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54. TITULO

DERIVADOS EPIMERIZADOS DE POLISACARIDO K5 CON UN GRADO MUY ALTO

DE SULFATACION

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71. SOLICITANTE

GLYCORES 2000 S.r.l.

DOMICILIO

Milan, Italia.

74. APODERADO

EMILIO FERRERO

c.c. 438,019 de Usaquén.

22. BOGOTA, D.C.,

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SOLICITUD DE:



Patente de Invención.



Patente de Modelo de Utilidad.



Capítulo I.



Capítulo II.

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SOLICITANTE
(71)

Nombre: **GLYCORES 2000 S.r.l.**
sociedad constituida y existente bajo las
leyes de Italia
Dirección: **Via Mac Mahon, 43**
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IDENTIFICACION

C.C.



NIT



C.E.



Otro



Cual _____

Número: _____

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Título (54)

DERIVADOS EPIMERIZADOS DE POLISACARIDO K5 CON UN GRADO MUY ALTO
DE SULFATACIÓN

7

Clasificación Internacional (51) C08B 37/00, 37/08, 37/10, A61K 31/726, 31/727, 31/737 A61P35/04

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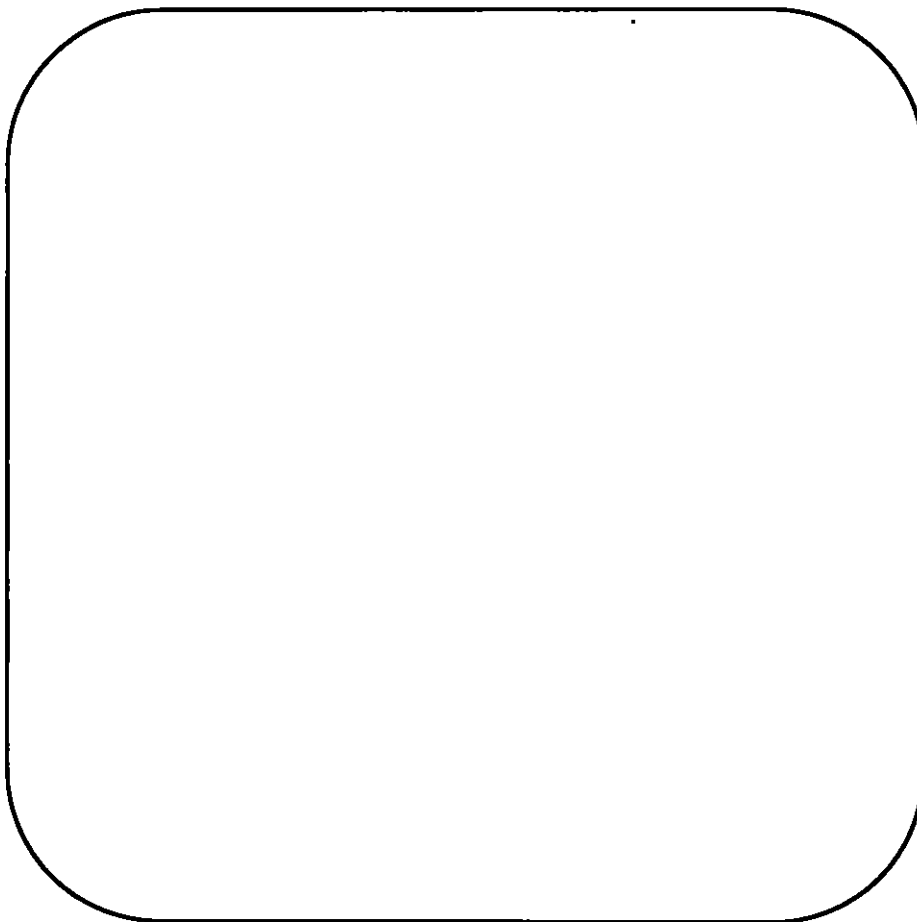
Comprobante de pago No.: 04-99.403Fecha: 27 de diciembre de 2004

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Anexos:

- ☒ Comprobante de pago de la tasa de presentación de la solicitud por \$ 422.000.00.
- ☐ Comprobante de pago por reivindicación de prioridad.
- ☐ Documento que acredite la existencia y representación legal cuando el solicitante sea persona jurídica.
- ☐ Poderes, si fuere el caso.
- ☐ Documento de cesión del inventor al solicitante o a su causante.
- ☐ Declaraciones.
- ☒ Copia de la solicitud internacional. (Resumen, descripción, reivindicaciones, dibujos)
- ☒ Traducción de la solicitud Internacional al castellano. (Resumen, descripción, reivindicaciones, dibujos)
- ☐ Copia del examen preliminar efectuado por la IPEA.
- ☐ Traducción del examen preliminar efectuado por la IPEA.
- ☐ De ser el caso, copia del contrato de acceso.
- ☐ De ser el caso, documento que acredite la licencia o autorización de uso de conocimientos tradicionales de las comunidades indígenas.
- ☐ De ser el caso, certificado de depósito del material biológico.
- ☐ Arte final 12x12
- ☐ De ser el caso, información sobre otras solicitudes de patente o títulos obtenidos en el extranjero por el mismo titular o su causante, relacionadas parcial o totalmente con la invención de esta solicitud.
- ☒ Otros: Formato PCT/IB/306 mediante el cual la Oficina Internacional de la OMPI notifica el registro del cambio de solicitante de los Inventores a la compañía GLYCORES 2000 S.r.l.

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FIGURA CARACTERISTICA:

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Solicito la concesión de la patente,

NOMBRE: EMILIO FERRERO

FIRMA :

C.C. 438.019 de Usaquén T.P. 31.929

DLM



PATENT COOPERATION TREATY

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PCT

From the INTERNATIONAL BUREAU

To:

SANTORO, Tiziana
Marietti, Gislone e Trupiano S.r.l.
Via Larga, 16
I-20122 Milan
Italy

NOTIFICATION OF THE RECORDING
OF A CHANGE

(PCT Rule 92bis.1 and
Administrative Instructions, Section 422)

Date of mailing (day/month/year) 05 November 2004 (05.11.2004)	IMPORTANT NOTIFICATION
Applicant's or agent's file reference 03420E45	
International application No. PCT/IB2003/002338	International filing date (day/month/year) 17 June 2003 (17.06.2003)

1. The following indications appeared on record concerning: ☒ the applicant ☐ the inventor ☐ the agent ☐ the common representative		
Name and Address	State of Nationality **	State of Residence IT
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	
2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning: ☒ the person ☐ the name ☐ the address ☐ the nationality ☐ the residence		
Name and Address GLYCORES 2000 S.r.l. Via Mac Mahon, 43 I-20155 Milan Italy	State of Nationality **	State of Residence IT
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	
3. Further observations, if necessary: Applicant indicated in box 2 has been added for "all designated states except US" and applicants ORESTE, Pasqua, Anna and ZOPPETTI, Giorgio become applicant/inventor for US only.		
4. A copy of this notification has been sent to: ☒ the receiving Office ☒ the designated Offices concerned ☐ the International Searching Authority ☐ the elected Offices concerned ☐ the International Preliminary Examining Authority ☐ other:		

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No. (41-22) 338.70.80	Authorized officer Sonia GARCIA MELLITI Telephone No. (41-22) 338 9919
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WO 03/106504 A1

(54) Title: EPIMERIZED DERIVATIVES OF K5 POLYSACCHARIDE WITH A VERY HIGH DEGREE OF SULFATION

(57) Abstract: A new method is described for the oversulfation of epiK5-N-sulfate to obtain an epiK5-amine-O-oversulfate with very high sulfation degree which, by subsequent N-sulfation, provides new epiK5-N,O-oversulfate-derivatives with a sulfation degree of at least 4, basically free of activity on the coagulation parameters and useful in the cosmetic or pharmaceutical field. Also described are new low molecular weight epiK5-N-sulfates useful as intermediates in the preparation of the corresponding LMW-epiK5-N,O-oversulfate-derivatives.

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Epimerized derivatives of K5 polysaccharide with a very high degree of sulfation.

OBJECT OF THE INVENTION

The present invention concerns new derivatives of K5 polysaccharide with a very high degree of sulfation, a process for their preparation, highly O-sulfated new intermediates useful in their synthesis and pharmaceutical compositions containing said derivatives of K5 polysaccharide as active ingredients basically free of activity on coagulation.

In particular, the invention refers to a process for the preparation of epiK5-N,O-oversulfates starting with a K5 polysaccharide, previously N-deacetylated, N-sulfated and C5-epimerized at least 20%, through O-oversulfation in suitable conditions and subsequent N-sulfation, to said epiK5-N,O-oversulfates of antiangiogenetic and antiviral activity and to new low molecular weight intermediates of epi-K5-N-sulfates.

BACKGROUND OF THE INVENTION

The glycosaminoglycans such as heparin, heparan sulfate, dermatan sulfate, chondroitin sulfate and hyaluronic acid are biopolymers that are industrially extracted from various animal organs.

In particular, heparin, mainly obtained by extraction from the intestinal mucous membrane of pigs or bovine lung, is a polydispersed copolymer with a molecular weight distribution from approximately 3,000 to approximately 30,000 D consisting of a chain mixture basically consisting of a uronic acid (glucuronic acid or iduronic acid) and of an amino sugar (glucosamine) linked by α -1 $\rightarrow$ 4 or β -1 $\rightarrow$ 4 bonds. In heparin, the uronic unit can be O-sulfated in position 2 and the glucosamine unit is N-acetylated or N-sulfated, 6-O-sulfated, and 3-O-sulfated in approximately 0.5% of the glucosamine units present.

The properties and natural biosynthesis of heparin in mammals have

been described by Lindahl et al., 1986 in Lane, D. and Lindahl, U. (Editors) "Heparin. Chemical and Biological Properties; Clinical Applications", Edward Arnold, London, Pages 159-190, by Lindahl, U., Feingold D. S. and Rodén L, 1986 TIBS, 11, 221-225 and by Conrad H. E. "Heparin Binding Proteins", Chapter 2: Structure of Heparinoids. Academic Press, 1998. The biosynthesis of heparin occurs starting with its precursor N-acetyl-heparosan consisting of a chain mixture consisting of the repetitive disaccharide unit glucuronyl- β -1 $\rightarrow$ 4-N-acetylglucosamine. Said precursor undergoes enzymatic modifications which partially hydrolyse the N-acetyl group, substituting it with an SO₃ group, epimerize the carboxyl in position 5 of a part of the glucuronic units converting them into iduronic units and introducing O-sulfate groups to get a product which, once extracted industrially, has approximately double the number of sulfate groups as regards carboxyl ones per disaccharide unit. These enzymatic modifications lead to, besides, the formation of the pentasaccharide region of a bond to antithrombin III (ATIII), called active pentasaccharide, which is the structure necessary for the high affinity bond of heparin to the ATIII and fundamental for anticoagulant and antithrombotic activity of the heparin itself. This pentasaccharide, present inside only some of the chains which form heparin, contains a sulfated glucosamine unit in position 3 and a glucuronic acid spaced out between disaccharides containing iduronic acids.

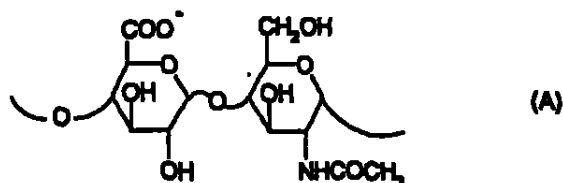
In nature, the formation of the active pentasaccharide is made possible by the epimerization reaction of the carboxyl of a part of the glucuronic units into iduronic units carried out by the glucuronyl-C5-epimerase (C5-epimerization) and by suitable sulfation which also leads to the introduction of a sulfate group onto the hydroxyl in position 3 of the glucosamine. More particularly, in nature the

formation of the active pentasaccharide is made possible by the fact that the C5-epimerization occurs in clusters, i.e. on portions of chains, and extensively, which results in a product that contains more iduronic units than glucuronic ones. Commercial heparin, in fact, contains approximately 70% of iduronic units and 30% of glucuronic units.

Alongside the main anticoagulant and antithrombotic activities, heparin also exercises antilipaemic, antiproliferative, antiviral, antitumorous and antimetastatic activities, but its use as a drug is hindered by the side effects due to the anticoagulant action which can cause bleeding.

PRIOR ART

It is known that the capsular K5 polysaccharide isolated from *Escherichia coli*, described by Vann W. F. et al., in *European Journal of Biochemistry*, 1981, 116, 359-364 ("Vann 1981"), consists of a chain mixture consisting of the repetitive disaccharide unit glucuronyl- β -1 $\rightarrow$ 4-N-acetyl glucosamine and therefore shows the same repetitive sequence (A)



of the N-acetyl-heparosan precursor of heparin. The capsular K5 polysaccharide, referred to hereafter as "K5 polysaccharide" or more simply "K5", was chemically modified by Lormeau et al. as described in US 5,550,116 and by Casu et al. as described in *Carbohydrate Research*, 1994, 263, 271-284. K5-O-sulfates having antitumorous, antimetastatic, antiviral, in particular anti-HIV activities are described in EP 333243 and WO 98/34958. The K5 was also modified chemically and enzymatically in order to obtain products having the same type

of *in vitro* biological activity on coagulation as that of heparin as extracted from animal organs (extractive heparin).

The attainment of the products having an activity on coagulation of the same type as that of extractive heparin occurs by processes which imitate that occurring in nature and envisage the entire key step of C5-epimerization with D-glucuronyl C5 epimerase.

The processes described in IT 1230785, WO 92/17507, WO 96/14425 and WO 97/43317 utilize K5 as the starting material. K5 originating from fermentation is subjected to N-deacetylation followed by N-sulfation and on the K5-N-sulfate thus obtained C5-epimerization with C5-epimerase in solution is performed, obtained either by chromatography of a solution of microsomal enzymes from mouse mastocytoma (IT 1230 785) or from bovine liver (WO 92/17507, WO 96/14425 and WO 97/43317).

The D-glucuronyl C5 epimerase from bovine liver was purified by Campbell, P. et al. in J. Biol. Chem., 1994, 269/43, 26953-26958 ("Campbell 1994") who also supplied its composition in amino acids and described its use in solution for the transformation of a K5-N-sulfate into the corresponding 30% epimerized product, demonstrating the formation of iduronic acid by HPLC method followed by total nitrous depolymerization to disaccharide.

The document WO 98/48006 describes the DNA sequence which codes for the D-glucuronyl C5 epimerase and a recombinant D-glucuronyl C5 epimerase, obtained from a recombinant expression vector containing said DNA, afterwards purified by Campbell et al. as shown by Jin-Ping L. et al. in J. Biol Chem. 2001, 276, 20069-20077 ("Jin-Ping 2001").

The complete C5-epimerase sequence was described by Crawford B. E. et al. in J. Biol. Ch m., 2001, 276(24), 21538-21543 (Crawford 2001).

The document WO 01/72848 describes a method for the preparation of N-deacetylated N-sulfate derivatives of K5 polysaccharide, at least 40% epimerized of iduronic acid as regards the total of the uronic acids, having a molecular weight from 2,000 to 30,000, containing from 25 to 50% of high affinity chains for ATIII and having an anticoagulant and antithrombotic activity expressed as HCII/antiXa ratio from 1.5 to 4. Said document describes the oversulfation of a K5-N-sulfate, 40-60% epimerized and shows that the product obtained, whose ^{13}C -RMN is illustrated, has a sulfate group content per disaccharide unit of 2-3.5. Repeating the aforesaid oversulfation in the conditions described and examining the ^{13}C -RMN it was ascertained that the product obtained is actually a free amine whose 6-O-sulfate content is 80-95%, that of 3-O-sulfate on the amino sugar is 30%, but whose sulfation degree is 3.2. It was also observed that in the conditions of oversulfation described in WO 01/72848 a degree of sulfation higher than 3.2 was not obtained. The document US 2002/0062019 describes a process for the preparation of epiK5-N,O-sulfates, active in the control of coagulation, having a degree of sulfation from 2.3 to 2.9 and a molecular weight from 2,000 to 30,000, or from 4,000 to 8,000, or from 18,000 to 30,000. The aforesaid process involves the steps: (p-a) an N-deacetylation of K5 polysaccharide and an N-sulfation of the resulting K5-amine, (p-b) an epimerization of K5-N-sulfate, (p-c) an O-oversulfation of epiK5-N-sulfate, (p-d) a partial O-desulfation, (p-e) a selective 6-O-sulfation, (p-f) an N-sulfation of the product thus obtained, any product obtained upon termination of one of the steps (p-b)-(p-f) able to be subjected to depolymerization. Said document describes an epiK5-N,O-sulfate having a molecular weight of 7,400, obtained by the aforesaid steps (p-a)-(p-f) followed by a nitrous depolymerization at the end of step (p-f), with a degree of

sulfation from 2.3 to 2.9.

The same document also describes a moiety of K5 with a molecular weight of approximately 5,000 which can also be subjected to steps (p-a) – (p-f).

In order to standardize the terminology and render the text more comprehensible, in the present description conventional terms or expressions will be used, in the singular or plural. In particular:

- by "K5" or "K5 polysaccharide" is meant the capsular polysaccharide from *Escherichia coli* obtained by fermentation, i.e. a chain mixture consisting of disaccharide units (A) optionally containing a double bond at the non-reducing end as shown above, in any case, prepared and purified according to the methods described in literature, in particular according to Vann 1981, according to Manzoni M. et al., Journal of Bioactive Compatible Polymers, 1996, 11, 301-311 ("Manzoni 1996") or according to the method described in WO 01/72848 and in WO 02/068447; it is obvious for a person skilled in the art that what is shown hereafter can be applied to any N-acetylheparosan;
- by "C5-epimerase" is meant the D-glucuronyl C-5 epimerase, extractive or recombinant, in any case prepared, isolated and purified, in particular as described in Campbell 1994, in WO 98/48006, in Jin-Ping L. et al. In J. Biol Chem. 2001, 276, 20069-20077 (Jin-Ping 2001") or in Crawford 2001;
- by K5-amine is meant at least 95% N-deacetylated K5, but in which N-acetyl groups are undetectable with a normal NMR apparatus;
- by "K5-N-sulfate" is meant at least 95% N-deacetylated and N-sulfate K5, normally 100%, since N-acetyl groups are undetectable with a normal NMR apparatus, as described hereafter;
- by "piK5" is meant the K5 and its derivatives in which 20-60% of the



glucuronic units is C5-epimerized to iduronic units

- by "epiK5-N-sulfate" is meant K5-N-sulfate in which 20-60% of the glucuronic units is C5-epimerized to iduronic units;
- by "epiK5-amine-O-oversulfate" is meant an epiK5-amine-O-sulfate with a sulfation degree of at least 3.4;
- by "epiK5-N,O-oversulfate" is meant an epiK5-amine-O-sulfated completely N-sulfated with a sulfation degree of at least 4;

In addition:

- the conventional terms and expressions herein defined above refer to a K5 as isolated after fermentation, generally with a molecular weight distribution from approximately 1,500 to approximately 50,000 with a mean molecular weight of 10,000- 25,000, advantageously of 15,000-25,000;
- the conventional terms and expressions herein defined above, when preceded by the acronym "LMW" (low molecular weight), for example LMW-K5-N-sulfate, LMW-epiK5-N-sulfate, indicate low molecular weight products, obtained by fractionation or by depolymerization of K5-N-sulfate and consisting of or derived from K5-N-sulfates having a mean molecular weight from approximately 1,500 to approximately 12,000, calculated on a 100% N-sulfated product;
- the conventional terms and expressions as herein defined above, when followed by "-derivative" indicate as a whole both the derivatives from native K5 and those of low molecular weight;
- by the term "approximately", referring to the molecular weight, is meant the molecular weight measured by viscometry $\pm$ the theoretical weight of a disaccharide unit, including the weight of the sodium, calculated as 461 in the case of an epiK5-N-sulfate-derivative and 806 in the case of an epiK5-N,O-oversulfated-derivative with a sulfation degree of 4.26;

- by the expression "preponderant species", is meant the compound which, in the mixture constituting the LMW-epiK5-N-sulfate, the LMW-epiK5-amine-O-oversulfate or the LMW-epiK5-N,O-oversulfate, is the most represented type, determined by the peak of the curve of the molecular weight measured by HPLC;
- unless otherwise specifically stated, by "degree of sulfation" is meant the SO_3/COO^- ratio, expressible also as the number of sulfate groups per disaccharide unit, measured with the conductometric method described by Casu B. et al. in Carbohydrate Research, 1975, 39, 168-176 (Casu 1975), the same utilized in WO 01/72848;
- by "conditions of O-oversulfation" is meant an extreme O-sulfation performed, for example, according to the Method C described by B. Casu et al. in Carbohydrate Research, 1994, 263, 271-284 (Casu 1994);
- by the term "alkyl" is meant a linear or branched alkyl, whereas "tetrabutylammonium" denotes the tetra-n-butylammonium group.

