Patents Act 1952

22 APR 1988
Melbourne

616403

APPLICATION FOR A STANDARD PATENT

(Combined Form – Convention and Non–Convention)

I/We ..PIERRE FABRE MEDICAMENT, a French Body Corporate,...
.....of 125 rue de la Faisanderie 75116 PARIS, FRANCE,...
.....

hereby apply for the grant of a Standard Patent for an invention entitled Novel Derivative of D.25, Process For Its Preparation, Its Use As An Immunostimulant,.... And Pharmaceutical Compositions Containing The Derivative .....

which is described in the accompanying ~~Provisional~~ Complete Specification.

2. This application is a convention application and is based on the application(s) for a patent or similar protection made –

Strike our para.2. for non-convention

in ..France.....
on ..22nd April, 1987....., numbered .....87 05690....., and
on ....., numbered ....., and
on ....., numbered .....

3. My/Our address for service is: Care of COWIE, THOMSON & CARTER, Patent Attorneys, of 71 Queens Road, Melbourne, Victoria 3004, Australia.

DATED this .....19th..... day of .....April,..... 1988.

To The Commissioner of Patents
COMMONWEALTH OF AUSTRALIA

COWIE, THOMSON & CARTER
By:
Patent Attorneys for
PIERRE FABRE MEDICAMENT.

COWIE, THOMSON & CARTER
Patent Attorneys
71 Queens Road, Melbourne,
Victoria, 3004, Australia

COMMONWEALTH OF AUSTRALIA

Patents Act 1952-1962

Forms 7 and 8
(Combined Form)

Declaration in Support of an Application for a Patent

In support of the *Convention** application made by PIERRE FABRE MEDICAMENT

for a patent for an invention entitled:
NOVEL DERIVATIVE OF D.25, PROCESS FOR ITS PREPARATION, ITS USE AS AN IMMUNOSTIMULANT, AND PHARMACEUTICAL COMPOSITIONS CONTAINING THE DERIVATIVE

I, ... Pierre FABRE ....., CHAIRMAN
(INSERT FULL NAME) (CAPACITY)

of and care of the applicant company do solemnly and sincerely declare as follows:

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

or

I am authorised by the applicant for the patent to make this declaration on its behalf.

Strike out Para 2, for non-convention

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

in ..... FRANCE .....

on the ..... 22nd ..... day of ..... APRIL ..... 1987 ....., No. 87 05690 .....,

by PIERRE FABRE MEDICAMENT ....., and

in .....

on the ..... day of ..... 19 ....., No. .....,

by ....., and

in .....

on the ..... day of ..... 19 ....., No. .....,

by .....

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

3. ~~I am~~ / We are ~~the actual inventor(s) of the invention~~.

or

of Lucien DUSSOURD, D'HINTERLAND, Domaine de Plombière, 81100 CASTRES, FRANCE
Gérard NORMIER, 23 rue Francis Poulenc, 81100 CASTRES, FRANCE
Anne-Marie PINEL, 22 rue des Capucins, 81100 CASTRES, FRANCE
.....
.....
.....

~~is~~ / are the actual inventor(s) of the invention and the facts upon which the applicant is entitled to make the application are as follows:- The said company is the assignee of the invention from the said actual inventors.

PIERRE FABRE MEDICAMENT
Société Anonyme
135 Rue de la Fauconnerie 75
17 Avenue Jean Moulin

Pierre FABRE
Chairman

DECLARED AT Castres this 22nd day of February 19 88

.....
Signature of Declarant

(12) PATENT ABRIDGMENT (11) Document No. AU-B-15233/88
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 616403

(54) Title
NOVEL DERIVATIVE OF D.25, PROCESS FOR ITS PREPARATION, ITS USE AS AN IMMUNOSTIMULANT, AND PHARMACEUTICAL COMPOSITIONS CONTAINING THE DERIVATIVE

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(71) Applicant(s)
PIERRE FABRE MEDICAMENT

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(56) Prior Art Documents
AU 71415/87 C08B 37/00
AU 21077/88 C08B 37/00

(57) The compounds according to the invention and their derivatives exhibit noteworthy immunostimulant properties and an absence of cytotoxicity. It is for this reason that the invention also relates to the use of the compounds and of the derivatives as an immunostimulant as well as to pharmaceutical compositions containing at least one compound or one derivative according to the present invention.

