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ABOGADOS

EDIFICIO SISKI

WEB SITE: www.cavelier.com
E-MAIL: cavelier@cavelier.com

CARRER
BOGOT,

SUPERINDUSTRIA Y COMERCIO

(57) (1) 347-3611

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Dependencia: 2000 DIVISION DE NUEVAS CREACIONES

Señora Jefe de la
DIVISION DE NUEVAS CREACIONES
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
E. S. D.

Referencia : Expediente No. 01.101.336

Solicitante : BOMBUND GRUPO ASESOR, S.L.

Asunto : Solicitud de patente para: "COMPUESTO
POLISACÁRIDO CON ACTIVIDAD
INMUNOESTIMULANTE"

Fecha de solicitud : 26 de noviembre de 2001.

TRAMITE: 002 - EVENTO: 001 - ACTUACIÓN: 329

1. Yo, Emilio FERRERO, obrando como apoderado de la solicitante en referencia, atentamente presento con este escrito la copia certificada de la solicitud de patente PCT No. PCT/IB00/01946 del 27 de noviembre de 2000, junto con su respectiva traducción de sus reivindicaciones y carátula, de la cual se reclama prioridad en esta solicitud de patente.

2. Ruego a la Señora Jefe se sirva aceptar este escrito con sus anexos, y se continúe con el trámite de la solicitud.

Bogotá, 21 MAR. 2002
Señora Jefe,



EMILIO FERRERO

T.P.A. No. 31.929

COLO 13059-801-001-0
MRE-8



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**PATENT COOPERATION TREATY (PCT)
TRAITÉ DE COOPÉRATION EN MATIÈRE DE BREVETS (PCT)**

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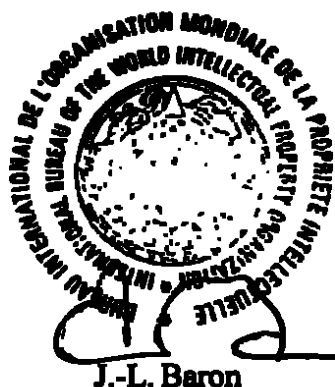
International Application No. } PCT / IB 00 / 0 1 9 4 6 International Filing Date } 27 NOVEMBER 2000
Demande internationale n° } Date du dépôt international } (27. 11. 00)

Geneva/Genève,

**12 DECEMBER 2001
(12. 12. 01)**

**International Bureau of the
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J.-L. Baron
Head, PCT Receiving Office Section
Chef de la section "office récepteur du PCT"

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

PCT / IB 00 / 01948	
International Application No.	
27 NOVEMBER 2000	(27. 11. 00)
International Filing Date	
INTERNATIONAL BUREAU OF WIPO	
PCT International Application	
Name of receiving Office and "PCT International Application"	
Applicant's or agent's file reference (if desired) (12 characters maximum) 2000412	

Box No. I TITLE OF INVENTION	
POLYSACCHARIDE COMPOUND HAVING IMMUNE STIMULATING ACTIVITY	
Box No. II APPLICANT	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)	
BOMSUND GRUPO ASESOR, S.L. C/Mallorca 235, ppic. 1º 08008 BARCELONA SPAIN	
☐ This person is also inventor.	
Telephone No.	
Facsimile No.	
Teleprinter No.	
State (that is, country) of nationality: ES	State (that is, country) of residence: ES
This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box	
Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)	
FRIAS PEÑA, José Manuel C/Mallorca 235, ppic. 1º 08008 BARCELONA SPAIN	
This person is: ☐ applicant only ☒ applicant and inventor ☐ inventor only (If this check-box is marked, do not fill in below.)	
State (that is, country) of nationality: ES	State (that is, country) of residence: ES
This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box	
☐ Further applicants and/or (further) inventors are indicated on a continuation sheet.	
Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE	
The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)	
CARPINTERO LÓPEZ, Francisco Herrero & Asociados, S.L. C/Alcalá, 35 28014 MADRID SPAIN	
Telephone No. +34915227420	
Facsimile No. +34915226249	
Teleprinter No.	
☐ Address for correspondence: Mark this check-box where no agent or common representative has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.	

Box No.V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) (mark the applicable check-boxes; at least one must be marked):

Regional Patent

- ☒ AP ARIPO Patent: GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, MZ Mozambique, SD Sudan, SL Sierra Leone, SZ Swaziland, TZ United Republic of Tanzania, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- ☒ EA Eurasian Patent: AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- ☒ EP European Patent: AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, CY Cyprus, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- ☒ OA OAPI Patent: BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, GW Guinea-Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT (if other kind of protection or treatment desired, specify on dotted line)

National Patent (if other kind of protection or treatment desired, specify on dotted line):

- | | |
|--|---|
| ☒ AE United Arab Emirates | ☒ LC Saint Lucia |
| ☒ AG Antigua and Barbuda | ☒ LK Sri Lanka |
| ☒ AL Albania | ☒ LR Liberia |
| ☒ AM Armenia | ☒ LS Lesotho |
| ☒ AT Austria | ☒ LT Lithuania |
| ☒ AU Australia | ☒ LU Luxembourg |
| ☒ AZ Azerbaijan | ☒ LV Latvia |
| ☒ BA Bosnia and Herzegovina | ☒ MA Morocco |
| ☒ BB Barbados | ☒ MD Republic of Moldova |
| ☒ BG Bulgaria | ☒ MG Madagascar |
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| ☒ BY Belarus | ☒ MN Mongolia |
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| ☒ CN China | ☒ NO Norway |
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| ☒ GD Grenada | ☒ TJ Tajikistan |
| ☒ GE Georgia | ☒ TM Turkmenistan |
| ☒ GH Ghana | ☒ TR Turkey |
| ☒ GM Gambia | ☒ TT Trinidad and Tobago |
| ☒ HR Croatia | ☒ TZ United Republic of Tanzania |
| ☒ HU Hungary | ☒ UA Ukraine |
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| ☒ IN India | ☒ UZ Uzbekistan |
| ☒ IS Iceland | ☒ VN Viet Nam |
| ☒ JP Japan | ☒ YU Yugoslavia |
| ☒ KE Kenya | ☒ ZA South Africa |
| ☒ KG Kyrgyzstan | ☒ ZW Zimbabwe |
| ☒ KP Democratic People's Republic of Korea | Check-box reserved for designating States which have become party to the PCT after issuance of this sheet |
| ☒ KR Republic of Korea | ☐ |
| ☒ KZ Kazakhstan | |

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. (Confirmation (including fees) must reach the receiving Office within the 15-month time limit.)

Box No. VI PRIORITY CLAIM				
☐ Further priority claims are indicated in the Supplemental Box.				
Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is:		
		national application: country	regional application: regional Office	international application: receiving Office
item (1)				
item (2)				
item (3)				

☐ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) (only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office) identified above as item(s):

* Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(1)). See Supplemental Box.

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) (if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):

ISA / EP

Request to use results of earlier search; reference to that search (if an earlier search has been carried out by or requested from the International Searching Authority):

Date (day/month/year)

Number

Country (or regional Office)

Box No. VIII CHECK LIST; LANGUAGE OF FILING

This international application contains the following number of sheets:

request : 3

description (excluding sequence listing part) : 12

claims : 3

abstract : 1

drawings : 21

sequence listing part of description :

Total number of sheets : 40

This international application is accompanied by the item(s) marked below:

1. ☐ fee calculation sheet2. ☐ separate signed power of attorney3. ☐ copy of general power of attorney; reference number, if any:4. ☐ statement explaining lack of signature5. ☐ priority document(s) identified in Box No. VI as item(s):6. ☐ translation of international application into (language):7. ☐ separate indications concerning deposited microorganism or other biological material8. ☐ nucleotide and/or amino acid sequence listing in computer readable form9. ☐ other (specify):

Figure of the drawings which should accompany the abstract:

Language of filing of the international application: ENGLISH

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which that person signs (if such capacity is not obvious from reading the request).



FRANCISCO CARPINTERO LOPEZ

For receiving Office use only	
1. Date of actual receipt of the purported international application: 27 NOV 2000	2. Drawings: ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:	
4. Date of timely receipt of the required corrections under PCT Article 11(2):	
5. International Searching Authority (if two or more are competent): ISA / EP	6. ☒ Transmittal of search copy delayed until search fee is paid.

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POLYSACCHARIDE COMPOUND HAVING IMMUNE STIMULATING ACTIVITY

5

FIELD OF THE INVENTION

The present invention relates to a polysaccharide compound of formula I which present immune stimulating activity, to a process for obtaining it, to its use in the treatment of immune suppressant diseases and to pharmaceutical compositions containing it.

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BACKGROUND OF THE INVENTION

The immune system can be defined as the collection of molecules, cells and organs whose complex interactions form an emergent system which is generally able to protect an individual both from outside invaders and its own altered cells.

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The immune system can be separated into two functionally distinct parts: those elements which are innate and those which are acquired. Innate immunity refers to immune elements which are non-specific and non-adaptive. They are able to distinguish foreign tissues/organisms but are unable to recognise a particular invader. They can be best divided in barriers (skin and mucosa), non-specific chemical agents (enzymes present in mucusal secretions, quinines and histamines) and non-specific effector cells (macrophages). Acquired immunity refers to elements which are specific and adaptive. They are able to distinguish foreign cells from self, and can distinguish one foreign antigen from another. Acquired immunity has memory. This allows immunisation and resistance to reinfection with the same microorganism. The cells which are responsible for the acquired immunity are lymphocytes, of which there are two subclasses: B and T-cells.

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The acquired immunity can be attained either by natural infection or vaccination (in an active way) or by administration of immune cells (in a passive way). While the first way presents long lasting effects and can be even permanent, the later is not long lasting.

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Patients presenting immune-suppressant diseases are treated with immune stimulants in order to activate their immune system. Different immune stimulants are

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Scheme 1



wherein $n = 7$ to 8 .

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polysaccharide by a employing a combination of separation techniques guided by bioassay.

5 A further aspect of the invention relates to the use of the present polysaccharide compound of formula I as therapeutic agent in the treatment of immune suppressant diseases such as cancer, tuberculosis, influenza, common cold, allergies, lupus erythematosus, psoriasis and AIDS.

10 A further aspect of the invention relates to pharmaceutical compositions comprising the polysaccharide compound of formula I.

The present application includes the present figures and tables:

Figure 1 is an schema of the isolation of PF2.

15 Figure 2 is an schema of the isolation of PF2R.

Figure 3 is the ^1H -RMN spectrum of PF2RS8A.

Figure 4 is the ^{13}C -NMR spectrum of PF2RS8A.

Figure 5 is the TLC of the hydrolysis products of PF2RS8A with TFA and other monosugars.

20 Figure 6 is an schema of the isolation of PF2RS8B2, i.e., the polysaccharide of formula I.

Figure 7 is the HPLC profile of PF2RS8B2 under evaporative light scattering detector.

Figure 8 is the HPLC profile of PF2RS8B2 under photodiode UV detector.

25 Figure 9 is the ^1H -RMN spectrum of PF2RS8B2.

Table 1 reflects the effect of samples PF2RS8A and PF2RS8B2 on Lymphocyte Transformation.

Figure 10 is the ^{13}C -NMR spectrum of PF2RS8B2, i.e., the polysaccharide of formula I.

30 Figure 11 is the DEPT-135 spectrum of PF2RS8B2.

Figure 12 is the DEPT-90 spectrum of PF2RS8B2.

Figure 13 is the HMQC spectrum of PF2RS8B2.

Figure 14 is the ^1H - ^1H COSY spectrum of PF2RS8B2.

Figure 15 is the ^1H - ^1H COSY spectrum of PF2RS8B2.

35 Figure 16 is the HMQC spectrum of PF2RS8B2.

Table 2 reflects the ^{13}C -NMR spectrum of PF2RS8B2.

Figure 17 is the HMBC spectrum of PF2RS8B2.

Figure 18 is the HMBC spectrum of PF2RS8B2.

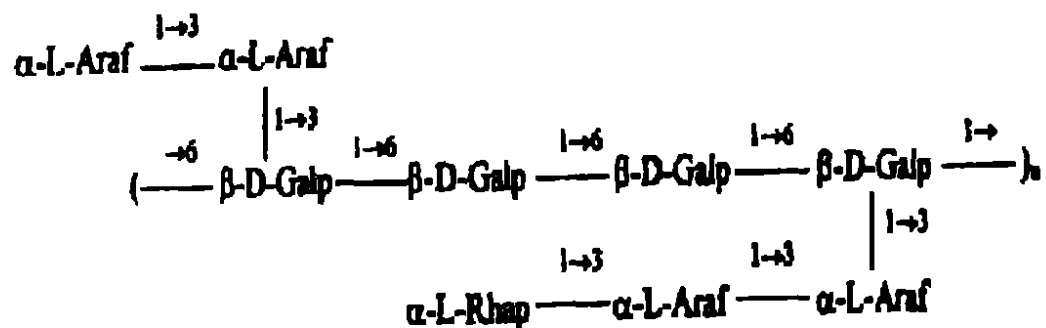
Figure 19 is the TLC of the hydrolysis products of PF2RS8B2 with TFA and other monosugars.

Figure 20 is the molecular weight measurement diagram.