SUMMARY OF THE INVENTION

It has now surprisingly been found that, unlike that which occurs with the processes described in IT 1230785, WO 92/17507, WO 96/14425, WO 97/43317, WO 01/72848 and US 2002/0062019, starting with an epiK5-N-sulfate it is possible to obtain an epiK5-amine-O-oversulfate with a greater degree of sulfation than every other epiK5-amine-O-sulfate described in literature, for example in WO 01/72848, by preparing the salt with tertiary or quaternary organic base of said piK5-N-sulfate taking care to let the reaction mixture to stand for a time period of 30-60 minutes maintaining the pH at approximately 7 with the same organic base and then treating the salt obtained with an O-sulfation reagent in the conditions of O-oversulfation.

Subjecting the epiK5-amine-O-oversulfates thus obtained to N-sulfation, new piK5- N,O-oversulfates are obtained which, unlike th

products described in IT 1230785, WO 92/17507, WO 96/14425, WO 97/43317, WO 01/72848 and US 2002/0062019, are free of activity on coagulation and useful for the preparation of medicines, particularly pharmaceutical compositions of antiangiogenetic and antiviral activity or of cosmetic compositions.

By depolymerization with nitrous acid of said epiK5-N,O-oversulfates new LMW-epiK5-N,Ooversulfates are obtained, free of activity on coagulation, and with antiangiogenetic and antiviral activity.

In preparing N,O-sulfate N-deacetylated derivatives of K5 polysaccharide, at least 40% epimerized of iduronic acid as regards the total of the uronic acids and having low molecular weight according to the method described in WO 01/72848, it was ascertained that the depolymerization of the product of high molecular weight obtained at the end of the final N-sulfation step of the process can give varying results since it generally produces some depolymerized products showing much lower activity, than that of high molecular weight products from which they arise, on all the coagulation parameters. It is assumed this takes place because degradation with nitrous acid is influenced by the presence of the sulfate groups. In particular the sulfates in position 3 of the glucosamine result in heterogeneous products, as described by Nagasawa et al. in *Thrombosis Research*, 1992, 65, 463-467 (Nagasawa 1992).

It has now been found that subjecting an epiK5-N-sulfate to nitrous depolymerization in which the iduronic acid content as regards the total of uronic acids is 20-60%, advantageously of 40-60%, preferably around 50%, LMW-epiK5-N-sulfates are obtained which constitute new effective intermediates for the preparation of LMW-epiK5-N,O-oversulfates having a high degree of activity on different biological

parameters, with or without activity on coagulation parameters. In particular, it was found that it is possible to depolymerize an epiK5-N-sulfate so as to obtain new LMW-epiK5-N-sulfates of mean molecular weight from approximately 2,000 to approximately 4,000, more particularly specific LMW-epiK5-N-sulfates consisting of mixtures in which the predominant compound is a decasaccharide or a dodecasaccharide or a tetradecasaccharide. Also these LMW-epiK5-N-sulfates, otherwise unobtainable, have interesting biological properties and are useful intermediates for the preparation of LMW-epiK5-N,O-oversulfated of antiviral and/or antiangiogenetic activity and surprisingly free of activity on coagulation.

By subjecting a LMW-epiK5-N-sulfate to the aforesaid method of sulfication with a tertiary or quaternary organic base, taking care to let the reaction mixture to stand for a time period of 30-60 minutes maintaining the pH at approximately 7 with the same organic base and then treating the salt obtained with an O-sulfation reagent in the conditions of O-oversulfation, new LMW-epiK5-amine-O-oversulfates are obtained. By subjecting the LMW-epiK5-amine-O-oversulfate to N-sulfation, new N-sulfated and O-oversulfated derivatives (LMW-epiK5-N,O-oversulfates) are obtained, surprisingly free of activity on coagulation and of antiviral and/or antiangiogenetic activity, useful for the preparation of pharmaceutical or cosmetic compositions.

These LMW-epiK5-N-sulfates are obtained starting with a K5-N-sulfate with an epimerization reaction with an isolated and purified recombinant C5-epimerase, immobilized on a solid support, at a temperature of approximately 30°C and at a pH of approximately 7 for 12-24 hours in the presence of a bivalent cation selected among calcium, magnesium, barium and manganese and a subsequent nitrous depolymerization reaction of the epimerized product thus

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obtained, or vice versa.

Surprisingly, from observations made on the course of the epimerization reaction in the aforesaid conditions, it is possible to assume that, contrary to that occurring in nature in the biosynthesis of heparin, ordinary and not "cluster" type C5-epimerization of the substrate occurs every 2 glucuronic acid units which leads to epi-K5-N-sulfate-derivatives characterized by a repetitive tetrasaccharide unit consisting of two glucosamine units separated by a glucuronic unit and followed by an iduronic unit or vice versa.

DETAILED DESCRIPTION

Thus, according to one of its aspects, the present invention provides a process for the preparation of epiK5-N,O-oversulfate-derivatives, characterized in that

(a) a K5-N-sulfate-derivative, in acidic form, is treated with a tertiary or quaternary organic base, letting the reaction mixture to stand for a time period of 30-60 minutes, maintaining the pH of the solution at a value of 7 by addition of the same tertiary or quaternary organic base, and its salt is isolated with said organic base;

(b) said salt of organic base of said epiK5-N-sulfate-derivative is treated with an O-sulfation reagent in the conditions of O-oversulfation;

(c) the epi-K5-amine-O-oversulfate-derivative thus obtained is treated with a reagent of N-sulfation and the epiK5-N,O-oversulfate-derivative is isolated.

Generally, the epiK5-N,O-oversulfate-derivative is isolated in sodium salt form and optionally said sodium salt is transformed into another chemically or pharmaceutically acceptable salt.

In this context, the term "chemically acceptable" refers to a cation usable in chemical synthesis, such as the sodium, ammonium, (C₁-

C₄)tetraalkylammonium ion, or for the purification of the product, whereas "pharmaceutically acceptable" is self-explanatory.

Advantageous cations are those derived from alkaline metals, alkaline-earth metals, ammonium, (C₁-C₄)tetraalkylammonium, aluminum and zinc. Preferred cations are the sodium, calcium and tetrabutylammonium ions.

According to an advantageous manner of procedure, step (a) is carried out by passing a solution of the sodium salt of epiK5-N-sulfate-derivative, i.e. of K5 polysaccharide, previously N-deacetylated, N-sulfated, normally 100%, and 20-60% C5-epimerized and optionally depolymerized with nitrous acid, having a mean molecular weight from approximately 1,000 to approximately 25,000, advantageously from approximately 1,500 to approximately 25,000, through an acid ionic exchange resin, for example of the type IR-120 H⁺, collecting the eluate also including the washing water of the resin and neutralizing the eluate with tertiary or quaternary organic base, preferably with an aqueous solution of tetrabutylammonium hydroxide. The solution is let to stand for 1 hour, maintaining its pH at 7 by addition of the same base and the salt thus obtained is isolated by lyophilization.

In step (b), the O-oversulfation occurs by using an excess of O-sulfating agent and working at a temperature from 20 to 70°C for a time period of up to 24 hours in an aprotic polar solvent.

Advantageously, the salt with a tertiary or quaternary organic base of the epiK5-N-sulfate-derivative, i.e. of K5 polysaccharide, previously N-deacetylated, N-sulfated preferably 100%, and 20-60% C5-epimerized and optionally depolymerized with nitrous acid, having a mean molecular weight from approximately 1,000 to approximately 25,000, advantageously from approximately 1,500 to approximately 25,000 as isolated in step (a), is dissolved in dimethylformamid and treated with

2 – 10 mol s of an O-sulfation reagent for every free hydroxyl at a temperature of 40-60°C for 10-20 hours. As an O-sulfation reagent is advantageously used the pyridine.SO₃ adduct in a quantity of 2.5 – 5 moles, preferably 2.5 – 4 moles per free hydroxyl per disaccharide and the reaction is advantageously carried out at 50-60°C, preferably at 55°C, overnight. The product obtained upon termination of the reaction is isolated by addition of 0.1-1 volume of water and neutralization, preferably with sodium hydroxide, precipitation with a saturated sodium chloride solution in acetone, filtration and possible ultrafiltration.

The product thus obtained is generally the sodium salt of an epiK5-amine-O-oversulfate-derivative having an iduronic acid content of 20-60% of the total of the uronic acids, having a mean molecular weight from approximately 3,500 to approximately 40,000, advantageously from approximately 4,500 to approximately 40,000 and a sulfation degree of at least 3.4, advantageously of at least 3.5, more advantageously from 3.55 to 4, preferably from 3.55 to 3.8. The sodium salt thus obtained can be converted into another salt. By way of example an exchange with the calcium ion can be performed working with ultrafiltration membranes.

In step (c), the epiK5-amine-O-oversulfated-derivative with a very high degree of sulfation is N-sulfated using the known N-sulfation methods in literature.

In practice, the N-sulfation is performed by treating an aqueous solution containing the epiK5-amine-O-oversulfated-derivative originating from step (b) with sodium carbonate and an agent of N-sulfation, for example a (C₁-C₄)trialkylamine.SO₃ or pyridine.SO₃ adduct, maintaining the mixture at 30-50°C for 8-24 hours and isolating the desired epiK5-N,O-oversulfate-derivative, for example by

diafiltration. Optionally the step of N-sulfation is repeated until obtaining more than 95% substitution, preferably complete.

The new epiK5-N,O-oversulfate-derivatives thus obtained are generally in their sodium salt form. Said sodium salt can be converted into another chemically or pharmaceutically acceptable salt. Particularly advantageous salts are those of alkaline metals, alkaline-earth metals, of ammonium, (C₁-C₄)tetraalkylammonium, aluminum and zinc. Preferred are the salts of sodium, calcium and tetrabutylammonium.

The starting epiK5-N-sulfates subjected to step (a) of the process of the present invention are derived from a K5 polysaccharide, previously N-deacetylated, N-sulfated virtually 100%, and 20-60% C5-epimerized, advantageously 40-60%, and optionally depolymerized with nitrous acid, having a mean molecular weight from approximately 1,000 to approximately 25,000, advantageously from approximately 1,500 to approximately 25,000. Preferably, said starting material is an epi-K5-N-sulfate having a mean molecular weight between 10,000 and 25,000 or a LMW-epiK5-N-sulfate having a mean molecular weight from approximately 1,000 to approximately 12,000, advantageously from approximately 1,000 to approximately 10,000, preferably between approximately 1,500 and approximately 8,000.

The epiK5-N-sulfates, prepared by C5-epimerization of K5-N-sulfates, are well known in literature and widely described for example in WO 92/17507, WO 01/72848, WO 98/14425, WO 97/43317 or US 2002/0062019. Their preparation by C-5 epimerization of the glucuronic unit of K5-N-sulfate with a D-glucuronyl C5 epimerase was described in documents cited herein above.

A LMWepiK5-N-sulfate having an iduronic unit content of approximately 20%, obtained by N-deacetylation, N-sulfation and C5-

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epimerization of a moiety of K5 having a mean molecular weight of 5,000 is described in WO 92/17507. However such LMW-K5-N-sulfate contains a considerable quantity of acetyl groups.

An epiK5-N-sulfate with an iduronic acid content of 40-60%, particularly advantageous as a starting material, is that obtained by epimerization of a K5-N-sulfate virtually free of acetyl groups, in turn prepared from particularly pure K5, in particular not containing lipophilic substances, described in WO 02/068477. According to a preferential manner of procedure, by epimerization a K5-N-sulfate is used obtained from a K5 free of lipophilic substances like that described in WO 02/068477 and the C5 epimerization is performed with a D-glucuronyl C5-epimerase that is isolated, purified and immobilized on a solid support, at a pH of approximately 7, at a temperature of approximately 30°C and for a time period of 12-24 hours in the presence of at least one bivalent ion selected among calcium, magnesium, barium and manganese.

The LMW-epiK5-N-sulfates having a higher content of iduronic units, in particular 40-60%, preferably 50-55%, are new, particularly advantageous products as starting materials in the preparation of LMW-epiK5-N,O-oversulfate-derivatives.

The LMW-epiK5-N-sulfates as shown above are prepared by a process characterized in that a K5-N-sulfate is subjected, in any one order,

- (i) to C5-epimerization with a D-glucuronyl C5-epimerase that is isolated, purified and in solution or immobilized on a solid support, at a pH of approximately 7, at a temperature of approximately 30°C and for a time period of 12-24 hours in the presence of at least one bivalent ion selected among calcium, magnesium, barium and manganese; and
- (ii) to a nitrous depolymerization optionally followed by

reduction, normally with sodium borohydride.

The expression "in any order" shows that the process can be indifferently carried out both in the direction (i)-(ii), i.e. in the sequence shown above, as well as in reverse direction, i.e. also in the direction (ii)-(i), subjecting the K5-N-sulfate at first to the nitrous depolymerization reaction, optionally followed by reduction with sodium borohydride, and afterwards to C5-epimerization in the aforesaid conditions. The preferred order is in the direction (i)→(ii). The sequence (ii)-(i) is preferably utilized starting with LMW-K5-N-sulfates having a mean molecular weight of more than 4,000, preferably starting with approximately 6,000. For example, one can determine the amount of sodium nitrite which, starting with 1 g of epiK5-N-sulfate, allows the attainment of a LMW-epiK5-N-sulfate with a molecular weight of more than 4,000, in particular of at least 6,000, so as to obtain useful intermediates for the preparation of LMWepiK5-N,O-oversulfates. In fact, in this case, in step (ii) the percentage of optimum epimerization is obtained.

According to a preferential aspect of the invention, the C5-epimerase is immobilized on an inert solid support.

The C5-epimerase, preferably recombinant, isolated and purified for example according to Campbell 1994, WO 98/48006, Jin-Ping 2001 or Crawford 2001, is immobilized on an inert support in the presence of the substrate, i.e. in the presence of starting K5-N-sulfate-derivative or in the presence of LMW-K5-N-sulfate, advantageously having a mean molecular weight of more than 4,000, preferably of at least 6,000. The immobilization is performed according to conventional methods, for example as described in WO 01/72848.

The C-5epimerization reaction is carried out by recirculating 20-1,000 ml of a 25 mM HEPES solution at a pH of approximately 7 containing

0.001-10 g of substrate (K5-N-sulfate or LMW-K5-N-sulfate, preferably with a molecular weight of more than 4,000, in particular of at least 6,000) and a cation selected among calcium, magnesium, barium and manganese at a concentration of between 10 and 60 mM through a column containing from 1.2×10^7 to 3×10^{11} cpm of the immobilized enzyme, maintaining the pH at approximately 7 at approximately 30°C, at a flow of 30-220 ml/hour for a time period of 12-24 hours, advantageously 15-24 hours.

Preferably said solution is recirculated at a flow of approximately 200 ml/hour overnight (15-20 hours). The product obtained is purified and separated according to known methods, for example by ultrafiltration and precipitation with ethanol. The product thus obtained is either consisting of epiK5-N-sulfate (and in such case is dissolved in water and subjected to depolymerization) or LMW-epiK5-N-sulfate (in such case it constitutes the end product). The percentage of epimerization, in practice the amount of iduronic units as regards the glucuronic ones, is calculated using ^1H -RMN according to the method described in WO 96/4425.

The nitrous depolymerization reaction is carried out according to known methods by the depolymerization of heparin, for example according to the method described in EP 37319, in WO 82/03627 or according to the method by depolymerization of a K5-N-sulfate described in EP 544592, but starting with a K5-N-sulfate or an epiK5-N-sulfate containing from 0 to no more than 10%, preferably no more than 5%, of acetyl groups. Preferably, the depolymerization, performed with sodium nitrite and hydrochloric acid on an epiK5-N-sulfate virtually free of acetyl groups, is followed by *in situ* reduction with sodium borohydride.

In practice, a cold aqueous solution of epiK5-N-sulfate is brought to

acid pH (approximately 2) with hydrochloric acid and, still cold, treated with sodium nitrite maintaining the temperature (approximately 4°C) and the pH (approximately 2) constant and, upon termination of depolymerization (approximately 15 - 30 minutes) the solution is neutralized with sodium hydroxide and treated, still at approximately 4°C, with an aqueous solution of sodium borohydride. Upon termination of the reduction (approximately 4 hours) the excess sodium borohydride is destroyed with hydrochloric acid, the solution is neutralized with sodium hydroxide and the depolymerized (and reduced) product is isolated according to known methods, for example by straightforward precipitation with ethanol or acetone. The product obtained upon termination of depolymerization can be either a LMW-epiK5-N-sulfate (in such case it constitutes the end product) or a LMW-K5-N-sulfate (and in such case is directly subjected to C5-epimerization as herein shown above, after isolation or also in solution without being previously isolated), in particular when it has a mean molecular weight of more than 4,000, preferably of at least 6,000, or is utilized to prepare antiangiogenetic and antiviral activity LMW-K5-N,O-oversulfated. By appropriately controlling the depolymerization reaction, in particular using different amounts of sodium nitrite/hydrochloric acid, are obtained LMW-K5-N-sulfates or LMW-epiK5-N-sulfates having a mean molecular weight in the entire interval from approximately 1,500 to approximately 12,000, advantageously from approximately 1,500 to approximately 10,000, preferably from approximately 1,500 to approximately 7,500, calculated at the ^{13}C -RMN spectrum through the integration of the signal attributed to the C2 of 2,5-anhydromannitol with that of the anomeric carbon of the glucosamine inside the polysaccharide chain. According to a general manner of procedure, starting for example

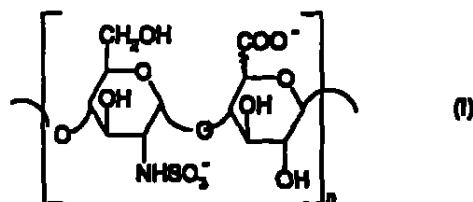
with 1 g of piK5-N-sulfate, the starting product is dissolved in 100-200 ml of deionized water and thermostated at 4 °C. Then an amount of sodium nitrite is added so as to obtain the desired mean molecular weight, for example from approximately 2,000 to approximately 4,000. Therefore, starting with an epiK5-N-sulfate having a molecular weight of 20,000 measured with the HPLC method equipped with a BioRad BioSil 250 column and using a heparin standard of known molecular weight, will require the addition of 330 to 480 mg of sodium nitrite dissolved in a 0.2 % aqueous solution. The solution containing the epiK5-N-sulfate and the sodium nitrite, kept at 4°C, is brought to pH 2 through the addition of 0.1 N HCl cooled to 4°C. It is left to react under slow agitation for 20-40 minutes, then is neutralized with 0.1 N NaOH. The product obtained is brought to room temperature and treated with reducing agent such as for example sodium borohydride (250-500 mg dissolved in 50-100 ml of water) and left to react for 4-8 hours. The excess sodium borohydride is eliminated bringing the pH to 5-5.5 with 0.1 N HCl and let to stand for a further 2-4 hours. In the end it is neutralized with 0.1 N NaOH and the product is recovered by precipitation with acetone or ethanol after having concentrated the product by evaporation at reduced pressure.

