



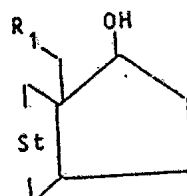
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METHOD OF PREPARING 17-OXOSTEROIDS
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- (57) Claim

1. Process for the production of 17-oxosteroids by fermentative oxidation of 17 β -hydroxy steroids, characterized in that for the fermentation, a bacterial culture of the species *Mycobacterium spec. NRRL B-3805*, *Mycobacterium spec. NRRL B-3683*, *Mycobacterium phlei NRRL B-8154* or *Mycobacterium fortuitum NRRL B-8153* is used.

2. Process for the production of 17-oxosteroids by fermentative oxidation of 17 β -hydroxy steroids according to claim 1, wherein a 17 β -hydroxy steroid of general formula I



(I),

in which

R_1 represents a hydrogen atom or a methyl group and
 St symbolizes the radical of an estran-17 β -ol derivative,
containing up to 5 noncumulated double bonds, substituted in the

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3-position by a phenolic hydroxy group, a lower alkoxy group, a lower 1-alkoxy-alkoxy group, a 2-tetrahydropyranyloxy group, or a lower alkylendioxy group, as well as optionally in 5,10-position by an epoxy group and in 19-position optionally by a methyl group,
is oxidized.

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(51) Internationale Patentklassifikation 5 : C12P 33/16	AI	(11) Internationale Veröffentlichungsnummer: WO 92/03572 (43) Internationales Veröffentlichungsdatum: 5. März 1992 (05.03.92)
(21) Internationales Aktenzeichen: PCT/DE91/00628 (22) Internationales Anmeldedatum: 1. August 1991 (01.08.91) (30) Prioritätsdaten: P 40 26 462.9 18. August 1990 (18.08.90) DE (71) Anmelder (für alle Bestimmungsstaaten ausser US): SCHE- RING AKTIENGESELLSCHAFT BERLIN UND BERGKAMEN [DE/DE]; Müllerstr. 170-178, D-1000 Berlin 65 (DE). (72) Erfinder; und (75) Erfinder/Anmelder (nur für US) : WEBER, Alfred [DE/ DE]; Schützallee 56, D-1000 Berlin 37 (DE). KENNEK- KE, Mario [DE/DE]; Taubertstr. 31 f, D-1000 Berlin 33 (DE). KLAGES, Uwe [DE/DE]; Schramberger Str. 19, D-1000 Berlin 28 (DE). NICKISCH, Klaus [DE/DE]; Alt Lichtenrade 11, D-1000 Berlin 49 (DE). ROHDE, Ralph [DE/DE]; Schwatlostr. 14, D-1000 Berlin 45 (DE).		(81) Bestimmungsstaaten: AT (europäisches Patent), AU, BE (europäisches Patent), CA, CH (europäisches Patent), DE (europäisches Patent), DK (europäisches Patent), ES (europäisches Patent), FI, FR (europäisches Patent), GB (europäisches Patent), GR (europäisches Patent), HU, IT (europäisches Patent), JP, LU (europäisches Patent), NL (europäisches Patent), SE (europäisches Patent), SU*, US. Veröffentlicht <i>Mit internationalem Recherchenbericht.</i> 650467
(54) Title: METHOD OF PREPARING 17-OXOSTEROIDS (54) Bezeichnung: VERFAHREN ZUR HERSTELLUNG VON 17-OXOSTEROIDEN (57) Abstract <p>Described is a method of preparing 17-oxosteroids by the fermentative oxidation of 17β-hydroxysteroids, characterized in that a bacterial culture of the species Mycobacterium spec. NRRL B-3805, Mycobacterium spec. NRRL B-3683, Mycobacterium phlei NRRL B-8154 or Mycobacterium fortuitum NRRL B-8153 is used for the fermentation.</p> (57) Zusammenfassung <p>Es wird ein Verfahren zur Herstellung von 17-Oxosteroiden durch fermentative Oxidation von 17β-Hydroxysteroiden beschrieben, welches dadurch gekennzeichnet ist, daß man zur Fermentation eine Bakterienkultur der Spezies Mycobacterium spec. NRRL B-3805, Mycobacterium spec. NRRL B-3683, Mycobacterium phlei NRRL B-8154 oder Mycobacterium fortuitum NRRL B-8153 verwendet.</p>		

Process for the Production of 17-Oxosteroids

The invention relates to a process for the production of 17-oxosteroids by fermentative oxidation of 17 β -hydroxy steroids.

