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AUSTRALIA
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NOTICE OF ENTITLEMENT

We, **SmithKline Beecham Corporation**, the applicant/Nominated Person in respect of Application No. 31452/93 state the following:-

The Nominated Person is entitled to the grant of the patent because the Nominated Person derives title to the invention from the inventors by assignment.

The Nominated Person is entitled to claim priority from the application listed in the declaration under Article 8 of the PCT because the Nominated Person is the assignee of the applicants in respect of the application listed in the declaration under Article 8 of the PCT, and because that application was the first application made in a Convention country in respect of the invention.

DATED this SEVENTEENTH day of JUNE 1994



.....
a member of the firm of
DAVIES COLLISON
CAVE for and on behalf
of the applicant(s)

(DCC ref: 1668002)

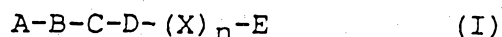


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HEMOREGULATORY PEPTIDES
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- (56) Prior Art Documents
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- (57) Claim

1. A compound of formula I:



wherein:

A is picolinic acid, pyroglutamic acid, proline or L-pipecolinic acid,

B is serine, glutamic acid, aspartic acid, threonine, cysteine or tyrosine;

C is aspartic acid or glutamic acid;

D is aminobutyric acid, alanine, propargyl glycine, glycine, serine, ~~cysteine~~ or β alanine;

X is tyrosine or lysine;

n is 0 or 1;

E is $-N(R_1)-R_2R_3$ or E is glycine, lysine, ornithine, tyrosine, para-aminophenylalanine or the carboxamide or hydroxymethyl derivative thereof;

R_1 , R_4 and R_5 are independently hydrogen, C_{1-5} alkyl, phenyl, naphthyl or C_{5-7} cycloalkyl;

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R₂ is C₁₋₆ alkyl, phenyl, naphthyl or C₅₋₇ cycloalkyl;

R₃ is -NR₄R₅ or aziridinyl, pyrrolidinyl, imidazolyl, pyrrolyl, piperidinyl, piperazinyl, morphollinyl, pyrrolidinyl or pyrralinyll; provided the compound of formula (I) is not p-Glu-Glu-Asp-Cys-Lys;

or a pharmaceutically acceptable salt thereof.

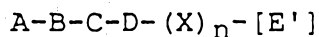
5. A compound of claim 1 selected from the group consisting of:

Pic-Glu-Asp-Abu-Lys	(SEQ ID NO: 2)
pGlu-Glu-Asp-Abu-Tyr-Lys	(SEQ ID NO: 4)
pGlu-Glu-Asp-Abu-Lys	(SEQ ID NO: 1)
Pic-Ser-Asp-Abu-Lys	(SEQ ID NO: 3)

7. A method of inhibiting the myelopoietic system which comprises administering to a subject in need thereof, an effective amount of a compound of claims 1 to 5.

8. A process for the preparation of a compound of the formula I as defined in claim 1, which process comprises

a) coupling protected amino acids to form a suitably protected compound of the formula



wherein:

A is picolinic acid, pyroglutamic acid, proline or L-pipecolinic acid,

B is serine, glutamic acid, aspartic acid, threonine, cysteine or tyrosine;

C is aspartic acid or glutamic acid;

D is aminobutyric acid, alanine, propargyl glycine, glycine, serine, ~~cysteine~~ or β alanine;

X is tyrosine or lysine;

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n is 0 or 1;

[E'] is a chloromethyl, methylbenzhydrylamine, benzhydrylamine or phenylacetamidomethyl resin or E is -
N(R₁)-R₂R₃ or glycine, lysine, ornithine, tyrosine, para-aminophenylalanine or the carboxamide or hydroxymethyl derivative thereof.

b) removing any protecting groups, and if necessary, cleaving the peptide from the resin; and

c) optionally forming a pharmaceutically acceptable salt thereof.

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(21) International Application Number: PCT/US92/10070 (22) International Filing Date: 24 November 1992 (24.11.92) (30) Priority data: 799,465 26 November 1991 (26.11.91) US (60) Parent Application or Grant (63) Related by Continuation US 799,465 (CIP) Filed on 26 November 1991 (26.11.91) (71) Applicant (for all designated States except US): SMITH-KLINE BEECHAM CORPORATION [US/US]; One Franklin Plaza, P.O. Box 7929, Philadelphia, PA 19101 (US).		(72) Inventors; and (75) Inventors/Applicants (for US only) : BHATNAGAR, Pradip, Kumar [US/US]; 300 South Balderston Drive, Exton, PA 19341 (US). HUFFMAN, William, Francis [US/US]; 40 Crest Avenue, Malvern, PA 19335 (US). (74) Agents: HALL, Linda, E. et al.; SmithKline Beecham Corporation, Corporate Patents - U.S., UW2220, 709 Swedeland Road, P.O. Box 1538, King of Prussia, PA 19406-0939 (US). (81) Designated States: AT, AU, BB, BG, BR, CA, CH, CS, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, PL, PT, RO, RU, SD, SE, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG). Published <i>With international search report.</i> 66 6345
(54) Title: HEMOREGULATORY PEPTIDES (57) Abstract The present invention relates to novel peptides which have hemoregulatory activities and can be used to inhibit the myelopoietic system of humans and animals.		

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HEMOREGULATORY PEPTIDESField of the Invention

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The present invention relates to novel peptides which have hemoregulatory activities and can be used to inhibit the myelopoietic system of humans and animals.