Similarly, the amount of sodium nitrite can be determined which, starting with 1 g of K5-N-sulfate or epiK5-N-sulfate, allows the attainment of a LMW-K5-N-sulfate or a LMW-epiK5-N-sulfate with a mean molecular weight from approximately 4,000 to approximately 12,000, advantageously from approximately 4,000 to approximately 7,500, in particular of 6,000-7,500.

The LMW-epiK5-N-sulfate thus obtained, with an iduronic acid content from 20 to 60%, advantageously from 40 to 60%, preferably of 50-55% and virtually free of NH₂ and N-acetyl groups, having a mean

molecular weight from approximately 1,500 to approximately 12,000, advantageously from approximately 1,500 to approximately 10,000, preferably from approximately 1,500 to approximately 7,500 and their chemically or pharmaceutically acceptable salts constitute new products useful as particularly interesting starting materials in the preparation of LMW- epiK5-N,O-oversulfates, but also themselves useful as active ingredients of pharmaceutical or cosmetic compositions and constitute an additional aspect of the present invention.

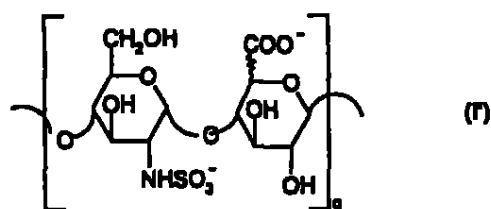
Advantageously, the starting materials in the preparation of the epiK5-N,O-oversulfate-derivatives of the present invention are epiK5-N-sulfate-derivatives consisting of a chain mixture in which at least 90% of said chains have the formula I



in which the uronic units are 20-60% consisting of iduronic acid, n is a integer from 2 to 100, advantageously from 3 to 100 and the corresponding cation is chemically or pharmaceutically acceptable. More advantageously, said starting epiK5-N-sulfate-derivatives are consisting of a chain mixture in which at least 90% of said chains have the formula I in which the uronic units are 40-60% consisting of iduronic acid, n is a integer from 2 to 100, advantageously from 3 to 100 and the corresponding cation is chemically acceptable. Preferred starting materials are LMW-epiK5-N-sulfates as shown above, consisting of a chain mixture in which at least 90% of said chains have the formula I in which the uronic units are 20-60% comprised advantageously 40-60%, preferably 50-55%, of iduronic acid, n is a integer from 2 to 20,

advantageously from 3 to 15 and the corresponding cation is chemically acceptable.

In practice, said preferred LMW-epiK5-N-sulfates are consisting of a chain mixture in which at least 90% of said chains have the formula I'

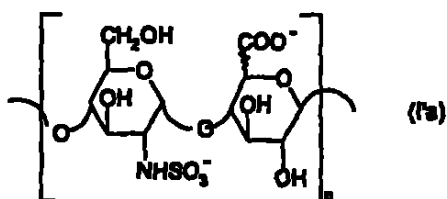


in which the uronic units are 20-60% comprised, advantageously 40-60%, preferably 50-55% of iduronic acid, q is a integer from 2 to 20, advantageously from 3 to 15, and the corresponding cation is chemically or pharmaceutically acceptable.

In this context, the term "chemically" refers to a cation usable in chemical synthesis, such as sodium, ammonium, (C₁-C₄)tetraalkylammonium ions, or for the purification of the product.

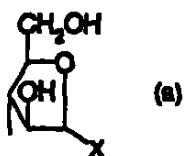
Advantageous cations are those derived from alkaline metals, alkaline-earth metals, ammonium, (C₁-C₄)tetraalkylammonium, aluminum and zinc. Preferred cations are the sodium, calcium and tetrabutylammonium ions.

Particularly interesting are the LMW-epiK5-N-sulfates consisting of a chain mixture in which at least 90% of said chains have the formula I' herein above, obtained by nitrous depolymerization of the corresponding epiK5-N-sulfates shown above and subsequent possible reduction for example with sodium borohydride. Among these, are preferred the LMW-epiK5-N-sulfates consisting of a chain mixture in which the preponderant species has the formula I'a

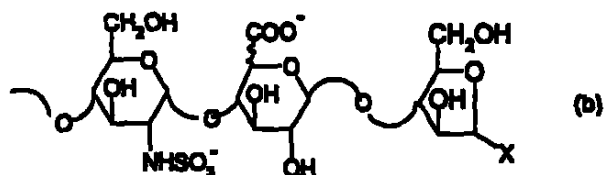


in which the uronic units are 60-40% consisting of glucuronic acid and 40% to 60% of iduronic acid, p is a integer from 4 to 8. The mean molecular weight of these products is from approximately 2000 to approximately 4000 and the corresponding cation is chemically or pharmaceutically acceptable.

The origin of these epiK5-N-sulfates from a step of nitrous depolymerization involves, at the reducing end of the majority of the chains in said chain mixture, the presence of a 2,5-anhydromannose unit or, in the case of reduction with for example sodium borohydride, of 2,5-anhydromannitol of structure (a)



In which X represents a formyl group or a hydroxymethyl group. Therefore, the reducing end of the majority (60-70% of the chains) is actually represented by the structure (b)

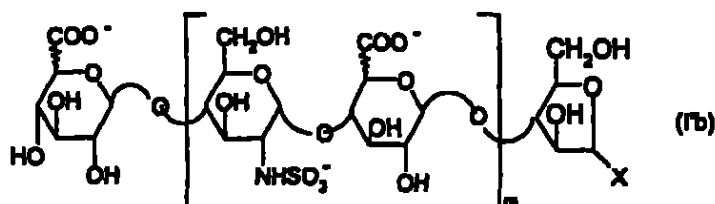


in which X is as defined above.

The presence of the structure (a) does not have any influence on the chemical characteristics of the epiK5-N-sulfates and their derivatives since any sulfations would lead to a possible introduction of one or two sulfate groups which would not however significantly move the

sulfation degree of the O-sulfated derivatives. It is however preferable that the nitrous depolymerization is followed by reduction for example with sodium borohydride since, according to the process of the present invention, said LMW-epiK5-N-sulfates are subjected to sulfation and acylation reactions whose influence, of the 2,5-anhydromannose radical of structure (a), is unknown on the formyl group in which X represents formyl. Besides, the presence of structure (a) does not influence the biological activity of the products, as demonstrated by Østergaard P. B. et al. in Thrombosis Research, 1987, 45, 739-749 (Østergaard 1987) for the heparins of low molecular weight.

Particularly advantageous LMW-epiK5-N-sulfates according to the present invention are consisting of chain mixtures in which the preponderant species is a compound of formula I'b



in which X is formyl or, preferably, hydroxymethyl, m is 4, 5 or 6, the corresponding cation is one chemically or pharmaceutically acceptable ion and the glucuronic and iduronic units are present alternately, starting with a glucuronic or iduronic unit. In such case the glucuronic/iduronic ratio is from 45/55 to 55/45, i.e. approximately 50/50.

The use of the C5-epimerase, preferably recombinant, preferably immobilized on a solid support in the conditions shown above therefore allows not the "cluster" epimerization of K5-N-sulfate derivatives into epiK5-N-sulfate-derivatives as occurs in nature, but the ordinary type.

Thus, according to another of its aspects, the present invention

provides the use of the isolated and purified C5-epimerase, for the conversion of a K5-N-sulfate-derivative into a corresponding epiK5-N-sulfate-derivative characterized by a repetitive tetrasaccharide unit consisting of two glucosamine units separated by a glucuronic unit and followed by an iduronic unit or separated by an iduronic unit and followed by a glucuronic unit.

Said epimerization occurs optimally if carried out on a K5-N-sulfate-derivative having a mean molecular weight of more than 4,000, preferably from 6,000 to 7,500.

According to the present invention, the starting epiK5-N-sulfate-derivatives, preferably 100% N-sulfated (in particular the epiK5-N-sulfate-derivatives consisting of chain mixtures in which at least 90% of said chains have the formula I or I' or in which the preponderant species has the formula I'a or I'b where X is hydroxymethyl), are subjected to the aforesaid steps (a) and (b), upon termination of which are isolated the corresponding, new epiK5-amine-O-oversulfate-derivatives, in which the amine is not substituted, normally in sodium salt form, which can be transformed into another chemically or pharmaceutically acceptable salt. Particularly advantageous salts are those of alkaline metals, alkaline-earth metals, ammonium, (C₁-C₄)tetraalkylammonium, aluminum and zinc and, among these, the salts of sodium, calcium and tetrabutylammonium are preferred.

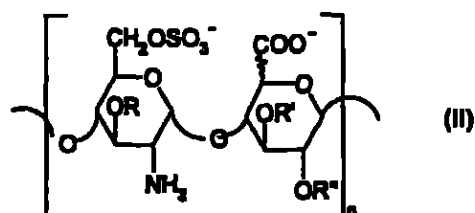
Thus, according to another of its aspects, the present invention refers to new epiK5-amine-O-oversulfate-derivatives and their chemically or pharmaceutically acceptable salts, obtainable by a process characterized in that

(a) an epiK5-N-sulfate-derivative, in acidic form, is treated with a tertiary or quaternary organic base, letting the reaction mixture to stand for a time period of 30-60 minutes, maintaining the pH of the

solution at a value of 7 by addition of said tertiary or quaternary organic base and its salt is isolated with said organic base;

(b) said salt of organic base of said epiK5-N-sulfate-derivative is treated with an O-sulfation reagent in the conditions of O-oversulfation and the epiK5-amine-O-oversulfate-derivative is isolated.

Using, as advantageous starting materials of step (a), epiK5-N-sulfate-derivatives consisting of a chain mixture in which at least 90% of said chains has the aforesaid formula I, in which the uronic units are 20-60% consisting of iduronic acid, n is a integer from 3 to 100 and the corresponding cation is chemically or pharmaceutically acceptable, at the end of step (b) an epiK5-amine-O-oversulfate-derivative is obtained consisting of a chain mixture in which at least 90% of said chains have the formula II



in which the uronic units are 20-60% consisting of iduronic acid, n is a integer from 2 to 100, preferably from 3 to 100, R, R' and R'' are hydrogen or SO₃-, for a sulfation degree of at least 3.4, advantageously of at least 3.5, more advantageously from 3.55 to 4, preferably from 3.55 to 3.8 and the corresponding cation is chemically or pharmaceutically acceptable.

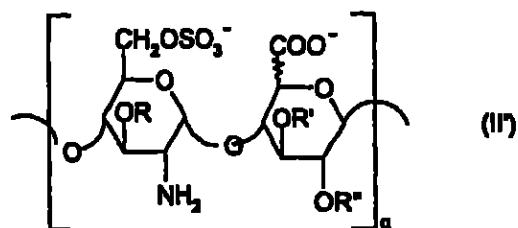
These epiK5-amine-O-oversulfate-derivatives with a very high degree of sulfation are new products useful as intermediates in the preparation of their N-sulfate or N-(C₂-C₄)acylated derivatives basically free of activity on the coagulation parameters, but having other interesting pharmacological properties.

Advantageous epiK5-amine-O-oversulfate-derivatives with a very high

degree of sulfation are consisting of a chain mixture in which at least 90% of said chains have the formula II in which the uronic units are 40-60% consisting of iduronic acid, n is a integer from 2 to 100, preferably from 3 to 100, with a mean molecular weight from approximately 2,000 to approximately 40,000, advantageously from approximately 4,500 to approximately 40,000, R is at least 40%, preferably 50-80% SO_3^- , R' and R'' are both SO_3^- or one is hydrogen and the other is 5-10% SO_3^- in monosulfate glucuronic acid and 10-15% SO_3^- in monosulfate iduronic acid, the degree of sulfation is more than 3.4, advantageously of at least 3.5, more advantageously from 3.55 to 4, preferably from 3.55 to 3.8, and the corresponding cation is chemically or pharmaceutically acceptable.

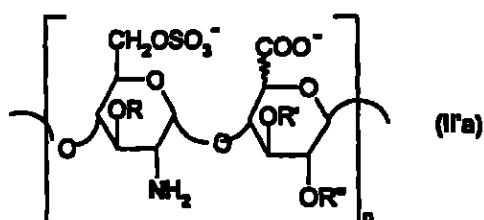
Preferred epiK5-amine-O-oversulfate-derivatives with a very high degree of sulfation are LMW-epiK5-amine-O-oversulfates consisting of a chain mixture in which at least 90% of said chains have the formula II in which the uronic units are comprised 40-60%, preferably 50-55%, of iduronic acid, R is at least 40%, advantageously 50-80%, preferably approximately 65% SO_3^- , R' and R'' are both SO_3^- or one is hydrogen and the other is 5-10% SO_3^- in glucuronic acid and 10-15% SO_3^- in iduronic acid, n is a integer from 2 to 20, advantageously from 3 to 15, with a mean molecular weight from approximately 4,000 to approximately 8,000 and the corresponding cation is chemically or pharmaceutically acceptable.

In practice, said preferred LMW-epiK5-amine-O-oversulfates are consisting of a chain mixture in which at least 90% of said chains have the formula II'



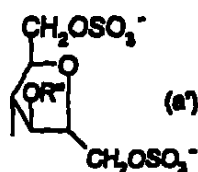
in which the uronic units are 20-60% consisting of iduronic acid, q is a integer from 2 to 20, advantageously from 3 to 15, R , R' and R'' are hydrogen or SO_3^- , for a sulfation degree of at least 3.4, advantageously of at least 3.5, more advantageously from 3.55 to 4, preferably from 3.55 to 3.8, and the corresponding cation is on chemically or pharmaceutically acceptable ion.

Among these LMW-epiK5-amine-O-oversulfates are preferred those consisting of a chain mixture in which the preponderant species has the formula II'a



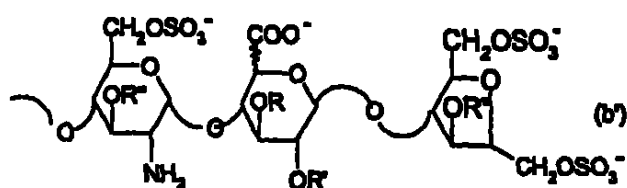
in which the uronic units are 20-60% consisting of iduronic acid, p is a integer from 4 to 8, R , R' and R'' are as defined above, the degree of sulfation is at least 3.4, advantageously of at least 3.5, more advantageously from 3.55 to 4, preferably from 3.55 to 3.8 and the corresponding cation is chemically or pharmaceutically acceptable.

The origin of the new LMW-epiK5-amine-O-oversulfates from LMW-epiK5-sulfates obtained by nitrous depolymerization and subsequent reduction with, for example, sodium borohydride, involves, at the reducing end of the majority of the chains in said chain mixture, the presence of a 2,5-anhydromannitol sulfated unit of structure (a')



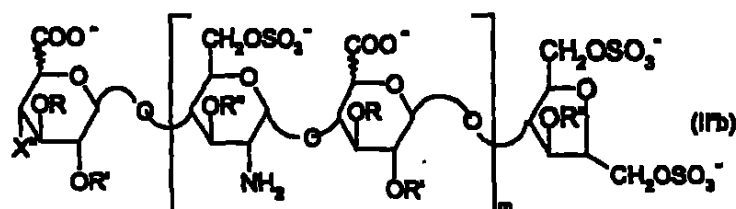
in which R'' represents hydrogen or SO₃.

Thus, the reducing end of the majority of the chains in said chain mixture is represented by the structure (b')



in which the uronic unit can be glucuronic or iduronic.

Among the aforesaid new LMW-epiK5-amine-O-oversulfates, are preferred those consisting of mixtures in which the preponderant species is a compound of formula II'b



in which the uronic units are 40-60% consisting of iduronic acid, m is 4, 5 or 6, R, R' and R'' are hydrogen or SO₃, X'' is OH or OSO₃, for a sulfation degree of at least 3.4, advantageously of at least 3.5, more advantageously from 3.55 to 4, preferably from 3.55 to 3.8, the iduronic units being present alternately, starting with a glucuronic or iduronic unit, and the corresponding cation is one chemically or pharmaceutically acceptable ion.

All these epiK5-amine-O-oversulfated-derivatives with a very high degree of sulfation are new products which are useful intermediates for the preparation of the new N-substituted epiK5-amine-O-

oversulfated derivatives and therefore constitute an additional aspect of the present invention.

In particular, according to another of its aspects, the invention concerns the use of the aforesaid epiK5-amine-O-oversulfated-derivatives with a very high degree of sulfation for the preparation of new N-substituted epiK5-amine-O-oversulfated-derivatives, in particular N-sulfated or N-acylated.

Upon termination of step (c) of the process of the present invention, consisting of an N-sulfation of the epiK5-amine-O-oversulfate-derivatives obtained at the end of step (b) (in particular the epiK5-amine-O-oversulfate-derivatives consisting of chain mixtures in which at least 90% of said chains have the formula II or II' or in which the preponderant species has the formula II'a or II'b) epiK5-N,O-oversulfate-derivatives are obtained whose iduronic acid content is 20-60% of the total of the uronic acids and whose sulfation degree is at least 4, preferably from 4 to 4.6.

Thus, according to another of its aspects, the present invention provides new N-deacetylated derivatives of K5 polysaccharide, O-sulfated and N-sulfated, C5-epimerized to iduronic acid in at least 20% of the total of the uronic units, having a mean molecular weight from approximately 2,000 to approximately 45,000, a sulfation degree of at least 4, said derivatives being basically inactive on the coagulation parameters.

Similarly to that stated above, said new derivatives are, as a whole, denoted by the general term "epiK5-N,O-oversulfate-derivatives", independently of their molecular weight.

In particular, the mean molecular weight is between approximately 2,000 to approximately 45,000 since said derivatives originate either from an epi-K5-N-sulfate obtained by N-deacetylation and N-sulfation

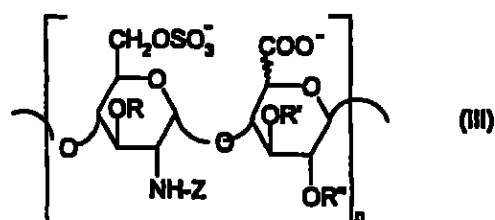
of K5 by fermentation or by the nitrous depolymerization of the latter. By controlling said nitrous depolymerization it is possible to obtain low molecular weight derivatives in virtually all the aforesaid interval. However, for use of the derivatives of the present invention as pharmaceutical or cosmetic products it is advantageous to prepare low molecular weight derivatives, with a mean molecular weight from approximately 2,000 to approximately 16,000, advantageously from approximately 3,500 to approximately 13,000 with a molecular weight distribution of between approximately 1,000 and approximately 15,000, preferably from approximately 4,500 to approximately 9,000, with a molecular weight distribution from approximately 2,000 to approximately 10,000, or of high molecular weight derivatives, originating from the unfractionated K5, with a mean molecular weight of between approximately 20,000 and approximately 45,000, with a molecular weight distribution from approximately 2,000 to approximately 70,000.

In the epiK5-N,O-oversulfate-derivatives of the present invention the degree of sulfation is very high, preferably from 4 to 4.6, the nitrogen of the glucosamine being virtually 100% sulfated. Besides, the epiK5-N,O-oversulfate-derivatives are 100% 6-O-sulfated and 50-80% 3-O-sulfated in their glucosamine units, 5-10% 3-O-monosulfated in glucuronic units, 10-15% O-monosulfated in iduronic units and 2,3-di-O-sulfated in the remaining uronic units, considering that the degree of sulfation is at least 4.