DETAILED DESCRIPTION OF THE INVENTION

As mentioned above, the first aspect of the invention relates to a polysaccharide compound of formula I:

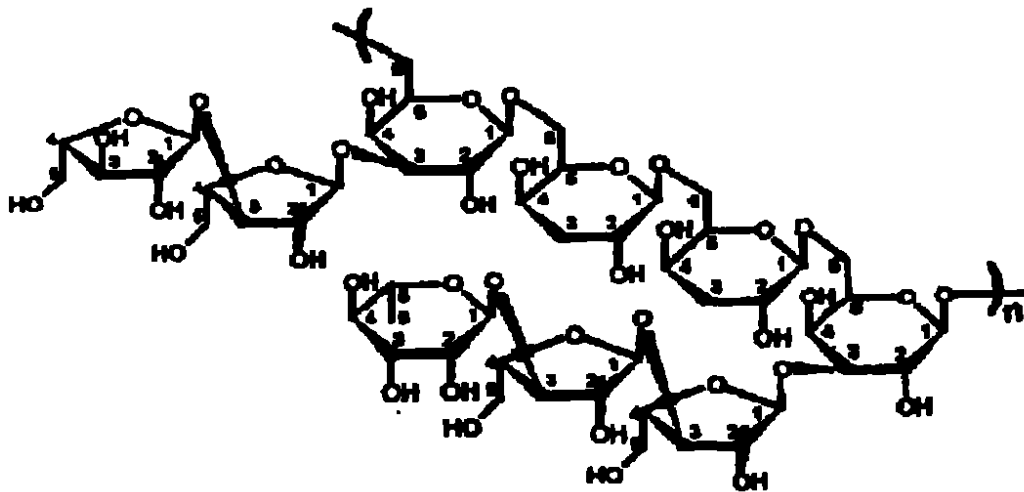
Scheme 1



wherein $n = 7$ to 8

The configurational structure of the compound of formula I is:

Scheme II



4

Figure 18 is the HMBC spectrum of PF2RS8B2.

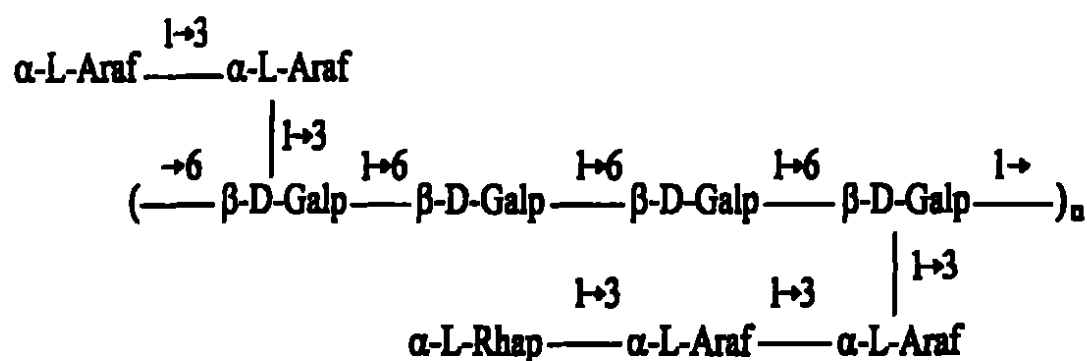
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Figure 20 is the molecular weight measurement diagram.

DETAILED DESCRIPTION OF THE INVENTION

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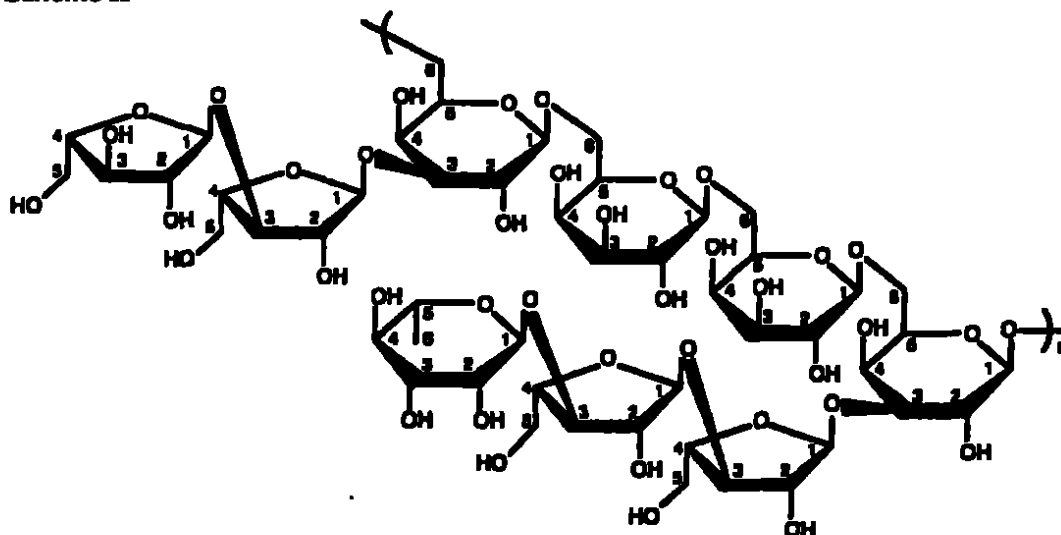
Scheme 1



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The configurational structure of the compound of formula I is:

Scheme II



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wherein $n = 7$ to 8

The polysaccharide compound present a mean molecular weight of about 10,000.

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The second aspect of the invention relates to the method for obtaining the polysaccharide compound of formula I. This method comprises firstly, obtaining an aqueous extract from the plant *Calendula officinalis* according to a method disclosed in a copending patent application of ours entitled "process for preparing aqueous
10 extracts of plants and extracts so obtained" and filed simultaneously with the present application of serial number ——— and, secondly, the isolation of the polysaccharide by a combination of separation techniques guided by bioassay.

The aqueous extract of the *Calendula officinalis* is obtained by subjecting
15 the flowers of this plant to the following process:

- a) Decontamination of the flowers
- b) Comminuting the flowers.
- c) Treatment of the comminuted flowers with a laser radiation.
- 20 d) Suspension of the mixture obtained in step c) in water.
- e) Maceration of the suspension obtained in step d).
- f) Separation of the resulting liquid.

The decontamination (step a) is effected by washing the flowers of
25 *Calendula officinalis* with water. The amount of water employed in this step is not determinant, and can be varied depending on the contamination state of the plant. Although higher and lower temperatures are not discarded, the water temperature should be between 10 and 40 °C, preferably between 20 and 35°C, and most preferably 28°C. A washing tunnel can be employed to facilitate this step. Both the
30 amount of water and the residence time of the plant in the washing tunnel is not determinant, and can therefore be varied depending on the contamination state of the plant. The washing step can be carried out several times, with a drying step in between. This drying step is preferably effected by placing the plant in the sun.

35 Once the flowers have been thoroughly decontaminated, they are comminuted (step b) by conventional methods such as a comminuting machine or

even manually. Although higher and lower temperatures are not discarded, the temperature at which the flowers are comminuted should be between 10 and 40 °C.

The comminuted flowers are next subjected to a treatment with laser radiation (step c). As source of the laser radiation, a red linear laser diode with a capability of harmonic generation in wavelengths within the range of 150 to 810 nm is preferably employed. The wavelength of the laser radiation is more preferably of 200 to 400 nm and most preferably of 250 nm. The power of the laser radiation is preferably of 1 to 60 watts, more preferably of 10 to 30 watts and most preferably of 20 watts. The spot is preferably of 1 to 6 mm, more preferably of 2 to 5 mm and most preferably of 4 mm of diameter. The comminuted plant is exposed to the laser radiation so that the whole or most of the mixture is irradiated. This is achieved either by displacing manually the laser generator through the comminuted plant, or by passing the comminuted matter on a conveyor belt through a set of several laser generators. Preferably each kilogram of the comminuted matter is treated with the laser radiation for a period of 3 to 10 minutes, more preferably for a period of 5 minutes. Although higher and lower temperatures are not discarded, the temperature at which the comminuted plant is treated with the laser radiation should be between 10 and 40 °C.

The laser treated matter is next suspended in water (step d). Any commercial mineral water can be employed in this step. The suspension is effected so that 50 to 300, preferably 100 to 250, grams of the laser treated matter are present per litre of water. Although higher and lower temperatures are not discarded, the temperature at which the comminuted plant is suspended in water should be between 10 and 40 °C.

The suspension is then kept for a period of between 5 to 20 days, preferably of 7 to 15 days, at a temperature of 2 to 10 °C, preferably of 4 to 8°C, so that maceration of the mixture takes place (step e).

Finally, after the maceration step, a separation of the liquid phase from the solid phase is effected (step f). The solids can be pressed to facilitate the separation. The separation can be achieved by decantation alone or, preferably, by decantation followed by filtration. The filtration is preferably effected under pressure. Most preferably three consecutive press-filtrations are effected with filters of 5 µm, 1 µm

and 0.22 μ m. Although higher and lower temperatures are not discarded, the temperature at which the separation is effected should be between 10 and 40 °C.

An aqueous extract of an ochre colour is finally obtained.

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The extract thus obtained is next subjected to an isolation process which comprises precipitation with methanol, centrifugation and chromatographic separation guided by bioassay. The bioassay employed were performed in vitro by adding the samples to lymphocytes isolated from mice according to the literature reference
10 *Mux. W. et al., Journal of Natural Products, vol. 54, no. 6, pp. 1531-1542 (1991)*. The incorporation of thymidine, which means replication of DNA, was monitored. This incorporation is indicative both of an increase in lymphocyte number and an increase in lymphocyte activity. These separation techniques employed include repeated precipitation with ethanol, centrifugation, dialysis and/or column chromatography.
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Thus, 112 litres of the aqueous extract previously obtained were lyophilised to yield 800 g of a tan coloured powder ("PF2"). This powder was fractionated into a MeOH-soluble and a MeOH-insoluble residue (PF2R, 350 g), which exhibit
20 lymphocyte transformation activity. A portion (80 g) of this material was subjected to MeOH precipitation, centrifugation, dialysis and/or column chromatography on Sephadex DEAE led to the isolation of a number of crystalline materials, which were determined to be inactive inorganic salts (figure 1).

25 A portion (270 g) of PF2R which was subjected to precipitation with MeOH to final concentrations of 25, 50 and 67% led to active precipitates. The most active material was the precipitate obtained from the 50% MeOH solution (PF2RS8 51.2 g, LT= +1059 %). Work up of a 5 g portion of PF2RS8 solubilised in water, followed by centrifugation, and chromatographic separation of the supernatant on
30 Sephadex G-25 led to the active polysaccharide enriched fraction designated as PF2RS8A (0.13 g, LT = +1074 %). Subsequently, an identical fraction designated as PF2RS8A' (0.3 g) was obtained from a second portion (6 g) of the 50 % MeOH precipitate. As indicated in figure 2, a number of other fractions and crystalline materials were isolated, however, none showed the level of LT activity of PF2RS8A.

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PF2RS8A was characterised by spectroscopic (^1H , ^{13}C -NMR, and DEPT spectra) and chemical analysis (hydrolysis with TFA and analysis on silica gel TLC). See Figures 3, 4 and 5.

More active polysaccharide mixture PF2RS8A (1.4 g) was isolated from the remaining 50% MeOH precipitate (PF2RS8). At the same time, work-up of a portion (2.0 g) of the 67% MeOH insoluble fraction (PF2RS9; LT = +735%) led to the isolation of a polysaccharide mixture (PF2RS9A, 0.3g), which was showed by ^1H -NMR analysis to be identical as PF2RS8A. Thus, PF2RS9A (0.2 g) was combined with PF2RS8A (1.4 g), and the mixture, designated as "PF2S8B", was subjected to further separation with sephadex G-50 (20-80 mm) as showed in figure 6. Elution with water yielded six fractions (PF2RS8B1, 2, 3, 4, 5 and 6), which on HPLC analysis showed that only the major isolate PF2RS8B2 to be homogeneous using both evaporative light scattering and UV detection (figures 7 and 8). ^1H and ^{13}C -NMR spectral analysis (figures 9 and 10) showed spectra of this isolate to be very similar to those obtained for PF2RS8A (figures 3 and 4), which implied that it is the major polysaccharide in the mixture. The fraction PF2RS8B2 consists therefore of the polysaccharide of formula I and water. The latter can be eliminated by methods known in the art.

Bioassay of the homogeneous isolate PF2RS8B2 and its parent mixture PF2RS8A in the same assay showed lymphocyte transformation (LT) activities of 6203% and 3532%, respectively (Table 1). The higher level of activity showed by the isolate implied that this polysaccharide is the major active constituent for the biological effect of the present extract.

