594449

APPLICATION ACCEPTED AND AMENDMENTS ALLOWED 3-1-90

SPRUSON & FERGUSON

COMMONWEALTH OF AUSTRALIA

PATENTS ACT 1952

CONVENTION APPLICATION FOR A STANDARD PATENT

We, ELLEM INDUSTRIA FARMACEUTICA S.p.A.,

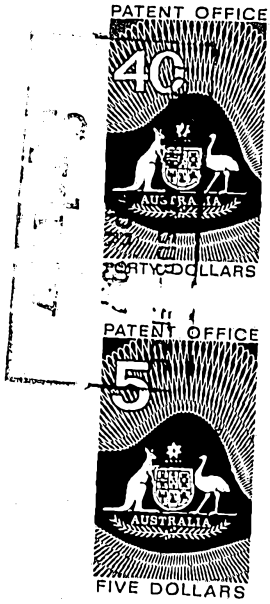
of Corso di Porta Ticinese 89, 20123 Milan, Italy

hereby apply for the grant of a standard patent for an invention entitled:

71183/87

"TRIPEPTIDE WITH IMMUNOSTIMULATING ACTIVITY"

which is described in the accompanying complete specification.



DETAILS OF BASIC APPLICATION

Number of Basic Application:-
20026 A/86

Name of Convention Country in which Basic Application was filed:-
ITALY

Date of Basic application:-
9 APRIL 1986

Our address for service is:-

C/- Spruson & Ferguson
Patent Attorneys
Level 33 St Martins Tower
31 Market Street
Sydney New South Wales Australia

DATED this EIGHTH day of APRIL 1987

ELLEM INDUSTRIA FARMACEUTICA S.p.A.

FEE STAMP TO VALUE OF $145 ATTACHED
MAIL OFFICER

By: M.J. Anderson
Registered Patent Attorney.

LODGED AT SUB-OFFICE
- 8 APR 1987
Sydney

TO: THE COMMISSIONER OF PATENTS
AUSTRALIA

SBR:ALB:83W

Spruson & Ferguson

AUSTRALIA
CONVENTION
STANDARD
& PETTY PATENT
DECLARATION
SFP4

COMMONWEALTH OF AUSTRALIA

THE PATENTS ACT 1952

DECLARATION IN SUPPORT OF A CONVENTION APPLICATION FOR A PATENT

In support of the Convention Application made for a patent for an invention entitled:

Title of Invention: "TRIPEPTIDE WITH IMMUNOSTIMULATING ACTIVITY"

Full name(s) and address(es) of Declarant(s): I/We Roberto Bianchi

Care of Ellem Industria Farmaceutica S.p.A., Corso di Porta Ticinese 89, 20123 Milan, Italy

do solemnly and sincerely declare as follows:-

Full name(s) of Applicant(s)

1. ~~I am/We are the applicant(s) for the patent~~

(or, in the case of an application by a body corporate)

1. I am/We ~~are~~ authorised by

ELLEM INDUSTRIA FARMACEUTICA S.p.A.

the applicant(~~s~~) for the patent to make this declaration on its/~~their~~ behalf.

2. The basic application(~~s~~) as defined by Section 141 of the Act was/~~were~~ made

Basic Country(ies): in ITALY

Priority Date(s): on 9 APRIL 1986

Basic Applicant(s): by ELLEM INDUSTRIA FARMACEUTICA S.p.A.

3. ~~I am/We are the actual inventor(s) of the invention referred to in the basic application(s)~~

Full name(s) and address(es) of inventor(s)

(or where a person other than the inventor is the applicant)

3. BRUNETTO BRUNETTI, Care of Ellem Industria Farmaceutica S.p.A., Corso di Porta Ticinese 89, 20123 Milan, and MARCO PRADA of via Caravaggio 31, Casalpusterlengo, ~~of~~ Milan, both in Italy

(respectively)

~~is~~/are the actual inventor(s) of the invention and the facts upon which the applicant(~~s~~) is/~~are~~ entitled to make the application are as follows:

Set out how Applicant(s) derive title from actual inventor(s) e.g. The Applicant(s) is/are the assignee(s) of the invention from the inventor(s)

ELLEM INDUSTRIA FARMACEUTICA S.p.A. is entitled by Contract of Employment between the inventors as employees and ELLEM INDUSTRIA FARMACEUTICA S.p.A. as employer, as a person who would be entitled to have the patent assigned to it if a patent were granted upon an application made by the inventors.