Processes for fermentative production of 17-oxosteroids have been known for a long time. As early as 1938, A. Vercellone and L. Momoli describe a process for the production of 4-androstene-3,17-dione by fermentation of 5-androstene-3 β ,17 β -diol (Ber. 71, 1938, 152-155). This process provides high yields of process product, when the suitable microorganisms are selected (Czech 87.068; ref. C.A. 54, 1960, 2441). There are also a great number of publications in which is described that 17-oxosteroids (such as 4-androstene-3,17-dione, 1,4-androstadiene-3,17-dione or 3-hydroxy-1,3,5(10)-estratrien-1-one) can be produced while achieving high yields by fermentative side chain catabolism of substrates, which carry a hydrocarbon chain in 17 β -position.

Only very little is known about processes with whose help it is possible to oxidize 17 β -hydroxy steroids microbiologically in good yield to 17-oxysteroids, without an additional conversion of the steroid taking place. M. Welsh and C. Heusghem describe a process that is to make it possible to convert estradiol with a yield of 95% to estrone, but this process cannot be done over again, since the microorganism used by these authors is not available to the public (Compt. Rend. Soc. Biol., 142, 1948, 1074).

But there is a considerable demand for making such a fermentative process available, since the chemical processes for oxidation are quite expensive. In addition, these chemical



processes suffer from the shortcoming that they are performed by reagents such as pyridine and sulfur trioxide, whose environment-compatible disposal is quite problematical.

It has now been found that 17-oxosteroids surprisingly can be produced in high yields from 17 β -hydroxy steroids, if bacterial cultures of the species *Mycobacterium spec.* NRRL B-3805, *Mycobacterium spec.* NRRL B-3683, *Mycobacterium phlei* NRRL B-8154 or *Mycobacterium fortuitum* NRRL B-8153 are used for fermentation of the 17 β -hydroxy steroids.

The process according to the invention is performed under the same fermentation conditions, which are also used with these bacterial cultures in the known microbiological conversions.

Under the culture conditions usually used for these bacterial cultures, submerged cultures are cultivated in a suitable nutrient medium with aeration. Then, the substrate (dissolved in a suitable solvent or in emulsified form) is added to the cultures and fermented, until a maximum substrate conversion is reached.

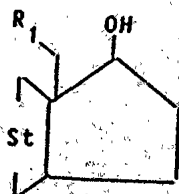
Suitable substrate solvents are, for example, methanol, ethanol, glycol monomethyl ether, dimethylformamide or dimethylsulfoxide. The emulsification of the substrate can be brought about, for example, by the latter being sprayed in micronized form or dissolved in a water-miscible solvent (such as methanol, ethanol, acetone, glycol monomethyl ether, dimethylformamide or dimethylsulfoxide) under strong turbulence in (preferably decalcified) water, which contains the usual emulsifying aids. Suitable emulsifying aids are nonionogenic



emulsifiers, such as, for example, ethylenoxy adducts or fatty acid esters of polyglycols. As suitable emulsifiers, the commercially available wetting agents Tegin^(R), Tween^(R) and Span^(R) can be mentioned as examples.

The optimum substrate concentration, substrate addition time and fermentation period depend on the type of substrate and microorganism used and fermentation conditions. These values, as is generally necessary in microbiological steroid conversions, have to be determined in the individual case by preliminary tests, as they are familiar to one skilled in the art.