The active polysaccharide PF2RS8B2 (polysaccharide of formula I) was elucidated by spectroscopic (^1H , ^{13}C -NMR, HMQC and 2D ^1H - ^1H COSY spectra) and chemical analysis (hydrolysis with TFA and analysis on silica gel TLC). Thus, The ^1H -RMN signals (Figure 9) at δ 5.3 and 3.2 ppm is indicative of a polysaccharide. The ^{13}C -NMR signals (Figures 10-12) at δ 111.9 (d), 110.1 (d) ppm were assigned to the anomeric carbons of 1 $\rightarrow$ 3 linked α -L-arabinofuranose and terminal α -L-arabinofuranose (expressed Araf and Araf' respectively). The signals at δ 106.1 (d) and 105.9 (d) ppm were assigned to the anomeric carbons of 1 $\rightarrow$ 6 linked β -D-galactopyranose and 1 $\rightarrow$ 3, 1 $\rightarrow$ 6 linked β -D-galactopyranose (expressed as Galp' and Galp respectively), whereas, the signal at δ 100.2 (d) ppm was assigned to

the anomeric carbons of α -L-rhamnopyranose (expressed as Rhap). The anomeric proton signals (H-1) were also easily recognised due to their relative low-field shift in the ^1H -RMN spectra. The direct correlation between proton and carbon-13 signals observed in HMQC spectrum (Figure 13) determined the ^1H -RMN signals of δ 5.08 (brs), 5.23 (brs), 4.47 (d, $J=7.9$ Hz), 4.53 (d, $J=7.3$ Hz) and 5.1 (brs) ppm to be the anomeric protons of α -L-arabinofuranose (Araf'), α -L-arabinofuranose (Araf), β -D-galactopyranose (Galp'), β -D-galactopyranose (Galp) and α -L-rhamnopyranose (Rhap). Employing these signals as reference, other proton signals could be traced by analysing 2D ^1H - ^1H COSY spectra (figures 14,15). Similarly, the corresponding carbon signals were defined by HMQC spectra (figures 13, 16 and Table 2). The sequencing of the sugar units was determined as follows. The down field shift of the signal at C-3 of Araf (δ 79.4) and Galp (δ 82.8), and the signals at C-6 of Galp and Galp' (δ 69.2) suggested that these carbons were connected to other sugar units. The observation of long-range correlations between C-1 of Araf and C-6 of Galp and Galp' in the HMBC spectrum (Figure 17) suggested a 1 $\rightarrow$ 6 linked β -D-galactopyranose backbone. The Araf was 1 $\rightarrow$ 3 linked to Galp by the observation of long-range correlation between C-1 of Araf and C-3 of Galp (Figure 18). From the ^1H -RMN spectrum (Figure 9), the ratio of the sugars was calculated according to the integration values of anomeric protons peaks to be Araf:Araf':Galp:Galp':Rhap/ 3:1:2:2:1. PF2RS8B2 was therefore elucidated as a large branched chain polysaccharide with the primary structure as shown in formula I (scheme I). Scheme II is a representation of the same structure showing the stereochemical configuration of the individual sugars.

To confirm the identities of the constituent sugars, PF2RS8B2 was hydrolysed with TFA (0.5 M, 100-120 $^{\circ}\text{C}$) followed by TLC analysis (Figure 19). The presence of the major sugars α -L-arabinofuranose and β -D-galactopyranose was easily discerned, whereas the presence of the minor sugar, α -L-rhamnopyranose, was detected with more difficulties, probably due to the quantity of the hydrolysate applied to the TLC plate.

The molecular weight of the PF2RS8B2 was estimated by gel permeation (size exclusion) chromatography. The mean molecular weight thus estimated of PF2RS8B2 is of 10,000 (See figure 20).

The polysaccharide compound of formula I unexpectedly presents very high activity as immune stimulator, as reflected in the example below. The third aspect of the invention relates, thus, to the use of the polysaccharide compound of formula I as therapeutic agent in the treatment of immune-suppressant diseases such as cancer, tuberculosis, influenza, common cold, allergies, lupus erythematosus, psoriasis and AIDS. Non limitative examples of cancers are hepatic carcinoma, lung cancer, kidney cancer, colon cancer, breast cancer, prostate cancer or prostatic adenocarcinoma; brain cancers such as astrocytoma and glioblastoma; cervix cancer and blade cancer.

The fourth aspect of the invention relates to pharmaceutical compositions comprising the polysaccharide compound of formula I.

The polysaccharide according to the present invention can be administrated either separately as a pure substance or in the form of pharmaceutical preparations, even though the compound of the invention is preferably administered in a combination. The drug combination is preferably in the form of a formulation which (1) contains the polysaccharide according to the invention alone; (2) contains one or more appropriate binders, carriers and/or further auxiliary materials, and (3) may further contain additional therapeutically active substances.

The carriers materials, binders and/or auxiliary materials must be pharmaceutically and pharmacologically tolerable, so that they can be combined with the other components of the formulation or preparation and do not exert adverse effects on the organism treated.

The formulations include those which are suitable for oral or parenteral (including subcutaneous, intradermal, intramuscular and intravenous) administration, even though the best route of administration is dependent on the patient's status.

The formulations can be in the form of single doses. The formulations are prepared according to methods known in the field of pharmacology. The appropriate quantities of active substances suitable for administration may vary as a function of the particularly field therapy. In general, the active substance concentration in a single-dose formulation is 5% to 95% of the total formulation.

The invention provided by the application is illustrated by the examples presented hereinbelow.

Example 1: Preparation of an aqueous extract of flowers of *Calendula officinalis* according to the method of the invention.

500 g. of flowers of *Calendula officinalis* are placed in a wash tunnel and subjected to a thorough wash with water at about 28°C. The flowers are next comminuted with a comminuting machine. The resultant 500 g of comminuted matter are subjected to a treatment with a red linear laser diode with a capability of harmonic generation in a wavelength of 250 nm, a power of 20 watts and a spot of 4 mm of diameter. The treatment is effected by manually displacing the laser generator through the comminuted matter during 2.5 minutes, so that the whole or most of the mixture is irradiated. The laser treated matter is next suspended in 2 litres of water at a temperature of about 20 °C. The suspension is then kept for 12 days at a temperature of 4 °C. Finally, the separation of the liquid and the solid phase is effected, first by decantation of the liquid (the solids are pressed to facilitate the separation), and then, by three consecutive press-filtrations with filters of 5, 1 and 0.22 µm at a temperature of about 20 °C. The process yields approximately 1.7 litres of a solution (aqueous extract) of an ochre colour.

Example 2: The isolation of the polysaccharide of formula I is effected as disclosed on pages 7-8 of the description.

Example 3: The polysaccharide of formula I was tested in order to establish its activity as immune stimulator by quantifying the lymphocyte transformation activity (LTA). By lymphocyte transformation activity it is meant the fact that the lymphocytes are transformed from a dormant to an active state, which is necessary to fight diseases through an immunological mechanism, or to restore the immune system, which might be weakened by different factors. The tests were performed according to the literature reference *Max. W. et al., Journal of Natural Products, vol. 54, no. 6, pp. 1531-1542 (1991)*, by adding in vitro a solution of the polysaccharide of the invention to lymphocytes isolated from mice. The incorporation of thymidine, which means replication of DNA, was monitored. This incorporation is indicative both of an increase in lymphocyte number and an increase in lymphocyte activity.

The polysaccharide of formula I presents a LTA of +6203 % with respect to non stimulated lymphocytes.

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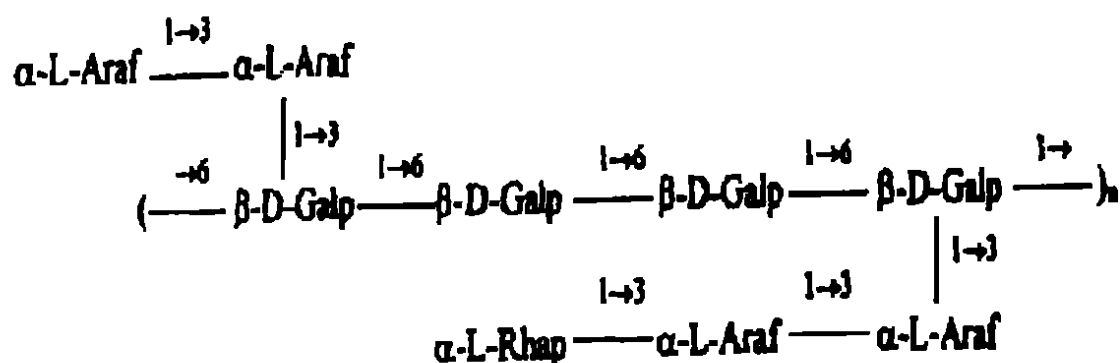
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CLAIMS

1. Polysaccharide according to the formula I,



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- 25

- i) Separation of the solid phase from the liquid phase.
j) Precipitating the solid phase obtained in step i) with methanol to final concentrations of 25%, 50% and 67%.
k) Solubilising in water the precipitates obtained in step j) at 50% and 67%, centrifugating and effecting a chromatographic separation of the supernatant.
l) Identifying the active fraction by bioassay.
m) Effecting a second chromatographic separation of the active fraction.
n) Identifying the active fraction by bioassay.
o) elimination of the eluent.
4. Method according to claims 2 and 3, wherein the treatment with the laser radiation is effected with a red linear laser diode with a capability of harmonic generation in wavelengths within the range of 150 to 810 nm, a power of 1 to 60 watts and a spot of 1 to 6 mm of diameter.
5. Method according to claim 4, wherein the wavelength is within the range of 200 to 400 nm, preferably 250 nm, the power of 20 watts and the spot of 4 mm of diameter.
6. Method according to any precedent claim, wherein each Kilogram of the comminuted matter is treated with the laser radiation for a period of 3 to 10 minutes, preferably for a period of 5 minutes.
7. Compound defined in claim 1 for use as therapeutic agent.
8. Compound according to claim 1 for use in the treatment of immune suppressant diseases.
9. Compound according to claim 1 for use in the treatment of immune-suppressant diseases such as cancer, tuberculosis, influenza, common cold, allergies, lupus erythematosus, psoriasis and AIDS.
10. Compound according to claim 1 for use in the treatment of hepatic carcinoma, lung cancer, kidney cancer, colon cancer, breast cancer, prostate cancer or prostatic adenocarcinoma; brain cancers such as strocytoma and glioblastoma;

cervix cancer and blade cancer.

11. Use of the compound defined in claim 1 for the manufacture of a medicament.

12. Use according to claim 11 for the manufacture of a medicament for treating immune suppressant diseases .

13. Use according to claim 12 for the manufacture of a medicament for treating cancer, tuberculosis, influenza, common cold, allergies, lupus erythematosus, psoriasis and AIDS.

14. Use according to claim 13 for the manufacture of a medicament for treating hepatic carcinoma, lung cancer, kidney cancer, colon cancer, breast cancer, prostate cancer or prostatic adenocarcinoma; brain cancers such as strocytoma and glioblastoma; cervix cancer and blade cancer.

15. A pharmaceutical preparation comprising a compound according to claim 1.

16. The pharmaceutical preparation according to claim 15 further including a pharmaceutically acceptable vehicle.

17. The pharmaceutical preparation of claims 15 and 16 further including at least another pharmaceutically active compound.

POLYSACCHARIDE COMPOUND HAVING IMMUNE STIMULATING ACTIVITY

ABSTRACT

The present invention relates to a polysaccharide compounds of formula I which present immune stimulating activity, a process for their preparation, their use in immune-suppressant diseases and pharmaceutical compositions containing them.