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Background of the Invention

A variety of regulatory messengers and modifiers such as colony stimulating factors, interferons, and different types of peptides are responsible for the regulation of myelopoiesis. Metcalf, Cell, 43:5 (1985); Baserga R., Foa P., Metcalf D, Polli EE (eds), Biological Regulation of Cell Proliferation (1986); Nicola et al., J. Cell Physiol. 128:501 (1986), Zoumbos et al., Proyr. Hemat. 1:341 and 14:201 (1986); Werner et al., Experientia 42:521 (1986). Over twenty years ago, Rytomaa and Kivieniemi Cell Tissue Kinet 1:329-340 (1968); Rytomaa et al., Control of Cellular Growth in Adult Organisms pp 106-138 (1967) reported that extracts of mature granulocytes (granulocytic chalone) could specifically inhibit rat myelopoietic cell proliferation in cover slip cultures.

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Later, they demonstrated that the factor, which had a molecular weight less than 3,000 daltons, was able to induce the regression of a transplantable rat granulocytic leukemia, as well as retard the growth of leukemia cells in humans. Paukovits and others extracted a similar factor from rat bone marrow cells and showed that it inhibited the titrated thymidine uptake of bone marrow cells, Paukovits, W.R., Cell Tissue Kinet 4:539-547 (1971); Naturforsch 37:1297 (1982). In 1979, Boll et al., Acta Haematologica 6:130 (1979) demonstrated the inhibitory effects of rat granulocytic extracts on human bone marrow cells in culture and a number of other researchers demonstrated that this crude granulocytic extract inhibited the development of g-CFUC and/or gm-CFUC in vitro from rodent bone marrow cells.

This biological agent was termed a granulocyte chalone which, according to this theoretical concept, should be an endogenous inhibitor of cell proliferation acting in the same tissue as it was secreted. The material obtained from crude extracts was found to be non-species specific but highly tissue specific. Furthermore, it was found to be nontoxic and to have reversible activities.

In 1982, a synthetic hemoregulatory pentapeptide was reported to have a selective inhibitory effect on myelopoietic cells both in vitro and in vivo, where the main effect seems to be on myelopoietic stem cells (CFU-gm), Paukovits et al., Z. Naturforsch 37:1297 (1982) and U.S. Patent No. 4,499,081. This peptide is believed to be an analogue of a naturally occurring granulopoiesis inhibition factor which has been found in minute quantities in bone marrow extracts.

We have now found certain synthetic peptides which have a selective inhibitory effect on myelopoietic cells in vitro. These peptides, by inhibiting haematopoiesis, and, in particular, granulopoiesis tend

to prevent quiescent cells from entering into cell division and so becoming susceptible to attack by cytotoxic anti-cancer drugs. In addition to providing a protective function in therapy using cytotoxic drugs, the peptides may also be used to arrest proliferation of cancer cells related to the myelopoietic system, i.e. myeloid leukemia.

Summary of the Invention

This invention comprises peptides, hereinafter represented as formula (I), which have hemoregulatory activities and can be used to inhibit haematopoiesis.

The peptides are useful in providing a protective function in therapy using irradiation and/or cytotoxic drugs, and may also be used to arrest proliferation of cancer cells related to the myelopoietic system, for example, in the treatment of myeloid leukemia. The peptides may also be used in many clinical situations where it is desirable to alter hemopoiesis.

These compounds may also be used in combination with the dimers of ~~co-pending U.S. Application No. 071,547,730~~, ^{Australian Patent No. 638468,} incorporated by reference

herein, to provide alternating peaks of high and low activity in the bone marrow cells, thus augmenting the natural circadian rhythm of haematopoiesis. In this way, cytostatic therapy can be given at periods of low bone marrow activity, thus reducing the risk of bone marrow damage, while regeneration will be promoted by the succeeding peak of activity. This invention is also a pharmaceutical composition, which comprises a compound of formula (I) and a pharmaceutically acceptable carrier.

This invention further constitutes a method for inhibiting the myelopoietic system of an animal, including humans, which comprises administering to an



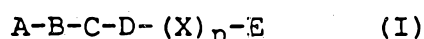
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animal in need thereof, an effective amount of a compound of formula (I).

Detailed Description of the Invention

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The peptides of this invention are illustrated by the formula (I):



10 wherein:

A is picolinic acid, pyroglutamic acid, proline or L-pipecolinic acid,

B is serine, glutamic acid, aspartic acid, threonine, cysteine or tyrosine;

15 C is aspartic acid or glutamic acid;

D is aminobutyric acid, alanine, propargyl glycine, glycine, serine, ~~cysteine~~ or β alanine;

X is tyrosine or lysine;

n is 0 or 1;

20 E is $-N(R_1)-R_2R_3$ or E is glycine, lysine, ornithine, tyrosine, para-aminophenylalanine or the carboxamide or hydroxymethyl derivative thereof;

R_1 , R_4 and R_5 are independently hydrogen, C_{1-5} alkyl, phenyl, naphthyl or C_{5-7} cycloalkyl;

25 R_2 is C_{1-6} alkyl, phenyl, naphthyl or C_{5-7} cycloalkyl;30 R_3 is $-NR_4R_5$ or aziridinyl, pyrrolidinyl, imidazolyl, pyrrolyl, piperidinyl, piperazinyl, morphollinyl, pyrrolidinyl or pyrrolinyl; provided the compound of formula (I) is not p-Glu-Glu-Asp-Cys-Lys; or a pharmaceutically acceptable salt thereof.

35 Also included in this invention are pharmaceutically acceptable salt complexes of the compounds of this invention. It should be noted in formula (I) E comprises the terminal carboxyl group of the amino acid residue corresponding to glycine, lysine, ornithine para-aminophenylalanine or the carboxamide or



hydroxy methyl derivatives thereof or E is the amide group of the $-N(R_1)-R_2R_3$ group.

