PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :		(11) International Publication Number: WO 92/07575
A61K 35/78, 35/74	A1	(43) International Publication Date: 14 May 1992 (14.05.92)

(21) International Application Number: PCT/GB91/01885

(22) International Filing Date: 28 October 1991 (28.10.91)

(30) Priority data:
9023337.0 26 October 1990 (26.10.90) GB

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(81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CI (OAPI patent), CM (OAPI patent), DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL, NL (European patent), NO, PL, RO, SD, SE, SE (European patent), SN (OAPI patent), SU+,TD (OAPI patent), TG (OAPI patent), US.

Published
With international search report.

(54) Title: ANTIMICROBIAL COMPOSITION

(57) Abstract

An antimicrobial composition comprising an antimicrobial material derived from the plant family Allium together with non-pathogenic microorganisms of at least one species, in which the antimicrobial material is isolated from the plant material in such a way (for example, by freeze-drying of whole cloves) that the material comprises alliin and alliinase and is substantially free of allicin. The composition is useful for combatting pathogenic microorganisms in animal gastrointestinal tract or for treatment of silage.

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+ DESIGNATIONS OF "SU"

Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.

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Antimicrobial Composition

The present invention is concerned with antimicrobial compositions, and the use thereof in the health/nutrition industry as dietary supplements, and as silage treatment agents.

It is known that antimicrobial materials can be extracted from the plant family Allium (which includes edible plants such as onions, chives, shallots, leek and garlic). The antimicrobial properties of garlic are well documented and garlic juice has been shown to inhibit the growth of a variety of pathogenic micro-organisms including Staphylococcus, Klebsiella, Proteus, Escherichia coli and Salmonella.

The antibacterial activity of garlic is attributed very largely to a compound known as allicin. About 0.24% (w/w) of each garlic clove consists of a compound known as alliin which is a non-odoriferous derivative of the amino acid cysteine. Intracellularly separated from the alliin in the garlic clove is an enzyme known as alliinase.

When the garlic clove is crushed, the alliinase comes into contact with the alliin to produce the odorous, unstable, water-soluble substance known as allicin (diallyl thiosulphinate).

Allicin decomposes readily to form a variety of intermediate products ultimately consisting of a mixture of allyl sulphides. This degradation process occurs at room temperature but is very much faster at elevated temperatures. The degradation products of allicin (i.e. the diallyl sulphides) have very little antimicrobial activity.

The mode of action of the allicin is largely unknown, but it is thought to act by blocking important metabolic enzymes, particularly those containing reactive SH groups. Alternatively, it may act by disrupting the microbial cell metabolism by interfering with protein function or by binding to cysteine and glutathione and inhibiting their activity.

The antifungal activity of garlic appears to be a combination of the effects of allicin and a specifically antifungal breakdown product of allicin known as ajoene. It has been found that lipid synthesis in C. albicans can be completely inhibited in the presence of garlic extract.

Dried (or freeze-dried) garlic powder is also known, which is prepared by crushing garlic cloves and then drying the crushed material. This results in reaction of alliin with alliinase to produce allicin.

We have now discovered that improved antimicrobial materials can be obtained from plant sources if special procedures are adopted which have been found to avoid reaction between alliin and allinase.

According to the invention, there is provided an antimicrobial composition which comprises dried non-pathogenic micro-organisms in combination with an antimicrobial material derived from the plant family Allium, the antimicrobial material comprising alliin and alliinase and being substantially free of allicin.

Known antibiotic compositions used to treat gastrointestinal disorders tend to have broad spectrum activities and act on both pathogenic and beneficial organisms in the gastrointestinal tract, leaving the gut susceptible to reinfection by pathogenic organisms. In contrast, the antimicrobial composition according to the invention acts selectively against pathogenic organisms, attacking pathogenic micro-organisms but not beneficial micro-organisms.

The antimicrobial composition according to the invention preferably comprises a carrier which is substantially unaffected by culture growth of pathogenic micro-organisms thereon (which carrier may be the antimicrobial material, the non-pathogenic micro-organisms, or another material which does not substantially interact with either said antimicrobial material or with said non-pathogenic micro-organisms).