To perform the process according to the invention, 17 β -hydroxy steroids of general formula I



(I),

in which

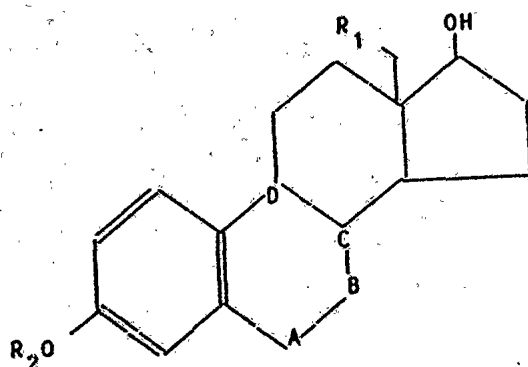
R₁ represents a hydrogen atom or a methyl group and

St symbolizes the radical of an estran-17 β -ol derivative, containing up to 5 noncumulated double bonds, substituted in the 3-position by a phenolic hydroxy group, a lower alkoxy group, a lower 1-alkoxy-alkoxy group, a 2-tetrahydropyranyloxy group, or a lower alkylendioxy group, as well as optionally in 5,10-position by an epoxy group and in 19-position optionally by a methyl group, are preferably used as substrates.



By a lower alkoxy group is preferably to be understood a group which carries up to 4 carbon atoms. Suitable alkoxy groups are, for example, the methoxy group or the tert-butyloxy group. By a lower 1-alkoxy-alkoxy group is preferably to be understood a lower alkoxy-methoxy group with an alkoxy group having up to 4 carbon atoms. For example, the 1-methoxy-methoxy group can be mentioned. By a lower alkylendioxy group is preferably to be understood a grouping having 2 to 6 carbon atoms such as, for example, the ethylenedioxy group or the 2,2-dimethyl-propylenedioxy group.

Substrates, which are suitable to perform the process according to the invention are, for example, estran-17 β -ol derivatives of general formula Ia



(Ia),

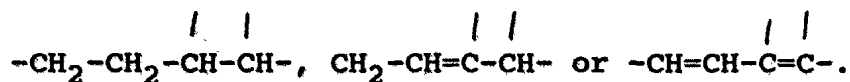
in which

R_1 has the above-mentioned meaning,

R_2 represents a hydrogen atom or an alkyl group containing up to 4 carbon atoms and

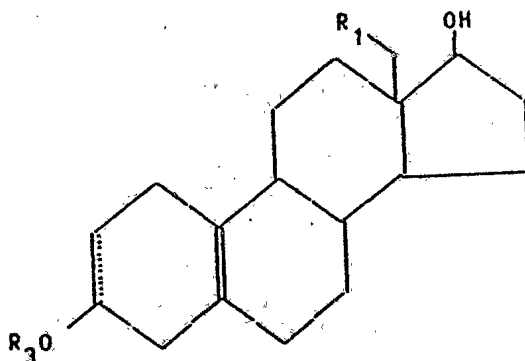
-A-B-C-D- symbolizes a grouping of partial formula





The estran-17-one derivatives formed from these substrates or the 3-hydroxy compounds obtained from this by ether cleavage are, as is known, pharmacologically effective and can, moreover, as is known, be reduced to the corresponding 17 α -hydroxy compounds or be converted to the corresponding 17 β -hydroxy-17 α -ethinyl compounds.

Further, suitable substrates are estrane derivatives of general formula Ib



(Ib),

in which

..... symbolizes a single bond or a double bond,

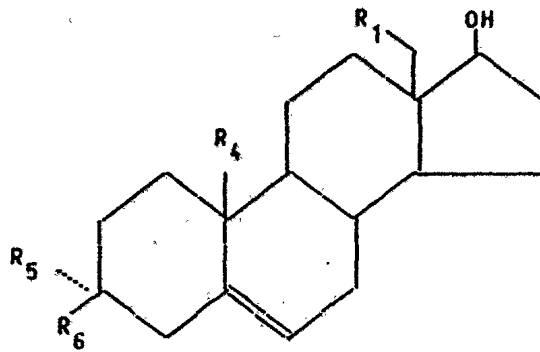
R₁ has the above-mentioned meaning,

R₃ represents an alkyl group with at most 4 carbon atoms.