The abbreviations and symbols commonly used in the art are used herein to describe the peptides:

5	ala	= alanine
	pGlu	= pyroglutamic acid
	Pro	= proline
	Glu	= glutamic acid
	Asp	= aspartic acid
10	Tyr	= tyrosine
	Pic	= picolinic acid
	Ppc	= L-pipecolinic acid
	Abu	= aminobutyric acid
	Ppg	= propargyl glycine
15	Gly	= glycine
	Orn	= ornithine
	p-(NH ₂)Phe	= para-aminophenylalanine
	Hna	= 2,6-diamino-4-hexynoic acid
	Lys	= lysine
20	Cad	= cadaverine

In accordance with conventional representation, the amino terminus is on the left and the carboxy terminus is on the right. All chiral amino acids may be in the D or L absolute configuration.

25 The amino terminus may be protected by acylation. Examples of such protecting groups are, t-butoxycarbonyl (t-Boc), CH₃CO and Ar-CO (Ar = benzyl, or phenyl).

The C-terminus may be carboxy as in the case of
30 the natural amino acid or the carboxamide ($-\overset{\text{O}}{\parallel}\text{CONH}_2$) or hydroxymethyl ($-\text{CH}_2\text{-OH}$).

Preferred compounds are those in which:

35 A is pyroglutamic acid or picolinic acid;
B is glutamic acid or serine;

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C is aspartic acid;
D is aminobutyric acid;
E is glycine or lysine; and
n is 0;

- 5 and the chiral amino acids are in the L absolute configuration.

Especially preferred is pGlu-Glu-Asp-Abu-Lys (SEQ ID NO: 1) and Pic-Ser-Asp-Abu-Lys (SEQ ID NO: 3). The peptides of the invention are prepared by the solid
10 phase technique of Merrifield, J. Am. Chem. Soc., 85, 2149 (1964), or solution methods known to the art may be successfully employed. The methods of peptide synthesis generally set forth in J.M. Stewart and J.D. Young, "Solid Phase Peptide Synthesis", Pierce Chemical
15 Company, Rockford, IL (1984) or M. Bodansky, Y.A. Klauser and M.A. Ondetti, "Peptide Synthesis", John Wiley & Sons, Inc., New York, NY. (1976) may be used to produce the peptides of this invention and are incorporated herein by reference.

20 Each amino acid or peptide is suitably protected as known in the peptide art. For example, the α -fluoroenylmethyloxycarbonyl group (Fmoc) or t-butoxycarbonyl (t-Boc) group are preferred for protection of the amino group, especially at the α -
25 position. A suitably substituted carbobenzoxy group may be used for the ϵ -amino group of lysine and benzyl group for the β and γ carboxy groups of Asp and Glu respectively. Suitable substitution of the carbobenzoxy protecting group is ortho and/or para substitution with
30 chloro, bromo, nitro or methyl, and is used to modify the reactivity of the protective group. Except for the t-Boc group, the protective groups are, most conveniently, those which are not removed by mild acid treatment. These protective groups are removed by such
35 methods as catalytic hydrogenation, sodium in liquid ammonia or HF treatment as known in the art.

If solid phase synthetic methods are used, the peptide is built up sequentially starting from the carboxy terminus and working toward the amino terminus of the peptide. Solid phase synthesis is begun by covalently attaching the C terminus of a protected amino acid to a suitable resin, such as benzhydrylamine resin (BHA), methylbenzhydrylamine resin (MBHA) or chloromethyl resin (CMR), as is generally set forth in U.S. Patent No. 4,244,946 or phenyl acid amino methyl resin (PAM). A BHA or MBHA support resin is used if the carboxy terminus of the product peptide is to be a carboxamide. ACMR or PAM resin is generally used if the carboxy terminus of the product peptide is to be a carboxy group, although this may also be used to produce a carboxamide or ester.

The protective group on the α -amino group is removed by mild acid treatment (i.e. trifluoroacetic acid). Suitable deprotection, neutralization and coupling cycles known in the art are used to sequentially add amino acids without isolation of the intermediate, until the desired peptide has been formed. The completed peptide may then be deblocked and/or split from the carrying resin in any order.

Treatment of a resin supported peptide with HF or HBr/acetic acid splits the peptide from the resin and produces the carboxy terminal amino acid as a carboxylic acid or carboxamide.

If an ester is desired, the CMR or Pam resin may be treated with an appropriate alcohol, such as methyl, ethyl, propyl, butyl or benzyl alcohol, in the presence of triethylamine to cleave the peptide from the resin and product the ester directly.

Esters of the peptides of this invention may also be prepared by conventional methods from the carboxylic acid precursor. Typically, the carboxylic acid is treated with an alcohol in the presence of an acid catalyst. Alternatively, the carboxylic acid may

be converted to an activated acyl intermediate, such as an acid halide, and treated with an alcohol, preferably in the presence of a base.

The preferred method for cleaving a peptide
5 from the support resin is to treat the resin supported peptide with anhydrous HF in the presence of a suitable cation scavenger, such as anisole or dimethoxybenzene. This method simultaneously removes all protecting groups, except a thioalkyl group protecting sulfur, and
10 splits the peptide from the resin. Peptides hydrolyzed in this way from the CMR and Pam resins are carboxylic acids, those split from the BHA resin are obtained as carboxamides.