The composition according to the invention may be used for therapeutic treatment of certain microbial mediated diseases, particularly those of the gastrointestinal tract, e.g. enteric disease caused by E. coli, rotavirus and Candida spp and microbially mediated diseases of the urinogenital tract, e.g. where Candida albicans is implicated, and/or for the improvement of the health and well being of the subject being treated.

The present invention encompasses the selective use of micro-organisms of any of the genera Lactobacillus, Bifidobacteria, Streptococcus and Pedioccus in combiantion with the antimicrobial composition according to the invention.

The microbial component of the composition of the present invention provides a source of beneficial micro-organisms which will effectively compete with any potential pathogens present in the gastrointestinal tract, and should therefore be capable of colonising the small intestine of the host when the composition is used for administration to animals.

The gastrointestinal tract of the neonatal animal is sterile at birth but it rapidly becomes colonised by the micro-organisms prevailing in the natural environment. It is essential that the organisms that colonise the gastrointestinal tract at that stage are the beneficial organisms which will effectively prevent the establishment of pathogens. However, the pathogens tend to grow more rapidly than the beneficial organisms and this is one reason why disease occurs in young animals.

Preferably the microbial component present in the compposition according to the invention comprises at least one strain of a host-specific, non-pathogenic Gram-positive bacteria which:

1. attaches to the epithelial tissue of the small intestine of the host;
2. is resistant to at least 3% (w/w) bile salts;
3. has a doubling time of less than two hours in vitro; and
4. is a homo- or hetero-fermentative lactic acid producer with greater than 40% by molarity of the acid produced thereby being lactic acid.

The microbial component preferably substantially retains its stability and characteristic features after being subjected to a typical production strategy of fermentation, centrifugation and spray- or freeze-drying. The microbial component is combined according to the invention with the antimicrobial material in such a way that allicin production is not triggered until the composition is put into use.

A preferred microbial component for use in the composition according to the invention is of the species Lactobacilius acidophilus or Lactobacillus plantarum.

The plant extract component of the composition according to the present invention is typically obtained either from the drying of substantially the entire bulb of the relevant Allium species (which includes many well known edible plants such as onions, chives, shallots, leeks and garlic). The preferred source of the plant extract is Allium sativum (garlic).

The antimicrobial activity of the antibacterial material present in the composition according to the invention has been found to be highly selective. We have found that there is no adverse effect on selected members of the beneficial lactobacillus microbial population of the gastrointestinal tract.

It is preferred that fresh substantially whole garlic cloves are freeze dried to produce garlic granules or powder, for use in the composition according to the invention. The freeze drying process entails the rapid freezing of the substantially whole cloves followed by gently drying from that frozen state at temperatures between -25 and -5 degrees Celsius.

In the composition according to the invention, more than one species of micro-organism may be present. The dosage of micro-organisms depends largely on the target host and the treatment required, but the dosage of micro-organisms in any 24 hours period is typically from 1×10^7 to 1×10^{12}. The dosage of the antimicrobial material also depends upon the target host and treatment required, but the dosage of fresh garlic equivalent administered over a 24 hour period is generally between 1 and 10,000 mg. The formulation may be provided in any suitable form, such as a powder, in powder tablet, capsule or similar form, or as a powder which is reconstituted with water.

According to the present invention there is also provided a method of therapeutic treatment which comprises administering to an animal, simultaneously or successively, an antimicrobial material derived from the plant family Allium, which comprises alliin and alliinase and is substantially free of allicin, and at least one species of non-pathogenic micro-organisms, such that the antimicrobial material acts selectively against pathogenic micro-organisms present in the animal.

There is further provided a kit for combatting pathogenic micro-organisms in the gastrointestinal tract of animals or for treatment of silage, said kit comprising a first receptacle containing an antimicrobial material derived from the plant family Allium, and a second receptacle containing at least one species of non-pathogenic micro-organisms, in which the antimicrobial material comprises alliin and alliinase and is substantially free of allicin.

It is also intended that lower dosage levels (sub-therapeutic amounts) of the composition according to the present invention may be used in the animal feed industry as a growth promoting agent, in a similar way to the way in which sub-therapeutic levels of antibiotics are currently used to perform such a function.