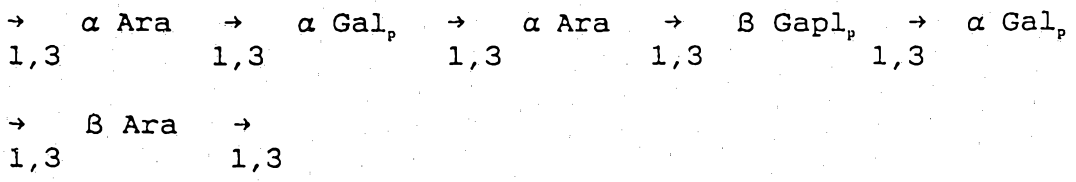
CLAIM

1. A polysaccharide compound which is chosen from among compounds derived from D.25 – a polysaccharide extracted from bacterial membrane proteoglycane of Klebsellia pneumonia – in which compounds the galactofuranose residues (Gal_f) of the linear polysaccharide chain of said D.25 have been converted wholly or at least partly to arabinose.

2. A polysaccharide compound according to claim 1 wherein all the galactofuranose residues of the linear polysaccharide chain have been converted to arabinose and which is defined by the monomer:

$$\xrightarrow[1,3]{} \beta\ Gal_p \xrightarrow[1,3]{} \beta\ Ara \xrightarrow[1,3]{} \alpha\ Gal_p \xrightarrow[1,3]{} \beta\ Gal_p$$

.../2



in which Gal$_p$ is galactopyranose (in the α and ß forms) and Ara is arabinose (in the α and ß forms).

P/00/011

AUSTRALIA

616403

Form 10

PATENTS ACT 1952-1973

COMPLETE SPECIFICATION

(ORIGINAL)

FOR OFFICE USE

Class:

Int. Cl:

Application Number:
Lodged:

Complete Specification—Lodged:
Accepted:
Published:

Priority:

Related Art:

TO BE COMPLETED BY APPLICANT

Name of Applicant: PIERRE FABRE MEDICAMENT, a French Body Corporate,

of 125 rue de la Faisanderie 75116 PARIS, FRANCE.

Address of Applicant:

Actual Inventor: Lucien Dussourd D'hinterland, Gerard Normier & Anne-Marie Pinel

Address for Service:

COWIE, THOMSON & CARTER
PATENT & TRADEMARK ATTORNEYS
71 QUEENS ROAD
MELBOURNE, 3004, AUSTRALIA

Complete Specification for the invention entitled: NOVEL DERIVATIVE OF D.25, PROCESS FOR ITS PREPARATION, ITS USE AS AN IMMUNOSTIMULANT, AND PHARMACEUTICAL COMPOSITIONS CONTAINING THE DERIVATIVE

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

*Note: The description is to be typed in double spacing, pica type face, in an area not exceeding 250 mm in depth and 180 mm in width, on tough white paper of good quality and it is to be inserted inside this form.

11710/76-L

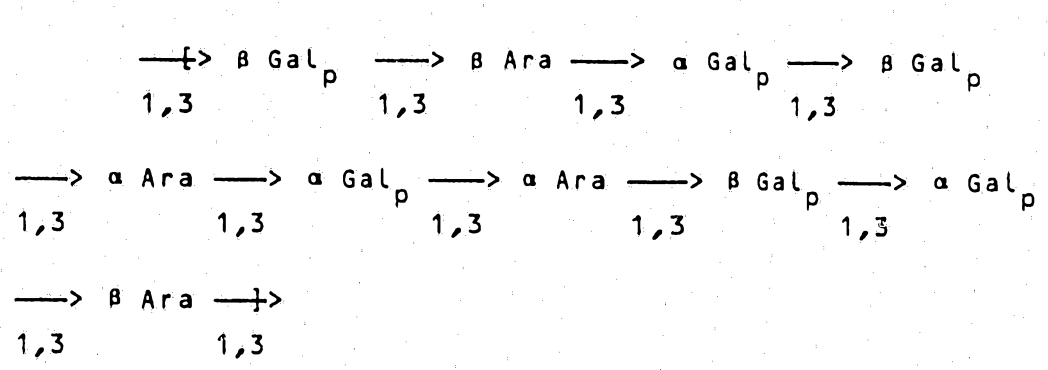
C. J. Thompson, Commonwealth Government Printer, Canberra

The present invention relates to novel compounds derived from D.25, to the process for their preparation and to the pharmaceutical compositions in which they are present.