Formula I,
wherein n = 7 to 8

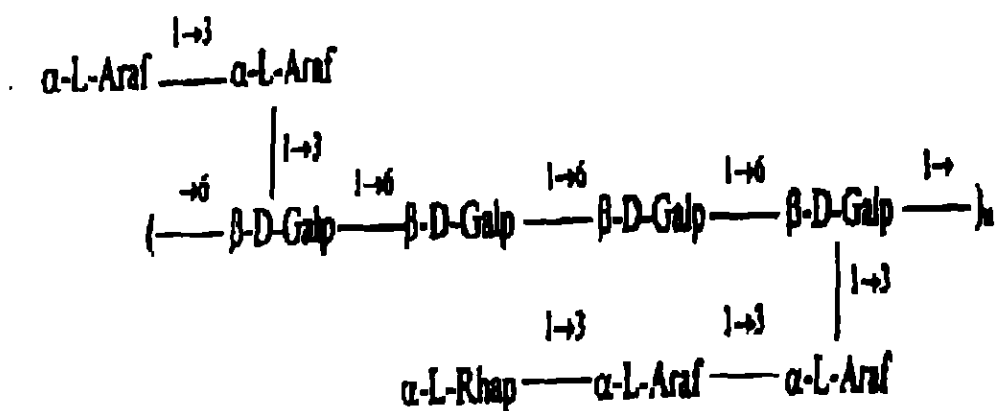
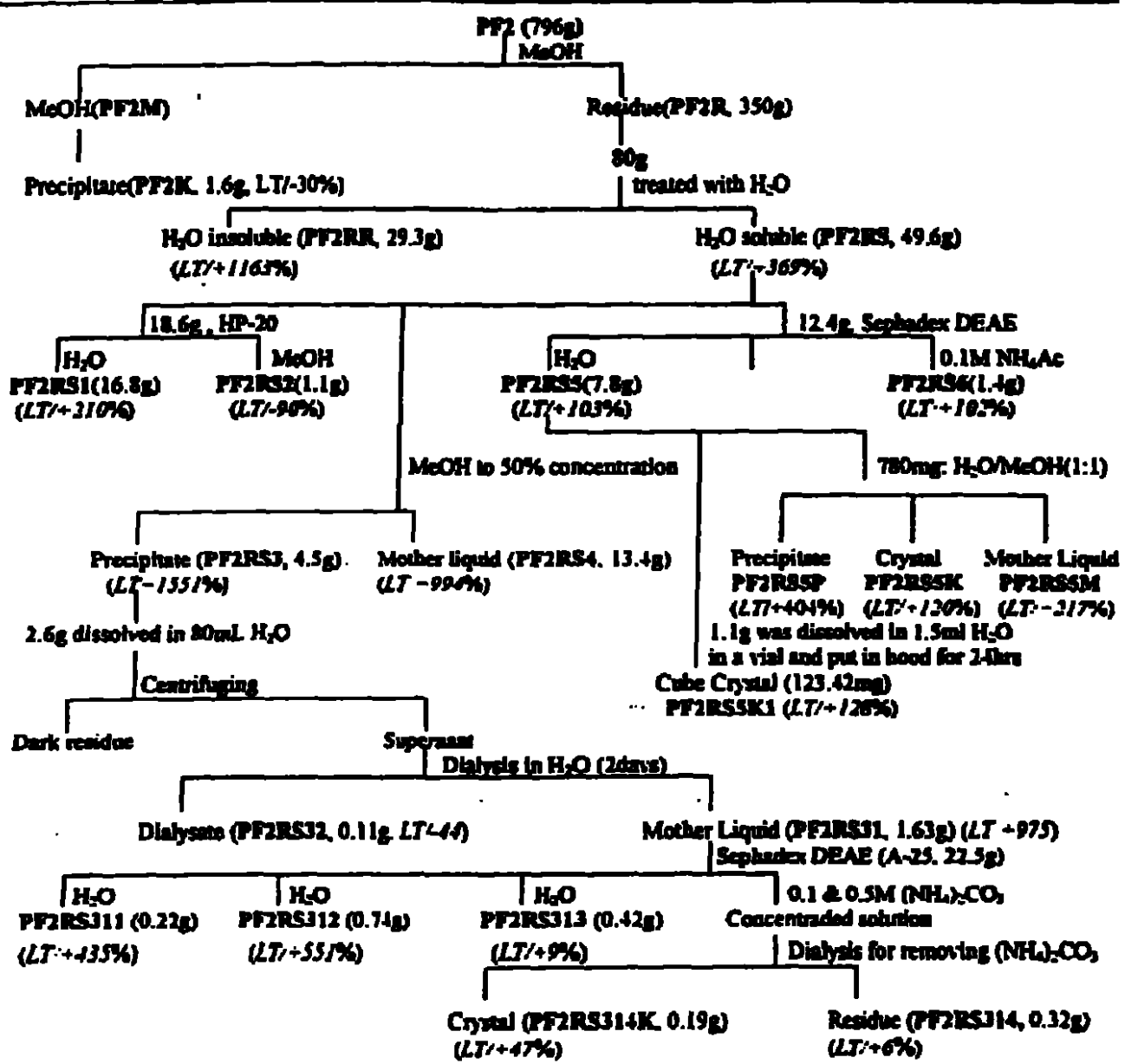
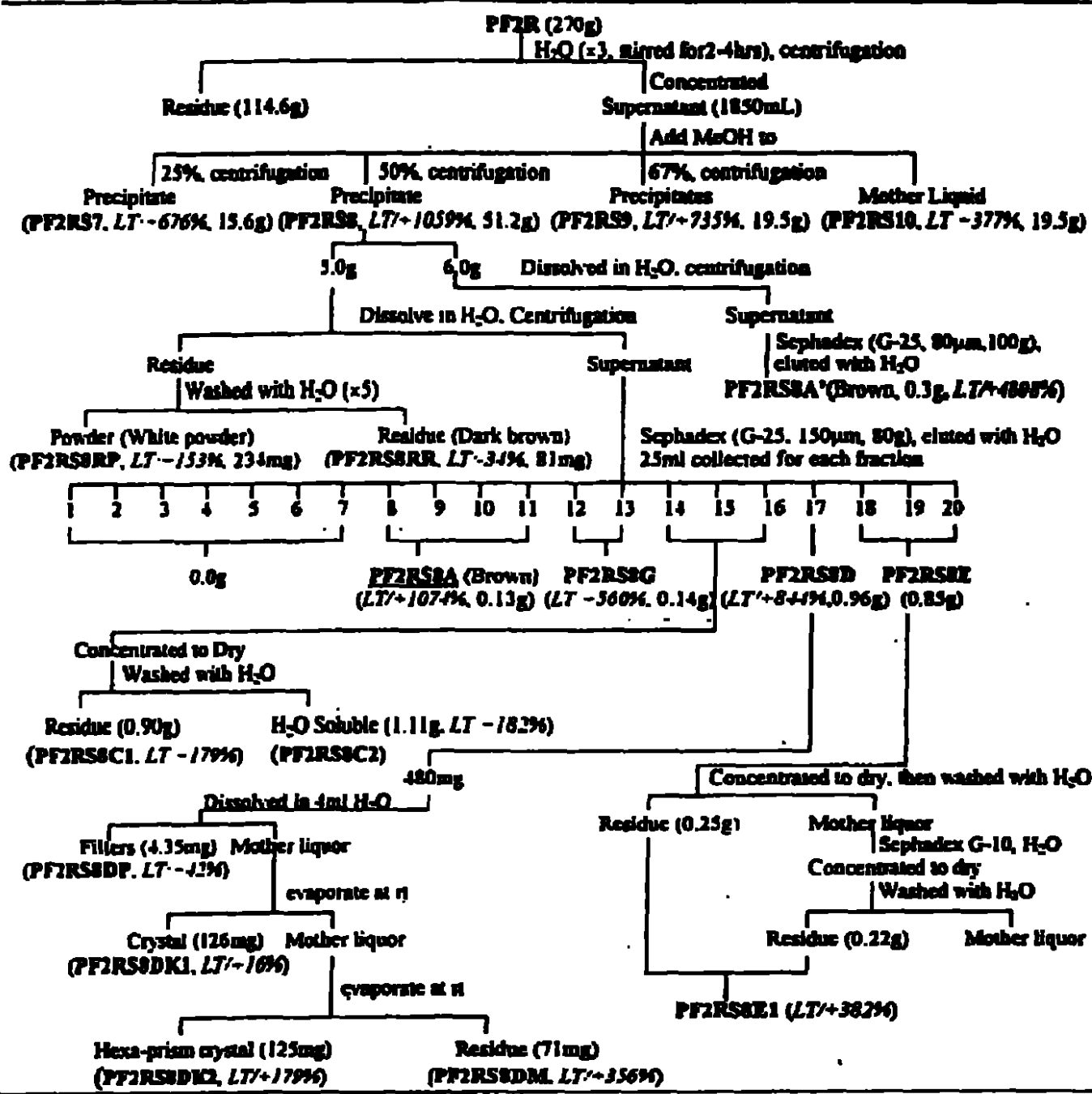


Figure 1. Bioactivity-directed phytochemical isolation for PF2



LT: Lymphocyte transformation activity

Figure 2. Bioactivity-directed phytochemical isolation for PF2R



LT: Lymphocyte transformation activity

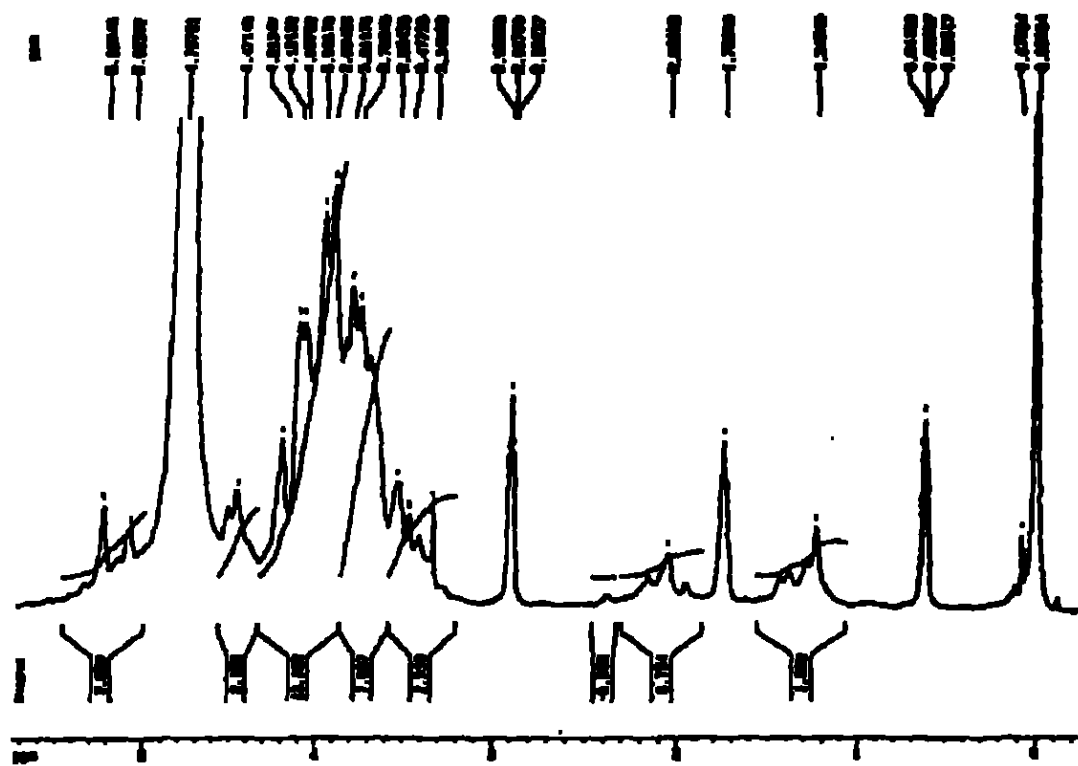


Figure 3. The ¹H NMR spectrum of PF2RS8A (500MHz, D₂O, referred to DSS)

4/21

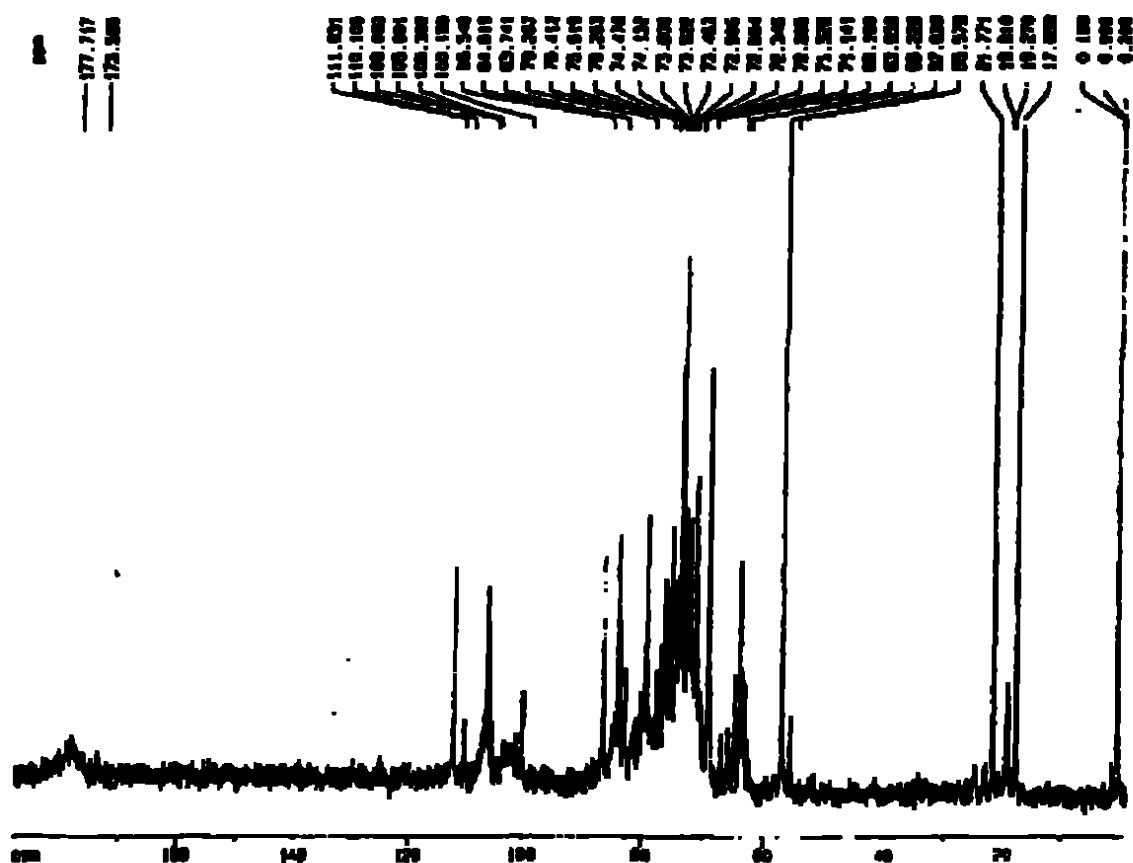
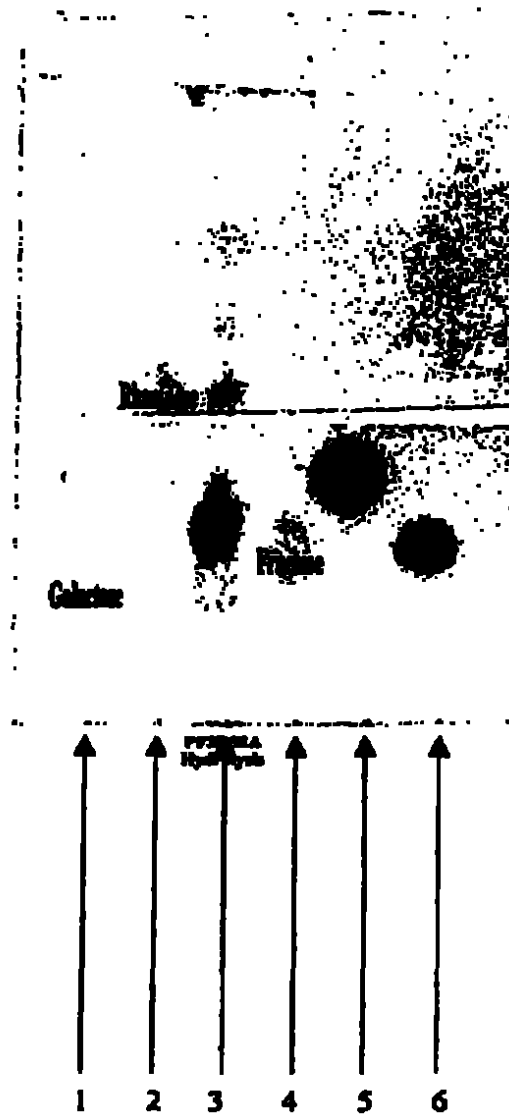