The composition according to the invention may be used where physiological, emotional or environmental stresses are placed on the patient which profoundly alters the balance of the microflora. This may include events such as post-antibiotic therapy, post-drug therapy and menstruation in human cases, and weaning in the case of domestic animals.

In addition to its activity against pathogenic bacteria, the composition according to the invention has antiviral properties and in vitro activity against influenza B virus. This is particularly important in view of the fact that a large percentage of the cases of diarrhoea in young animals have been attributed to strains of rotavirus rather than to bacterial sources.

Ensilage is a major way of adequately preserving forage as a winter feed. The ensilage process basically allows lactic acid bacteria to convert sugars in the forage to acids, notably lactic acid. The production of this acid and the resultant pH drop results in a material which stabilises at about pH 4.0 where further spoilage is prevented by the silage being kept in anaerobic/microaerophilic conditions.

A major problem in silage is aerobic spoilage which occurs when it is exposed to the air at time of feeding out to the livestock.

This aerobic spoilage is mainly caused by yeasts and moulds which multiply to a large degree before the lactic fermentation of the silage has been completed. This higher population level is benign when the silage is anaerobic, but when exposed to the air, the yeast and mould population rapidly multiply, utilising residual sugar and lactic acid, and producing ethanol and acetic acid, which is undesirable.

The addition of lactic acid cultures to forage to encourage and hasten the ensilage process is now well established. The species normally used is Lactobacillus plantarum. By adding the composition according to the invention at the time of ensilage, the proliferation of the yeast and mould population is dramatically reduced whilst not interfering with the desirable ensilage fermentation. The end result is that when opened to the air during feeding out, there is a much greater delay in the onset of aerobic spoilage.

The following Examples are given by way of illustration only.

EXAMPLE 1

Whole garlic cloves were peeled to remove the brittle outer skin. The clove was left entirely intact (cutting the scar causes some allicin to be produced). The cloves were then frozen to -30°C. Primary freeze drying took place with the product at temperatures between -25°C and -5°C.

When the primary freeze drying phase was completed, the temperature of the garlic cloves rose rapidly to +20°C where it remained whilst secondary drying took place to remove desorbed water. This took approximately 2 hours, the entire drying process taking between 12 and 24 hours.

The resulting freeze-dried powder contained 1.55% alliin and had allicin potency (as measured by the size of zone of inhibition of the yeast Candida Albicans) of 15mm.

Similar results, but with slightly less allicin potency, were obtained by cutting off the scar at the end of each clove to accelerate moisture loss during the freeze-drying process.

Again similar results were obtained by cryogenically crushing, dicing or milling of the frozen whole cloves, reaction between alliin and alliinase being substantially prevented by ensuring that the temperatures did not exceed 20°C at any point.

The results achieved using freeze-dried whole garlic cloves are compared with those from crushed garlic in the following table.

	Alliin (Micrograms/gram Powder)	Allicin Potency
Whole cloves freeze dried (according to the invention)	1.55%	15
Crushed garlic 4°C 30 mins Freeze Dried	0.42%	13
Crushed garlic 4°C 2 hours Freeze dried	0.11%	12
Crushed garlic 4°C 24 hours Freeze dried	Trace	9.5
Crushed garlic 20°C 30 mins Freeze dried	0.04%	11
Crushed garlic 20°C 2 hours Freeze dried	Trace	10
Crushed garlic 20°C 24 hours Freeze dried	Trace	6

The above table demonstrates the rapid reduction in the level of alliin after disintegration of a garlic clove, and the concomitant decrease on the allicin potency as demonstrated by a bioassay method against the yeast Candida albicans.

The results show that allicin is rapidly produced from alliin and alliinase even at low temperatures. Also the antimicrobial activity of the preparation is decreased after allicin is formed and this decrease is dependent both upon time and temperature.

In the extreme cases above, garlic cloves which were crushed and kept for 24 hours at 20°C before freeze drying had only 40% of the activity of garlic which was freeze dried as the whole clove.

The Allicin potency (measured in mm) was assayed as follows:

Equidistant wells were bored in agar plates containing MRS (de Mann, Rogosa and Sharpe) medium. The plates were then seeded with a lawn of Candida Albicans.