Advantageous epiK5-N,O-oversulfate-derivatives according to the present invention are obtained through epiK5-amine-O-oversulfate-derivatives in turn prepared from epiK5-N-sulfate-derivatives consisting of a chain mixture in which at least 90% of said chains has the aforesaid formula I, in which the uronic units are 20-60% consisting of

iduronic acid, n is a integer from 2 to 100, advantageously from 3 to 100 and the corresponding cation is chemically or pharmaceutically acceptable.

In such case, the new epiK5-N,O-oversulfate-derivatives consisting of chain mixtures in which at least 90% of said chains have the formula III



in which the uronic units are 20-60% consisting of iduronic acid, n is a integer from 2 to 100, preferably from 3 to 100, R , R' and R'' are hydrogen or SO_3 , Z is SO_3 , the degree of sulfation is at least 4, preferably from 4 to 4.6 and the corresponding cation is chemically or pharmaceutically acceptable.

Said cations are advantageously those of alkaline metals, alkaline-earth metals, ammonium, $(\text{C}_1\text{-C}_4)$ tetraalkylammonium, aluminum and zinc and, among these, preferably the salts of sodium, calcium and tetrabutylammonium.

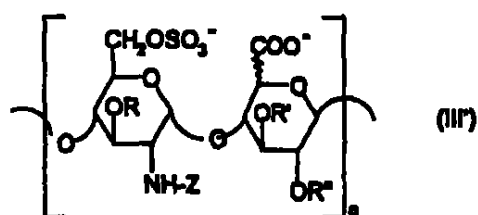
Among the aforesaid new epiK5-amine- N,O-oversulfate-derivatives, those consisting of chain mixtures in which at least 90% of said chains has the aforesaid formula III in which R is SO_3 in 50%-80%, preferably in approximately 65% of said chains and the degree of sulfation is at least 4, advantageously is from 4 to 4.6, preferably from 4 to 4.3.

Advantageous epiK5-N,O-oversulfate-derivatives with a very high degree of sulfation are consisting of a chain mixture in which at least 90% of said chains have the formula III, in which Z is SO_3 , the uronic units are 40-60% consisting of iduronic acid, n is a integer from 2 to 100, preferably from 3 to 100, with a mean molecular weight from approximately 2,000 to approximately 45,000, advantageously from

approximately 4,500 to approximately 45,000, R is at least 40%, preferably 50-80% SO_3^- , R' and R'' are both SO_3^- or one is hydrogen and the other is 5-10% SO_3^- in monosulfate glucuronic acid and 10-15% SO_3^- in monosulfate iduronic acid, the degree of sulfation is at least 4, from 4 to 4.6 and the corresponding cation is chemically or pharmaceutically acceptable.

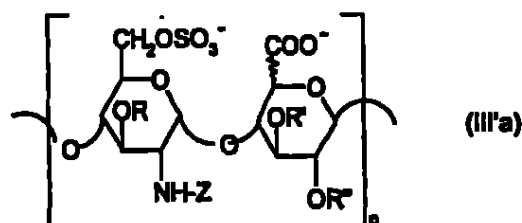
Preferred N-substituted epiK5-amine-O-oversulfated-derivatives are LMW-epiK5-amine-O-oversulfated consisting of a chain mixture in which at least 90% of said chains have the formula III in which the uronic units are 40-60% comprised, preferably 50-55%, of iduronic acid, R is at least 40%, advantageously 50-80%, preferably approximately 65% SO_3^- , R' and R'' are both SO_3^- or one is hydrogen and the other is 5-10% SO_3^- in glucuronic acid and 10-15% SO_3^- in iduronic acid, Z is 100% SO_3^- or (C2-C4)acyl, n is a integer from 2 to 20, preferably from 3 to 15, with a mean molecular weight from approximately 4,000 to approximately 8,500 and the corresponding cation is chemically or pharmaceutically acceptable.

In practice, said preferred epiK5-N,O-sulfate-derivatives with a very high degree of sulfation are consisting of a chain mixture in which at least 90% of said chains have the formula III'



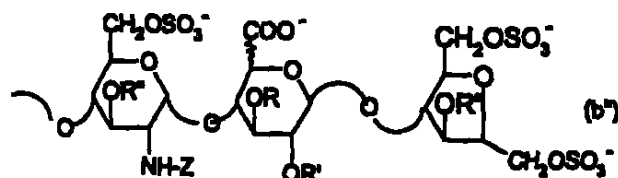
in which the uronic units are 20-60% consisting of iduronic acid, q is a integer from 2 to 20, advantageously from 3 to 15, R, R' and R'' represent hydrogen or an SO_3^- group, Z is SO_3^- , for a sulfation degree of at least 4, preferably from 4 to 4.6 and the corresponding cation is one chemically or pharmaceutically acceptable ion.

Particularly interesting are chain mixtures of formula III' in which the uronic units are 40-60% comprised, preferably 50-55%, of iduronic acid, R is at least 40%, advantageously 50-80%, preferably approximately 65% SO_3^- , R' and R'' are both SO_3^- or one is hydrogen and the other is 5-10% SO_3^- in glucuronic acid and 10-15% SO_3^- in iduronic acid, n is an integer from 2 to 20, advantageously from 3 to 15, with a mean molecular weight from approximately 2,000 to approximately 16,000, advantageously from approximately 3,500 to approximately 13,000, preferably from approximately 4,500 to approximately 9,000 and the corresponding cation is chemically or pharmaceutically acceptable. Among these LMW-epiK5-N,O-oversulfates, are advantageous those consisting of a chain mixture in which the preponderant species has the formula III'a



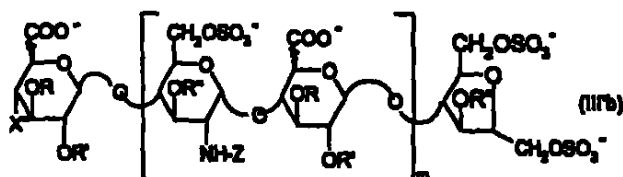
in which the uronic units are 20-60% consisting of iduronic acid, p is an integer from 4 to 8, Z is SO_3^- , R, R' and R'' are hydrogen or SO_3^- , for a sulfation degree of at least 4, preferably from 4 to 4.6 and the corresponding cation is chemically or pharmaceutically acceptable. The origin of the new LMW-epiK5-N,O-oversulfates from LMW-epiK5-sulfates obtained by nitrous depolymerization and subsequent reduction with, for example, sodium borohydride involves, at the reducing end of the majority of the chains in said chain mixture, the presence of a sulfated 2,5-anhydromannitol unit of structure (a') as shown above, in which R'' represents hydrogen or SO_3^- . Thus, the reducing end of the majority of the chains in said chain

mixture is represented by the structure (b'')



in which Z represents SO_3^- and the uronic unit can be glucuronic or iduronic.

Among the aforesaid new LMW-epiK5-N,O-oversulfates, are preferred those consisting of mixtures in which the preponderant species is a compound of formula III''b

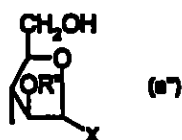


in which R, R' and R'' are hydrogen or SO_3^- , Z is SO_3^- , X'' is OH or OSO_3^- , m is 4, 5 or 6, for a sulfation degree of at least 4, preferably from 4 to 4.6, the uronic units are present alternately, starting with a glucuronic or iduronic unit, and the corresponding cation is one chemically or pharmaceutically acceptable ion. Said cations are advantageously those of alkaline metals, alkaline-earth metals, ammonium, (C₁-C₄)tetraalkylammonium, aluminum and zinc and, among these, preferably the ions of sodium, calcium and tetrabutylammonium.

If an epiK5 is used as a starting epiK5-derivative of the process of the present invention, i.e. a K5 polysaccharide, previously N-deacetylated, N-sulfated normally 100%, and 20-60% C5-epimerized and not depolymerized, upon termination of step (c) an epiK5-N,O-oversulfate is isolated which can be subjected to nitrous depolymerization and possible, subsequent reduction with, for example, sodium borohydride to obtain the corresponding LMW-epiK5-N,O-oversulfate having the

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same degree of sulfation. In particular, LMW-epiK5-N,O-oversulfates are obtained consisting of a chain mixture in which at least 90% of said chains have the formula III' or III'a, in which the uronic units are 20-60% consisting of iduronic acid, q, R, R' R" and Z have the meaning defined above, for a sulfation degree of at least 4, preferably from 4 to 4.6 and the corresponding cation is one chemically or pharmaceutically acceptable ion. In such case, the origin of these LMW-epiK5-N,O-oversulfates from a depolymerization reaction and possible, subsequent reduction with, for example, sodium borohydride involves, at the reducing end of the majority of the chains in said chain mixture, the presence of a 2,5-anhydromanno unit of structure (a'')



in which X is formyl or hydroxymethyl and R" represents hydrogen or SO₃.

The new epiK5-N,O-oversulfate-derivatives, especially in their salt form, are highly anionic products able to capture the free radicals and are utilizable in the cosmetics industry as adjuvants against hair loss or to prepare "anti-ageing" creams and, in the pharmaceutical industry, as products for the treatment of dermatitis. Besides, the epiK5-N,O-oversulfate-derivatives of the present invention, in particular the LMW-epiK5-N,O-oversulfates possess antiangiogenic and antiviral activity and therefore constitute active ingredients for the preparation of medicines.

Thus, according to one of its additional aspects, the present invention provides pharmaceutical compositions including, as one of their active ingredients, a pharmacologically active amount of an epiK5-

N,O-oversulfate-derivative as shown above or of one of its pharmaceutically acceptable salts, in mixture with a pharmaceutical excipient.

In the pharmaceutical compositions of the present invention for oral, subcutaneous, intravenous, transdermal or topical administration, the active ingredients are preferably administered in the form of dosage units, in mixture with the classic pharmaceutical excipients or vehicles. The posology can vary widely depending on the age, weight, and the health condition of the patient. This posology includes the administration of a dose from 1 to 1000 mg, advantageously from 10 to 750 mg, preferably 250 to 500 mg from one to three times a day by intravenous, subcutaneous, oral, transdermal or topical administration. The pharmaceutical compositions of the present invention are formulated with the classic excipients suitable for different ways of administration. Particularly advantageous are the formulations in the form of creams, ointments, liniments, gels, foams, balsams, vaginal pessaries, suppositories, solutions or suspensions suitable for local administration.

Advantageously, the compositions of the present invention include, as one of its active ingredients, an epiK5-N,O-oversulfate-derivative obtainable starting with an epiK5-derivative according to steps (a), (b) and (c) of the process described above, or starting with an epiK5 not depolymerized, according to steps (a), (b) and (c) of the process described above, with possible subsequent nitrous depolymerization after step (c), or one of its pharmaceutically acceptable salts, in mixture with a pharmaceutical excipient. Advantageously, said epiK5-N,O-oversulfate-derivative consists of a chain mixture in which at least 90% of said chains have the formula III or III' or in which the preponderant species is a compound of formula III'a or III'b. Preferred

active ingredient is a LMW-epiK5-N,O-oversulfat having a sulfation degree of at least 4, preferably from 4 to 4.6, advantageously having a mean molecular weight from approximately 3,500 to approximately 11,000, more advantageously from approximately 3,500 to approximately 5,200 and basically free of N-acetyl groups.

Finally, according to another of its aspects, the present invention provides a cosmetic composition including an effective amount of an epiK5-N,O-oversulfate-derivative or one of its pharmaceutically acceptable salts, in mixture with a cosmetic excipient.

A salt selected from the group consisting of salts of sodium, potassium, calcium, magnesium, aluminum and zinc of the epiK5-N,O-oversulfat derivatives, in particular those consisting of chain mixtures in which at least 90% of said chains have the formula III or III' or in which the preponderant species has the formula III'a or III'b, constitutes an effective active ingredient of the pharmaceutical or cosmetic compositions of the present invention.

The following examples illustrate the invention without however limiting it.

PREPARATION I

Preparation of K5 polysaccharide from Escherichia coli

At first fermentation is carried out in an Erlenmeyer flask using the following medium:

Fat-free soya meal	2	g/l
K ₂ HPO ₄	9.7	g/l
KH ₂ PO ₄	2	g/l
MgCl ₂	0.11	g/l
Sodium citrate	0.5	g/l
Ammonium sulfate	1	g/l
Glucose	2	g/l

Spring water 1000 ml

pH = 7.3

The medium is sterilized at 120°C for 20 minutes. The glucose is prepared separately in solution form which is sterilized at 120°C for 30 minutes and added sterilely to the medium. The Erlenmeyer flask is inoculated with a suspension of *E. coli* cells BI 8337/41 (O10:K5:H4) originating from a slant kept in Tryptic soy agar, and incubated at 37°C for 24 hours under controlled agitation (160 rpm, 6 cm stroke). The bacterial growth is measured by counting the cells using a microscope. In a subsequent operation, a 14 l Chemap-Braun fermenter containing the same medium as above, is 0.1% inoculated with the culture of the Erlenmeyer flask as above and fermentation is performed by aeration of 1 vvm, (vvm = volume of air per volume of liquid per minute) 400 rpm agitation and temperature of 37°C for 18 hours. During fermentation are measured the pH, the oxygen, the glucose residue, K5 polysaccharide produced and bacterial growth. At the end of fermentation the temperature is brought to 80°C for 10 minutes. The cells are separated from the medium through centrifugation at 10,000 rpm and the supernatant is ultrafiltered using an SS 316 (MST) module fitted with PES membrane with nominal cut-off of 800 and 10,000 D to reduce the volume to 1/5. K5 polysaccharide is then precipitated by addition of 4 volumes of acetone at 4°C and left to settle overnight at 4°C. Finally it is recovered by centrifugation at 10,000 rpm for 20 minutes or filtration. Deproteinization of the solid obtained is carried out by using a type II protease from *Aspergillus oryzae* in a buffer of 0.1 M NaCl and 0.15 M EDTA at pH 8 containing SDS (0.5% sodium dodecyl sulfate) (10 mg/l of filtrate) at 37°C for 90 minutes. The solution obtained is ultrafiltered on model SS 316 with membrane at a nominal cut-off of 10,000 D with 2 extractions with 1M

NaCl and washed with water until disappearance of absorbance in the ultrafiltrate. K5 polysaccharide is then precipitated with acetone and a yield of 850 mg per litre of fermenter is obtained. The purity of the polysaccharide obtained is measured through the determination of the uronic acids (carbazole method), proton and carbon 13 NMR, UV and protein content. The purity is more than 80%.

The polysaccharide obtained is composed of two moieties of different molecular weight, respectively 30,000 and 5,000 D as emerges from the determination by HPLC using a Pharmacia 75 HR column and a single moiety with a retention time of approximately 9 minutes using two seriate columns of Bio-sil SEC 250 (Bio Rad) and Na₂SO₄ as mobile phase at room temperature and a flow of 0.5 ml/minute. The measurement is performed against a standard curve obtained with moieties of heparin of known molecular weight.

The ¹H- RMN spectrum of the purified K5 thus obtained shows different signals attributable to methyls of lipophilic substances.

PREPARATION II

Purification of K5

In 100 ml of a saturated aqueous solution of sodium chloride and thermostated at 4°C is dissolved 1 g of K5 obtained at the end of PREPARATION I and to the solution thus obtained are added 3 volumes of cold isopropanol. The saline concentration of the solution is brought to 3 M by addition of the calculated amount of a saturated sodium chloride solution and the solution obtained is left in a cold environment (approximately 4°C) overnight. The precipitate which forms is separated by centrifugation at 10,000 rpm for 20 minutes and the purity of the product is checked by dialysis overnight and subsequent examination of the ¹H-RMN spectrum, from which signals in the region under 1.5ppm must be absent. Optionally, the operation of

dissolution in water saturated with NaCl and precipitation with isopropanol is repeated. The precipitate is dissolved in water and ultrafiltered on a Miniplat Millipore membrane 10,000 D cut off until disappearance of the salts. Thus a K5 having a purity of at least 99% is obtained from whose ^1H -RMN spectrum no traces of lipophilic impurities result in the region under 1.5 ppm.

PREPARATION III

Preparation of a K5-N- sulfate

(i) N-Deacetylation

Ten grams of pure K5 polysaccharide prepared as described in PREPARATION II are dissolved in 1000 ml of 2N sodium hydroxide and the solution thus prepared is left at 60 °C for 24 hours. The solution is brought to room temperature then to neutral pH (pH7) with 6N hydrochloric acid.

(ii) N-sulfation

To the solution containing the deacetylated K5, kept at 40 °C, are added 16 g of sodium carbonate and afterwards and in 4 hours, 16 g of pyridine. SO_3 . At the end of the reaction, after 24 hours, the solution is brought to room temperature, then to pH 6.5-7 with a 5% solution of hydrochloric acid. The product is purified from salts by diafiltration using a 1,000 D helically wound membrane (prepscale cartridge-Millipore). The process is terminated when the conductivity of the permeate is less than 1000 μS , preferably less than 100 μS . The intradialysis is reduced until a 10% concentration of the polysaccharide is obtained using the same in concentration dialysis system. The concentrated solution is dried by lyophilization. Upon ^{13}C -RMN spectrum analysis N-acetyl or NH_2 residues do not appear.

PREPARATION IV

LMW-K5-N-sulfate

The product obtained as described in Example 1, steps (i) and (ii), of WO 02/068477 is depolymerized by the degradation method with nitrous acid and subsequent reduction of the aldehyde which forms. One continues by dissolving 1 g of K5-N-sulfate in 200 ml of distilled water and adding it with 480 mg of sodium nitrite dissolved in 240 ml of distilled water. The solution is then brought to 4°C and the pH to 2 with 0.1 N HCl and maintained for 30 minutes. At the end of the reaction the solution is brought to pH 7 with 0.1 M NaOH and then to room temperature. The solution is then added with 450 mg. of NaBH₄ and left to react for 4 hours. The excess NaBH₄ is eliminated with HCl bringing the pH to 5-6. The product, neutralized with 0.1 M NaOH, is recovered by precipitation with 3 volumes of acetone at 4°C, filtration with filtering funnel and dried at 40° C in a vacuum oven. 900 mg. of LMW-K5-N-sulfate are obtained with a mean molecular weight of approximately 2,000, consisting of a chain mixture in which the preponderant species is a compound of formula I'b in which m is 4 and the uronic units are those of glucuronic acid.

EXAMPLE 1

LMW-epiK5-N-sulfate. Sequence (i) → (ii)

(i) Epimerization to epiK5-N-sulfate

Ten grams of K5-N-sulfate obtained as described in Example 1, steps (i) and (ii), of WO 02/068477, from whose ¹H-RMN spectrum, signals concerning acetyl groups or NH₂ do not appear, are dissolved in 600 ml of 25 mM HEPES buffer at pH 7, containing CaCl₂ at a concentration of 50 mM and the solution thus obtained is made to recirculate through a 50 ml column filled with Sepharose 4B resin containing 5 g of recombinant C5-epimerase (WO 96/14425) immobilized as described in Example 1 of WO 01/72848. The reaction is carried out at 30°C at pH 7 with a flow of 200 ml/h for 24 hours. The

product obtained is purified by ultrafiltration and precipitation with ethanol. Thus an epiK5-N-sulfate is obtained whose iduronic acid content is 54%.

(ii) Depolymerization of epiK5-N-sulfate.

To a solution of 1 g of the product thus obtained, in 25 ml of distilled water, are added 230 mg of sodium nitrite dissolved in 115 ml of distilled water. The solution is then brought to 4°C and the pH to 2 with 0.1 N HCl and maintained for 30 minutes. At the end of the reaction the solution is brought to room temperature and the pH to 7 with 0.1 M NaOH. The solution is then added with 450 mg. of NaBH₄ and left to react for 4 hours. The product is recovered by precipitation with 3 volumes of acetone at 4°C, filtration with filtering funnel and dried at 40° C in a vacuum oven. 900 mg of LMW-epiK5-N-sulfate are obtained with an iduronic acid content of 54% and a molecular weight distribution from 1,000 to 4,000, measured with HPLC method.