Figure 4. The ^{13}C NMR spectrum of PF2RS8A (125MHz, D_2O , referred to DSS)

5/21

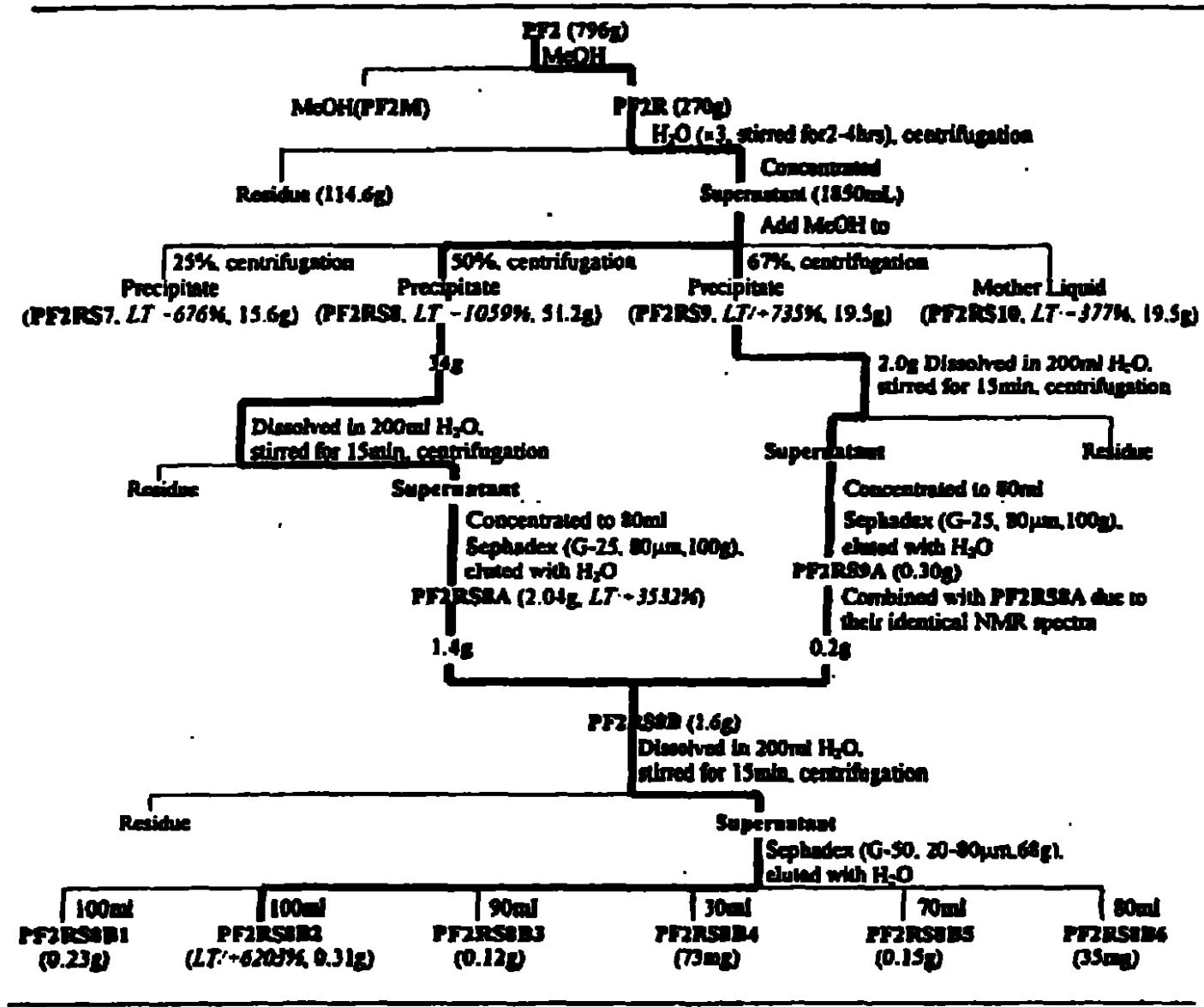


1. Galactose; 2. Rhamnose; 3. The hydrolysis products of PF2RS8A; 4. Fructose; 5. Xylose; 6. Glucose.

Figure 5. TLC of hydrolysis products of PF2RS8A with TFA and other monosugars.

6/21

Figure 6. Bioactivity-directed phytochemical isolation of active polysaccharide



7/21

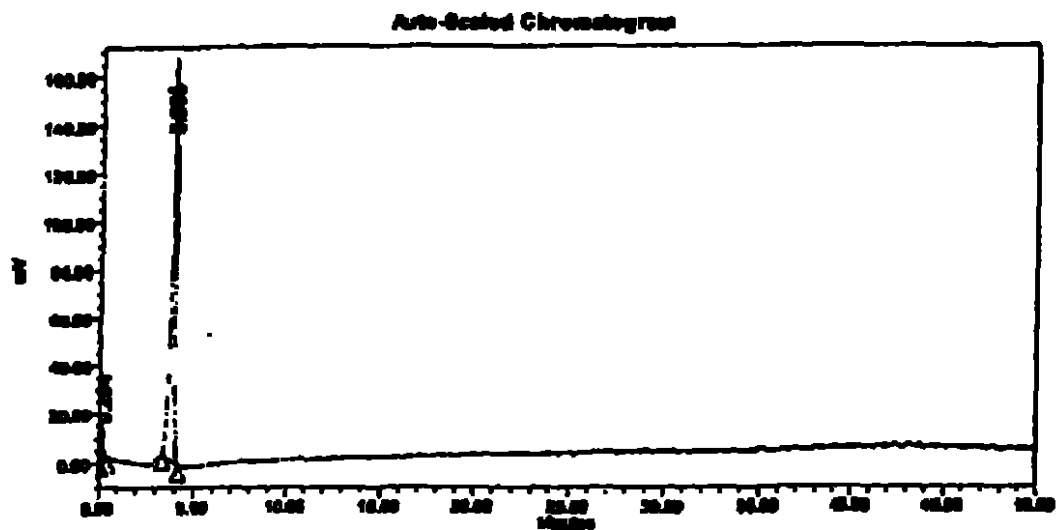


Figure 7. The HPLC profile of PF2RS8B2 under evaporative light scattering detector (Watrex GMB 200, 250x8mm, 5 μ m, 1.0ml/min, H₂O)

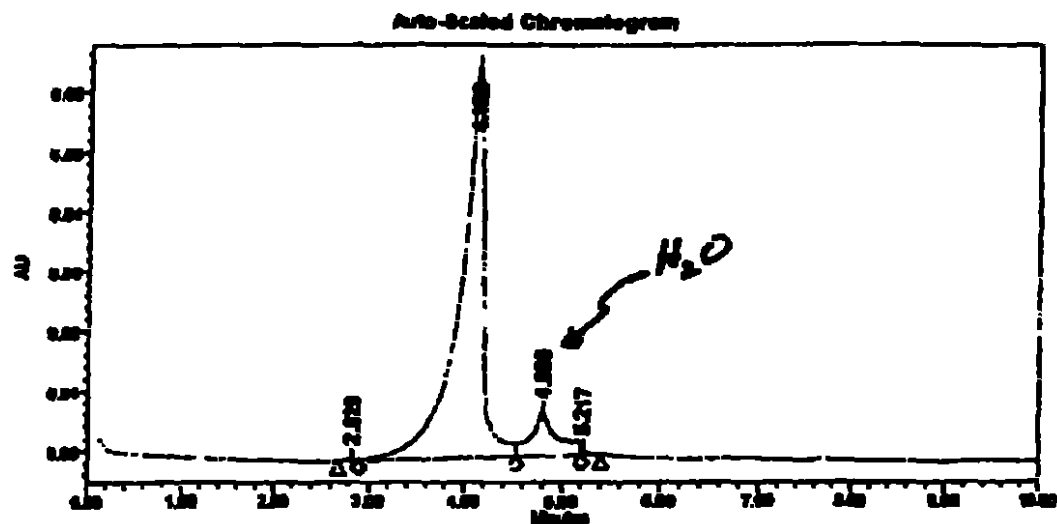


Figure 8. The HPLC profile of PF2RS8B2 under photodiode UV detector (Watrex GMB 200, 250x8mm, 5 μ m, 1.0ml/min, H₂O)

8/21

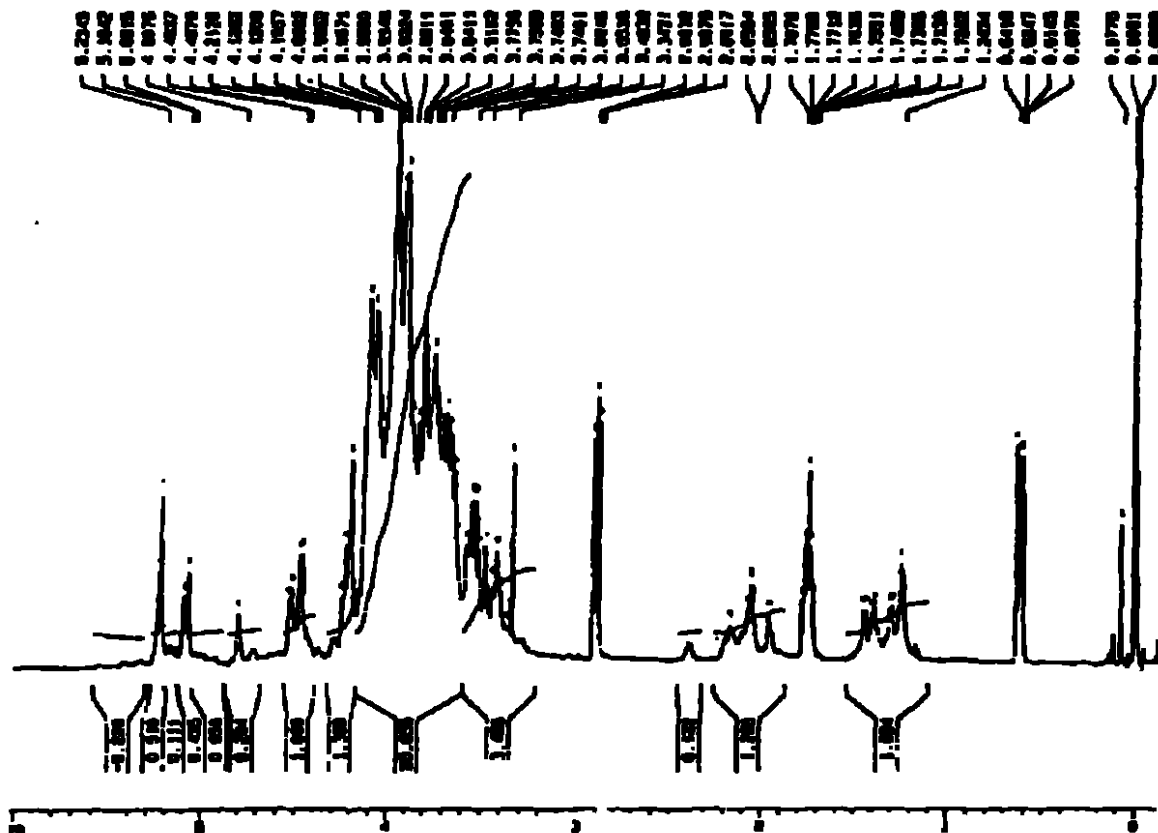


Figure 9. The ^1H NMR spectrum of PF2RS8B2 (500MHz, D_2O , referred to DSS)

9/21

Table 1. Effect of Samples PF2RS8A and PF2RS5B2 on Lymphocyte Transformation (Mouse SMC, Female)

Concentration of Compound	Sample							
	No ConA				No ConA			
	PF2RS8A		PF2RS5B2					
	Mean±S.D	%	Mean±S.D.	%	Mean±S.D.	%	Mean±S.D.	%
0	165 ± 31	0	197 ± 51	0				
0.01	311 ± 41	+91	205 ± 49	+24				
0.1	371 ± 51	+127	361 ± 76	+83				
1	556 ± 26	+237	649 ± 48	+229				
10	3829 ± 380	+2220	2348 ± 305	+1092				
100	5993 ± 1760	+3532	12417 ± 3153	+6203				
Con A Bug/ml	1762± 349		1507 ± 250					