An overnight mixture of the organism was prepared and the agar plates were flooded with standardised concentrations of the mixture and the excess culture was removed. The lawns of the various cultures were allowed to dry and then the garlic preparations (at 20 mg/ml) were added to the wells in equal concentrations.

The efficacy, or allicin potency, was detected as a zone of clearing around the well containing the garlic and the comparative activities could be assessed by the diameters of the zones (in mm) in inhibition.

The test was repeated for several other micro-organisms, using freeze dried whole garlic cloves according to the invention, at 50 mg/ml; the results are given in the following table:

Organism	Diameter of Zone (mm)
E. coli	56
C. albicans	42
S. faecium	No inhibition
S. aureus	40
L. acidophilus	No inhibition
L. plantarum	No inhibition

This showed that the freeze dried garlic had a significant inhibitory effect on the growth of the pathogenic organisms viz. E. coli, C. albicans and Staph aureus. However, the freeze dried garlic preparation did not have any adverse effects on the growth of "beneficial" intestinal organisms such as L. acidophilus, S. faecium or L. plantarum and can therefore be combined therewith to produce compositions according to the invention.

The organisms L. acidophilus and L. plantarum referred to above were isolated from the pig's gut were identified and the Gram positive organisms were selected for further study.

The chosen organisms were put through a screening procedure and five strains of Lactobacillus were finally selected on the basis of their performance. These strains were not fully identified but were:

Lactobacillus acidophilus

Lactobacillus delbruekii

Lactobacillus plantarum

Lactobacillus sp

Lactobacillus sp

On the basis of these results, the organisms were subjected to the production regime and two of the strains showed good survival through the process; these were the L. acidophilus and L. plantarum referred to above.

EXAMPLE 2

The Antimicrobial effect of the Composition In Vitro.

The effects of the composition were tested in vitro using the plate/well method previously described. The composition was prepared according to the formulation used for field trial studies and was added to the wells directly. The antimicrobial activity was tested against C. albicans (which was used as the reference organism for testing antimicrobial activity) and E. coli, which is a strain known to be pathogenic to pigs and so provides a good indication of the potential activity of the composition.

Organism	Diameter of Zone (mm)
C. albicans	15
E. coli	9
L. acidophilus	No inhibition
L. plantarum	No inhibition

The zones observed during this study were smaller than those observed previously because the garlic concentration (of the composition) was 20 mg/ml compared with the 50 mg/ml used in the previous study.

EXAMPLE 3

Effect of strains of Lactobacillus on the growth of C. albicans In Vitro.

It is very difficult to demonstrate the potentially inhibitory activity of Lactobacillus in the laboratory. However, a system has been developed which involved the growth of various strains of Lactobacillus at 30°C for 24 hours and then

spinning down to obtain the supernatants which were filter sterilised and used as the aqueous phase for fresh batches of media. The plates which were prepared were inoculated with a pathogenic strain of Candida and the effects of the Lactobacillus growth media on the growth of the yeast were observed.

The results are shown in the following table:

Organism	Inhibition of Growth (%)
L 1	40
L 4	58
L 5	0
L 6	69
L 7	99
L 8	99
L 9	100
L10	98
L11	0
Acidic pH	63

As can be seen, using this modified in vitro method for assessing the inhibition of C. albicans, several strains of Lactobacillus can inhibit the growth of the pathogenic yeast.

EXAMPLE 4

Population Growth of Yeasts and Moulds of Grass Silage Exposed to Air for Feeding to Livestock

	Number of Yeasts & Moulds Immediately on Exposure to air	After 72 hrs Exposure to air
Freeze dried Alliin and Alliinase* (100mg/kg)	5.0×10^2/g	6.5×10^4/g
Freeze dried Lactobacillus Plantarum* (1.0×10^9/kg)		
Freeze dried Lactobacillus Plantarum* Applied at 1.0×10^9/kg)	2.3×10^4/g	1.47×10^7/g
Control	1.9×10^4/g	$>2 \times 10^8$/g

*All treatments applied at time of ensiling.

All the above silages had satisfactory fermentation profile with pH values ranging from 3.8 - 4.1.