4. The basic application(~~s~~) referred to in paragraph 2 of this Declaration was/~~were~~ the first application(~~s~~) made in a Convention country in respect of the invention (~~s~~) the subject of the application.

Declared at Milan this 30 day of March 19 87

Industria Farmaceutica S. p. A.
L'Amministratore Delegato

(Roberto BIANCHI)

SFP4 To: The Commissioner of Patents Signature of Declarant(s) 11/81

(12) PATENT ABRIDGMENT (11) Document No. AU-B-71183/87
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 594449

(54) Title
IMMUNOSTIMULATING TRIPEPTIDE

International Patent Classification(s)
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(71) Applicant(s)
ELLEM INDUSTRIA FARMACEUTICA S.P.A.

(72) Inventor(s)
BRUNETTO BRUNETTI; MARCO PRADA

(74) Attorney or Agent
SPRUSON & FERGUSON

(56) Prior Art Documents
GB 2189490
AU 84799/82 C07C 103/52

(57) Claim

1. The tripeptide H-Arg-Lys-Glu-OH or pharmaceutically acceptable salts thereof.

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FORM 10

SPRUSON & FERGUSON

COMMONWEALTH OF AUSTRALIA

PATENTS ACT 1952

COMPLETE SPECIFICATION

(ORIGINAL)

FOR OFFICE USE:

71183/87

This document contains the amendments made under Section 49 and is correct for printing.

Class Int. Class

Complete Specification Lodged:

Accepted:

Published:

Priority:

Related Art:

This document contains the amendments allowed under Section 43 (2) by the Supervising Examiner on

..... and is correct for printing

: :

Name of Applicant: ELLEM INDUSTRIA FARMACEUTICA S.p.A.

Address of Applicant: Corso di Porta Ticinese 89,
20123 Milan, Italy

Actual Inventor(s): BRUNETTO BRUNETTI and MARCO PRADA

Address for Service: Spruson & Ferguson, Patent Attorneys,
Level 33 St Martins Tower,
31 Market Street, Sydney,
New South Wales, 2000, Australia

Complete Specification for the invention entitled:

"TRIPEPTIDE WITH IMMUNOSTIMULATING ACTIVITY"

The following statement is a full description of this invention, including the best method of performing it known to us

SBR:ALB:83W

ABSTRACT

The tripeptide Arg-Lys-Glu, synthetized by conventional solution methods, and its salts display immunostimulating activity both on maturation of immature T cells and on T cell function.

SUMMARY OF INVENTION

According to a first embodiment of this invention there is provided the tripeptide H-Arg-Lys-Glu-OH or pharmaceutically acceptable salts thereof.

According to a second embodiment of this invention there is provided a pharmaceutical composition comprising the tripeptide of the first embodiment together with a pharmaceutically acceptable carrier, diluent, excipient and/or adjuvant.

According to a third embodiment of this invention there is provided a method of treating primary and secondary deficiencies of an immune system in a mammal requiring such treatment, comprising administering to said mammal an effective amount of a tripeptide of the first embodiment and/or a pharmaceutical composition of the second embodiment.

CHEMICAL CHARACTERISTICS OF THE TRIPEPTIDE H-Arg-Lys-Glu-OH

MOLECULAR WEIGHT: 431.52

OPTICAL ROTATION: $[d]_D^{20}$ = 5.13. (c= 1, acetic acid)

HPLC ANALYSIS:

The tripeptide has been analyzed by means of ion-pairing HPLC, according to the separation conditions here described:

Eluent: NaH_2PO_4 0.05M pH 4.3 + SDS 5 x 10^{-4} M, MeOH; 50:50.