The product called D.25 is the polysaccharide extracted from bacterial membrane proteoglycanes, comprising essentially galactose units and having a molecular weight of 30 ± 10 KD. This polysaccharide has been described in detail in French Patent No. 84/13,844 filed on 10th September 1984. This polysaccharide possesses immunostimulant properties, especially in respect of the induction of endogenous interferon and the activation of NK (Natural Killer) cells. This polysaccharide is preferentially isolated from a non-capsulated and non-pathogenic strain of Klebsiella pneumoniae biotype a, deposited in the National Collection of the Pasteur Institute under number 145.I.IP.

The present invention relates to compounds derived from D.25, in which at least a part of the galactofuranose residues (Gal_f) of the linear polysaccharide chain of the D.25 have been converted to arabinose, preferably without any other modification of the initial product. In these compounds, the galactopyranose residues (Gal_p) are preferably preserved.

Among these compounds, one is particularly interesting. This is the compound in which all the galactofuranose residues of the linear polysaccharide chain have been converted to arabinose and which is defined by the following monomer:



in which Gal_p is galactopyranose (α and β forms) and Ara is arabinose (α and β forms).

The invention also relates to the derivatives of the above compounds, namely, in particular:

- semisynthetic derivatives
- labeled derivatives and
- conjugated derivatives.

Among the semisynthetic derivatives, there may be mentioned the amides, esters, ethers, salts or quaternary ammonium derivatives with acids, amines, amides or alcohols.

The derivatives of the compounds according to the present invention can also be compounds labeled by any suitable method, for example by means of radioactive elements such as I^{125} or Tc^{99}, or by using fluorescent or magnetic compounds. Thanks to this type of labeling, the products in question can be detected in vivo or ex vivo.

The derivatives of the compounds according to the invention can also be conjugated with chemical compounds capable of improving their activity or which can bring them close to particular sites, especially of the immune system, so as to enable them to improve the activity of the conjugated chemical product.

A process for obtaining the compounds of the present invention comprises subjecting D.25 to a periodate oxidation followed by a reduction.

The oxidation is preferably carried out with the aid of sodium metaperiodate. The reduction can be carried out with the aid of $NaBH_4$, optionally used in excess.

The derivatives of the compounds can be obtained by known methods, namely a labeling method or a conjugation method.

The compounds according to the invention and their derivatives exhibit noteworthy immunostimulant properties and an absence of cytotoxicity. It is for this reason that the invention also relates to the use of the compounds and of the derivatives as an immunostimulant as well as to pharmaceutical compositions containing at least one compound or one derivative according to the present

invention.

The compounds and above all the labeled derivatives according to the invention can also be used for diagnostics, in particular for detecting certain elements of the immune system.

Other characteristics and advantages of the present invention will be clear on reading the detailed description which now follows.

Example 1: Isolation of the crude membrane proteoglycane.

The biomass of the strain of Klebsiella pneumoniae 145.I.IP is dispersed in ice-cold Tris-HCl buffer (10 mM, pH 7.0) containing NaCl (0.15 M) and is then subjected to mechanical grinding intended to break the cell walls. The bacterial lysate is clarified by continuous centrifuging at 15,000 g and the supernatant liquor is collected. The latter is treated by adding acetic acid, in the cold, until pH 4.2 is reached, so as to remove the impurities (nucleic acids and heavy proteins) by precipitation. The precipitate of impurities is removed by continuous centrifuging at 15,000 g. The limpid supernatant liquor is collected and then neutralized with NaOH.

The solution is then dialyzed, and is thereafter concentrated by ultrafiltration on a membrane with a cutoff at 10,000 Daltons.

The concentrated solution obtained at this stage corresponds to the crude membrane proteoglycane.

Example 2: Isolation of the crude polysaccharide fraction.

This consists of a controlled alkaline hydrolysis intended to depolymerize the crude membrane proteoglycane to liberate the polysaccharide fraction. To the concentrated and dialyzed solution of crude membrane proteoglycane obtained above is added NaOH to give a final NaOH concentration of 0.5 M. Thereafter hydrolysis is carried out for 1 hour at 56°C. After rapid cooling, the solution is neutralized with HCl.