EXAMPLE 2

LMW-epiK5-N-sulfate. Sequence (ii) → (i)

(ii) Depolymerization of K5-N-sulfate

2 g of K5-N-sulfate, obtained as described in Example 1, steps (i) and (ii), of WO 02/068477, is depolymerized as described in PREPARATION I, using 100 mg of sodium nitrite and 300 mg of sodium borohydride. 1.8 g of LMW-K5-N-sulfate are obtained with a mean molecular weight of 5,000.

(i) Epimerization of LMW-K5-N-sulfate

1 g of LMW-K5 N-sulfate obtained in step (ii) herein above is treated as described in step (i) of the Example 1. An epimerized product is obtained with an iduronic acid/glucuronic acid ratio of 44/56 against a ratio of 0/100 of the starting product, with a molecular weight distribution from 2,000 to 10,000 and with a mean molecular weight of

5,000 D. The yield, calculated by measuring the content of uronic acids against a standard with the carbazole method (Bitter and Muir, Anal. Biochem. 1971, 39, 88-92) is 90%.

EXAMPLE 3

LMW-epiK5-N-sulfate. Sequence (i)→(ii)

(i) Epimerization of K5-N-sulfate

A 2g amount of K5 N-sulfate, obtained as described in Example 1, steps (i) and (ii), of WO 02/068477, is dissolved in 120 ml of 25 mM HEPES buffer, pH 7, containing 50 mM CaCl₂. The solution obtained is made to recirculate through a 50 ml column filled with the resin containing the immobilized enzyme obtained as described in WO 96/14425. This operation is carried out at 30°C with a flow of 200 ml/h for 24 hours. The product obtained is purified through ultrafiltration on a 1000 D membrane and passing over an IR 120 H⁺ ionic exchange column, neutralizing the eluate with 1N NaOH. The sample is recovered by precipitation with ethanol or acetone. An epimerized product is obtained with an iduronic acid/glucuronic acid ratio of 55/45 against a ratio of 0/100 of the starting product. The percentage of epimerization was calculated with ¹H-RMN according to the method described in WO 96/14425. The yield, calculated by measuring the content of uronic acids against a standard with the carbazole method (Bitter and Muir Anal. Biochem. 39, 88-92-1971) is 90%.

(ii) Depolymerization of epi-K5-N-sulfate

One gram of product obtained in step (a) is depolymerized by the degradation method with nitrous acid and subsequent reduction of the aldehyde which forms. In particular one continues by dissolving the product in 25 ml of distilled water and adding it with 230 mg of sodium nitrite dissolved in 115 ml of distilled water. The solution is then

brought to 4°C and the pH to 2 with 0.1 N HCl and maintained for 30 minutes. At the end of the reaction the solution is brought to room temperature and the pH to 7 with 0.1 M NaOH. The solution is then added with 450 mg. of NaBH₄ and left to react for 4 hours. The product is recovered by precipitation with 3 volumes of acetone at 4°C, filtration with filtering funnel and dried at 40° C in a vacuum oven. 900 mg of LMW-epiK5-N-sulfate are obtained with a molecular weight distribution measured with HPLC method which ranges from 1,000 to 4,000 and with a glucuronic unit content of 45% and an iduronic unit content of 55%.

EXAMPLE 4

EpiK5-N,O-oversulfate

(a) Tetrabutylammonium salt of epiK5-N-sulfate

A solution in 40 ml of water of 400 mg of epiK5-N-sulfate, as obtained at the end of step (i) of the Example 1, is thermostated at 4°C, then passed over IR 120⁺ ionic exchange resin preconditioned with water at 4°C. The eluate obtained, consisting of 100 ml of a solution at pH 1.94, is neutralized with a 15% solution of tetrabutylammonium hydroxide and left at room temperature for one hour, maintaining the pH at 7 by addition of 15% tetrabutylammonium hydroxide and finally is lyophilized. Thus 805 mg of tetrabutylammonium salt of epiK5-N-sulfate are obtained.

(b) Epi-K5-amine-O-oversulfate

A solution containing 805 mg of the salt thus obtained in 30 ml of dimethylformamide is set at 55°C and treated with 30 ml of dimethylformamide containing 2.26 g of pyridine.SO₃ adduct. The reaction at 55°C is continued overnight then 60 ml of water are added to the mixture. After neutralization with 1N NaOH, the product is precipitated with 3 volumes of acetone saturated with NaCl and set

at 4°C overnight. The precipitate is recovered by filtration on G4 and then ultrafiltered with 1000 D TFF Millipore system and dried at reduced pressure. 550 mg of epi-K5-amine-O-oversulfated are obtained having a content of iduronic acid of 54%, of glucosamine-6-O-sulfate of 100%, of glucosamine 3-O-sulfate of 60%, of monosulfate glucuronic acid of 10%, of monosulfate iduronic acid of 15%, the rest of the uronic units being disulfated, with a sulfation degree of 3.55 measured with the conductometric method according to Casu et al. 1975.

(c) EpiK5-amine-O-oversulfated-N-sulfate

To a solution of 250 mg of the epi-K5-amine-O-oversulfated obtained in step (b) in 15 ml of water are added 400 mg of sodium carbonate, then to the mixture thus obtained are added 400 mg of pyridine.SO₃ adduct in solid form a little at a time in 4 hours. The reaction mixture is kept at 55°C overnight, then is stopped bringing the pH to 7 with 0.1N HCl. After ultrafiltration on a 1000 D membrane are added 3 volumes of acetone saturated with sodium chloride and the precipitate is recovered by centrifugation at 5000 rpm for 5'. Thus 244 mg of epiK5-N,O-oversulfate are obtained whose sulfation degree, measured with conductometric method according to Casu et al. 1975, is 4.25. By the analysis of the ¹H-RMN spectrum it results that the epiK5-N,O-oversulfate thus obtained has an iduronic acid content of 54%, 6-O-sulfate of 100%, N-sulfate of 100%, glucosamine 3-O-sulfate of 60%, monosulfate glucuronic acid of 10%, monosulfate iduronic acid of 15%, the rest of the uronic units being disulfated. From the ¹H-RMN spectrum is therefore calculated a sulfation degree of 4.35 which, considering the margins of error of the methods, corresponds to the sulfation degree of epiK5-amine-O-oversulfated obtained upon termination of step (b), 100% N-sulfated. It is therefore assumed that,

beyond a certain percentage of sulfate groups, the strong anionic nature of the product can lead to an underestimation of the degree of sulfation determined with the conductometric method.



CLAIMS

1. Process for the preparation of an epiK5-N,O-oversulfate-derivative, characterized in that
 - (a) an epiK5-N-sulfate-derivative having a mean molecular weight of between approximately 1,500 and approximately 25,000, in acidic form, is treated with tertiary or quaternary organic base, letting the reaction mixture to stand for a time period of 30-60 minutes at a pH of approximately 7 and its salt is isolated with said organic base;
 - (b) said salt of organic base of said epiK5-N-sulfate-derivative is treated with an O-sulfation reagent in the conditions of O-oversulfation;
 - (c) a salt of tertiary or quaternary organic base of epiK5-amine-O-oversulfate-derivative thus obtained is treated with a reagent of N-sulfation and the epiK5-N,O-oversulfate-derivative thus obtained is isolated.
2. Process according to claim 1, characterized in that said epiK5-N,O-oversulfate-derivative is isolated in sodium salt form and optionally transformed into another chemically or pharmaceutically acceptable salt.
3. Process according to anyone of claims 1 and 2, characterized in that, in step (a) tetrabutylammonium hydroxide is used as an organic base.
4. Process according to anyone of claims from 1 to 3, characterized in that, in step (b) the O-oversulfation is carried out in dimethylformamide using 2-4 moles of O-sulfation reagent per available OH per disaccharide at a temperature of 40-60°C for 15-20 hours.
5. Process according to anyone of claims from 1 to 4, characterized in that an epiK5-N-sulfate-derivative is used as starting material having a

mean molecular weight from approximately 1,000 to approximately 25,000.

6. Process according to claim 5, characterized in that said starting epiK5-N-sulfate-derivative is 40-60% C5-epimerized.

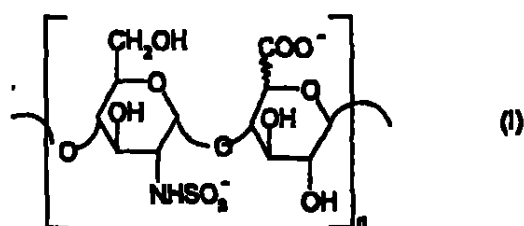
7. Process according to anyone of claims from 1 to 6, characterized in that said starting epiK5-N-sulfate-derivative has a mean molecular weight from approximately 1,500 to approximately 25,000.

8. Process according to claim 7, characterized in that said starting epiK5-N-sulfate-derivative has a mean molecular weight between 10,000 and 25,000.

9. Process according to anyone of claims from 1 to 6, characterized in that said starting material has a mean molecular weight from approximately 1,000 to approximately 12,000.

10. Process according to claim 9, characterized in that said starting material has a mean molecular weight from approximately 1,500 to approximately 8,000.

11. Process according to claim 5, characterized in that an epiK5-N-sulfate-derivative is used as starting material consisting of a chain mixture in which at least 90% of said chains have the formula I



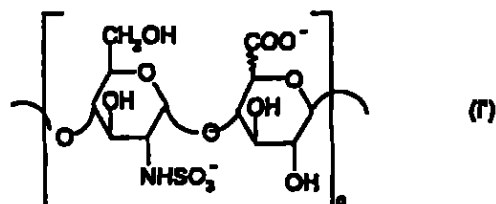
in which the uronic units are 20-60% consisting of iduronic acid, n is a integer from 2 to 100 and the corresponding cation is chemically or pharmaceutically acceptable.

12. Process according to claim 11, characterized in that said starting material consists of a chain mixture in which at least 90% of said chains

have the formula I, in which the uronic units are 40-60% consisting of iduronic acid.

13. Process according to anyone of claims 11 and 12, characterized in that, in the formula I, n represents a integer from 3 to 100.

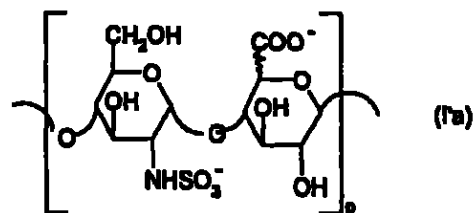
14. Process according to claim 5, characterized in that said starting material consists of a chain mixture in which at least 90% of said chains have the formula I'



in which the uronic units are 20-60% consisting of iduronic acid, q is a integer from 2 to 20 and the corresponding cation is chemically or pharmaceutically acceptable.

15. Process according to claim 14, characterized in that said starting material consists of a chain mixture in which at least 90% of said chains have the formula I', in which n is a integer from 3 to 15.

16. Process according to claim 5, characterized in that said starting material consists of a chain mixture in which the preponderant species has the formula I'a

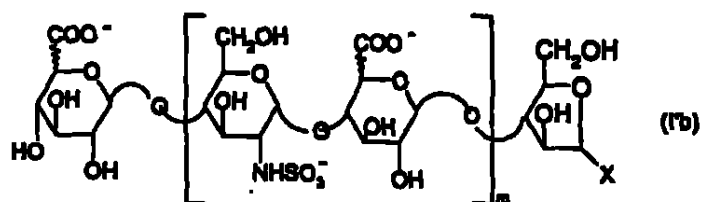


in which the uronic units are 60-40% consisting of glucuronic acid and 40% to 60% of iduronic acid, p is a integer from 4 to 8 and the corresponding cation is chemically or pharmaceutically acceptable.

17. Process according to claim 16, characterized in that the mean

molecular weight of said starting material is from approximately 2000 to approximately 4000.

18. Process according to anyone of claims 16 and 17, characterized in that said starting material consists of a chain mixture in which the preponderant species has the formula I'b



in which X is hydroxymethyl, m is 4, 5 or 6 and the glucuronic and iduronic units are present alternately, starting with a glucuronic or iduronic unit.

19. Process according to anyone of claims from 5 to 18, characterized in that said starting material comes from an N-deacetylation and from an N-sulfation of a K5 that is basically free of lipophilic substances.

20. An epiK5-N,O-oversulfate-derivative obtainable according to the process of claims from 1 to 19.

21. An epiK5-N,O-oversulfate-derivative having an iduronic acid content of 20-60%, a mean molecular weight from approximately 2,000 to approximately 45,000 and a sulfation degree of at least 4, or one of its chemically or pharmaceutically acceptable salts, said derivative being basically inactive on the coagulation parameters.

22. An epiK5-N,O-oversulfate-derivative according to claim 21, whose mean molecular weight is between approximately 15,000 and approximately 45,000.

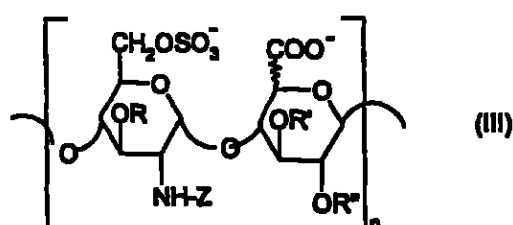
23. An epiK5-N,O-oversulfate-derivative according to claim 21, whose mean molecular weight is between approximately 4,500 and approximately 8,500.

24. An epiK5-N,O-oversulfate-derivative according to anyone of

claims from 21 to 23, characterized in that said degree of sulfation is from 4 to 4.6.

25. An epiK5-N,O-oversulfate-derivative according to anyone of claims from 21 to 24, characterized in that it is 100% 6-O-sulfated and 50-80% 3-O-sulfated in its glucosamine units, 5-10% O-monosulfated in glucuronic units, 10-15% 3-O-monosulfated in iduronic units and 2,3-di-O-sulfated in the remaining uronic units.

26. An epiK5-N,O-oversulfate-derivative according to anyone of claims from 21 to 25, characterized in that it consists of a chain mixture in which at least 90% of said chains have the formula III



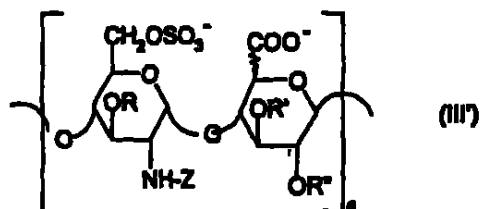
in which the uronic units are 20-60% consisting of iduronic acid, R, R', R'' represent hydrogen or SO₃, R being SO₃ in at least 40% of said chain mixture, Z is an SO₃ group, n is a integer from 2 to 100, the degree of sulfation is at least 4 and the corresponding cation is chemically or pharmaceutically acceptable.

27. An epiK5-N,O-oversulfate-derivative according to claim 26, characterized in that it consists of a chain mixture in which at least 90% of said chains have the formula III, in which the uronic units are 40-60% iduronic acid.

28. An epiK5-N,O-oversulfate-derivative according to anyone of claims 26 and 27, characterized in that it consists of a chain mixture in which at least 90% of said chains have the formula III, in which n is a integer from 3 to 100.

29. An epiK5-N,O-oversulfate-derivative according to anyone of

claims from 26 to 28, characterized in that it is a LMW-epiK5-N,O-oversulfate consisting of a chain mixture in which at least 90% of said chains have the formula III'



in which the uronic units are 20-60% consisting of iduronic acid, q is a integer from 2 to 20, R , R' and R'' represent hydrogen or an SO_3^- group, Z is SO_3^- , for a sulfation degree of at least 4, from 4 to 4.6, and the corresponding cation is one chemically or pharmaceutically acceptable ion.

30. A LMW-epiK5-N,O-oversulfate according to claim 29, characterized in that it consists of a chain mixture in which at least 90% of said chains have the formula III' in which q is a integer from 3 to 15.

31. A LMW-epiK5-N,O-oversulfate according to claim 30, characterized in that it consists of a chain mixture in which at least 90% of said chains have the formula III' in which the uronic units are 40-60% consisting of iduronic acid.

32. A LMW-epiK5-N,O-oversulfate according to claim 31 characterized in that its iduronic acid content is 50-55%.

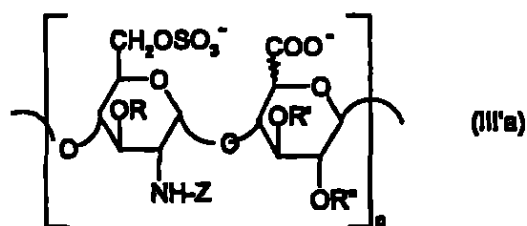
33. A LMW-epiK5-N,O-oversulfate according to anyone of claims from 29 to 32, characterized in that it consists of a chain mixture in which at least 90% of said chains have the formula III' in which R is at least 40% SO_3^- , R' and R'' are both SO_3^- or one is hydrogen and the other is 5-10% SO_3^- in glucuronic acid and 10-15% SO_3^- in iduronic acid.

34. A LMW-epiK5-N,O-oversulfate according to claim 33 characterized in that it has a mean molecular weight from approximately 2,000 to approximately 16,000.

35. A LMW-epiK5-N,O-oversulfate according to claim 34 characterized in that it has a molecular weight from approximately 4,500 to approximately 9,000.

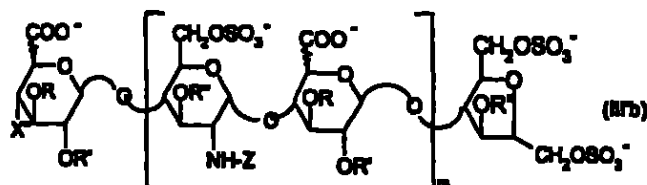
36. A LMW-epiK5-N,O-oversulfate according to anyone of claims from 33 to 35, characterized in that it consists of a chain mixture in which at least 90% of said chains have the formula III' in which R is 50-80% SO_3^- .

37. A LMW-epiK5-N,O-oversulfate according to anyone of claims from 32 to 36, characterized in that it consists of a chain mixture in which the preponderant species has the formula III'a



in which the uronic units are 20-60% consisting of iduronic acid, p is a integer from 4 to 8, Z is SO_3^- , R, R' and R'' are hydrogen or SO_3^- , for a degree of sulfation from 4 to 4.6 and the corresponding cation is chemically or pharmaceutically acceptable.

38. A LMW-epiK5-N,O-oversulfate according to anyone of claims from 33 to 37, characterized in that it consists of a chain mixture in which the preponderant species has the formula III'b



in which R, R' and R'' are hydrogen or SO_3^- , Z is SO_3^- , X'' is OH or OSO_3^- , m is 4, 5 or 6, for a degree of sulfation from 4 to 4.6, the glucuronic and iduronic units are present alternately, starting with a glucuronic or iduronic unit, and the corresponding cation is one chemically or pharmaceutically acceptable ion.

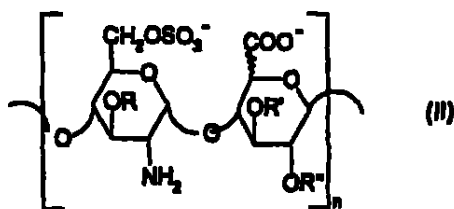
39. An epiK5-N,O-oversulfate-derivative according to anyone of claims from 20 to 38, characterized in that said chemically or pharmaceutically acceptable salt or cation is the salt or cation of an alkaline metal, alkaline-earth metal, ammonium, (C₁-C₄)tetraalkylammonium, aluminum and zinc.

40. An epiK5-N,O-oversulfate-derivative according to claim 39, characterized in that said chemically or pharmaceutically acceptable salt or cation is the salt or cation of sodium, calcium or tetrabutylammonium.

41. An epiK5-amine-O-oversulfate-derivative, one of its chemically or pharmaceutically acceptable salts, obtainable according to steps (a) and (b) of the claim 1, isolated in sodium salt form and, optionally, transformed into another chemically or pharmaceutically acceptable salt.