Modification of the terminal amino group of
15 the peptide is accomplished by alkylation or acylation by methods generally known in the art. These modifications may be carried out upon the amino acid prior to incorporation into the peptide, or upon the peptide after it has been synthesized and the terminal
20 amino group liberated, but before the protecting groups have been removed.

Typically, acylation is carried out upon the free amino group using the acyl halide, anhydride or activated ester, of the corresponding alkyl or aryl
25 acid, in the presence of a tertiary amine. Mono-alkylation is carried out most conveniently by reductive alkylation of the amino group with an appropriate aliphatic aldehyde or ketone in the presence of a mild reducing agent, such as lithium or sodium cyanoboro-
30 hydride. Dialkylation may be carried out by treating the amino group with an excess of an alkyl halide in the presence of a base.

Solution synthesis of peptides is accomplished using conventional methods used to form amide bonds.
35 Typically, a protected t-Boc amino acid which has a free carboxyl group is coupled to a protected amino acid which has a free amino group using a suitable coupling

agent, such as N,N'-dicyclohexyl carbodiimide (DCC), optionally in the presence of catalysts such as 1-hydroxybenzotriazole (HOBT) or dimethylamino pyridine (DMAP). Other methods, such as the formation of
5 activated esters, anhydrides or acid halides, of the free carboxyl of a protected t-Boc-amino- acid, and subsequent reaction with the free amine of a protected amino acid, optionally in the presence of a base, are also suitable. For example, a protected Boc-amino acid
10 or peptide is treated in an anhydrous solvent, such as methylene chloride or tetrahydrofuran (THF), in the presence of a base, such as N-methyl morpholine, DMAP (dimethylaminopyridine) or a trialkyl amine, with isobutyl chloroformate to form the "activated
15 anhydride", which is subsequently reacted with the free amine of another protected amino acid or peptide. The peptide formed by these methods may be deprotected selectively, using conventional techniques, at the amino or carboxy terminus and coupled to other peptides or
20 amino acids using similar techniques. After the peptide has been completed, the protecting groups may be removed as hereinbefore described, such as by hydrogenation in the presence of a palladium or platinum catalyst, treatment with sodium in liquid ammonia, hydrofluoric
25 acid or alkali.

If the final peptide, after it has been deprotected, contains a basic group, an acid addition salt may be prepared. Acid addition salts of the peptides are prepared in a standard manner in a suitable
30 solvent from the parent compound and a slight excess of an acid, such as hydrochloric, hydrobromic, sulfuric, phosphoric, acetic, maleic, succinic or methanesulfonic. The acetate salt for is especially useful. If the final peptide contains an acidic group, cationic salts may be
35 prepared. Typically the parent compound is treated with a slight excess of an alkaline reagent, such as a hydroxide, carbonate or alkoxide, containing the

appropriate cation. Cations such as Na^+ , K^+ , Ca^{++} and NH_4^+ are examples of cations present in pharmaceutically acceptable salts. Na^+ and NH_4^+ are especially preferred.

In general, in order to exert an inhibitory effect, the peptides of the invention may be administered to human patients by injection in the dose range of about 0.5 ng to about 10 mg, for example about 5-500 ng, or orally in the dose range of about 50 ng to about 5 mg, for example about 0.1 ng to 1 mg per 70 kg body weight per day; if administered by infusion or similar techniques, the dose may be in the range of about 0.005 ng to about 10 mg per 70 kg body weight, for example about 0.03 ng to 1 mg over six days. In principle, it is desirable to produce a concentration of the peptide of about 10^{-15}M to about 10^{-5}M in the extracellular fluid of the patient.

According to a still further feature of the present invention there are provided pharmaceutical compositions comprising as active ingredient one or more compound of formula (I) as hereinbefore defined or physiologically compatible salts thereof, in association with a pharmaceutical carrier or excipient. The compositions according to the invention may be presented, for example, in a form suitable for oral, nasal, parenteral or rectal administration.

As used herein, the term "pharmaceutical" includes veterinary applications of the invention. These peptides may be encapsulated, tableted or prepared in an emulsion or syrup for oral administration. Pharmaceutically acceptable solid or liquid carriers may be added to enhance or stabilize the composition, or to facilitate preparation of the composition. Liquid carriers include syrup, peanut oil, olive oil, glycerin, saline and water. Solid carriers include starch, lactose, calcium sulfate dihydrate, terra alba, magnesium stearate or stearic acid, talc, pectin,

acacia, agar or gelatin. The carrier may also include a sustained release material such a glyceryl monostearate or glyceryl distearate, alone or with a wax. The amount of solid carrier varies but, preferably will be
5 between about 20 mg to about 1 g per dosage unit. The pharmaceutical preparations are made following the conventional techniques of pharmacy involving milling, mixing, granulating, and compressing, when necessary, for tablet forms; or milling, mixing and filling for
10 hard gelatin capsule forms. Capsules containing one or several active ingredients may be produced, for example, by mixing the active ingredients with inert carriers, such as lactose or sorbitol, and filling the mixture into gelatin capsules. When a liquid carrier is used,
15 the preparation will be in the form of a syrup, elixir, emulsion or an aqueous or non-aqueous suspension. Such a liquid formulation may be administered directly p.o. or filled into a soft gelatin capsule. Organ specific carrier systems may also be used.