Visually, the control sample was showing signs of mould growth at the end of 72 hours. The sample treated with L. plantarum alone was visually not as appealing as the sample with alliin-alliinase and L. plantarum applied together.

CLAIMS:

1. An antimicrobial composition which comprises dried non-pathogenic micro-organisms in combination with an antimicrobial material derived from the plant family Allium, said antimicrobial material comprising alliin and alliinase and being substantially free of allicin.

2. A composition according to claim 1, which further comprises a carrier which is substantially unaffected by culture growth of pathogenic micro-organisms thereon, and which does not substantially interact with either said antimicrobial material or with said non-pathogenic micro-organisms.

3. A composition according to claim 1 or 2, wherein said non-pathogenic micro-organisms comprise any of the genera Lactobacillus, Bifidobacteria, Streptococcus or Pediococcus.

4. A composition according to any of claims 1 to 3, wherein said species of said non-pathogenic micro-organisms comprises Lactobacillus acidophilus, Streptococcus faecum or Lactobacillus plantarum.

5. A composition according to any of claims 1 to 4, wherein said antimicrobial material is derived from a plant source of said plant family by freeze-drying of substantially whole cloves thereof.

6. A composition according to any of claims 1 to 5, wherein said plant family comprises Allium sativum.

7. A composition according to any of claims 1 to 6, wherein said non-pathogenic micro-organisms are freeze-dried.

8. A kit for combatting pathogenic micro-organisms in the gastrointestinal tract of animals or for treatment of silage, said kit comprising a first receptacle containing an antimicrobial material derived from the plant family Allium, and a second receptacle containing at least one species of non-pathogenic micro-organisms, characterised in that the antimicrobial material comprises alliin and alliinase and is substantially free of allicin.

9. A method of treatment of silage, which comprises administering thereto a composition according to any of claims 1 to 7.

10. A method of therapeutic treatment which comprises administering to an animal patient, either simultaneously or successively, an antimicrobial material derived from the plant family Allium, which comprises alliin and alliinase and is substantially free of allicin, and at least one species of non-pathogenic micro-organisms, such that the antimicrobial material acts selectively against pathogenic micro-organisms present in the patient.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 91/01885

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: A 61 K 35/78, A 61 K 35/74

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	A 61 K

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

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]
Y	Dialog Information Services, File 350, WPI 74-80, Dialog accession no. 000988351, LEHNER AG: "Stabilized garlic powder - produced by powdering deep-frozen garlic and freeze-drying", CH 540013, A, 7344 (Basic)	1-3,5,7-10
Y	Dialog Information Services, File 350, WPI 74-80, Dialog accession no. 000889166, FORM E: "Stable garlic powder - produced by deep freezing", DE 2101880, A, 7231 (Basic)	1-3,5-7-10
Y	Dialog Information Services, File 155, Medline 66-91, Dialog accession no. 03049637, Tynecka Z et al: "The fungistatic activity of garlic (Allium sativum L.) in vitro", Ann Univ Mariae Curie SklodowskaMed 1975, 30 p 5-13	1-3,5,7-10

* Special categories of cited documents:[10]

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

"E" earlier document but published on or after the international filing date

"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)

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

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"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
28th January 1992	12 FEB 1992
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Caroline Palmerantz

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Dialog Information Services, File 351, WPI 81-91, Dialog accession no. 004840940, RIKEN KAGAKU KOGYO: "Growth accelerator for bifidobacterium etc. comprises garlic extracts e.g. oxoamidine(rtm), peptone sodium chloride which are added to culture", JP 61260023, A, 861118, 8652 (Basic)	1-3,5
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Y	EP, A2, 0302300 (MICROLIFE TECHNICS, INC.) 8 February 1989, see especially claims 1 and 33, page 3 line 57, page 10 example 5	1-3,5,7-10
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FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim number X....1.0.., because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39.1(iv): Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

2. [ ] Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the the claims. It is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additonal fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional seach fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.PCT/GB 91/01885

SA 52701

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 European Patent Office EDP file on 31/10/91
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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A2- 0302300	08/02/89	AU-B-	604900	03/01/91
		AU-D-	1662288	09/02/89
		JP-A-	1086883	31/03/89