Flow rate: 1 ml/min

Detection: 225 nm

Injection volume: 20 mcl

Sample: 20 mcg

Column: u Bondapack C18 (waters), 300 x 3.9 mm

The following instrumentation was used:

Liquid chromatograph: SERIES 4 (Perkin Elmer)

Injection valve: Reodyne mod. 7125-075, with a 20 ul loop

Detector: Spectrophometer LC 95 (Perkin Elmer)

Computing integrator: Data Station 3600 (Perkin Elmer)

The figure shows the HPLC profile of the tripeptide.

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RESISTANCE TO THE IN VITRO SIMULATED GASTRIC AMBIENT

The tripeptide is resistant to the in vitro simulated gastric ambient. In this study the gastric simulated juice USP XXI (HCl + pepsin) has been used at 37 C for 5 hrs.

SYNTHESIS OF H-Arg-Lys-Glu-OH

ε Z(Cl)

Boc-Lys (0.1 mole) dissolved in methylene chloride and cooled to 0 C was added to N-Methylmorpholine (0.1 mole).

The solution was cooled to -15 C +/-1 isobutyl chloroformate (0.1 mole) was added under stirring while maintaining the temperature at -15 C. After stirring the reaction mixture for 15 minutes at this temperature, a precooled solution of glutamic acid-dibenzyl ester-p-tosylate (0.1 mole) and N-

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methylmorpholine (NMM) (0.1 mole) in dimethyl formamide was added slowly and the reaction mixture stirred overnight. Solvents were removed under reduced pressure and the residue was taken up in ethyl acetate. The ethyl acetate was washed with water, 1N-hydrochloric acid, water 5% sodium bicarbonate solution and water. It was dried ower sodium sulphate and solvent removed under reduced pressure. The product is syrup. TLC System CHCl3:MeOH:HOAc (90:8:2). 95% pure: Yield 80%.

(1) was deblocked with 50% trifluoro acetic acid-methylene chloride mixture (1:1), 10 ml per gram, for half and hour. It was evaporated under reduced pressure, triturated with ether, filtered, washed with ether and dried in vacuo.

Yield 98%.

εZ(Cl)εOBe

The TFA-Lys--Glu--$^{\varepsilon}$OBe was neutralized with NMM and coupled to Z3-Arg in dimethyl formamide-tetra-hydrofuran mixture using NMM and isobutyl chloroformate and worked up as in (1).

Yield 60%. TLC System CHCl3:MeOH (92:8). One major spot.

The above tripeptide was hydrogenated in acetic acid-water methanol mixture in presence of pd/c until its completion. It was filtered from catalyst and the filtrate was evaporated in vacuo.

The product, tripeptide, was purified by counter current distribution using system N-butanol: acetic acid: water (4:1:5)

Yield 50%. TLC System butanol: acetic acid: water: pyridine (32:6:22:20). One major spot. HPLC 97%.

BIOLOGICAL ACTIVITIES

1.A. IN VITRO INDUCTION OF THY 1.2 ANTIGEN

The capacity of Arg-Lys-Glu, hereinafter denoted as ELS1, to induce in vitro the differentiation of mouse T cell precursors into lymphocytes expressing T cell markers has been tested by evidencing the induction of Thy 1.2 membrane antigen.

MATERIAL AND METHODS

MICE: 8 week-old athymic (nu/nu) mice outbred on C3H/He back-

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ground, maintained under specific pathogen-free conditions, were used.

PREPARATION OF THE CELLS: mice were killed by cervical dislocation. Spleens were aseptically removed and finely minced with forceps in Hank's balanced salt solution (HBSS) (Gibco Ltd, Paisley, Scotland). Splenocytes, washed and resuspended in 199 medium (Gibco Ltd) supplemented with 1% BSA (Boehringer Mannheim) and gentamycin (100 ug/ml) were incubated for 45 minutes in equilibrated nylon wool columns according to the method of Julius et al. (Eur. J. Immunol. 3, 645, 1973). The effluent cell populations enriched with precursor T cells, were used in the bioassay.