The neutralized solution is clarified with filtration on a filter press and then concentrated by ultrafiltration on a membrane with a cutoff at 10,000 Daltons.

Example 3: Purification of the polysaccharide fraction.

The concentrate obtained in the preceding example is subjected to a first enzymatic treatment with the lysozyme, intended to destroy the mureine residues which may persist during the preparation. The hydrolysis is carried out for 2 hours at ambient temperature in a Tris-HCl buffer (10 mM, pH 8.0) containing EDTA, Na_2 (4 mM) and 0.1 mg/ml of lysozyme.

After the action of the lysozyme, the contaminating proteins are removed by proteolysis under the following conditions: the pH of the solution is adjusted to 7.0 and 0.1 mg/ml of proteinase K is then added. The incubation is continued for 2 hours at 37°C, with stirring.

The polysaccharide is then isolated by precipitation with alcohol. Three volumes of ethyl alcohol are added at ambient temperature. After 30 minutes' stirring, the precipitate is collected by filtration. It is redissolved in distilled water and the solution is filtered over a membrane of porosity 0.45 µm.

The residual contaminants of the polysaccharide, originating from the enzymatic hydrolyses, are removed by molecular sieve chromatography on a Pharmacia industrial column with SEPHACRYL S200 HR gel. The volume of sample deposited represents 5% of the gel volume. Elution is carried out with distilled water at a linear flow rate of 5 cm/hour.

The purified polysaccharide peak, detected by continuous measurement of the refractive index, is collected and concentrated by ultrafiltration on a membrane with a cutoff at 10,000 Daltons, to 1/5 of the initial volume.

The concentrated solution obtained at this stage corresponds to the purified polysaccharide D.25.

Example 4: Preparation of the novel polysaccharide.

The preceding solution is diluted so as to give 20 g of polysaccharide per liter of solution. 0.1 M sodium acetate is then added and the pH is adjusted to 3.8.

Thereafter, 15 g of sodium metaperiodate per liter of solution are added, after which stirring is continued for 48 hours in the dark, at a temperature of 15°C.

The excess metaperiodate is then removed by

precipitation with barium hydroxide, in the form of a concentrated solution added gradually, with stirring, until the precipitation has ended. The precipitate thus formed is removed by simple filtration.

To the above filtrate are then added 21.6 g of $NaBH_4$, after which the mixture is left to react for 18 hours at ambient temperature. The excess $NaBH_4$ is then destroyed by adding acetic acid until the mixture is neutral.

The solution obtained is dialyzed, concentrated on a membrane with a cutoff at 10,000 Daltons and then lyophilized. The lyophilisate thus obtained, constitutes the novel D.25 derivative of the present invention.

Example 5: Checking the absence of cytotoxicity.

The cytotoxicity is measured by in vitro incubation of various concentrations of the derivative of the present invention in a culture of YAC-1 cells labeled with ^{51}Cr. After 4 hours' incubation, the yield of ^{51}Cr liberated by the lysis of the cells is measured in the supernatant liquor. The results are expressed in terms of the percentage of cells lysed in comparison with a reference culture.

The spontaneous lysis in the reference culture is about 5%.

The results are reported in Table I below.

TABLE I

Concentration in µg/ml of novel derivative in ^{51}Cr labeled YAC-1 cell culture	% of cell lysis
0.05	- 0.2
0.1	1.0
0.5	0.3
1.0	- 1.0
5.0	0.3
10.0	- 0.4
50.0	- 0.1
100.0	- 1.3

The results show clearly that the product is completely devoid of cytotoxicity.

Example 6: Activation of the NK cells in vitro.

Effector cells (mouse spleen cells) are preincubated

for 6 hours in an RPMI-1640 medium containing 5% of calf fetus serum with various concentrations of the derivative to be tested.

The NK activity is measured on 10,000 YAC-1 target cells labeled with ^{51}Cr in each well, with a ratio of effector cells/target cells of 100/1.

After 4 hours' incubation, the amount of ^{51}Cr liberated into the supernatant liquor by lysis of the cells is measured with a gamma counter.

The results are given in Table II below.