20 Alternately pharmaceutical compositions of the peptides of this invention, or derivatives thereof, may be formulated as solutions of lyophilized powders for parenteral administration. Powders may be reconstituted by addition of a suitable diluent or other
25 pharmaceutically acceptable carrier prior to use. The liquid formulation is generally a buffered, isotonic, aqueous solution. Examples of suitable diluents are normal isotonic saline solution, standard 5% dextrose in water or buffered sodium or ammonium acetate solution.
30 Such formulation is especially suitable for parenteral administration, but may also be used for oral administration and contained in a metered dose inhaler or nebulizer for insufflation. It may be desirable to add excipients such as polyvinylpyrrolidone, gelatin,
35 hydroxy cellulose, acacia, polyethylene glycol, mannitol, sodium chloride or sodium citrate.

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For rectal administration, a pulverized powder of the peptides of this invention may be combined with excipients such as cocoa butter, glycerin, gelatin or polyethylene glycols and molded into a suppository. The pulverized powders may also be compounded with an oily preparation, gel, cream or emulsion, buffered or unbuffered, and administered through a transdermal patch.

Nasal sprays may be formulated similarly in aqueous solution and packed into spray containers either with an aerosol propellant or provided with means for manual compression.

Dosage units containing the compounds of this invention preferably contain 1 mg-100 mg, for example 0.1-50 mg of the peptide of formula (I) or salt thereof.

According to a still further feature of the present invention there is provided a method of inhibition of myelopoiesis which comprises administering an effective amount of a pharmaceutical composition as hereinbefore defined to a subject.

No unacceptable toxicological effects are expected when compounds of the invention are administered in accordance with the present invention.

25 Biological Assays

A. In Vitro Hematopoietic Assay

The following in vitro assay system quantitates positive or negative effects of the compounds of this invention on the entrance of granulocyte-macrophage progenitor cells (CFU-GM) into cell cycle following pulse exposure in vitro. Under soft agar culture conditions in the presence of appropriate hematopoietic growth factors, colonies of granulocytes and macrophages can be quantitated and arise specifically from the proliferation of single CFU-GM. In order to proliferate, CFU-GM must enter S-phase of the cell cycle. Exposure of bone marrow cells to ³H-

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Thymidine prior to the soft agar culture eliminates those CFU-GM which are actively proliferating, ie, are in S-phase of the cell cycle. This effect is quantitated by a decrease in total CFU-GM colonies

5 quantitated in the agar assay. In comparison to controls, the degree of decrease or increase in colony counts provides an index of stimulation or inhibition, respectively, of CFU-GM proliferation.

Procedure:

- 10 Femoral bone marrow cells are obtained and suspended at a concentration of $6-9 \times 10^6$ cells/ml in McCoy's 5A medium supplemented with 10% fetal bovine serum, antibiotics, essential and non-essential amino acids, MEM vitamins, serine, asparagine, glutamine,
- 15 sodium pyruvate and sodium bicarbonate. Replicate cultures are pulsed for 2 hours with compounds of the invention over a concentration range of 1 pg to 1 mg per ml final concentration and incubated at 37°C in an atmosphere of 7% CO₂ in humidified air. Following
- 20 incubation, the cultures are washed three times with assay medium to remove peptides, and exposed to 560 uCi of ³H-Thymidine (Specific Activity=20 Ci/mMole) for 20 minutes. After exposure, 0.5 ml of cold Thymidine (1 mg/ml stock solution) is added to each tube to stop the
- 25 reaction. Cultures are washed three times and plated in the CFU-GM agar assay at a concentration of 75,000 cells per culture in media as described above and containing 0.3% agar and 10% v/v medium conditioned by spleen cells exposed to pokeweed mitogen as a source of hematopoietic
- 30 growth factors. The cultures are incubated at 37°C, 7% CO₂ in humidified air for 5-7 days and CFU-GM colonies enumerated microscopically. The percentage of CFU-GM in S-phase is calculated by dividing colony counts from ³H-Thymidine treated cultures by those of untreated control
- 35 cultures. These values are compared to similarly derived numbers from peptide exposed and untreated cells.

B. Measurement of Superoxide Formation

Polymorphonuclear neutrophils (PMNs) are the first-line defense against many invading pathogens including *C. albicans*. The assessment of ex vivo candidacidal activity of PMNs from treated animals is important in determining the mode of action of therapeutic compounds in the *C. albicans* animal model. Superoxide is the major initial product of oxygen reduction in the respiratory burst of activated PMNs and the various species of oxygen radicals generated during the respiratory burst are directly or indirectly cytotoxic to invading pathogens. Therefore, the measurement of superoxide formation and release during stimulation is important in the assessment of the oxidative metabolism and subsequent cidal efficiency of phagocytic cells.

Procedure:

The compound or control is administered to normal mice IP daily for 7 days. Peripheral blood from individual treated animals was collected and pooled. PB-PMNs were purified by standard separation methods; (Boyum, A. 1968. "Isolation of mononuclear cells and granulocytes from human peripheral blood" Scand. J. Clin. Lab Invest. 21: 77-89); and cell suspensions from the different groups were standardized to contain equivalent numbers of PMNs/ml. For candidacidal assays sufficient PB-PMNs were added to quadruplicate tubes containing *C. albicans* yeasts and 10% normal mouse serum in balanced salt solution to result in a final E:T ratio of 10:1. PB-PMNs were omitted from control tubes. The tubes were incubated at 37°C for 1 hr. The phagocytes were lysed and aliquots were plated onto agar plates. The plates were incubated for 48 hrs at which time the number of colony forming units (CFU) were enumerated and the percent reduction in CFU for each sample determined.

