or T	threonine
Z	benzyloxycarbonyl
Boc	t-butoxycarbonyl
OBzl	benzyloxy
Ac	acetyl

Other abbreviations used include :

DMF	dimethylformamide
DCM	dichloromethane
NMM	N-methylmorpholine
TFA	trifluoroacetic acid
TLC	thin layer chromatography
FAB	fast atom bombardment

The invention relates to peptides having the general formula I

H - W - Ser - Asp - Lys - X - OH I

wherein X represents a Pro or Lys residue or a valence bond ; and W represents a Thr or Pro residue or a valence bond ; with the proviso that if X represents a Pro residue then W also represents a Pro residue.

The peptides included within the general formula I may be listed as follows :

Ser-Asp-Lys	(i)
Ser-Asp-Lys-Lys	(ii)
Pro-Ser-Asp-Lys-Lys	(iii)
Thr-Ser-Asp-Lys-Lys	(iv)
Pro-Ser-Asp-Lys	(v)
Thr-Ser-Asp-Lys	(vi)
Pro-Ser-Asp-Lys-Pro	(vii)

The invention includes pharmaceutically acceptable salts of the peptides of the general formula I.

This tripeptide (i), on which the other peptides of the invention are based, does not appear to have been isolated or prepared to date. However, these sequences of amino-acids have been found in various living proteins, for example Ser-Asp-Lys in lymphocyte T membrane protein, where its action appears when a portion of a lymphocyte marker such as lymphocyte CD_2 as well as α- or γ-subunit of T-receptor, which comprise the specific sequence which takes a position in regard of certain specific receptors of the lymphocyte ; these amino-acid sequences may be also found in various proteins such as proteins of viruses and

also in certain proteins of the complement or tumour necrosis factor α.

Accordingly, it was particularly interesting to investigate the activity of the specific amino-acid sequences and, according to the invention, it has been found that they play active roles in the field of immuno-modulation as evidenced by the immunologic reports.

This invention also relates to a preparation process of these peptides conducted by usual routes through the following reaction sequence :

I Boc K(Z) - X( ) - OH

II Boc D(OBzl) - K(Z) - X( ) - OH

III Boc S(Bzl) - D(OBzl) - K(Z) - X( ) - OH

IV Boc W( ) - S(Bzl) - D(OBzl) - K(Z) - X( ) - OH

with the conventions that :

a) the brackets following W or X are void when W and X stand for Pro, that the brackets following W contain Bzl when W stands for Thr and that the brackets following X contain Z when X stands for Lys, and

b) when W and/or X stand for a valence bond, the corresponding step is omitted.

When a therapeutically acceptable salt is prepared, e.g. acetyl, the salification should be conducted before the final deprotection step.

The solvents herewith used are of Purex quality. DMF is stored on 3 and 4 Å-molecular sieve.

TLCs are run on commercial analytic plates. Plates have been observed at 254 nm and revealed with a ninhydrine solution and according to Pataki. Following solvents are used :

A : DCM/methanol (8/2 by volume)

B : n-butanol/AcOH/water (4/1/1 by volume)

and

C : n-butanol/AcOH/water/pyridine (1/1/1/1 by volume).

Intermediate products are purified on a silica column when necessary (silica 60 A (40-60 μ) SDS), they are characterized by their mass spectrum (molecular peak or MH^+), by fast atom bombardment (FAB).

Final products are purified by HPLC with a semi-preparative column (7.2 mm x 250 mm) C_{18}ODS Hypersil (Trade Mark) 10 µ SFCC, with isocratic mode or with gradient.

Analysis of amino-acids are carried out after acid hydrolysis of the peptide with 6N HCl, at 110°C for 12 hours on an HPLC apparatus.

This invention relates, finally, to therapeutic compositions of matter, the active ingredient therein being a sufficient amount of the said peptides associated to carriers suitable for the selected administration route.

The invention will be better understood from the description of the preparation of Ser-Asp-Lys (i).

SYNTHESIS OF PEPTIDES

Synthesis have been carried out step by step with the mixed anhydride method, using isobutyl chloroformate or by the active esters method, using the esters of N-hydroxy-

succinimide. Protected amino-acids are commercial ones.

Deprotection

The Boc group is cleaved by acidolysis using TFA : the peptide is treated with a mixture (1/1 by volume) of DCM/TFA (10 to 30 equivalents) for 1 to 2 hours, at room temperature.

The Z, Bzl or OBzl groups are hydrogenolysed. The peptide, in solution in a mixture of methanol/water (9/1 by volume), is hydrogenolysed in presence of Pd/C at 10%.

Acetylation

Acetylation is carried out by reacting acetylimidazole on the trifluoroacetate of the amine neutralized by triethylamine in DMF.