42. An epiK5-amine-O-oversulfate-derivative whose iduronic acid content is 20-60% of the total of the uronic acids, having a mean molecular weight from approximately 2,000 to approximately 40,000 and a sulfation degree of at least 3.4, or one of its chemically or pharmaceutically acceptable salts.

43. An epiK5-amine-O-oversulfate-derivative according to claim 42, characterized in that said epiK5-amine-O-oversulfate-derivative consists of a chain mixture in which at least 90% of said chains have the formula II



in which the uronic units are 20-60% consisting of Iduronic acid, n is a integer from 2 to 100, R, R' and R'' are hydrogen or SO₃, the degree of

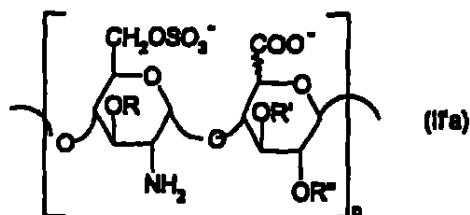
sulfation is at least 3.4 and the corresponding cation is chemically or pharmaceutically acceptable.

44. An epiK5-amine-O-oversulfated-derivative according to claim 43, characterized in that, in the formula II, n represents a integer from 3 to 100.

45. An epiK5-amine-O- oversulfated-derivative according to anyone of claims from 42 to 44, characterized in that it consists of a chain mixture in which at least 90% of said chains have the formula II in which the uronic units are 40-60% consisting of iduronic acid, with a mean molecular weight from approximately 2,000 to approximately 40,000, R is at least 40%, SO₃⁻, R' and R'' are both SO₃⁻ or one is hydrogen and the other is 5-10% SO₃⁻ in monosulfate glucuronic acid and 10-15% SO₃⁻ in monosulfate iduronic acid.

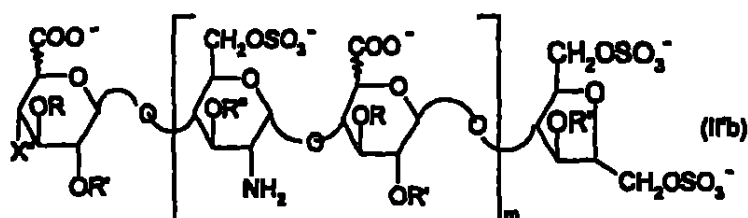
46. An epiK5-amine-O-oversulfate-derivative according to anyone of claims from 42 to 45, characterized in that it is a LMW-epiK5-amine-O-oversulfate consisting of a chain mixture in which at least 90% of said chains have the formula II in which the uronic units are 40-60% consisting of iduronic acid, R is at least 40%, SO₃⁻, R' and R'' are both SO₃⁻ or one is hydrogen and the other is 5-10% SO₃⁻ in glucuronic acid and 10-15% SO₃⁻ in iduronic acid, n is a integer from 3 to 15, with a mean molecular weight from approximately 4,000 to approximately 8,000 and the corresponding cation is chemically or pharmaceutically acceptable.

47. An epiK5-amine-O-oversulfate-derivative according to anyone of claims from 42 to 46, characterized in that it is a LMWepiK5-amine-O-oversulfate consisting of a chain mixture in which the preponderant species has the formula II'a



in which the uronic units are 20-60% consisting of iduronic acid, p is a integer from 4 to 8, R , R' and R'' are hydrogen or SO_3 , the degree of sulfation is at least 3.4.

48. A LMW epiK5-amine-O-oversulfate according to claim 47 characterized in that it consists of a chain mixture in which the preponderant species is a compound of formula II'b



In which the uronic units are 40-60% consisting of iduronic acid, m is 4, 5 or 6, R , R' and R'' are hydrogen or SO_3 , X'' is OH or OSO_3 , for a sulfation degree of at least 3.4, the iduronic units being present alternately, starting with a glucuronic or iduronic unit.

49. A LMW-epiK5-N-sulfate virtually free of NH_2 and N-acetyl groups, having an iduronic acid content from 20 to 60% and a mean molecular weight from approximately 1,500 to approximately 12,000, or one of its chemically or pharmaceutically acceptable salts.

50. A LMW-epiK5-N-sulfate according to claim 49, characterized in that the iduronic acid content is from 40 to 60% and the mean molecular weight is from approximately 1,500 to approximately 10,000.

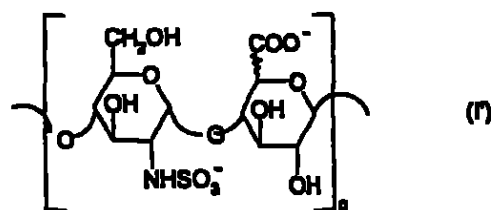
51. A LMW-epiK5-N-sulfate according to claim 49, characterized in that the iduronic acid content is 50-55% and the mean molecular weight is from approximately 1,500 to approximately 7,500.

52. A LMW-epiK5-N-sulfate according to anyone of claims from 49 to 51, obtained by a process characterized in that a K5-N-sulfate is subjected, in any one order,

(i) to C5-epimerization with a D-glucuronyl C5-epimerase isolated, purified and in solution or immobilized on a solid support, at a pH of approximately 7, at a temperature of approximately 30°C and for a time period of 12-24 hours in the presence of at least one bivalent ion selected among calcium, magnesium, barium and manganese; and
(ii) to nitrous depolymerization optionally followed by reduction.

53. A LMW-epiK5-N-sulfate according to claim 52, characterized in that, in step (ii), the depolymerization is followed by reduction with sodium borohydride.

54. A LMW-epiK5-N-sulfate according to claim 53, characterized in that it consists of a chain mixture in which at least 90% of said chains have the formula I'



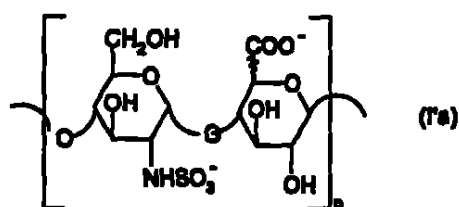
in which the uronic units are 20-60% consisting of iduronic acid, q is a integer from 2 to 20 and the corresponding cation is chemically or pharmaceutically acceptable.

55. A LMW-epiK5-N-sulfate according to claim 54, characterized in that it consists of a chain mixture in which at least 90% of said chains have the formula I', in which the uronic units are 40-60% iduronic acid.

56. A LMW-epiK5-N-sulfate according to anyone of claims 54 and 55, characterized in that it consists of a chain mixture in which at least 90% of said chains have the formula I', in which n is a integer from 3 to 15.

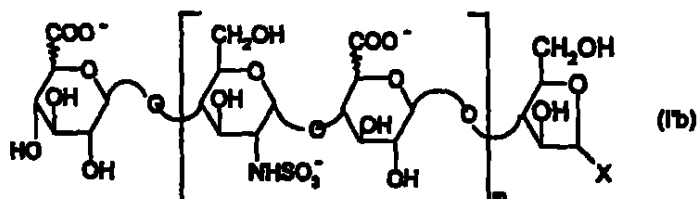
57. A LMW-epiK5-N-sulfate according to anyone of claims 54 and 55,

characterized in that it consists of a chain mixture in which the preponderant species has the formula I'a



in which the uronic units are 60-40% consisting of glucuronic acid and 40% to 60% iduronic acid, p is a integer from 4 to 8 and the corresponding cation is chemically or pharmaceutically acceptable.

58. A LMW-epiK5-N-sulfate according to anyone of claims from 55 to 57, characterized in that it consists of a chain mixture in which the preponderant species has the formula I'b



In which X is hydroxymethyl, m is 4, 5 or 6, the corresponding cation is one chemically or pharmaceutically acceptable ion and the glucuronic and iduronic units are present alternately, starting with a glucuronic or iduronic unit.

59. A LMW-epiK5-N-sulfate according to anyone of claims from 49 to 58, characterized in that said salt or cation is selected among those of alkaline metals, alkaline-earth metals, ammonium, (C₁-C₄)tetraalkylammonium, aluminum and zinc.

60. A LMW-epiK5-N-sulfate according to claim 59, characterized in that said salt or cation is selected among those of sodium, calcium and tetrabutylammonium.

61. Process for the preparation of a LMW-epiK5-N-sulfate,

characterized in that a K5-N-sulfate is subjected, in any one order,

- (i) to C5-epimerization with a D-glucuronyl C5-epimerase isolated, purified and in solution or immobilized on a solid support, at a pH of approximately 7, at a temperature of approximately 30°C and for a time period of 12-24 hours in the presence of at least one bivalent ion selected among calcium, magnesium, barium and manganese; and
- (ii) to nitrous depolymerization optionally followed by reduction.

62. Process according to claim 61, characterized in that it is carried out in the order (i)-(ii).

63. Process according to claim 61, characterized in that it is carried out in the order (ii)-(i).

64. Process according to claim 63, characterized in that the product obtained upon termination of the depolymerization is a LMW-K5-N-sulfate which is directly subjected to C5-epimerization.

65. Process according to claim 64, characterized in that said LMW-K5-N-sulfate has a mean molecular weight of more than 4,000.

66. A pharmaceutical composition including, as one of its active ingredients, a pharmacologically active amount of an epiK5-N,O-oversulfate-derivative according to anyone of claims from 20 to 40 in mixture with a pharmaceutical excipient.

67. A cosmetic composition including an effective amount of an epiK5-N,O-oversulfate-derivative according to anyone of claims from 20 to 40, in mixture with a cosmetic excipient.

68. Use of the isolated and purified C5-epimerase for the conversion of a K5-N-sulfate-derivative into a corresponding epiK5-N-sulfate-derivative characterized by a repetitive tetrasaccharide unit consisting of two glucosamine units separated by a glucuronic unit and followed by an iduronic unit or separated by an iduronic unit and followed by a glucuronic unit.

69. Use according to claim 68, characterized in that said K5-N-sulfate-derivative has a mean molecular weight of more than 4,000.

70. Use according to claim 69, characterized in that said mean molecular weight is from 6,000 to 7,500.

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB 03/02338

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C08B37/00 C08B37/08 C08B37/10 A61K31/726 A61K31/727
A61K31/737

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C08B

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

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

WPI Data, PAJ, CHEM ABS Data, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01 72848 A (CIPOLLETTI GIOVANNI ;PASQUA ORESTE (IT); INALCO SPA (IT); ZOPPETTI) 4 October 2001 (2001-10-04) cited in the application page 5, line 7 -page 10, line 8 claims; examples	1-45, 66-68
A	WO 97 43317 A (CIPOLLETTI GIOVANNI ;PASQUA ORESTE (IT); INALCO SPA (IT); ZOPPETTI) 20 November 1997 (1997-11-20) cited in the application page 3, line 3 -page 7, line 14 examples claims	1-45, 66-68
	-/-	

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents:

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- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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- "Z" document member of the same patent family

Date of the actual completion of the international search

29 October 2003

Date of mailing of the international search report

05/11/2003

Name and mailing address of the ISA

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Authorized officer

Mazet, J-F

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB 03/02338

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NAGGI A ET AL: "TOWARD A BIOTECHNOLOGICAL HEPARIN THROUGH COMBINED CHEMICAL AND ENZYMATIC MODIFICATION OF THE ESCHERICHIA COLI K5 POLYSACCHARIDE" SEMINARS IN THROMBOSIS AND HEMOSTASIS, STUTTGART, DE, vol. 27, no. 5, 2001, pages 437-443, XP008004483 ISSN: 0094-6176 the whole document	1,20,21, 41,42, 49,61,68
X	WO 98 42754 A (TORRI GIANGIACOMO ;CASU BENITO (IT); INALCO SPA (IT); NAGGI ANNAMA) 1 October 1998 (1998-10-01)	41-48
A	page 4, line 4 - line 18 page 9, line 26 -page 11, line 24; examples 7-17 claims	1-40
X	CAMPBELL P ET AL: "BIOSYNTHESIS OF HEPARIN/HEPARAN SULFATE PURIFICATION OF THE D-GLUCURONYL C-5 EPIMERASE FROM BOVINE LIVER" JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, US, vol. 269, no. 43, 28 October 1994 (1994-10-28), pages 26953-26958, XP002902546 ISSN: 0021-9258 cited in the application abstract	68-70
P,A	WO 02 50125 A (PASQUA ORESTE ;ZOPPETTI GIORGIO (IT)) 27 June 2002 (2002-06-27) the whole document	1-70
A	WO 92 17507 A (ITALFARMACO SPA ;MAX PLANCK INST FUER IMMUNBIOL (DE); TUBBY DAVID) 15 October 1992 (1992-10-15) cited in the application claims; examples	1-70
A	WO 96 14425 A (INALCO SPA ;ZOPPETTI GIORGIO (IT); PASQUA ORESTE (IT); CIPOLLETTI) 17 May 1996 (1996-05-17) cited in the application	
A	US 2002/062019 A1 (ORESTE PASQUA ET AL) 23 May 2002 (2002-05-23) cited in the application	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB 03/02338

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0172848 A	04-10-2001	IT MI20000665 A1	01-10-2001
		AU 4651001 A	08-10-2001
		CA 2404478 A1	04-10-2001
		CN 1422283 T	04-06-2003
		WO 0172848 A1	04-10-2001
		EP 1268559 A1	02-01-2003
		US 2003023079 A1	30-01-2003
		US 2002062019 A1	23-05-2002
WO 9743317 A	20-11-1997	IT MI960956 A1	10-11-1997
		AT 210154 T	15-12-2001
		AU 3026597 A	05-12-1997
		DE 69708862 D1	17-01-2002
		DE 69708862 T2	14-08-2002
		DK 897393 T3	02-04-2002
		WO 9743317 A1	20-11-1997
		EP 0897393 A1	24-02-1999
		ES 2167748 T3	16-05-2002
		US 6162797 A	19-12-2000
WO 9842754 A	01-10-1998	IT MI970678 A1	24-09-1998
		AT 220410 T	15-07-2002
		AU 7040598 A	20-10-1998
		DE 69806468 D1	14-08-2002
		DE 69806468 T2	27-02-2003
		DK 970130 T3	04-11-2002
		WO 9842754 A2	01-10-1998
		EP 0970130 A2	12-01-2000
		ES 2180161 T3	01-02-2003
		JP 2001518133 T	09-10-2001
		US 6197943 B1	06-03-2001
WO 0250125 A	27-06-2002	US 2002062019 A1	23-05-2002
		AU 2235802 A	01-07-2002
		CA 2432150 A1	27-06-2002
		WO 0250125 A2	27-06-2002
WO 9217507 A	15-10-1992	GB 2254083 A	30-09-1992
		AU 1430892 A	02-11-1992
		CA 2107124 A1	29-09-1992
		EP 0577665 A1	12-01-1994
		FI 934141 A	22-09-1993
		WO 9217507 A1	15-10-1992
		GB 2286193 A	09-08-1995
		HU 67208 A2	28-02-1995
		IE 920982 A1	07-10-1992
		IT 1254564 B	25-09-1995
		JP 7501684 T	23-02-1995
		NO 933440 A	26-10-1993
		PT 100316 A	30-07-1993
		ZA 9202313 A	02-08-1993
WO 9614425 A	17-05-1996	IT 1271057 B	26-05-1997
		AT 195348 T	15-08-2000
		AU 3926195 A	31-05-1996
		CA 2204366 A1	17-05-1996
		CN 1162339 A , B	15-10-1997
		DE 69518333 D1	14-09-2000

INTERNATIONAL SEARCH REPORT

International application No

PCT/IB 03/02338

Information on patent family members

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9614425 A		DE 69518333 T2	22-02-2001
		DK 789777 T3	06-11-2000
		WO 9614425 A1	17-05-1996
		EP 0789777 A1	20-08-1997
		ES 2150589 T3	01-12-2000
		JP 10508204 T	18-08-1998
		PT 789777 T	31-01-2001
		US 5958899 A	28-09-1999
US 2002062019 A1	23-05-2002	IT MI20000665 A1	01-10-2001
		AU 2235802 A	01-07-2002
		CA 2432150 A1	27-06-2002
		WO 0250125 A2	27-06-2002
		AU 4651001 A	08-10-2001
		CA 2404478 A1	04-10-2001
		CN 1422283 T	04-06-2003
		WO 0172848 A1	04-10-2001
		EP 1268559 A1	02-01-2003
		US 2003023079 A1	30-01-2003

From the INTERNATIONAL SEARCHING AUTHORITY

PCTNOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL SEARCH REPORT
OR THE DECLARATION

(PCT Rule 44.1)

To:

MARIETTI, GISLON E TRUPIANO S.R.L.
Attn. Santoro, Tiziana
Via Larga, 16
20122 Milano
ITALY

Date of mailing
(day/month/year)

05/11/2003

Applicant's or agent's file reference

03420E45

FOR FURTHER ACTION

See paragraphs 1 and 4 below

International application No.

PCT/IB 03/02338

International filing date
(day/month/year)

17/06/2003

Applicant

ORESTE, Pasqua Anna

1. ☒ The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:

The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. ☐ The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. ☐ With regard to the protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. Further action(s): The applicant is reminded of the following:

Shortly after 18 months from the priority date, the International application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the International application, or of the priority claim, must reach the International Bureau as provided in Rules 90bis.1 and 90bis.3, respectively, before the completion of the technical preparations for International publication.

Within 19 months from the priority date, a demand for International preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within 20 months from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority



European Patent Office, P.B. 5818 Patentlaan 2
NL-2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Alicja Van der Heijden

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When?

Within 2 months from the date of transmittal of the international search report or 18 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been filed, see below.

How?

Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped), whether

- (i) the claim is unchanged;
- (ii) the claim is cancelled;
- (iii) the claim is new;
- (iv) the claim replaces one or more claims as filed;
- (v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."
2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."
3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."
4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 48.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international application is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 03420E45	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/IB 03/ 02338	International filing date (day/month/year) 17/06/2003	(Earliest) Priority Date (day/month/year) 18/06/2002
Applicant ORESTE, Pasqua Anna		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 05 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

- a. With regard to the language, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

- b. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of the sequence listing:

☐ contained in the international application in written form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. ☐ Certain claims were found unsearchable (See Box I).

3. ☐ Unity of invention is lacking (see Box II).

4. With regard to the title,

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5. With regard to the abstract,

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the drawings to be published with the abstract is Figure No.

☐ as suggested by the applicant.

☐ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

☐ None of the figures.