Superoxide dismutase-inhibitable superoxide anion production was quantitated in a microtiter

ferricytochrome c reduction assay (1.5×10^5 PB-PMN/well) in 0.5% gelatin/balanced salt solution during exposure to soluble and particulate stimuli (phorbol 12-myristate 13-acetate (PMA) and serum-opsonized *C. albicans*, respectively). The nmoles of ferricytochrome c reduced/well (quadruplicate wells/pooled group) was determined following a 1 hr incubation. Baseline superoxide activity was determined in the absence of any stimuli. PMA and *Candida* stimulated activity were corrected for spontaneous superoxide production prior to data calculation. Data for PB-PMNs from each treatment group are expressed as the percent of control superoxide where control activity is that observed for PB-PMNs from PBS/serum-treated mice.

The examples which follow serve to illustrate this invention. The Examples are intended to in no way limit the scope of this invention, but are provided to show how to make and use the compounds of this invention.

In the examples, all temperatures are in degrees Centigrade. Amino acid analysis were performed upon a Dionex Autoion 100. Analysis for peptide content is based upon Amino Acid Analysis. FAB mass spectra were performed upon a VG ZAB mass spectrometer using fast atom bombardment. The abbreviations used are as follows:

Abu	=	Aminobutyric acid
Ala	=	alanine
Asp	=	aspartic acid
t-Boc	=	tert. butyloxy carbonyl
Cad	=	cadaverine
Cl-Z	=	p-chlorobenzyloxy carbonyl (Z = benzyloxy carbonyl)
DCC	=	dicyclohexyl carbodiimide
DCM	=	dichloromethane
DIEA	=	diisopropylethyl amine

	EDC	=	(N-ethyl-N'-) 3-dimethylaminopropyl)
			carbodiimide
	Glu	=	glutamic acid
	p-Glu	=	pyroglutamic acid
5	Tyr	=	tyrosine
	Hna	=	diaminohexynoic acid
	HOBT	=	hydroxybenzotriazole
	Lys	=	lysine
	NMP	=	N-methyl-2-pyrrolidinone
10	Pic	=	picolenic acid
	Pro	=	proline
	Ppg	=	propargyl glycine
	Gln	=	glutamine
	Gly	=	glycine
15	Orn	=	ornithine

EXAMPLE 1

(pGlu-Glu-Asp-Abu-Lys): (SEQ ID NO. 1)

Mol. Form C₂₄H₃₈N₆O₁₁ Mol. Wt. 585.6

20

800 mg of BOC-Lys (Cl-Z)-PAM resin (0.5 mM, substitution = 0.63 mM/g) was charged in an ABI Model 430 A Peptide Synthesizer. The target peptide was synthesized according to manufacturer suggested standard protocols using 2 mM of each amino acid. (Applied Biosystems Model 430A Peptide Synthesizer Users Manual Version 1-30, February 1987, Part number 000066). After completion of the synthesis, the resin peptide was washed with DCM and dried (1.665 g). The peptide resin (1.2 g) was charged in a cleavage apparatus and cleaved using 10 ml HF and 1 ml anisole at -15°C for two hrs. After removal of HF, the resin was extensively washed with ether and the peptide extracted in glacial acetic acid. Most of the acetic acid was removed on a rotovap and the residue was diluted with water and lyophilized. Two hundred twenty milligrams of crude peptide were obtained. The pure peptide (130 mg) was obtained after

35

column chromatography on a C-18 "Bond Elute"® column using acetonitrile/water/trifluoroacetic acid (TFA) buffer.

Amino acid analysis:

- 5 Asp 1.0 (1), Glu 1.98 (2), Abu 1.12(1), Lys 1.06 (1)
FAB/MS MH⁺ 587.5

EXAMPLE 2

(Pic-Glu-Asp-Abu-Lys) (SEQ ID NO: 2)

- 10 Mol. Form C₂₅H₃₆N₆O₁₀ Mol. Wt 580.6:

- 800 mg of BOC-Lys (Cl-Z)-PAM resin (0.5 mM/substitution 0.63 mM/g) was charged in an ABI Model 430 A Peptide Synthesizer. The target peptide was synthesized
15 according to manufacturer suggested standard protocols using 2 mm of each amino acid. After completion of the synthesis, the resin peptide was washed with DCM and dried (1.6g). The peptide resin (884 mg) was charged in the HF cleavage apparatus and cleaved using 10 ml HF and
20 1 ml anisole at -15°C for two hrs. After removal of HF, the resin was extensively washed with ether and the peptide was extracted in glacial acetic acid. Most of the acetic acid was removed on a rotavap and the residue was diluted with water and lyophilized. Two hundred and
25 fifty-one milligrams of crude peptide were obtained. After purification on a C-18 "Bond Elute"® column using acetonitrile/water/TFA buffer, 141 mg of pure peptide (HPLC purity 97%) was obtained.

Amino acid analysis:

- 30 Asp 1.0 (1), Glu 1.03 (1), Abu 1.12 (1), Lys 1.17 (1).
Pic is not identified by amino acid analysis.
FAB/MS 581.1

EXAMPLE 3

(Pic-Ser-Asp-Abu-Lys) (SEQ ID NO: 3)

Mol. Form. $C_{23}H_{34}N_6O_9$ Mol. Wt 538.56:

5

Eight hundred fifty mg BOC-Lys (Cl-Z)-PAM resin (0.56 mm of resin) was charged in an ABI Model 430 A Peptide Synthesizer. The target peptide was synthesized according to manufacturer suggested standard protocols using 2 mm of each amino acid. After completion of the synthesis, the resin peptide was washed with DCM and dried (1.038 g). The peptide resin (1.03 g) was charged in the HF cleavage apparatus and cleaved using 10 ml HF and 1 ml anisole at -15°C for two hrs. After removal of HF, the resin was extensively washed with ether and the peptide was extracted in trifluoroacetic acid. Most of the trifluoroacetic acid was removed on a rotavap and the residue was diluted with water and lyophilized. Two hundred and ninety-eight milligrams of crude peptide was obtained. After purification on a C-18 "Bond Elute"® column using acetonitrile/water/TFA buffer, 178 mg of pure peptide (HPLC purity 97%) was obtained.