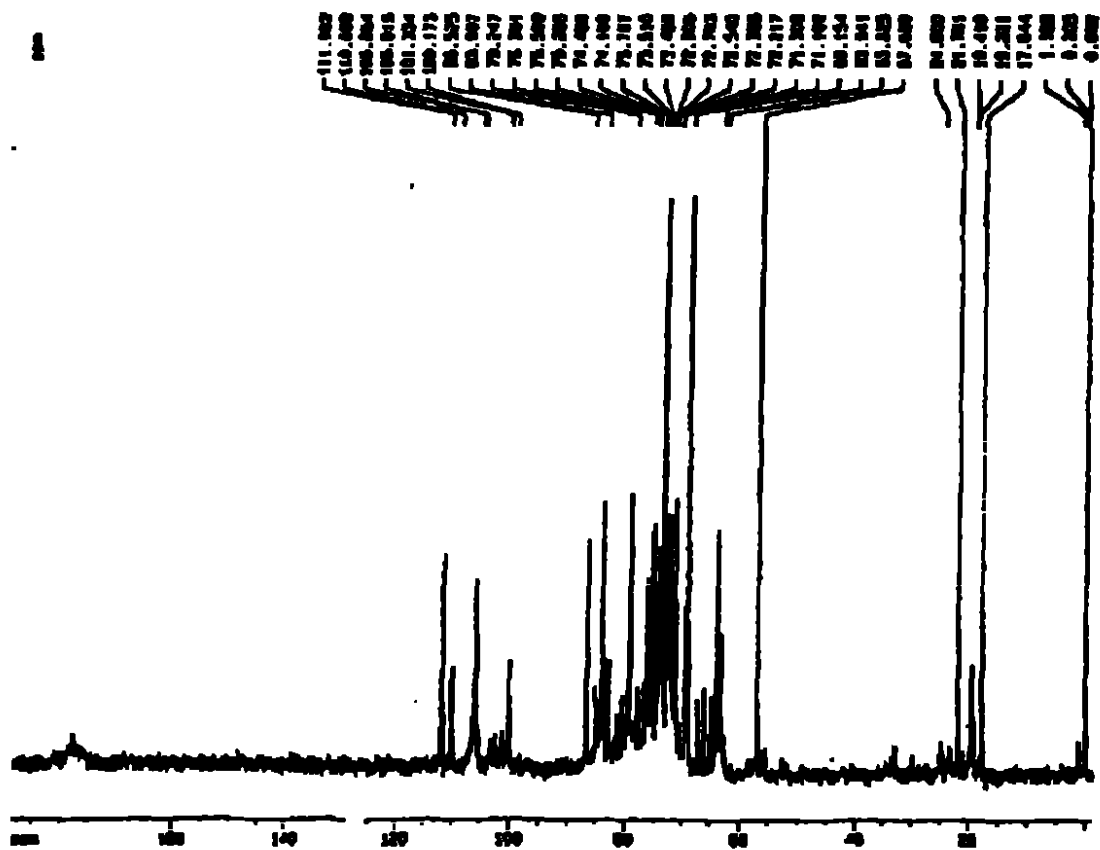


Figure 10. The ^{13}C NMR spectrum of PF2RS8B2 (125MHz, D_2O , referred to DSS)

87

11/21



Figure 11. The DEPT-135 spectrum of PF2RS8B2 (125MHz, D₂O, referred to DSS)

12/21

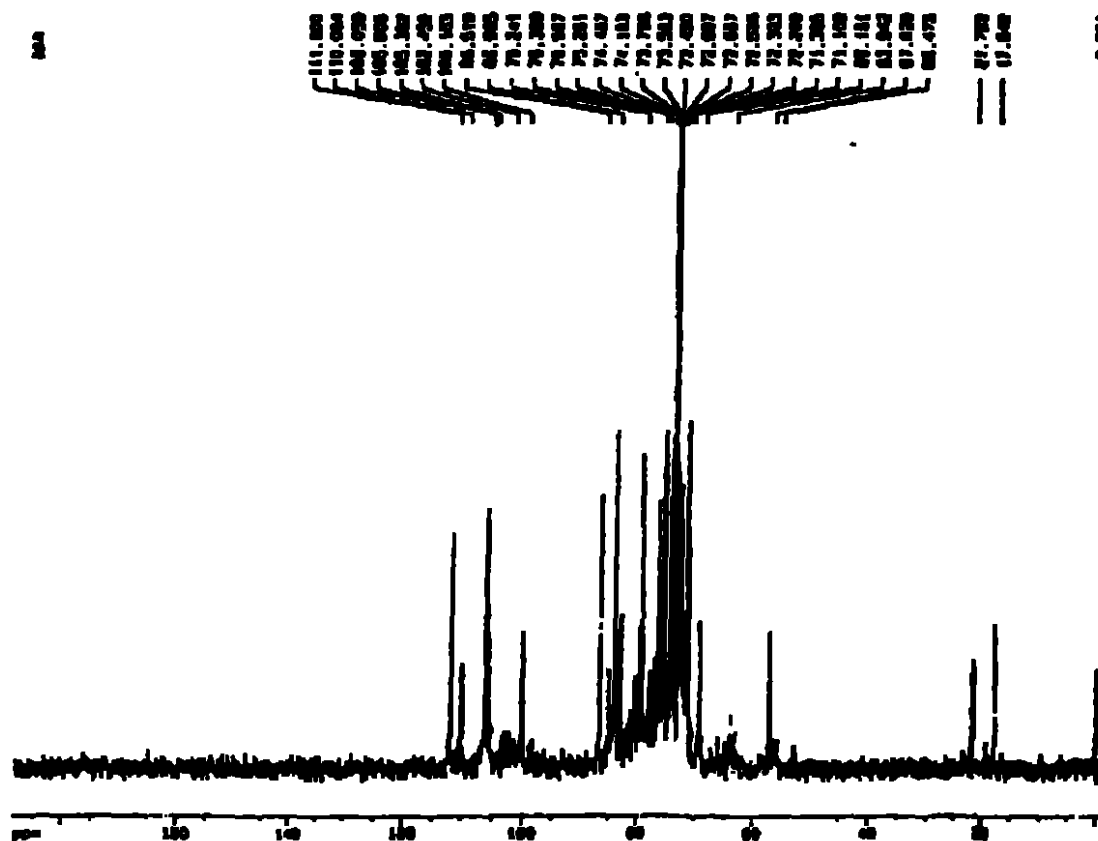


Figure 12 The DEPT-90 spectrum of PF2RS8B2 (125MHz, D₂O, referred to DSS)

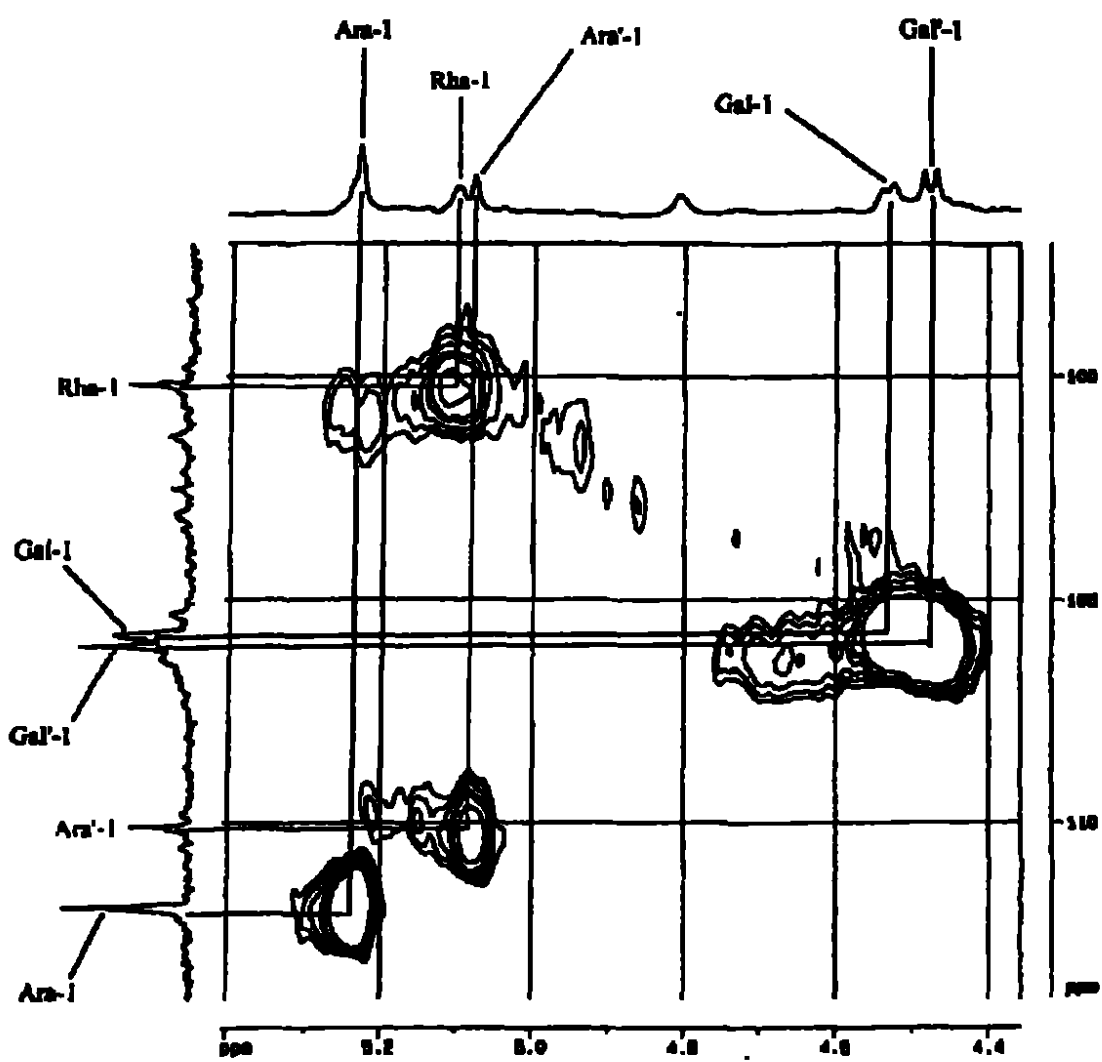


Figure 13. The HMQC spectrum of PF2RS8B2 (500MHz, D₂O)

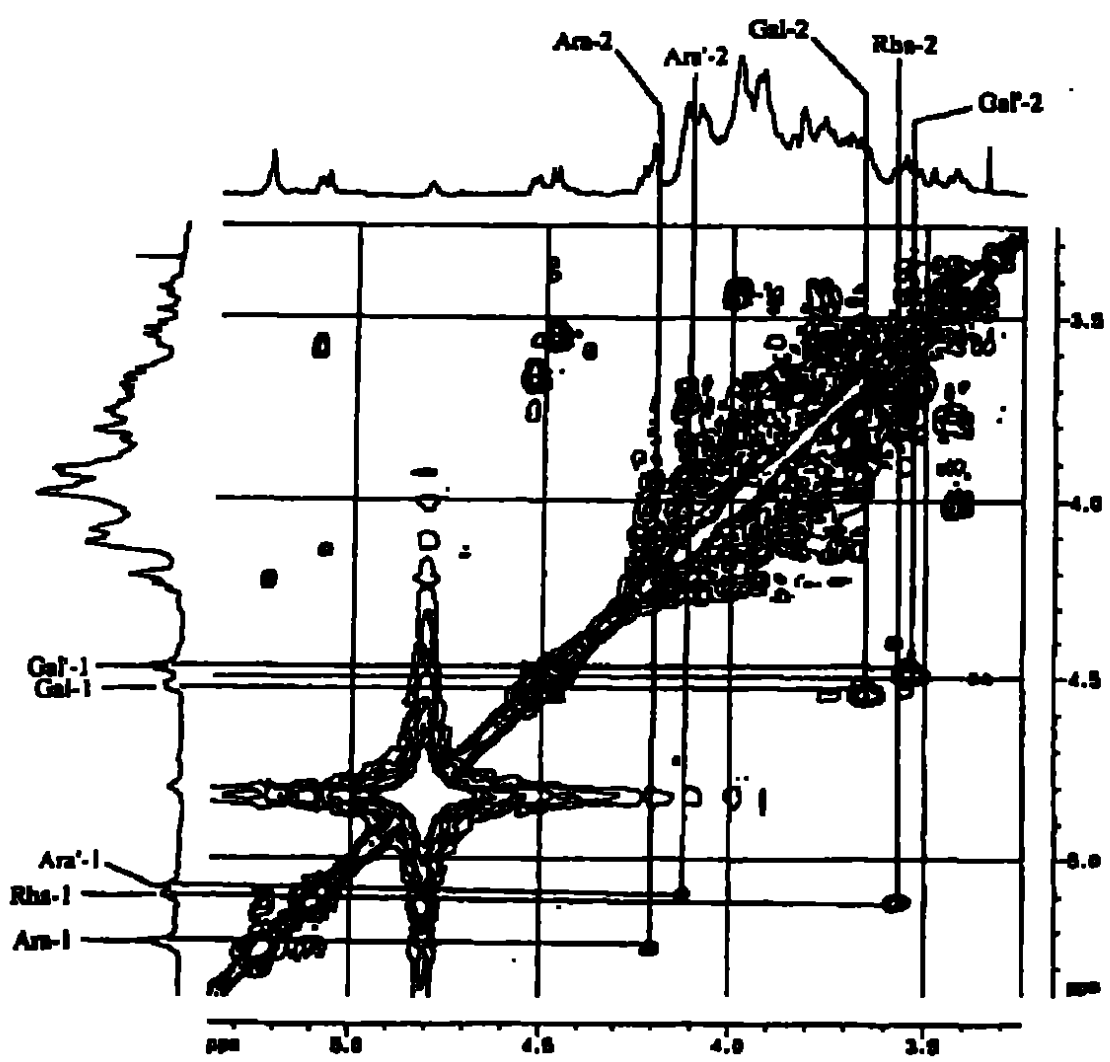


Figure 14. The ^1H - ^1H COSY spectrum of PF2RS8B2 (500MHz, D_2O)