The 17-oxosteroids formed from these compounds can, as is known, be used, for example, to produce the pharmacologically effective 17 α -ethinyl-17 β -hydroxy-4-estren-3-one or its 18-methyl homologs.

Substrates worth mentioning are also those of general formula Ic





(Ic),

in which

R₁ has the above-mentioned meaning,

R₄ represents a hydrogen atom or a methyl group,

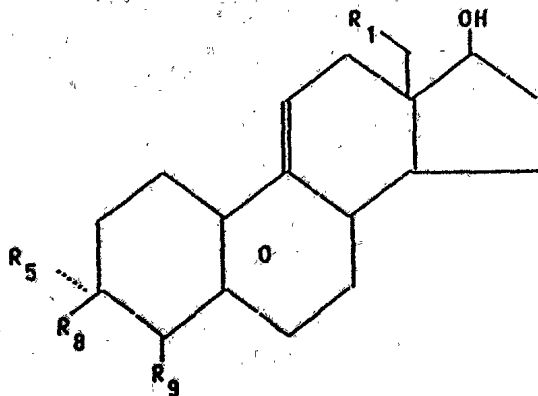
R₅ and R₆ together symbolize a lower alkyldioxy group or

R₅ means a hydrogen atom and

R₆ represents a lower alkoxy group, a lower 1-alkoxyalkoxy group or a 2-tetrahydropyranyl group.

The 17-oxosteroids obtained from these compounds can be further processed in the same way as those of the estran-17 β -ol derivatives of general formula Ic; but, on the other hand, they can also be used for the production of pregnane derivatives, as is well known to one skilled in the art.

Finally, also worth mentioning are estran-17 β -ol derivatives of general formula Id



(Id),



in which

R_1 has the above-mentioned meaning,

R_9 symbolizes a hydrogen atom and R_7 and R_8 together symbolize a lower alkylendioxy group or

R_7 and R_9 mean two hydrogen atoms or together mean a carbon-carbon bond and

R_8 represents a lower alkoxy group, a lower 1-alkoxyalkoxy group or a 2-tetrahydropyranyloxy group.

The ketones obtained from these compounds can be converted to antigestagenically active steroids according to the process, as described in EP-A 0129499.

The following embodiments are used to explain the process according to the invention in more detail.



Example 1

a) A 500 ml Erlenmeyer with 100 ml of sterile nutrient medium containing

1 % yeast extract

0.45 % $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$

0.34 % KH_2PO_4

0.2 % Tween 80

with a pH of 6.7 is inoculated with a suspension of a *Mycobacterium spec.* NRRL B-3683 culture and shaken for 72 hours at 30°C with 180 revolutions per minute.

b) 50 Erlenmeyers (100 ml) with 20 ml of sterile nutrient medium each containing

0.5 % corn steep liquor

0.05 % glucose monohydrate

0.2 % yeast extract

-- adjusted to pH 7.0 --

are inoculated with 1 ml of *Mycobacterium spec.*-growing culture each and incubated for 24 hours on a rotary shaker with 220 revolutions per minute at 30°C.

Then, 0.02 g of 3,3-(2,2-dimethyltrimethylenedioxy)-5,10 α -epoxy-5 α -estr-9(11)-en-17 β -ol dissolved in 0.2 ml of dimethylformamide and sterilized by filtration is added to each culture and fermented for another 48 hours at 30°C.

c) The combined cultures are extracted with methyl isobutyl ketone and the extract is concentrated by evaporation under vacuum in a rotary evaporator at a maximum of 50°C. Then, a



purification takes place by chromatography on a silica gel column.

0.85 g of 3,3-(2,2-dimethyltrimethylenedioxy)-5,10 α -epoxy-5 α -estr-9(11)-en-17 β -one, which is identical with an authentic sample according to HPLC, is thus obtained.

Example 2

a) The production of the growing culture takes place as indicated in example 1a).

b) 50 Erlenmeyers (100 ml) with 20 ml of sterile nutrient medium each containing

2.5 % corn steep liquor

0.25 % soybean flour

0.3 % $(\text{NH}_4)_2\text{HPO}_4$

0.25 % Tween 80

-- adjusted to pH 6.5 --

are inoculated with 1 ml of the Mycobacterium-spec.-growing culture each and incubated for 24 hours on a rotary shaker with 220 revolutions per minute at 30°C.