Preparation of Boc D(OBzl) K(z) 1

The ester of the N-hydroxysuccinimide of Boc D(OBzl) (1 mole) is dissolved in 2.5 ml of DMF. K(Z) (1.1 eq.) in suspension is added to this solution, put on an ice-bath and 1.1 eq. of triethylamine is added unter stirring. Stirring is maintained for 24 hours. The reaction mixture is evaporated under reduced pressure. The residue is then treated with ethyl acetate and a solution of 0.5 N HCl. The organic phase is washed with water, then with a saturated solution of sodium chloride, dried on sodium sulphate and finally evaporated under reduced pressure. The residue is chromatographed on a silica gel column and eluted with a mixture of DCM/methanol (95/5 by volume). The homogeneous fractions from TLC are collected. Yield : 0.540 g.

Preparation of Boc S(Bzl) D(OBzl) K(Z) 2

0.5 mM of 1 is treated with a mixture of 1.4 cm^3 of TFA/DCM (1/1 by volume) (20 equivalents). After 60 minutes at room temperature, the solvents are evaporated off under reduced pressure. The residue is treated twice with DCM and dried under reduced pressure in the presence of KOH.

The ester of the N-hydroxysuccinimide of Boc S(Bzl) (1.2 eq.) and the trifluoroacetate previously obtained are dissolved in 1.3 ml of DMF. One equivalent of triethylamine is added under magnetic stirring. The stirring is maintained for 24 hours. The reaction mixture is treated as for 1. The residue is chromatographed on silica gel and the resulting product is eluted with a mixture of diethylether/ methanol (8/2 by volume). The homogeneous fractions from TLC are collected (yield : 0.318 g, 83 %). When triturating with pentane, a solid is obtained (0.27 g). Mass Spectrometry (MS) (FAB) MH^+, 763 were calculated ; 763 and 763.100 were found (MH^+ - Boc).

Preparation of NAc S(Bzl) D(OBzl) K(Z) 3

The derivative 2 is deprotected by a solution 3.1N HCl (20 equivalents) in tetrahydrofuran. The solution is stirred at room temperature for 6 1/2 hours. The reaction mixture is evaporated and dried under reduced pressure for one night in the presence of KOH.

The resulting chlorhydrate and 1.1 equivalent of acetylimidazole are dissolved in 1 ml of DMF. The pH of this solution is brought to 7.5 with triethylamine. The mixture is stirred for 22 hours. The reaction mixture is then evaporated and the residue treated by ethyl acetate and a 0.5N solution of HCl. The organic phase is washed

with water and a saturated solution of sodium chloride and then dried on sodium sulphate.
MS FAB MH^+, 705 were calculated and 705 observed.

Preparation of NAc SDK 4

0.140 g of 3 (0,2 mM) dissolved in 3 ml of methanol and 1 ml of water are hydrogenolysed for 22 hours in the presence of 10% Pd/C. The catalyst is filtered off on a Celite (Trade Mark) pad and the solvent evaporated off under reduced pressure. Yield : 0.079 g. TLC shows 2 spots sensitive to ninhydrine.

The product is purified by HPLC, isocratic mode ; eluent : water : TFA 0.1 % ; flow rate 4 ml/mn ; t = 8 mn.

Analysis of amino-acids : S : 0.9 (1) ; D : 1.3 (1) ; K : 1.3 (1)

MS : FAB MH^+ 391 were calculated and 391 observed.

Preparation of SDK 5

The tripeptide 2 is initially treated with a mixture of TFA/DCM (1:1 by volume) for one hour and a half ; the solvents are then evaporated off under reduced pressure and the residue treated with toluene ; the solvent is evaporated off and the residue is hydrogenolysed in a solution of 3 ml of methanol and 0.3 ml of water, for 20 hours, at room temperature and in presence of 0.030 g of Pd/C. The catalyst is filtered on a Celite (Trade Mark) pad and the solvent is evaporated off under reduced pressure. Yield : 0.090 g.
MS MH^+ 349 were observed and 349 calculated.

5 is purified by HPLC, isocratic mode ; eluent : water, TFA 0.10 %, flow rate 2 ml/mn ; t = 6 mn.

Analysis of amino-acids : S : 1(1); D : 0.8(1);
K : 1.3(1)

The other peptides of the invention are prepared by the same technique as SDK.

The interest of the present invention will be exemplified by the following immunologic reports.