15/21

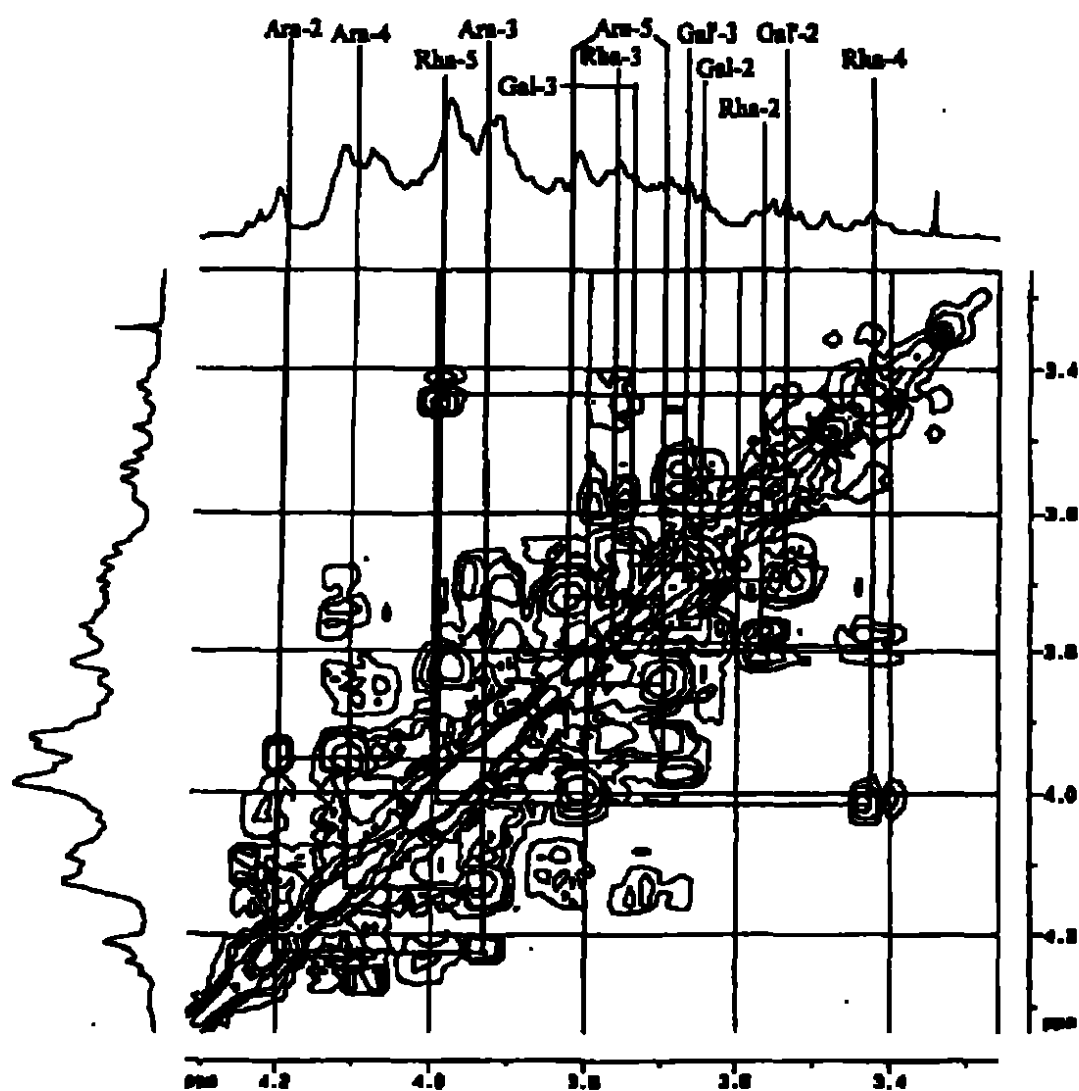


Figure 15 The ^1H - ^1H COSY spectrum of PF2RS8B2 (500MHz, D_2O)

16/21

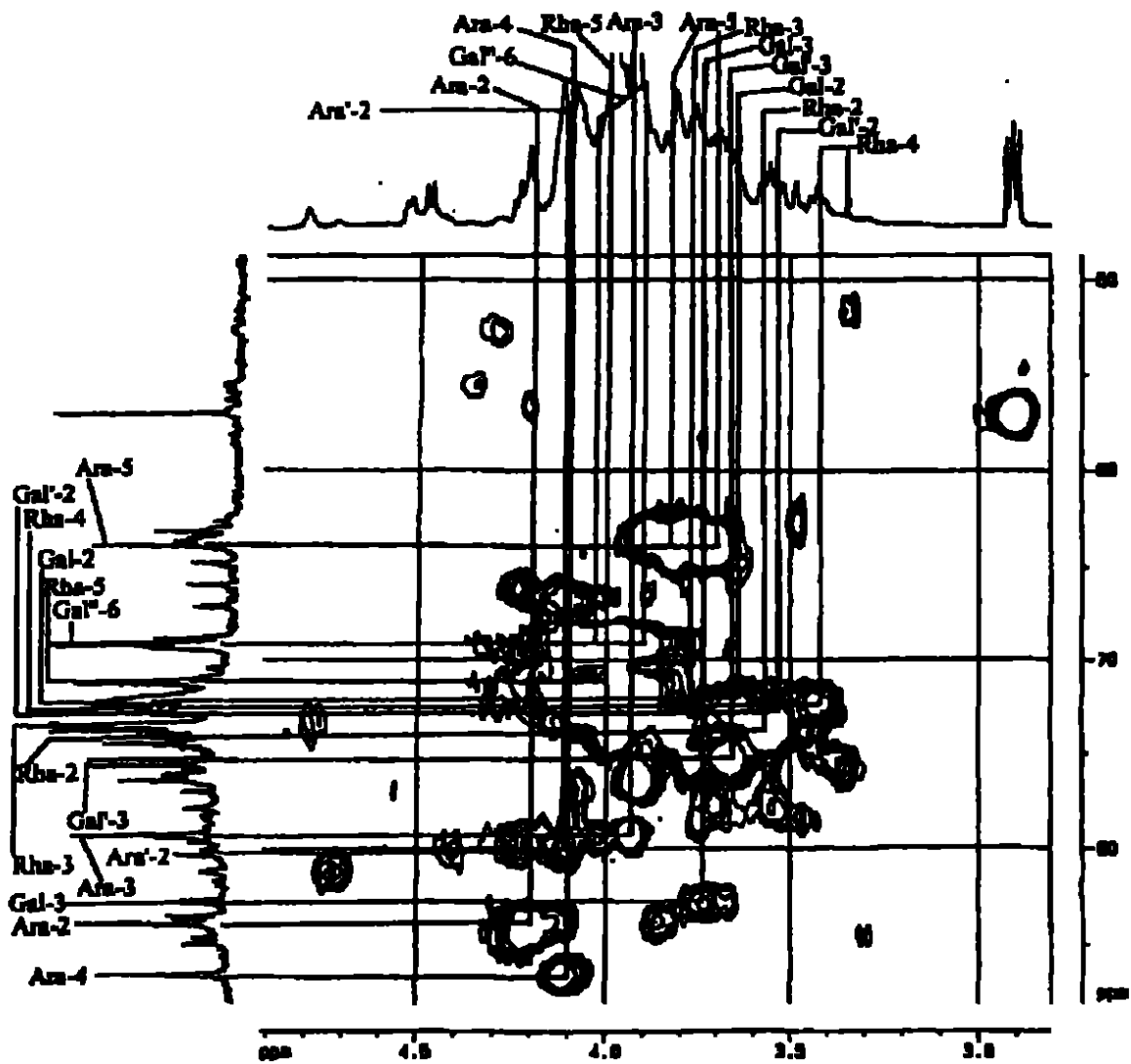


Figure 16, The HMQC spectrum of PF2RS8B2 (500MHz, D₂O)

17/21

Table 2. ^{13}C NMR data of PF2RS8B-2 (D_2O , referred to DSS, 125MHz)

Sugar linkages	C-1	C-2	C-3	C-4	C-5	C-6
Araf-(1→	110.1d	85.0d	74.5d	86.7d	63.2t	—
		84.7d				
→3)-Araf-(1→	111.9d	84.0d	79.4d	86.5d	63.9t	—
		83.9d			63.7t	
		83.7d			64.8t	
→6)-Galp-(1→	106.1d	72.9d	75.3d	72.2d	76.4d	69.2t
→3) \	105.9d	73.5d	82.8d	73.5d	76.1d	69.2t
Galp-(1→)	105.8d					
→6)						
Rhap-(1→	100.2d	72.2d	73.5d	72.5d	71.1d	19.2q

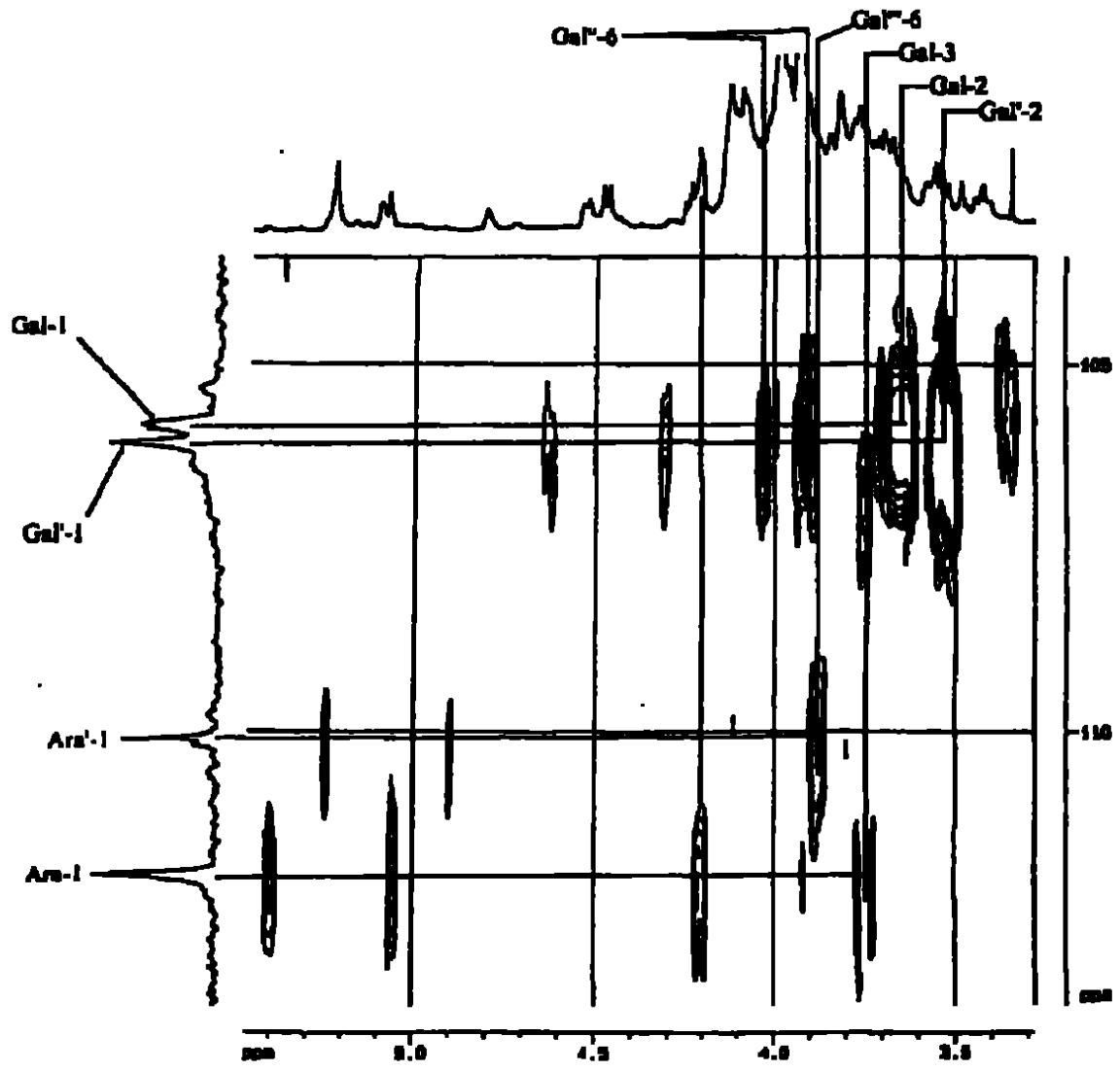


Figure 17. The HMBC spectrum of PF2RS8B2 (500MHz, D₂O)