Amino acid analysis:

Asp 1.0 (1), Ser 0.88 (1), Abu 1.12 (1), Lys 1.17 (1).

25 Pic is not identified by amino acid analysis.

EXAMPLE 4

(pGlu-Glu-Asp-Abu-Tyr-Lys): (SEQ ID NO: 4)

Mol. Form. $C_{33}H_{47}N_7O_{13}$ Mol. Wt 749.77

30

Eight hundred fifty mg BOC-Lys (Cl-Z)-PAM resin (0.56 mm of resin) was charged in ABI Model 430 A Peptide Synthesizer. The target peptide was synthesized according to the manufacturer's suggested standard protocols using 2 mm of each amino acid. After completion of the synthesis, the resin peptide was washed with DCM and dried (1.353 g). The peptide resin

(1.3 g) was charged in the HF cleavage apparatus and cleaved using 10 ml HF and 1 ml anisole at -15°C for two hrs. After removal of HF, the resin was extensively washed with ether and the peptide was extracted in
5 trifluoroacetic acid. Most of the trifluoroacetic acid was removed on a rotavap and the residue was diluted with water and lyophilized. Four hundred and fifty-four milligrams of crude peptide were obtained. The crude peptide (204.7 mg) was purified on a C-18 "Bond Elute"®
10 column using acetonitrile/water/TFA buffer. 95.2 mg of peptide (HPLC purity 90%) was obtained.
Amino acid analysis:
pGlu + Glu 2.02 (2), Asp 1.0 (1), Tyr 0.94 (1), Lys 1.03 (1)

EXAMPLES 5-11

15

By the methods given above the following peptides were made.

Example

- | | | | |
|----|----|-----------------------------|-----------------|
| 20 | 5 | p-Glu-Glu-Asp-Cys-Gly-OH | (SEQ ID NO: 5) |
| | 6 | pGlu-Glu-Asp-Ser-Gly | (SEQ ID NO: 6) |
| | 7 | Pic-Glu-Asp-Abu-Lys-OH | (SEQ ID NO: 7) |
| | 8 | Pic-Ser-Asp-Abu-Lys-OH | (SEQ ID NO: 8) |
| | 9 | pGlu-Glu-Asp-Abu-Lys-OH | (SEQ ID NO: 9) |
| | 10 | pGlu-Glu-Asp-Abu-Tyr-Lys-OH | (SEQ ID NO: 10) |
| 25 | 11 | pGlu-Glu-Asp-Abu-Lys-Tyr-OH | (SEQ ID NO: 11) |

EXAMPLE 12

Formulations for pharmaceutical use incorporating compounds of the present invention can be
30 prepared in various forms and with numerous excipients. Examples of such formulations are given below.

Tablets/Ingredients

Per Tablet

- | | | | |
|----|----|-------------------|-------|
| 35 | 1. | Active ingredient | 40 mg |
| | | (Cpd of Form. I) | |
| | 2. | Corn Starch | 20 mg |
| | 3. | Alginic acid | 20 mg |

20

- | | | |
|----|-----------------|---------------|
| 4. | Sodium alginate | 20 mg |
| 5. | Mg stearate | <u>1.3 mg</u> |
| | | 2.3 mg |

Procedure for tablets:

5

Step 1 Blend ingredients No. 1, No. 2, No. 3 and No. 4 in a suitable mixer/blender.

10

Step 2 Add sufficient water portion-wise to the blend from Step 1 with careful mixing after each addition. Such additions of water and mixing until the mass is of a consistency to permit its conversion to wet granules.

15

Step 3 The wet mass is converted to granules by passing it through an oscillating granulator using a No. 8 mesh (2.38 mm) screen.

20

Step 4 The wet granules are then dried in an oven at 140°F (60°C) until dry.

Step 5 The dry granules are lubricated with ingredient No. 5

25

Step 6 The lubricated granules are compressed on a suitable tablet press.

Parenteral Formulation

A pharmaceutical composition for parenteral administration is prepared by dissolving an appropriate amount of a compound of formula I in polyethylene glycol with heating. This solution is then diluted with water for injections Ph Eur. (to 100 ml). The solution is then sterilized by filtration through a 0.22 micron membrane filter and sealed in sterile containers.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

5

(i) APPLICANT: Bhatnagar, Pradip K
Huffman, William F

10

(ii) TITLE OF INVENTION: Hemoregulatory Peptides

(iii) NUMBER OF SEQUENCES: 11

(iv) CORRESPONDENCE ADDRESS:

15

(A) ADDRESSEE: SmithKline Beecham Corporation

(B) STREET: 709 Swedeland Road

(C) CITY: King of Prussia

(D) STATE: Pennsylvania

(E) COUNTRY: USA

(F) ZIP: 19406-0939

20

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

25

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

30

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 07/799465

(B) FILING DATE: 26-NOV-1991

35

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Hall, Linda E

(B) REGISTRATION NUMBER: 31763

(C) REFERENCE/DOCKET NUMBER: P50044-1

(ix) TELECOMMUNICATION INFORMATION:

5

(A) TELEPHONE: (215) 270-5016

(B) TELEFAX: (215) 270-5090

(2) INFORMATION FOR SEQ ID NO:1:

10

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: peptide

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Xaa Glu Asp Xaa Lys

1

5

25

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids

(B) TYPE: amino acid

30

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

23

Xaa Glu Asp Xaa Lys

1 5

(2) INFORMATION FOR SEQ ID NO:3:

5

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: peptide

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Xaa Ser Asp Xaa Lys

1 5

20 (2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 amino acids

(B) TYPE: amino acid

25

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Xaa Glu Asp Xaa Tyr Lys

1 5

35

(2) INFORMATION FOR SEQ ID NO:5:

24

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: peptide

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Xaa Glu Asp Cys Xaa

1

5

15 (2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids

(B) TYPE: amino acid

20

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Xaa Glu Asp Ser Gly

1

5

30

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids

35

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Xaa Glu Asp Xaa Xaa

1 5

10 (2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids

(B) TYPE: amino acid

15 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Xaa Ser Asp Xaa Xaa

1 5

25

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids

30 (B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Xaa Glu Asp Xaa Xaa

1 5

5 (2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 amino acids

(B) TYPE: amino acid

10 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Xaa Glu Asp Xaa Tyr Xaa

1 5

20

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 amino acids

25 (B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

30

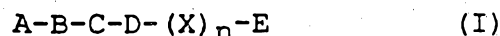
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Xaa Glu Asp Xaa Lys Xaa

35 1 5

CLAIMS:

1. A compound of formula I:



5

wherein:

A is picolinic acid, pyroglutamic acid, proline or L-pipecolinic acid,

10 B is serine, glutamic acid, aspartic acid, threonine, cysteine or tyrosine;

C is aspartic acid or glutamic acid;

D is aminobutyric acid, alanine, propargyl glycine, glycine, serine, ~~cysteine~~ or β alanine;

X is tyrosine or lysine;

15

n is 0 or 1;

E is $-N(R_1)-R_2R_3$ or E is glycine, lysine, ornithine, tyrosine, para-aminophenylalanine or the carboxamide or hydroxymethyl derivative thereof;

20 R_1 , R_4 and R_5 are independently hydrogen, C_{1-5} alkyl, phenyl, naphthyl or C_{5-7} cycloalkyl;

R_2 is C_{1-6} alkyl, phenyl, naphthyl or C_{5-7} cycloalkyl;

25 R_3 is $-NR_4R_5$ or aziridinyl, pyrrolidinyl, imidazolyl, pyrrolyl, piperidinyl, piperazinyl, morphollinyl, pyrrolidinyl or pyrrolinyl; provided the compound of formula (I) is not p-Glu-Glu-Asp-Cys-Lys;

or a pharmaceutically acceptable salt thereof.

30

2. A compound of claim 1 wherein A is pyroglutamic acid or picolinic acid; B is glutamic acid or serine; C is aspartic acid; D is aminobutyric acid; E is glycine or lysine and n is 0.

35

3. A compound of claims 1 or 2 wherein the chiral amino acids are in the L absolute configuration.



4. A compound of claim 1 wherein n is 1 and E is glycine or lysine.

5. A compound of claim 1 selected from the group consisting of:

Pic-Glu-Asp-Abu-Lys (SEQ ID NO: 2)
pGlu-Glu-Asp-Abu-Tyr-Lys (SEQ ID NO: 4)
pGlu-Glu-Asp-Abu-Lys (SEQ ID NO: 1)
10 Pic-Ser-Asp-Abu-Lys (SEQ ID NO: 3)

6. A pharmaceutical composition comprising a compound of claims 1-5 and a pharmaceutical acceptable carrier.

15

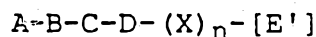
7. A method of inhibiting the myelopoietic system which comprises administering to a subject in need thereof, an effective amount of a compound of claims 1 to 5.

20

8. A process for the preparation of a compound of the formula I as defined in claim 1, which process comprises

a) coupling protected amino acids to form a suitably protected compound of the formula

25



wherein:

30 A is picolinic acid, pyroglutamic acid, proline or L-pipecolinic acid,

B is serine, glutamic acid, aspartic acid, threonine, cysteine or tyrosine;

C is aspartic acid or glutamic acid;

35 D is aminobutyric acid, alanine, propargyl glycine, glycine, serine, ~~cysteine~~ or β alanine;

X is tyrosine or lysine;



n is 0 or 1;

[E'] is a chloromethyl, methylbenzhydrylamine, benzhydrylamine or phenylacetamidomethyl resin or E is - N(R₁)-R₂R₃ or glycine, lysine, ornithine, tyrosine,

5 para-aminophenylalanine or the carboxamide or hydroxymethyl derivative thereof.

b) removing any protecting groups, and if necessary, cleaving the peptide from the resin; and

c) optionally forming a pharmaceutically
10 acceptable salt thereof.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/10070

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : A61K 37/02; C07K 5/10, 7/06

US CL : 530/330; 514/17, 18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/330; 514/17, 18

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Cas, Aps

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
<u>A</u> Y	US, A 4, 499, 081 (Laerum) 12 February 1985, see entire document.	<u>1-7</u> 8
<u>A</u> Y	WO, A, 90/02754, (Paulsen et al) 22 March 1990, see entire document.	<u>1-7</u> 8
<u>A</u> Y	WO, A, 90/02753 (Laerum) 22 March 1990, see entire document.	<u>1-7</u> 8

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to underpin the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be part of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"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
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"A" document member of the same patent family
"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	

Date of the actual completion of the international search

23 JANUARY 1993

Date of mailing of the international search report

12 FEB 1993

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