Then, 0.002 g of α -equilol dissolved in 0.2 ml of dimethylformamide and sterilized by filtration is added to each culture and fermented for another 72 hours at 30°C.

c) The isolation of the product takes place as described under 1c).

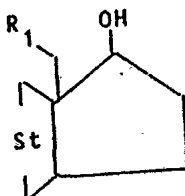
0.095 g of equilin, which is identical with an authentic sample according to HPLC, is thus obtained.



Claims

1. Process for the production of 17-oxosteroids by fermentative oxidation of 17 β -hydroxy steroids, characterized in that for the fermentation, a bacterial culture of the species *Mycobacterium spec.* NRRL B-3805, *Mycobacterium spec.* NRRL B-3683, *Mycobacterium phlei* NRRL B-8154 or *Mycobacterium fortuitum* NRRL B-8153 is used.

2. Process for the production of 17-oxosteroids by fermentative oxidation of 17 β -hydroxy steroids according to claim 1, wherein a 17 β -hydroxy steroid of general formula I



(I),

in which

R_1 represents a hydrogen atom or a methyl group and

St symbolizes the radical of an estran-17 β -ol derivative, containing up to 5 noncumulated double bonds, substituted in the 3-position by a phenolic hydroxy group, a lower alkoxy group, a lower 1-alkoxy-alkoxy group, a 2-tetrahydropyranyloxy group, or a lower alkylendioxy group, as well as optionally in 5,10-position by an epoxy group and in 19-position optionally by a methyl group,
is oxidized.



Abstract

A process for the production of 17-oxosteroids by fermentative oxidation of 17 β -hydroxy steroids is described, which is characterized in that for the fermentation, a bacterial culture of the species *Mycobacterium spec.* NRRL B-3805, *Mycobacterium spec.* NRRL B-3683, *Mycobacterium phlei* NRRL B-8154 or *Mycobacterium fortuitum* NRRL B-8153 is used.



INTERNATIONAL SEARCH REPORT

International Application No PCT/DE 91/00628

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. ⁵	C12P33/16	
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. ⁵	C12P; C12R	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category ⁶	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Cla'm No. ¹³
A	EP, A, 322081 (GIST-BROCADES) 28 June 1989	
A	EP, A, 129499 (SCHERING) 27 December 1984 (cited in the application)	
<p>* Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"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)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"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</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"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.</p> <p>"&" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
17 October 1991 (17.10.91)	25 October 1991 (25.10.91)	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE		

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

DE 9100628
SA 49669

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
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17/10/91

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-322081	28-06-89	JP-A- 2002395	08-01-90
EP-A-129499	27-12-84	DE-A- 3321826	20-12-84
		DE-A- 3413036	17-10-85
		AU-B- 572849	19-05-88
		AU-A- 2936184	20-12-84
		DE-A- 3468030	21-01-88
		JP-B- 2016760	18-04-90
		JP-A- 60041695	05-03-85
		JP-A- 2167296	27-06-90
		US-A- 4780461	25-10-88