19/21

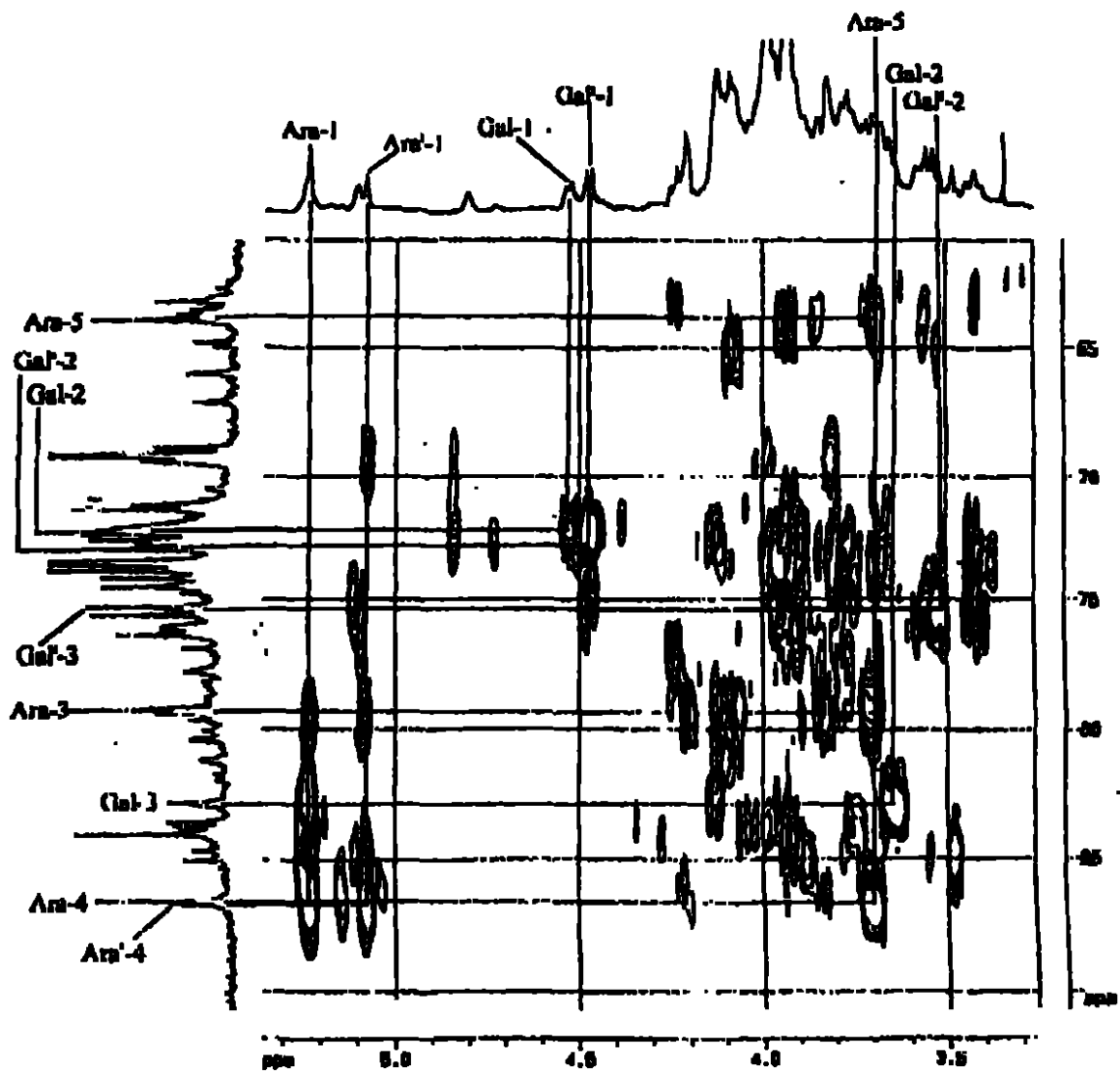
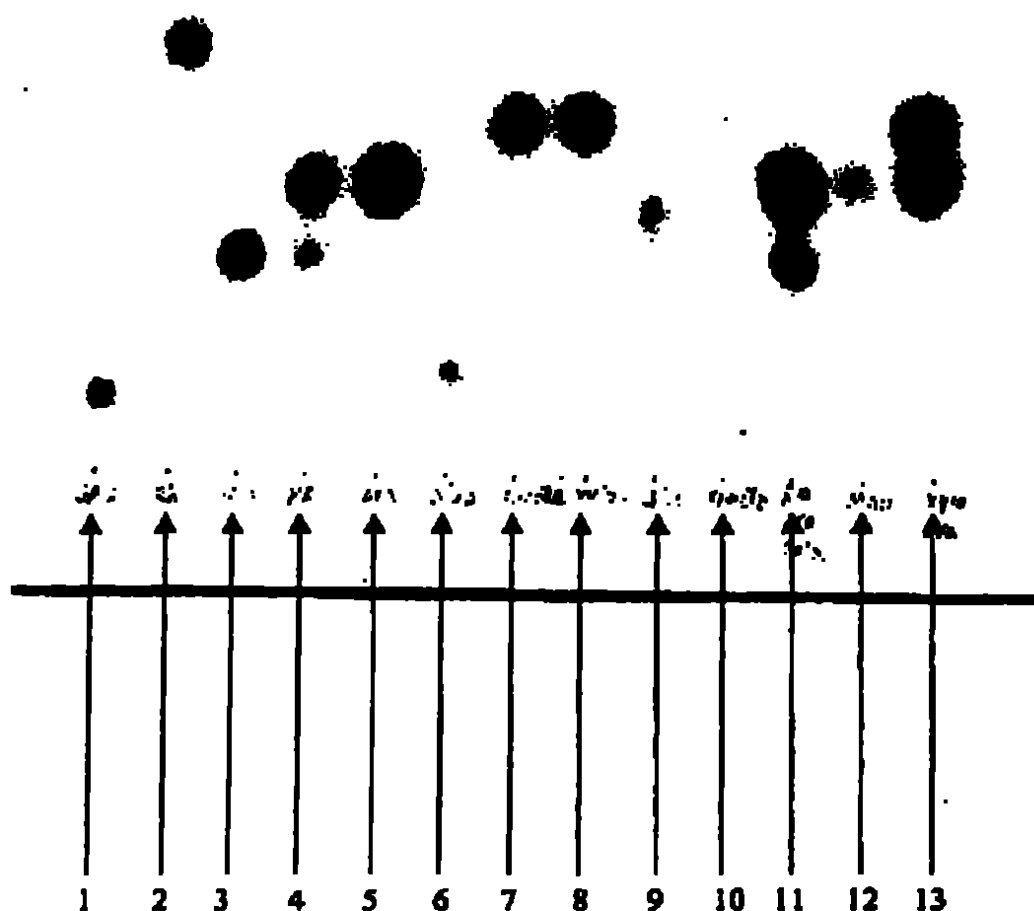


Figure 18. The HMBC spectrum of PF2RS8B2 (500MHz, D₂O)



1. D-Galacturonic acid; 2. L-(+)Rhamnose; 3. D-(+)Galactose; 4. Hydrolysis products of PF2RS8B-2; 5. L-(+)Arabinose; 6. D-Glucuronic acid; 7. L-(-)Fucose; 8. D-(+)Xylose; 9. D-(+)Glucose; 10. D-(-)Fructose; 11. Mixture of hydrolysis products of PF2RS8B-2, L-(+)Arabinose and D-(+)Galactose; 12. D-(+)Mannose; 13. Mixture of D-(+)Xylose and L-(+)Arabinose

Figure 19. TLC of hydrolysis products of PF2RS8B2 with TFA and other monosugars.

91

ORGANIZACIÓN MUNDIAL DE LA PROPIEDAD INTELECTUAL
34, chemin des Colombettes, Apartado postal 18, CH-1211
Ginebra 20 (Suiza)
Teléfono: (4122)338 91 11 e-mail: wipo.mail@wipo.int. -
Facsimil: (4122)733 54 28

TRATADO DE COOPERACIÓN EN MATERIA DE PATENTES (PCT)

**COPIA CERTIFICADA DE LA SOLICITUD INTERNACIONAL COMO FUE
PRESENTADA Y DE CUALQUIER CORRECCION EN LA MISMA**

Solicitud Internacional No. PCT/IB00/01946

Fecha de presentación internacional 27 de noviembre de 2000
(27.11.00)

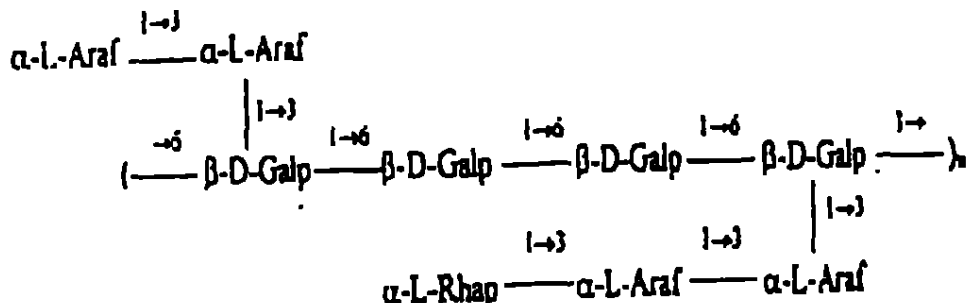
Ginebra,
Diciembre 12 de 2001
(12.12.01)

**Oficina Internacional de la
Organización Mundial de la
Propiedad Intelectual (OMPI)**

J.-L. Baron
Jefe de Sección de la oficina
receptora PCT

REIVINDICACIONES

1. El polisacárido de acuerdo con la fórmula I,



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en la que n = 7 a 8.

2. El procedimiento para obtener el compuesto definido en la reivindicación 1 que comprende:

- 10 1) separar las flores de la planta *Calendula officinalis*,
- 2) someterlas al siguiente procedimiento de extracción:
 - a) Descontaminación de las flores
 - b) Trituración de las flores.
 - c) Tratamiento de las flores trituradas con una
 - 15 radiación láser.
 - d) Suspensión de la mezcla obtenida en la etapa c) en agua.
 - e) Maceración de la suspensión obtenida en la etapa d).
 - 20 f) Separación del líquido resultante.

y 3) someter el líquido obtenido en la etapa f) a un procedimiento de aislamiento que comprende la precipitación con metanol, la centrifugación y la

25 separación cromatográfica controladas mediante bioensayo.

3. El procedimiento de acuerdo con la reivindicación 2, en el que la etapa 3) comprende:

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- g) Liofilización del líquido obtenido en la etapa f).
 - h) Precipitación de la materia liofilizada obtenida en la etapa g) con metanol.
 - 5 i) Separación de la fase líquida y la fase sólida.
 - j) Precipitación de la fase sólida obtenida en la etapa i) con metanol a unas concentraciones finales del 25%, del 50% y del 67%.
 - k) Solubilización en agua del precipitado obtenido en la etapa j) al 50% y al 67%, centrifugación y 10 realización de una separación cromatográfica del sobrenadante.
 - l) Identificación de la fracción activa mediante bioensayo.
 - 15 m) Realización de una segunda separación cromatográfica de la fracción activa.
 - n) Identificación de la fracción activa mediante bioensayo.
 - o) Eliminación del eluyente.
 - 20
4. El procedimiento de acuerdo con las reivindicaciones 2 y 3, en el que el tratamiento con la radiación láser se lleva a cabo con un láser diódico lineal rojo que tiene una capacidad de generación de armónicos con longitudes 25 de onda en el intervalo de 150 a 810 nm, una potencia de 1 a 60 vatios y una mancha focal de 1 a 6 mm de diámetro.
5. El procedimiento de acuerdo con la reivindicación 4, en el que la longitud de onda está en el intervalo de 200 30 a 400 nm, siendo preferiblemente de 250 nm, la potencia es de 20 vatios y la mancha focal es de 4 mm de diámetro.
6. El procedimiento de acuerdo con cualquier 35 reivindicación anterior, en el que se trata cada kilogramo de materia triturada con la radiación láser

durante un periodo de 3 a 10 minutos, preferiblemente durante un periodo de 5 minutos.

7. El compuesto definido en la reivindicación 1 para su uso como agente terapéutico.

8. El compuesto de acuerdo con la reivindicación 1 para su uso en el tratamiento de enfermedades inmunosupresoras.

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9. El compuesto de acuerdo con la reivindicación 1 para su uso en el tratamiento de enfermedades inmunosupresoras tales como cáncer, tuberculosis, gripe, resfriado común, alergias, lupus eritematoso, psoriasis y SIDA.

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10. El compuesto de acuerdo con la reivindicación 1 para su uso en el tratamiento de: carcinoma hepático, cáncer de pulmón, cáncer de riñón, cáncer de colon, cáncer de mama, cáncer de próstata o adenocarcinoma prostático; cánceres de cerebro tales como astrocitoma y glioblastoma; cáncer de cuello uterino y cáncer de vejiga.

11. El uso del compuesto definido en la reivindicación 1 para la fabricación de un medicamento.

12. El uso de acuerdo con la reivindicación 11 para la fabricación de un medicamento destinado al tratamiento de enfermedades inmunosupresoras.

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13. El uso de acuerdo con la reivindicación 12 para la fabricación de un medicamento destinado al tratamiento de: cáncer, tuberculosis, gripe, resfriado común, alergias, lupus eritematoso, psoriasis y SIDA.

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14. El uso de acuerdo con la reivindicación 13 para la
fabricación de un medicamento destinado al tratamiento
de: carcinoma hepático, cáncer de pulmón, cáncer de
riñón, cáncer de colon, cáncer de mama, cáncer de
5 próstata o adenocarcinoma prostático; cánceres de cerebro
tales como astrocitoma y glioblastoma; cáncer de cuello
uterino y cáncer de vejiga.

15. Una preparación farmacéutica que incluyen un
10 compuesto de acuerdo con la reivindicación 1.

16. La preparación farmacéutica de acuerdo con la
reivindicación 15 que incluye además un vehículo
farmacéuticamente aceptable.

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17. La preparación farmacéutica de las reivindicaciones
15 y 16 que incluye además al menos otro compuesto
farmacéuticamente activo.