ROSETTES PROTOCOL

Red blood cells were washed three times with RPMI 1640 or RBS or Hanks + antibiotic drugs, and centrifuged for 7 minutes at 3000 rpm. The cells sap were put in suspension, counted, and adjusted to 2.10^8 red blood cells per ml in a solution of RPMI containing 20% of Foetal Calf Serum (FCS) (4 ml formerly adsorbed on 1 ml of RBC sap, 30 minutes on an ice-bath, then centrifuged for 7 minutes at 3000 rpm and recovered in sterile conditions).

Jurkat T cells made viable with Trypan blue, were washed three times with RPMI 1640 or PBS or Hanks + antibiotic drugs and centrifuged for 7 minutes at 900 rpm. The cells sap were put in suspension, counted and adjusted to 2.10^6 Jurkat T cells per ml of solution of RPMI containing 20% of adsorbed Foetal Calf Serum.

2×10^{-7} molar solutions of SDK and of PSDKP were prepared by dissolving the peptides in RPMI 1640.

200 µl of the peptide solution and 100 µl of the Jurkat T cell preparation were placed in a tube and stood for 10 minutes on an ice-bath. 100 µl of the red blood cell preparation were then added, and the mixture was centrifuged for 5 minutes at 500 rpm and then stood overnight at 4°C.

The cells were carefully put back in suspension by tapping the tube, which was then stood for a while. 30 µl of Toluidine blue 1% was added to the tube to dye the lymphocytes, and the tube was again tapped gently. With the microscrope set at ocular : x 6
objective : x 7
read 3 Malassex hemocytometer per experimental point. Results are reported in the following table.

% ROSETTES (% INHIBITION)

Peptide	SDK	PSDKP
Control	63 ± 4	59 ± 4
10^{-4} M	38 ± 3 (-40%) *	37 ± 7 (-37%) **
10^{-5} M	45 ± 4 (-28%) ***	33 ± 3 (-44%) *
10^{-6} M	50 ± 2 (-22%) ***	27 ± 5 (-54%) *
10^{-7} M	49 ± 2 (-22%) *	45 ± 6 (-24%) ***
10^{-8} M	40 ± 2 (-36%) *	48 ± 2 (-19%) ***
10^{-9} M	43 ± 1 (-32%) *	48 ± 4 (-19%) ***
10^{-10} M	43 ± 3 (-32%) *	52 ± 5 (NS)
10^{-11} M	40 ± 1 (-36%) *	
10^{-12} M	44 ± 3 (-30%) *	
10^{-13} M	51 ± 7 (-19%) ***	
10^{-14} M	55 ± 2 (-14%) ***	
10^{-15} M	63 ± 4 (NS)	

( ) % Inhibition; (NS) = not significant
* : $\alpha < 0.001$
** : $\alpha < 0.01$
*** : $\alpha < 0.05$

ISOLATION OF HUMAN PERIPHERAL MONONUCLEAR CELLS

I - ISOLATION OF (nPMN)

Blood was diluted to 1/2 with PBS, and 35 ml was put on 15 ml of Ficoll-Hypaque. "Ficoll" is a Trade Mark. After centrifuging for 30 minutes at 1800 rpm at room temperature, the ring corresponding to mononuclear cells (MNC) was taken back with a Pasteur pipette. The cells were washed twice in PBS and centrifuged for 10 minutes at 1800 rpm, and adjusted to 2.10^6 cells/ml with RPMI 1640 "complete" medium containing 10% SVF.

II - REACTIVE PRODUCTS

PHA-M : Reconstitute a 5 ml bottle lyophylized with 5 ml of "complete" medium. Solution of 100%. Dilute to obtain a 0.4% solution.

Peptides : SDK : Tube 10^6 mole + 4.35 ml "complete" medium. Solution $2.3\ 10^{-4}$ M.

Then dilute successively : 450 µl "medium" + 50 µ l of dilution.

III - PROTOCOL

In 96-well sterilized boxes NUNC, there were placed

- 100 µ l of MNC at 2.10^6 cells/ml (2.10^5 cells)
- 50 µ l of PHA 0.4% (0.1% final)
- 50 µ l of medium
- 30 µ l of peptide (3.10^{-5} M)

Incubation was for 3 days at 37°C in air/CO_2 95/5. During the last 24 hours, a puls of [^{3}H] Thymidine : 1 µ ci/well was made. The wells were picked up on filters and counted with a counter.

Results are reported in the following Table.