INDUCTION BIOASSAY: 0.5×10^6 effluent cells in 0.1 ml medium were incubated at 37 C for 18 hours with 0.1 ml of tripeptide or medium alone. Cultures were done in duplicate. At the end of the incubation, the cells were washed with 0.87% ammonium chloride to lyse red cells and then with HBSS.

The induction of membrane Thy 1.2 antigen was determined by a direct immunofluorescence test.

DIRECT IMMUNOFLUORESCENCE: the cells were incubated at 4 C for 20 minutes with fluorescein-conjugated monoclonal antibody (Bio- Yeda) at 1:200 dilution. The mixture was centrifuged at 300 g for 5 minutes, washed twice in HBSS and then suspended for counting at the fluorescence microscope (Leitz Orthoplan). The difference in percentages of fluorescing cells between cultures with and without tripeptide gave the inducing activity of the product.

RESULTS

As shown in the table, the tripeptide induces the appearance of the marker Thy 1.2 on immature T cells with an optimum response at 1 mcg/ml. The dose-response relationship curve is bell-shaped, as both lower and higher concentrations of the

peptide provoke a smaller induction.

PEPTIDE CONCENTRATION (mcg/ml)	% THY 1.2+CELLS MEAN +/- S.E.	DIFFERENCE
0	11 +/- 1.6	-
0.0001	19 +/- 1.2	+ 8
0.001	34 +/- 3.3	+ 23
0.01	44 +/- 3.1	+ 33
0.1	50 +/- 1.2	+ 39
1	54 +/- 5.0	+ 43
10	45 +/- 4.9	+ 34
20	40 +/- 1.2	+ 29
50	28 +/- 4.5	+ 17
100	21 +/- 1.7	+ 10
200	16 +/- 2.4	+ 5

1.B IN VIVO INDUCTION OF THY 1.2 ANTIGEN

ELS1 was administered on 4 consecutive days after which the mice were rested for 24 hrs. and then the spleens were removed and cells were examined for expression of the Thy 1.2 antigen by fluorescence. The control mice were given Medium 199 (M 199), the medium in which the drug was dissolved. The mice had an average weight of about 24 g.

RESULTS

		% THY 1.2+ CELLS	
		Oral	i.p.
Control		3%	5%
ELS1	42 ug/kg	3%	6%
ELS1	420 ug/kg	5%	8%
ELS1	1055 ug/kg	7%	12%
ELS1	2110 ug/kg	15%	18%
ELS1	4220 ug/kg	14%	17%
ELS1	8440 ug/kg	15%	16%

The data show that ELS1 is able to induce the maturation of splenocytes after both oral and i.p. administration.
The optimal dosage is 2110 ug/kg while with higher dosages a plateau response is observed.

2. IN VITRO STIMULATION OF LYMPHOKINE PRODUCTION

MATERIAL AND METHODS

PREPARATION OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC).
Peripheral blood is obtained from healthy volunteers by venipuncture. The red blood cells are separated from white cells on Ficoll-Hipaque gradients. The buffy coat (PBMC) is removed and washed, and the cells are resuspended at $1x10^6$ cells/ml in RPMI 1640, supplemented with 1% penicillin/streptomycin, 1% glutamine and 1% heat inactivated fetal calf serum (FCS, 56 C 30 min).

PREPARATION OF GROWTH FACTOR

PBMC at $1x10^6$ cells/ml in 1% heat inactivated FCS are incubated with or without Phytohemmagglutinin (PHA) at 0.75% concentration v/v. The peptide to be tested is added at the concentration of 1 ug/ml to appropriate cultures. The incubation period is 18-24 hrs., at 37 C in a humidified atmosphere. The

cultures are then filtered through 0.22 mM filters and supernatants are examined for the presence of growth factors.

MEASUREMENT OF GROWTH FACTORS IN SUPERNATANTS

A. Test cells

The B cells used to test for the presence of B cell growth factor (BCGF) are long term cultured cell lines, maintained on BCGF, and are EBV negative. These cells are grown in serum free medium using Nutridoma (Boehringer Mannheim Biochemicals), and do not respond to IL-2.