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/92

INTERNATIONALER RECHERCHENBERICHT

Internationales Aktenzeichen

PCT/DE 91/00628

I. KLASSIFIKATION DES ANMELDUNGSGEGENSTANDS (bei mehreren Klassifikationssymbolen sind alle anzugeben) ⁶		
Nach der Internationalen Patentklassifikation (IPC) oder nach der nationalen Klassifikation und der IPC		
Int.Kl. 5	C12P33/16	
II. RECHERCHIERTE SACHGEBIETE		
Recherchierter Mindestprüfstoff ⁷		
Klassifikationssystem	Klassifikationssymbole	
Int.Kl. 5	C12P ; C12R	
Recherchierte nicht zum Mindestprüfstoff gehörende Veröffentlichungen, soweit diese unter die recherchierten Sachgebiete fallen ⁸		
III. EINSCHLAGIGE VERÖFFENTLICHUNGEN ⁹		
Art. ¹⁰	Kennzeichnung der Veröffentlichung ¹¹ , soweit erforderlich unter Angabe der maßgeblichen Teile ¹²	Betr. Anspruch Nr. ¹³
A	EP,A,322 081 (GIST-BROCADES) 28. Juni 1989 ---	
A	EP,A,129 499 (SCHERING) 27. Dezember 1984 in der Anmeldung erwähnt ---	
<p>¹⁰ Besondere Kategorien von angegebenen Veröffentlichungen:</p> <ul style="list-style-type: none"> ^A Veröffentlichung, die den allgemeinen Stand der Technik definiert, aber nicht als besonders bedeutsam anzusehen ist ^E älteres Dokument, das jedoch erst am oder nach dem internationalen Anmeldedatum veröffentlicht worden ist ^L Veröffentlichung, die geeignet ist, einen Prioritätsanspruch zweifelhaft erscheinen zu lassen, oder durch die das Veröffentlichungsdatum einer anderen im Recherchenbericht genannten Veröffentlichung belegt werden soll oder die aus einem anderen besonderen Grund angegeben ist (wie ausgeführt) ^O Veröffentlichung, die sich auf eine mündliche Offenbarung, eine Benutzung, eine Ausstellung oder andere Maßnahmen bezieht ^P Veröffentlichung, die vor dem internationalen Anmeldedatum, aber nach dem beanspruchten Prioritätsdatum veröffentlicht worden ist ^T Spätere Veröffentlichung, die nach dem internationalen Anmeldedatum oder dem Prioritätsdatum veröffentlicht worden ist und mit der Anmeldung nicht kollidiert, sondern nur zum Verständnis der der Erfindung zugrundeliegenden Prinzipien oder der ihr zugrundeliegenden Theorie angegeben ist ^X Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann nicht als neu oder auf erfinderischer Tätigkeit beruhend betrachtet werden ^Y Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann nicht als auf erfinderischer Tätigkeit beruhend betrachtet werden, wenn die Veröffentlichung mit einer oder mehreren anderen Veröffentlichungen dieser Kategorie in Verbindung gebracht wird und diese Verbindung für einen Fachmann naheliegend ist ^A Veröffentlichung, die Mitglied derselben Patentfamilie ist. 		
IV. BESCHEINIGUNG		
Datum des Abschlusses der internationalen Recherche	Abschließendes Datum des internationalen Recherchenberichts	
17. OKTOBER 1991	25. 10. 91	
Internationale Recherchenbehörde:	Unterschrift des bevollmächtigten Reklamations	
EUROPAISCHES PATENTAMT	C. TURMO	

**ANHANG ZUM INTERNATIONALEN RECHERCHENBERICHT
 ÜBER DIE INTERNATIONALE PATENTANMELDUNG NR.**

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 SA 49669

In diesem Anhang sind die Mitglieder der Patentfamilien der im obengenannten internationalen Recherchenbericht angeführten Patentdokumente angegeben.
 Die Angaben über die Familienmitglieder entsprechen dem Stand der Datei des Europäischen Patentamts am
 Diese Angaben dienen nur zur Unterrichtung und erfolgen ohne Gewähr.

17/10/91

Im Recherchenbericht angeführtes Patentdokument	Datum der Veröffentlichung	Mitglied(er) der Patentfamilie	Datum der Veröffentlichung
EP-A-322081	28-06-89	JP-A- 2002395	08-01-90
EP-A-129499	27-12-84	DE-A- 3321826	20-12-84
		DE-A- 3413036	17-10-85
		AU-B- 572849	19-05-88
		AU-A- 2936184	20-12-84
		DE-A- 3468030	21-01-88
		JP-B- 2016760	18-04-90
		JP-A- 60041695	05-03-85
		JP-A- 2167296	27-06-90
		US-A- 4780461	25-10-88

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Für nähere Einzelheiten zu diesem Anhang : siehe Amtsblatt des Europäischen Patentamts, Nr.12/82