Stimulation of T lymphocytes proliferation by Ser-Asp-Lys (SDK) and Pro-Ser-Asp-Lys-Pro (PSDKP)

PBL with 0.1 % PHA	DNA synthesis induced in MNC stimulated by 0.1 % PHA	
	cpm x 10^3/2.10^5 cells	% stimulation
Control (RPMI 1640)	35 ± 2	
+ Autologous RBC	44 ± 5	+ 26 % **
+ SDK 3.10^{-5} M	56 ± 5	+ 60 % *
Control (RPMI 1640)	40 ± 3	
+ SDK 3.10^{-10} M	52 ± 5	+ 30 % ***
Control (RPMI 1640)	38 ± 3	+ 42 % ***
+ PSDKP 3.10^{-5}M	54 ± 4	

* : $\mu < 0.001$

** : $\mu < 0.02$

*** : $\mu < 0.01$

MNC : Mononuclear Cell

HUMORAL RESPONSE

6 to 8 week old mice of B6D2F1 strain were sensitized, on day 0, with 0.2 ml of a sheep red blood cells suspension [10^8 cells] in a saline solution of Hanks.

The Ser-Asp-Lys and the Pro-Ser-Asp-Lys-Pro peptides (3 µg/kg) were injected in solution in physiological serum, by IP route, in 0.2 ml, at the appointed times.

On day + 4, the animals were sacrificed and the number of

B lymphocytes which secrete IgM immunoglobulines were counted by the hemolysis plaque technique (Jerne method).

8 mice / batch, 4 determinations / animal
remade test : 2 experiments / point.

Results are reported in the following Table.

Stimulation of the humoral response in mice to sheep red blood cells a T dependant antigen by Ser-Asp-Lys and Pro-Ser-Asp-Lys-Pro peptides (Number of P.F.C./10^6 cells)

PERIOD OF INJECTION	SUBSTANCES INJECTED		
	Saline	Ser-Asp-Lys	Pro-Ser-Asp-Lys-Pro
- 24 h	444 ± 44	644 ± 170 (+ 45) *	630 ± 142 (+ 42) *
0 h	459 ± 103	903 ± 133 (+ 97) *	915 ± 140 (+ 99) *
+ 24 h	407 ± 66	459 ± 103 (NS)	475 ± 82 (NS)
+ 48 h	407 ± 66	673 ± 110 (+ 65) *	589 ± 101 (+ 45) *
+ 72 h	459 ± 103	1073 ± 348 (+ 134) *	543 ± 112 (NS)

* : $\alpha < 0.001$

** : $\alpha < 0.01$

( )% stimulation; (NS) = not significant

TOXICITY

No toxicity was observed for any of the peptides according to the invention when administered per os, at the maximum administrable doses, to rats and mice. By

the IP route, no death was observed at 1 g/kg for the same animals.

PRESENTATION - POSOLOGY

Only Ser-Asp-Lys peptides may be administered orally and reach their target without substantial degradation; tablets and gelatine capsules are suitable with dosage units containing 10 mg of Ser-Asp-Lys. By this route, higher peptides need protected forms and higher unitary dosage (20 mg). Daily doses may be from 10 to 100 mg (SDK) or from 20 to 200 mg for higher peptides. By IP route, daily doses are from 1 to 10 mg for immediate action but sustained release forms (microcapsules, microspheres or the like) may contain up to 300 mg for long term release.

CLAIMS:

1. A peptide having the general formula

H - W - Ser - Asp - Lys - X - OH

wherein X represents a Pro or Lys residue or a valence bond; and W represents a Thr or Pro residue or a valence bond; with the proviso that if X represents a Pro residue then W also represents a Pro residue, or a therapeutically acceptable salt of such a peptide.

2. The peptide Ser-Asp-Lys

3. The peptide Ser-Asp-Lys-Lys.

4. The peptide Pro-Ser-Asp-Lys-Lys.

5. The peptide Thr-Ser-Asp-Lys

6. The peptide Pro-Ser-Asp-Lys.

7. The peptide Thr-Ser-Asp-Lys.

8. The peptide Pro-Ser-Asp-Lys-Pro.

9. A process for the preparation of a peptide according to claim 1, the process comprising conducting the following reaction sequence by conventional techniques:

I Boc K(Z) - X( ) - OH

II Boc D(OBzl) - K(Z) - X( ) - OH

III Boc S(Bzl) - D(OBzl) - K(Z) - X ( ) - OH

IV Boc W( ) - S(Bzl) - D(OBzl) - K(Z) - X( ) - OH

with the conventions that:

a) the brackets following W or X are void when W and X stand for Pro, that the brackets following W contain Bzl when W stands for Thr and that the brackets following X contain Z when X stands for Lys, and

b) when W and/or X stand for a valence bond, the corresponding step is omitted.

10. A process according to claim 9 performed by the mixed anhydride method, using isobutyl chloroformate.

11. A process according to claim 9 performed by the active esters method using the esters of N-hydroxy-succinimide.

* C L M S *

12. A therapeutic composition comprising a peptide according to any of claims 1 to 8 or a therapeutically acceptable salt thereof in admixture with a therapeutically acceptable diluent or carrier.

* C L M S *