TABLE II

Concentration in µg/ml	% lysis of the YAC-1 target cells
0-reference	18.4
0.1	21.6
1.0	31.6
10	47.4
100	40.8

The results confirm the very high stimulant power of the derivative on the activity of the NK cells "in vitro".

Example 7: Activation of the NK cells "in vitro" in the mouse. Comparison with polysaccharide D.25.

CBA/H mice aged 6 to 8 weeks are given an intraperitoneal injection of 100 µg of the derivative of the present invention one day or three days before measuring the activity of their spleen NK cells.

The NK activity is then measured as above in the "in vitro" test.

The results are shown in Table III below.

TABLE III

Product injected	% lysis of the target cells on Day + 1	on Day + 3
PBS-reference	25.2	18.2
D.25 (100 µg)	20.0	22.0
PS derivative (100 µg)	32.6	26.8

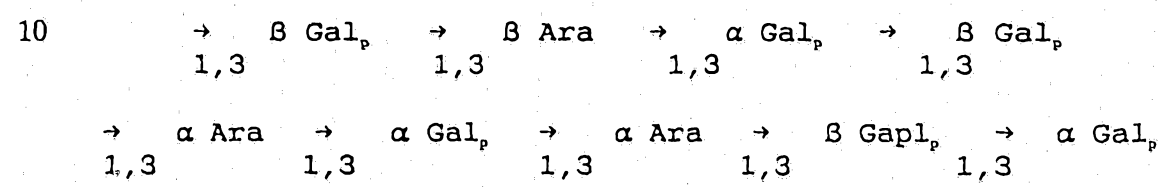
The results presented in the table show that the novel polysaccharide has a stimulating capacity for NK cells which is greater than that of D.25.

This activity manifests itself from the first day after injection and continues to Day + 3 while, under the same conditions, the activity of D.25 only appears on Day + 3.

The claims defining the invention are as follows:

1. A polysaccharide compound which is chosen from among compounds derived from D.25 – a polysaccharide extracted from bacterial membrane proteoglycane of Klebsellia pneumonia – in which compounds the galactofuranose residues (Gal_f) of the linear polysaccharide chain of said D.25 have been converted wholly or at least partly to arabinose.

2. A polysaccharide compound according to claim 1 wherein all the galactofuranose residues of the linear polysaccharide chain have been converted to arabinose and which is defined by the monomer:



in which Gal_p is galactopyranose (in the α and ß forms) and Ara is arabinose (in the α and ß forms).

3. Semisynthetic, labelled and conjugated derivatives of the polysaccharide compounds according to claim 1 or claim 2.

4. A semisynthetic derivative according to claim 3 chosen from among the amides, esters, ethers, salts or quaternary ammonium derivatives with an amine, an amide, an acid or an alcohol.

5. A polysaccharide compound or derivative thereof as claimed in any one of the preceding claims which is labelled.

6. A polysaccharide compound or derivative thereof according to any one of claims 1 to 4 which is conjugated with chemical compounds capable of improving the activity of said polysaccharides or derivatives thereof or which can bring said polysaccharides or derivatives thereof close to particular sites, especially of the immune system, enabling an improvement in the activity of the conjugated chemical product.

7. A process for obtaining a compound as claimed in claim 1 or 2, which

comprises:

(a) subjecting D.25 to a periodate oxidation and

(b) subjecting the compound resulting from (a) to a reduction.

8. The process as claimed in claim 7, wherein the said periodate oxidation is performed with a metaperiodate, optionally used in excess.

9. The process as claimed in claim 8, wherein sodium metaperiodate is used.

10. The process as claimed in any one of claims 7 to 9 wherein said reduction is performed with $NaBH_4$, optionally used in excess.

11. An immunostimulant comprising a compound as claimed in any one of claims 1 to 4.

12. A pharmaceutical composition which comprises by way of the active principle at least one compound as claimed in any one of claims 1 to 4, or its derivatives, in admixture or otherwise associated with a pharmaceutically acceptable diluent or carrier.

13. A polysaccharide compound or derivative thereof according to any one of claims 1 to 6, process for obtaining said compounds or derivatives, use thereof as an immunostimulant or as an active ingredient in pharmaceutical compositions substantially as hereinbefore described with reference to the examples.

DATED this 9th day of August, 1991.

PIERRE FABRE MEDICAMENT.

CARTER SMITH & BEADLE

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Camberwell, Victoria 3124,

Australia.