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C08B37/00 C08B37/08 C08B37/10 A61K31/726 A61K31/727
A61K31/737

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C08B

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

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

WPI Data, PAJ, CHEM ABS Data, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01 72848 A (CIPOLLETTI GIOVANNI ;PASQUA ORESTE (IT); INALCO SPA (IT); ZOPPETTI) 4 October 2001 (2001-10-04) cited in the application page 5, line 7 -page 10, line 8 claims; examples	1-45, 66-68
A	WO 97 43317 A (CIPOLLETTI GIOVANNI ;PASQUA ORESTE (IT); INALCO SPA (IT); ZOPPETTI) 20 November 1997 (1997-11-20) cited in the application page 3, line 3 -page 7, line 14 examples claims	1-45, 66-68



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *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
- *Z* document member of the same patent family

Date of the actual completion of the international search

29 October 2003

Date of mailing of the international search report

05/11/2003

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentplan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-3040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3018

Authorized officer

Mazet, J-F

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NAGGI A ET AL: "TOWARD A BIOTECHNOLOGICAL HEPARIN THROUGH COMBINED CHEMICAL AND ENZYMATIC MODIFICATION OF THE ESCHERICHIA COLI K5 POLYSACCHARIDE" SEMINARS IN THROMBOSIS AND HEMOSTASIS, STUTTGART, DE, vol. 27, no. 5, 2001, pages 437-443, XP008004483 ISSN: 0094-6176 the whole document	1,20,21, 41,42, 49,61,68
X	WO 98 42754 A (TORRI GIANGIACOMO ;CASU BENITO (IT); INALCO SPA (IT); NAGGI ANNAMA) 1 October 1998 (1998-10-01)	41-48
A	page 4, line 4 - line 18 page 9, line 26 -page 11, line 24; examples 7-17 claims	1-40
X	CAMPBELL P ET AL: "BIOSYNTHESIS OF HEPARIN/HEPARAN SULFATE PURIFICATION OF THE D-GLUCURONYL C-5 EPIMERASE FROM BOVINE LIVER" JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, US, vol. 269, no. 43, 28 October 1994 (1994-10-28), pages 26953-26958, XP002902546 ISSN: 0021-9258 cited in the application abstract	68-70
P,A	WO 02 50125 A (PASQUA ORESTE ;ZOPPETTI GIORGIO (IT)) 27 June 2002 (2002-06-27) the whole document	1-70
A	WO 92 17507 A (ITALFARMACO SPA ;MAX PLANCK INST FUER IMMUNBIOL (DE); TUBBY DAVID) 15 October 1992 (1992-10-15) cited in the application claims; examples	1-70
A	WO 96 14425 A (INALCO SPA ;ZOPPETTI GIORGIO (IT); PASQUA ORESTE (IT); CIPOLLETTI) 17 May 1996 (1996-05-17) cited in the application	
A	US 2002/062019 A1 (ORESTE PASQUA ET AL) 23 May 2002 (2002-05-23) cited in the application	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/IB 03/02338

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 0172848	A	04-10-2001	IT MI20000665 A1	01-10-2001
			AU 4651001 A	08-10-2001
			CA 2404478 A1	04-10-2001
			CN 1422283 T	04-06-2003
			WO 0172848 A1	04-10-2001
			EP 1268559 A1	02-01-2003
			US 2003023079 A1	30-01-2003
			US 2002062019 A1	23-05-2002
WO 9743317	A	20-11-1997	IT MI960956 A1	10-11-1997
			AT 210154 T	15-12-2001
			AU 3026597 A	05-12-1997
			DE 69708862 D1	17-01-2002
			DE 69708862 T2	14-08-2002
			DK 897393 T3	02-04-2002
			WO 9743317 A1	20-11-1997
			EP 0897393 A1	24-02-1999
			ES 2167748 T3	16-05-2002
			US 6162797 A	19-12-2000
WO 9842754	A	01-10-1998	IT M1970678 A1	24-09-1998
			AT 220410 T	15-07-2002
			AU 7040598 A	20-10-1998
			DE 69806468 D1	14-08-2002
			DE 69806468 T2	27-02-2003
			DK 970130 T3	04-11-2002
			WO 9842754 A2	01-10-1998
			EP 0970130 A2	12-01-2000
			ES 2180161 T3	01-02-2003
			JP 2001518133 T	09-10-2001
			US 6197943 B1	06-03-2001
WO 0250125	A	27-06-2002	US 2002062019 A1	23-05-2002
			AU 2235802 A	01-07-2002
			CA 2432150 A1	27-06-2002
			WO 0250125 A2	27-06-2002
WO 9217507	A	15-10-1992	GB 2254083 A	30-09-1992
			AU 1430892 A	02-11-1992
			CA 2107124 A1	29-09-1992
			EP 0577665 A1	12-01-1994
			FI 934141 A	22-09-1993
			WO 9217507 A1	15-10-1992
			GB 2286193 A	09-08-1995
			HU 67208 A2	28-02-1995
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			IT 1254564 B	25-09-1995
			JP 7501684 T	23-02-1995
			NO 933440 A	26-10-1993
			PT 100316 A	30-07-1993
			ZA 9202313 A	02-08-1993
WO 9614425	A	17-05-1996	IT 1271057 B	26-05-1997
			AT 195348 T	15-08-2000
			AU 3926195 A	31-05-1996
			CA 2204366 A1	17-05-1996
			CN 1162339 A ,B	15-10-1997
			DE 69518333 D1	14-09-2000

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/IB 03/02338

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9614425	A	DE 69518333 T2	22-02-2001
		DK 789777 T3	06-11-2000
		WO 9614425 A1	17-05-1996
		EP 0789777 A1	20-08-1997
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		US 5958899 A	28-09-1999
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		WO 0250125 A2	27-06-2002
		AU 4651001 A	08-10-2001
		CA 2404478 A1	04-10-2001
		CN 1422283 T	04-06-2003
		WO 0172848 A1	04-10-2001
		EP 1268559 A1	02-01-2003
		US 2003023079 A1	30-01-2003

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The demand must be filed directly with the competent International Preliminary Examining Authority or, if two or more Authorities are competent, with the one chosen by the applicant. The full name or two-letter code of that Authority may be indicated by the applicant on the line below:

IPEA/ _____

PCT

CHAPTER II

DEMAND

under Article 31 of the Patent Cooperation Treaty:
The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty and hereby elects all eligible States (except where otherwise indicated).

For International Preliminary Examining Authority use only		
Identification of IPEA	Date of receipt of DEMAND	
Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION		
Applicant's or agent's file reference 03420E45		
International application No. PCT/IB03/02338	International filing date (day/month/year) 17 June 2003 (17.06.03)	(Earliest) Priority date (day/month/year) 18 June 2002 (18.06.02)
Title of invention "K5 polysaccharide-epimerized derivatives having a very high degree of sulfation"		
Box No. II APPLICANT(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.) ORESTE, Pasqua Anna Via Mac Mahon, 107 I-20155 Milan Italy		Telephone No. +39-02-33101771
		Facsimile No. +39-02-34534495
		Teleprinter No.
		Applicant's registration No. with the Office
State (that is, country) of nationality: Italian	State (that is, country) of residence: Italy	
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.) ZOPPETTI, Giorgio Via Mac Mahon, 107 I-20155 Milan Italy		
State (that is, country) of nationality: Italian	State (that is, country) of residence: Italy	
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.) 		
State (that is, country) of nationality:	State (that is, country) of residence:	
☐ Further applicants are indicated on a continuation sheet.		

Sheet No. 2

International application No.
PCT/IB03/02338

Box No. III AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The following person is ☒ agent ☐ common representative

and ☒ has been appointed earlier and represents the applicant(s) also for international preliminary examination.

☐ is hereby appointed and any earlier appointment of (an) agent(s)/common representative is hereby revoked.

☐ is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority, in addition to the agent(s)/common representative appointed earlier.

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.)*

SANTORO, Tiziana
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Telephone No.

+39-02-86464387

Facsimile No.

+39-02-86463303

Teleprinter No.

Agent's registration No. with the Office

☐ Address for correspondence: Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Box No. IV BASIS FOR INTERNATIONAL PRELIMINARY EXAMINATION

Statement concerning amendments:*

1. The applicant wishes the international preliminary examination to start on the basis of:

☒ the international application as originally filed

the description ☒ as originally filed

☐ as amended under Article 34

the claims ☒ as originally filed

☐ as amended under Article 19 (together with any accompanying statement)

☐ as amended under Article 34.

the drawings ☐ as originally filed

☐ as amended under Article 34

2. ☐ The applicant wishes any amendment to the claims under Article 19 to be considered as reversed.

3. ☐ The applicant wishes the start of the international preliminary examination to be postponed until the expiration of 20 months from the priority date unless the International Preliminary Examining Authority receives a copy of any amendments made under Article 19 or a notice from the applicant that he does not wish to make such amendments (Rule 69.1(d)). *(This check-box may be marked only where the time limit under Article 19 has not yet expired.)*

* Where no check-box is marked, international preliminary examination will start on the basis of the international application as originally filed or, where a copy of amendments to the claims under Article 19 and/or amendments of the international application under Article 34 are received by the International Preliminary Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report, as so amended.

Language for the purposes of international preliminary examination: English.....

☐ which is the language in which the international application was filed.

☐ which is the language of a translation furnished for the purposes of international search.

☒ which is the language of publication of the international application.

☐ which is the language of the translation (to be) furnished for the purposes of international preliminary examination.

Box No. V ELECTION OF STATES

The applicant hereby elects all eligible States *(that is, all States which have been designated and which are bound by Chapter II of the PCT)*

excluding the following States which the applicant wishes not to elect:

Box No. VI CHECK LIST

The demand is accompanied by the following elements, in the language referred to in Box No. IV, for the purposes of international preliminary examination:

- | | | |
|--|---|--------|
| 1. translation of international application | : | sheets |
| 2. amendments under Article 34 | : | sheets |
| 3. copy (or, where required, translation) of amendments under Article 19 | : | sheets |
| 4. copy (or, where required, translation) of statement under Article 19 | : | sheets |
| 5. letter | : | sheets |
| 6. other (specify) | : | sheets |

For International Preliminary
Examining Authority use only

- | received | not received |
|--------------------------|--------------------------|
| ☐ | ☐ |
| ☐ | ☐ |
| ☐ | ☐ |
| ☐ | ☐ |
| ☐ | ☐ |
| ☐ | ☐ |

The demand is also accompanied by the item(s) marked below:

- | | |
|--|---|
| 1. ☒ fee calculation sheet | 5. ☐ statement explaining lack of signature |
| 2. ☐ original separate power of attorney | 6. ☐ sequence listings in computer readable form |
| 3. ☐ original general power of attorney | 7. ☐ tables in computer readable form related to sequence listings |
| 4. ☐ copy of general power of attorney; reference number, if any: | 8. ☐ other (specify): |

Box No. VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

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


SANTORO, Tiziana
(Agent)

For International Preliminary Examining Authority use only

1. Date of actual receipt of DEMAND:

2. Adjusted date of receipt of demand due to CORRECTIONS under Rule 60.1(b):

- | | |
|--|---|
| 3. ☐ The date of receipt of the demand is AFTER the expiration of 19 months from the priority date and item 4 or 5, below, does not apply. | ☐ The applicant has been informed accordingly. |
| 4. ☐ The date of receipt of the demand is WITHIN the period of 19 months from the priority date as extended by virtue of Rule 80.5. | |
| 5. ☐ Although the date of receipt of the demand is after the expiration of 19 months from the priority date, the delay in arrival is EXCUSED pursuant to Rule 82. | |

For International Bureau use only

Demand received from IPEA on:

PCT

REQUEST

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

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference
(if desired) (12 characters maximum) 03420E45

Box No. I TITLE OF INVENTION	
K5 polysaccharide-epimerized derivatives having a very high sulfation degree	
Box No. II APPLICANT ☒ This person is also inventor	
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.)	
ORESTE, Pasqua, Anna Via Mac Mahon, 43 20155 Milano Italy	
Telephone No. +39-02-33101771	
Facsimile No. +39-02-34534495	
Teleprinter No.	
Applicant's registration No. with the Office	
State (that is, country) of nationality: IT	State (that is, country) of residence: IT
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.)	
ZOPPETTI, Giorgio Via Mac Mahon, 43 20155 MILANO Italy	
This person is: ☐ applicant only ☒ applicant and inventor ☐ inventor only (If this check-box is marked, do not fill in below.)	
Applicant's registration No. with the Office	
State (that is, country) of nationality:	State (that is, country) of residence:
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.)	
SANTORO, Tiziana MARIETTI, GISLON e TRUPIANO S.r.l. Via Larga, 16 I-20122 Milan Italy	
Telephone No. +39-02-86464387	
Facsimile No. +39-02-86463303	
Teleprinter No.	
Agent's registration No. with the Office	
☐ Address for correspondence: Mark this check-box where no agent or common representative is/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

Mark the applicable check-boxes below; at least one must be marked.

The following designations are hereby made under Rule 4.9(a):

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, ZM Zambia, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT (if other kind of protection or treatment desired, specify on dotted line)
- ☒ 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, BG Bulgaria, CH & LI Switzerland and Liechtenstein, CY Cyprus, CZ Czech Republic, DE Germany, DK Denmark, EE Estonia, 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, SI Slovenia, SK Slovakia, TR Turkey, 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, GQ Equatorial 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 | ☒ GM Gambia | ☒ NZ New Zealand |
| ☒ AG Antigua and Barbuda | ☒ HR Croatia | ☒ OM Oman |
| ☒ AL Albania | ☒ HU Hungary | ☒ PH Philippines |
| ☒ AM Armenia | ☒ ID Indonesia | ☒ PL Poland |
| ☒ AT Austria | ☒ IL Israel | ☒ PT Portugal |
| ☒ AU Australia | ☒ IN India | ☒ RO Romania |
| ☒ AZ Azerbaijan | ☒ IS Iceland | ☒ RU Russian Federation |
| ☒ BA Bosnia and Herzegovina | ☒ JP Japan | ☒ SC Seychelles |
| ☒ BB Barbados | ☒ KE Kenya | ☒ SD Sudan |
| ☒ BG Bulgaria | ☒ KG Kyrgyzstan | ☒ SE Sweden |
| ☒ BR Brazil | ☒ KP Democratic People's Republic of Korea | ☒ SG Singapore |
| ☒ BY Belarus | ☒ KR Republic of Korea | ☒ SK Slovakia |
| ☒ BZ Belize | ☒ KZ Kazakhstan | ☒ SL Sierra Leone |
| ☒ CA Canada | ☒ LC Saint Lucia | ☒ TJ Tajikistan |
| ☒ CH & LI Switzerland and Liechtenstein | ☒ LK Sri Lanka | ☒ TM Turkmenistan |
| ☒ CN China | ☒ LR Liberia | ☒ TN Tunisia |
| ☒ CO Colombia | ☒ LS Lesotho | ☒ TR Turkey |
| ☒ CR Costa Rica | ☒ LT Lithuania | ☒ TT Trinidad and Tobago |
| ☒ CU Cuba | ☒ LU Luxembourg | ☒ TZ United Republic of Tanzania |
| ☒ CZ Czech Republic | ☒ LV Latvia | ☒ UA Ukraine |
| ☒ DE Germany | ☒ MA Morocco | ☒ UG Uganda |
| ☒ DK Denmark | ☒ MD Republic of Moldova | ☒ US United States of America |
| ☒ DM Dominica | ☒ MG Madagascar | ☒ UZ Uzbekistan |
| ☒ DZ Algeria | ☒ MK The former Yugoslav Republic of Macedonia | ☒ VC Saint Vincent and the Grenadines |
| ☒ EC Ecuador | ☒ MN Mongolia | ☒ VN Viet Nam |
| ☒ EE Estonia | ☒ MW Malawi | ☒ YU Yugoslavia |
| ☒ ES Spain | ☒ MX Mexico | ☒ ZA South Africa |
| ☒ FI Finland | ☒ MZ Mozambique | ☒ ZM Zambia |
| ☒ GB United Kingdom | ☒ NO Norway | ☒ ZW Zimbabwe |
| ☒ GD Grenada | | |
| ☒ GE Georgia | | |
| ☒ GH Ghana | | |

Check-boxes below reserved for designating States which have become party to the PCT after issuance of this sheet:

- ☒ NI Nicaragua
- ☒ PG Papua New Guinea

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.)

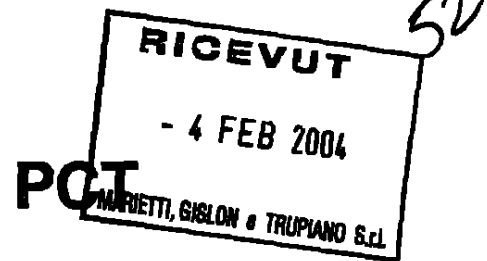
Box No. VI PRIORITY CLAIM				
The priority of the following earlier application(s) is hereby claimed:				
Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is:		
		national application: country or Member of WTO	regional application:* regional Office	international application: receiving Office
item (1) 18 June 2002 (18-06-2002)	MI2002A001345	IT		
item (2) 18 June 2002 (18-06-2002)	MI2002A001346	IT		
item (3) 27 August 2002 (27-08-2002)	MI2002A001854	IT		
item (4)				
item (5)				
☐ Further priority claims are indicated in the Supplemental Box.				
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 this international application is the receiving Office) identified above as: ☐ all items ☐ item (1) ☐ item (2) ☐ item (3) ☐ item (4) ☐ item (5) ☐ other, see Supplemental Box				
* Where the earlier application is an ARIPO application, indicate at least one country party to the Paris Convention for the Protection of Industrial Property or one Member of the World Trade Organization for which that earlier application was filed (Rule 4.10(b)(ii)):				
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 / EPO				
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 DECLARATIONS				
The following declarations are contained in Boxes Nos. VIII (i) to (v) (mark the applicable check-boxes below and indicate in the right column the number of each type of declaration):				Number of declarations
☐	Box No. VIII (i)	Declaration as to the identity of the inventor		:
☐	Box No. VIII (ii)	Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent		:
☐	Box No. VIII (iii)	Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application		:
☐	Box No. VIII (iv)	Declaration of inventorship (only for the purposes of the designation of the United States of America)		:
☐	Box No. VIII (v)	Declaration as to non-prejudicial disclosures or exceptions to lack of novelty		:

Box No. IX CHECK LIST; LANGUAGE OF FILING		
This international application contains: (a) In paper form, the following number of sheets : request (including declaration sheets) : 4 description (excluding sequence listings and/or tables related thereto) : 37 claims : 12 abstract : 1 drawings : Sub-total number of sheets : 54 sequence listings : tables related thereto : <i>(for both, actual number of sheets if filed in paper form, whether or not also filed in computer readable form; see (c) below)</i> Total number of sheets : 54 (b) ☐ only in computer readable form (Section 801(a)(i)) (i) ☐ sequence listings (ii) ☐ tables related thereto (c) ☐ also in computer readable form (Section 801(a)(ii)) (i) ☐ sequence listings (ii) ☐ tables related thereto Type and number of carriers (diskette, CD-ROM, CD-R or other) on which are contained the ☐ sequence listings: ☐ tables related thereto: <i>(additional copies to be indicated under items 9(ii) and/or 10(ii), in right column)</i>	This international application is accompanied by the following item(s) (mark the applicable check-boxes below and indicate in right column the number of each item): 1. ☒ fee calculation sheet : 1 2. ☐ original separate power of attorney will follow : 3. ☐ original general power of attorney : 4. ☐ copy of general power of attorney; reference number, if any: : 5. ☐ statement explaining lack of signature : 6. ☐ priority document(s) identified in Box No. VI as item(s): will follow : 7. ☐ translation of international application into (language): English will follow : 8. ☐ separate indications concerning deposited microorganism or other biological material : 9. ☐ sequence listings in computer readable form (indicate type and number of carriers) (i) ☐ copy submitted for the purposes of international search under Rule 13ter only (and not as part of the international application) : (ii) ☐ (only where check-box (b)(i) or (c)(i) is marked in left column) additional copies including, where applicable, the copy for the purposes of international search under Rule 13ter : (iii) ☐ together with relevant statement as to the identity of the copy or copies with the sequence listings mentioned in left column : 10. ☐ tables in computer readable form related to sequence listings (indicate type and number of carriers) (i) ☐ copy submitted for the purposes of international search under Section 802(b-quater) only (and not as part of the international application) : (ii) ☐ (only where check-box (b)(ii) or (c)(ii) is marked in left column) additional copies including, where applicable, the copy for the purposes of international search under Section 802(b-quater) : (iii) ☐ together with relevant statement as to the identity of the copy or copies with the tables mentioned in left column : 11. ☐ other (specify): :	
Figure of the drawings which should accompany the abstract:	Language of filing of the international application: Italian	
Box No. X SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE <i>Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).</i>  SANTORO, Tiziana		

For receiving Office use only	
1. Date of actual receipt of the purported international application: 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):	2. Drawings: ☐ received: ☐ not received:
5. International Searching Authority (if two or more are competent): ISA /	6. ☐ Transmittal of search copy delayed and/ search fee is paid
For International Bureau use only Date of receipt of the record copy by the International Bureau:	

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY



To:

Santoro, Tiziana
MARIETTI, GISLON & TRUPIANO S.R.L.
Via Larga, 16
20122 Milano
ITALIE

NOTIFICATION OF RECEIPT OF DEMAND BY COMPETENT INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

(PCT Rules 59.3(e) and 61.1(b), first sentence
and Administrative Instructions, Section 601(a))

Date of mailing
(day/month/year)

02-02-2004

Applicant's or agent's file reference
03420E45

IMPORTANT NOTIFICATION

International application No.