The T cells used to test for the presence of IL-2 are freshly isolated. They are initially stimulated with PHA (0.75%) and are maintained in culture for at least 10 days prior to use (to reduce background and establish their dependence on IL-2).

B. Preparation of Test cells for Use in Assay.

1. B cells are usually used 4 days after the last feeding with BCGF. They are washed 4 times in RPMI 1640 to remove any remaining BCGF, and adjusted to $15x10^4$ cells/ml in RPMI 1640 and Nutridoma (at 1% final concentration).

2. T cells are used 4 days after the last feeding with IL-2. They are washed 4x and adjusted to $50x10^4$ cells/ml in RPMI 1640 with 5% FCS.

C. Assay Procedures

1. Long term cultured B cells are incubated with various concentrations of supernatant from PBMC cultures, in 96 flat bottom microtiter plates. Each well has a total volume of 200 ul, consisting of 100 ul of B cells ($15x10^3$ cells) and 100 ul of supernatant. We examine the efficacy of our test B cells by incubating them with various concentrations of purified BCGF (Cellular Products, Inc. Buffalo, N.Y.).

The cultures are incubated for 24 hrs., after which 1 uCi of [^{3}H-Tdr] is added and then incubated additionally for 12 hrs. The cultures are then harvested and counted in a scintillat-

ion counter.

2. T cells are incubated in flat bottom wells. The total volume in each well is 200 ul, which includes $50x10^3$ T cells/well. The incubation period is 72 hrs which includes 12 hrs of labelling with [^{3}H-Tdr].

RESULTS

1) GROWTH FACTOR PRODUCTION

EXPERIMENT 1

BCGF ACTIVITY (C.P.M.)

% Sup.

Supt. from	3.05	6.25	12.5	25	50
PBL + PHA	424	1026	1674	3172	8392
PBL + PHA + ELS1	684	1658	2863	5600	7838

TCGF ACTIVITY (C.P.M.)

% Sup.

PBL + PHA	542	192	224	564	1144
PBL + PHA + ELS1	624	438	1062	1926	3296

EXPERIMENT 2

BCGF ACTIVITY (C.P.M.)

% Sup.

Supt. from	3.125	6.25	12.5	25	50
PBL + PHA	1369	2187	2894	4876	8104
PBL + PHA + ELS1	1586	2837	3994	7728	10886

TCGF ACTIVITY (C.P.M.)

% Sup.

PBL + PHA	1482	3146	4322	7184	9012
PBL + PHA + ELS1	1908	4424	6480	9329	11656

3. EFFECT ON RNA SYNTHESIS

EFFECT OF ELS1 ON RNA SYNTHESIS IN HUMAN T CELLS, AS OBSERVED BY INCORPORATION OF ^{3}H-URIDINE. COUNTS PER MINUTE (CPM). RESULTS OBTAINED AFTER 24 HRS. OF INCUBATION.

T 3732

T + PHA 20752

ELS1 Concentration ug/ml

	0.1	1	10	20
T + ELS1	5336	4868	5104	5272
T + ELS1 + PHA	32729	34966	34497	31764

4. EFFECT ON DNA SYNTHESIS

EFFECT OF ELS1 ON DNA SYNTHESIS IN HUMAN T CELLS AS OBSERVED BY INCORPORATION OF ^{3}H-THYMIDINE. COUNTS PER MINUTE (CPM). RESULTS OBTAINED AFTER 3 DAYS OF INCUBATION.

T 154

T + PHA 6076

ELS1 Concentration ug/ml

	0.01	0.1	1	10
T + ELS1	262	242	196	240
T + ELS1 + PHA	5908	6810	7264	9560

5. VITRO INCREASE OF CELL NUMBER

The tripeptide, added to cultures of either T lymphocytes or mixtures of T and B lymphocytes every fourth day at a concentration of 5 ug/ml for period of 30 days, is able to increase cell number with a maximum of + 50% with respect to control cultures, observed between day 10 and day 15 of the experiment.

TOXICOLOGICAL STUDIES

ACUTE TOXICITY

Acute toxicity studies carried out on mice and rats have shown that up to a dose of 1000 mg/Kg i.m. the tripeptide is totally devoid of toxic effects.

TOLERABILITY

Studies on rabbits and mice have shown that the product, at the dosage of 100 mg/Kg respectively i.v. and i.p., doesn't cause any hemodynamic modification and behavioral effect.

Particularly, pentobarbital-induced sleeping time shows only a slight increase.

ALLERGY-INDUCING ACTIVITY

The product, at the dosage of 100 mg/Kg i.m. doesn't induce any sensitization phenomena in the guinea-pig.

SALTS OF THE TRIPEPTIDE

The above mentioned researches have been carried out with an acetate salt of the tripeptide, however it is well known to the state of the art that similar results can be obtained using other salts, for istance trifluoroacetate, hydrochloride, sulfate.

The claims defining the invention are as follows:

1. The tripeptide H-Arg-Lys-Glu-OH or pharmaceutically acceptable salts thereof.

2. A pharmaceutical composition comprising the tripeptide as defined in claim 1 together with a pharmaceutically acceptable carrier, diluent, excipient and/or adjuvant.

3. A composition according to claim 2 wherein said composition is in the form of an injectable solution or oral formulation.

4. A process for manufacturing a pharmaceutical composition of claim 2 comprising mixing the tripeptide of claim 1 with a pharmaceutically acceptable carrier, diluent, excipient and/or adjuvant.

5. A method of treating primary and secondary deficiencies of an immune system in a mammal requiring such treatment, comprising administering to said mammal an effective amount of a tripeptide as defined in claim 1 and/or a composition as defined in claim 2 or claim 3.

6. A process for manufacturing the tripeptide of claim 1, substantially as herein described with reference to the SYNTHESIS example.

DATED this EIGHTH day of DECEMBER 1989
Ellem Industria Farmaceutica S.P.A.

Patent Attorneys for the Applicant
SPRUSON & FERGUSON

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MRC/170x

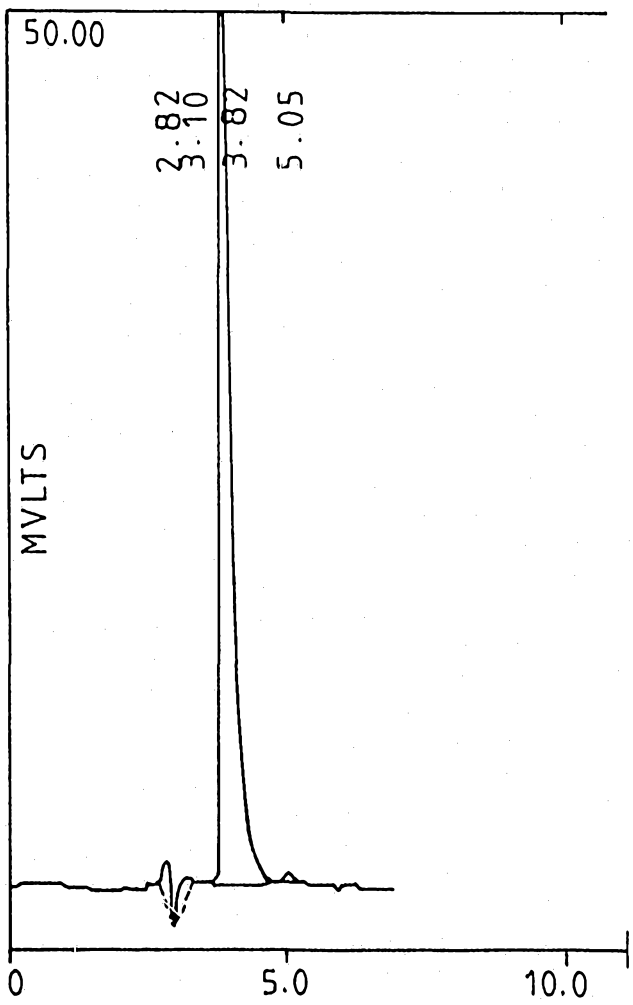

FIG. 1