PCT/IB03/02338

International filing date (day/month/year)

17/06/2003

Priority date (day/month/year)

18/06/2002

Applicant

ORESTE, Pasqua Anna

1. The applicant is hereby notified that this International Preliminary Examining Authority considers the following date as the date of receipt of the demand for international preliminary examination of the international application:

30/12/2003

2. This date of receipt is:

- ☒ the actual date of receipt of the demand by this Authority (Rule 61.1(b)).
☐ the actual date of receipt of the demand on behalf of this Authority (Rule 59.3(e)).
☐ the date on which this Authority has, in response to the invitation to correct defects in the demand (Form PCT/IPEA/404), received the required corrections.

3. ☐ **ATTENTION:** That date of receipt is after the expiration of 19 months from the priority date. Consequently, in respect of some Offices, the demand does not have the effect of postponing the entry into the national phase until 30 months from the priority date (or later in some Offices) (Article 39(1)) and the acts for entry into the national phase must therefore be performed within 20 months from the priority date (or later in some Offices). However, in respect of some other Offices, the time limit of 30 months (or later) may nevertheless apply. See the Annex to Form PCT/IB/301 and, for details about the applicable time limits, Office by Office, see the *PCT Applicant's Guide*, Volume II, National Chapters and the WIPO Internet site.

- ☐ (If applicable) This notification confirms the information given by telephone, facsimile transmission or in person on:

4. Only where paragraph 3 applies, a copy of this notification has been sent to the International Bureau.

Name and mailing address of the IPEA/



European Patent Office
D-80298 Munich
Tel. (+49-89) 2399-0, Tx: 523656 epmu d
Fax: (+49-89) 2399-4465

Authorized officer

CHURCHER G

Tel. (+49-89) 2399-2545



Form PCT/IPEA/402 (April 2002; reprint January 2004)

(28/01/2004)

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF RECEIPT OF
RECORD COPY

(PCT Rule 24.2(a))

To:

SANTORO, Tiziana
Marletti, Gislone & Trupiano S.r.l.
Via Larga, 16
I-20122 Milan
Italy

Date of mailing (day/month/year) 16 July 2003 (16.07.03)	IMPORTANT NOTIFICATION
Applicant's or agent's file reference 03420E45	International application No. PCT/IB03/02338

The applicant is hereby notified that the International Bureau has received the record copy of the international application as detailed below.

Name(s) of the applicant(s) and State(s) for which they are applicants:

ORESTE, Pasqua, Anna et al(all designated States)

International filing date : 17 June 2003 (17.06.03)

Priority date(s) claimed : 18 June 2002 (18.06.02)

18 June 2002 (18.06.02)

27 August 2002 (27.08.02)

Date of receipt of the record copy
by the International Bureau : 14 July 2003 (14.07.03)

List of designated Offices :

AP : GH,GM,KE,LS,MW,MZ,SD,SL,SZ,TZ,UG,ZM,ZW

EA : AM,AZ,BY,KG,KZ,MD,RU,TJ,TM

EP : AT,BE,BG,CH,CY,CZ,DE,DK,EE,ES,FI,FR,GB,GR,HU,IE,IT,LU,MC,NL,PT,RO,SE,SI,SK,TR

OA : BF,BJ,CF,CG,CI,CM,GA,GN,GQ,GW,ML,MR,NE,SN,TD,TG

National : AE,AG,AL,AM,AT,AU,AZ,BA,BB,BG,BR,BY,BZ,CA,CH,CN,CO,CR,CU,CZ,DE,DK,DM,DZ,
EC,EE,ES,FI,GB,GD,GE,GH,GM,HR,HU,ID,IL,IN,IS,JP,KE,KG,KP,KR,KZ,LC,LK,LR,LS,LT,LU,
LV,MA,MD,MG,MK,MN,MW,MX,MZ,NI,NO,NZ,OM,PG,PH,PL,PT,RO,RU,SC,SD,SE,SG,SK,SL,TJ,TM,
TN,TR,TT,TZ,UA,UG,US,UZ,VC,VN,YU,ZA,ZM,ZW

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer: Sonnla GARCIA MELLITI.
Facsimile No. (41-22) 338-7080	Telephone No. (41-22) 338 9819

5A

Continuation of Form PCT/IB/301

NOTIFICATION OF RECEIPT OF RECORD COPY

Date of mailing (day/month/year) 16 July 2003 (16.07.03)	IMPORTANT NOTIFICATION
Applicant's or agent's file reference 03420E45	International application No. PCT/IB03/02338

ATTENTION

The applicant should carefully check the data appearing in this Notification. In case of any discrepancy between these data and the indications in the international application, the applicant should immediately inform the International Bureau.

In addition, the applicant's attention is drawn to the information contained in the Annex, relating to:

- ☒ time limits for entry into the national phase - see updated Important Information (as of April 2002)
- ☐ confirmation of precautionary designations (if applicable)
- ☒ requirements regarding priority documents (if applicable)

A copy of this Notification is being sent to the receiving Office and to the International Searching Authority.

INFORMATION ON TIME LIMITS FOR ENTERING THE NATIONAL PHASE

The applicant is reminded that the "national phase" must be entered before each of the designated Offices indicated on the cover sheet of this Notification by paying national fees and furnishing translations, as prescribed by Articles 22 and 39 and the applicable national laws. In addition, the applicant may also have to comply with other special requirements applicable in certain Offices. It is the applicant's responsibility to ensure the necessary steps to enter the national phase are taken in a timely fashion. Most Offices do not issue reminders to applicants in connection with the entry into the national phase.

The applicable time limit for entering the national phase will, subject to what is said in the following paragraph, be 30 MONTHS from the priority date, not only in respect of any elected Office where a demand for international preliminary examination is filed before the expiration of 19 months from the priority date (see Article 39(1)), but also in respect of any designated Office, in the absence of filing of such demand, where Article 22(1) as modified with effect from 1 April 2002 applies in respect of that designated Office. For further details, see PCT Gazette No. 44/2001 of 1 November 2001, pages 18928, 18932 and 18934, as well as the PCT Newsletter, October and November 2001 and February 2002 issues.

In practice, time limits other than the 30-month time limit will continue to apply, for various periods of time, in respect of certain designated or elected Offices. For regular updates on the applicable time limits (20, 21, 30 or 31 months, or other time limit), Office by Office, refer to the PCT Gazette ("Section IV" part published on a weekly basis), to the PCT Newsletter (on a monthly basis) and to the relevant National Chapters in Volume II of the PCT Applicant's Guide (the paper version of which is updated usually twice a year and the Internet version of which is updated usually on a weekly basis). Finally, a cumulative table of all applicable time limits for entering the national phase is available from WIPO's Internet site, via links from various pages the site including those of the Gazette, Newsletter and Guide, at <http://www.wipo.int/pct/en/index.html>.

Information about the requirements for filing a demand for international preliminary examination is set out in the PCT Applicant's Guide, Volume I/A, Chapter IX. Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination (at present, all PCT Contracting States are bound by Chapter II).

CONFIRMATION OF PRECAUTIONARY DESIGNATIONS

This notification lists only specific designations made under Rule 4.9(a) in the request. It is important to check that these designations are correct. Errors in designations can be corrected where precautionary designations have been made under Rule 4.9(b). The applicant is hereby reminded that any precautionary designations may be confirmed according to Rule 4.9(c) before the expiration of 16 months from the priority date (this time limit may not be extended). If it is not confirmed, it will automatically be regarded as withdrawn by the applicant. There will be no reminder and no invitation. Confirmation of a designation consists of the filing of a notice specifying the designated State concerned (with indication of the kind of protection or treatment desired) and the payment of the designation and confirmation fees. The Notice of confirmation and payment must reach the receiving Office within the 16-month time limit.

REQUIREMENTS REGARDING PRIORITY DOCUMENTS

For applicants who have not yet complied with the requirements regarding priority documents, the following is recalled.

Where the priority of an earlier national, regional or international application is claimed, the applicant must submit a copy of the said earlier application, certified by the authority with which it was filed ("the priority document") to the receiving Office (which will transmit it to the International Bureau) or directly to the International Bureau, before the expiration of 16 months from the priority date, provided that any such priority document may still be submitted to the International Bureau before that date of international publication of the international application, in which case that document will be considered to have been received by the International Bureau on the last day of the 16-month time limit (Rule 17.1(a)).

Where the priority document is issued by the receiving Office, the applicant may, instead of submitting the priority document, request the receiving Office to prepare and transmit the priority document to the International Bureau. Such request must be made before the expiration of the 16-month time limit and may be subjected by the receiving Office to the payment of a fee (Rule 17.1(b)).

If the priority document concerned is not submitted to the International Bureau or if the request to the receiving Office to prepare and transmit the priority document has not been made (and the corresponding fee, if any, paid) within the applicable time limit indicated under the preceding paragraphs, any designated State may disregard the priority claim, provided that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within the time limit which is reasonable under the circumstances.

Where several priorities are claimed, the priority date to be considered for the purposes of computing the 16-month time limit is the filing date of the earliest application whose priority is claimed.

PATENT COOPERATION TREATY

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From the INTERNATIONAL BUREAU

PCT

NOTIFICATION CONCERNING
SUBMISSION OR TRANSMITTAL
OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

To:

SANTORO, Tiziana
Marietti, Gislon e Trupiano S.r.l.
Via Larga, 16
I-20122 Milan
Italy

Date of mailing (day/month/year) 18 August 2003 (18.08.03)	
Applicant's or agent's file reference 03420E45	IMPORTANT NOTIFICATION
International application No. PCT/IB03/02338	International filing date (day/month/year) 17 June 2003 (17.06.03)
International publication date (day/month/year) Not yet published	Priority date (day/month/year) 18 June 2002 (18.06.02)
Applicant ORESTE, Pasqua, Anna et al	

1. The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).
2. This updates and replaces any previously issued notification concerning submission or transmittal of priority documents.
3. An asterisk(*) appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b). In such a case, the attention of the applicant is directed to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.
4. The letters "NR" appearing in the right-hand column denote a priority document which was not received by the International Bureau or which the applicant did not request the receiving Office to prepare and transmit to the International Bureau, as provided by Rule 17.1(a) or (b), respectively. In such a case, the attention of the applicant is directed to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
18 June 2002 (18.06.02)	MI2002A001345	IT	07 Augu 2003 (07.08.03)
18 June 2002 (18.06.02)	MI2002A001346	IT	07 Augu 2003 (07.08.03)
27 Augu 2002 (27.08.02)	MI2002A001854	IT	07 Augu 2003 (07.08.03)

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No. (41-22) 338-7060	Authorized officer Sonia GARCIA MELLITI Telephone No. (41-22) 338 9819
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From the INTERNATIONAL BUREAU

PCT**NOTICE INFORMING THE APPLICANT OF THE
COMMUNICATION OF THE INTERNATIONAL
APPLICATION TO THE DESIGNATED OFFICES**

(PCT Rule 47.1(c), first sentence)

To:

SANTORO, Tiziana
Marietti, Gislone e Trupiano S.r.l.
Via Larga, 16
I-20122 Milan
ITALIEDate of mailing (day/month/year)
24 December 2003 (24.12.03)Applicant's or agent's file reference
03420E45**IMPORTANT NOTICE**International application No.
PCT/IB03/02338International filing date (day/month/year)
17 June 2003 (17.06.03)Priority date (day/month/year)
18 June 2002 (18.06.02)

Applicant

ORESTE, Pasqua, Anna et al

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this notice:

AU, AZ, BY, CH, CN, CO, DE, DZ, EP, HU, JP, KG, KP, KR, MD, MK, MZ, RU, TM, US

In accordance with Rule 47.1(c), third sentence, those Offices will accept the present notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:

AE, AG, AL, AM, AP, AT, BA, BB, BG, BR, BZ, CA, CR, CU, CZ, DK, DM, EA, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, ID, IL, IN, IS, KE, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MG, MN, MW, MX, NI, NO, NZ, OA, OM, PG, PH, PL, PT, RO, SC, SD, SE, SG, SK, SL, SY, TJ, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW

The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this notice is a copy of the international application as published by the International Bureau on 24 December 2003 (24.12.03) under No. WO 03/106504

4. **TIME LIMITS** for filing a demand for international preliminary examination and for entry into the national phase

The applicable time limit for entering the national phase will, subject to what is said in the following paragraph, be 30 MONTHS from the priority date, not only in respect of any elected Office if a demand for international preliminary examination is filed before the expiration of 19 months from the priority date, but also in respect of any designated Office, in the absence of filing of such demand, where Article 22(1) as modified with effect from 1 April 2002 applies in respect of that designated Office. For further details, see *PCT Gazette* No. 44/2001 of 1 November 2001, pages 19926, 19932 and 19934, as well as the *PCT Newsletter*, October and November 2001 and February 2002 issues.

In practice, time limits other than the 30-month time limit will continue to apply, for various periods of time, in respect of certain designated or elected Offices. For regular updates on the applicable time limits (20, 21, 30 or 31 months, or other time limit), Office by Office, refer to the *PCT Gazette*, the *PCT Newsletter* and the *PCT Applicant's Guide*, Volume II, National Chapters, all available from WIPO's Internet site, at <http://www.wipo.int/pct/en/index.html>.

For filing a demand for international preliminary examination, see the *PCT Applicant's Guide*, Volume I/A, Chapter IX. Only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination (at present, all PCT Contracting States are bound by Chapter II).

It is the applicant's sole responsibility to monitor all these time limits.

The applicant is hereby notified that, at the time of establishment of this Notice, the time limit under Rule 46.1 for making amendments under Article 19 has not yet expired and the International Bureau had received neither such amendments nor a declaration that the applicant does not wish to make amendments.

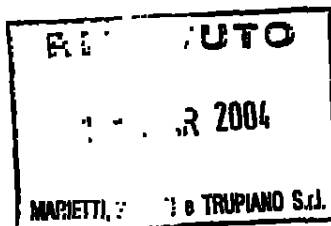
The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Authorized officer

Judith Zahra

Facsimile No.(41-22) 740.14.35

Telephone No.(41-22) 338.91.11



PATENT COOPERATION TREATY

PCT

From the INTERNATIONAL BUREAU

To:

SANTORO, Tiziana
 Marietti, Gislone e Trupiano S.r.l.
 Via Larga, 16
 I-20122 Milan
 Italy

**INFORMATION CONCERNING ELECTED
 OFFICES NOTIFIED OF THEIR ELECTION**

(PCT Rule 61.3)

Date of mailing (day/month/year) 02 March 2004 (02.03.2004)		IMPORTANT INFORMATION
Applicant's or agent's file reference 03420E45		
International application No. PCT/IB2003/002338	International filing date (day/month/year) 17 June 2003 (17.06.2003)	Priority date (day/month/year) 18 June 2002 (18.06.2002)
Applicant ORESTE, Pasqua, Anna et al		

1. The applicant is hereby informed that the International Bureau has, according to Article 31(7), notified each of the following Offices of its election:

EP : AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE,
 SI, SK, TR

National : BG, CA, CN, DE, GB, IL, JP, KP, KR, MN, NO, PL, RO, RU, SK, US

2. The following Offices have waived the requirement for the notification of their election; the notification will be sent to them by the International Bureau only upon their request:

AP : GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW

EA : AM, AZ, BY, KG, KZ, MD, RU, TJ, TM

OA : BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG

National : AE, AG, AL, AM, AT, AU, AZ, BA, BB, BR, BY, BZ, CH, CO, CR, CU, CZ, DK, DM, DZ, EC,
 EE, ES, FI, GD, GE, GH, GM, HR, HU, ID, IN, IS, KE, KG, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD,
 MG, MK, MW, MX, MZ, NI, NZ, OM, PG, PH, PT, SC, SD, SE, SG, SL, TJ, TM, TN, TR, TT, TZ, UA, UG,
 UZ, VC, VN, YU, ZA, ZM, ZW

3. Since the election(s) was (were) made after the expiration of 18 months from the priority date, the applicant is reminded that he must enter the national phase before the expiration of 20 months from the priority date before the Office(s) concerned. This must be done by paying the national fee(s) and furnishing, if prescribed, a translation of the international application (Article 22).

Some offices have fixed time limits expiring later than the above-mentioned time limit. For detailed information about the applicable time limits and the acts to be performed upon entry into the national phase before a particular Office, see Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer: Pascale CHOUQUER
Facsimile No. (41-22) 338-7080	Telephone No. (41-22) 338 8301

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

Santoro, Tiziana
MARIETTI, GISLON E TRUPIANO S.R.L.
Via Larga, 16
20122 Milano
ITALIE

PCT

RI EVUTO

- 4 FEB 2004

MARIETTI, GISLON e TRUPIANO S.r.l.

WRITTEN OPINION

(PCT Rule 66)

Date of mailing (day/month/year) 02/02/2004	
Applicant's or agent's file reference 03420E45	REPLY DUE within 2 / 00 months/days from the above date of mailing
International application No. PCT/IB03/02338	International filing date (day/month/year) 17/06/2003
	Priority date (day/month/year) 18/06/2002
International Patent Classification (IPC) or both national classification and IPC C08B37/00	
Applicant ORESTE, Pasqua Anna	

- This written opinion is the first drawn up by this International Preliminary Examining Authority.
- This opinion contains indications relating to the following items:
 - ☒ Basis of the opinion
 - ☐ Priority
 - ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
 - ☐ Lack of unity of invention
 - ☒ Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
 - ☐ Certain documents cited
 - ☐ Certain defects in the international application
 - ☐ Certain observations on the international application
- The applicant is hereby invited to reply to this opinion.

When? See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

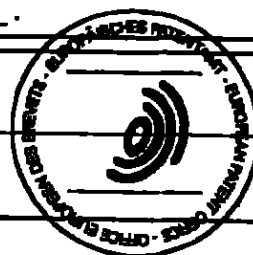
Also For an additional opportunity to submit amendments, see Rule 66.4.
For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4b1.
For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

- The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 18/10/2004

Name and mailing address of the IPEA/  European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465	Authorized officer Examiner Formalities officer (incl. extension of time limits) Tel. (+49-89) 2399 2828
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Form PCT/IPEA/408 (cover sheet) (march 2002)



I. Basis of the opinion

1. The basis of this written opinion is the application as originally filed.

V. Reasoned statement under Rule 66.2(a)(II) with regard to novelty, Inventive step or Industrial applicability

1. In light of the documents cited in the international search report, it is considered that the invention as defined in at least some of the claims does not appear to meet the criteria mentioned in Article 33(1) PCT, i.e. does not appear to be novel and/or to involve an inventive step (see international search report, in particular the documents cited X and/or Y and corresponding claims references).
2. If amendments are filed, the applicant should comply with the requirements of Rule 66.8 PCT and indicate the basis of the amendments in the documents of the application as originally filed (Article 34 (2) (b) PCT) otherwise these amendments may not be taken into consideration for the establishment of the international preliminary examination report. The attention of the applicant is drawn to the fact that if the application contains an unnecessary plurality of independent claims, no examination of any of the claims will be carried out.

NB: Should the applicant decide to request detailed substantive examination, then an international preliminary examination report will normally be established directly. Exceptionally the examiner may draw up a second written opinion, should this be explicitly requested.

Derivados epimerizados de polisacárido K5
con un grado muy alto de sulfatación

Objeto de la Invención

La presente invención se refiere a nuevos derivados del polisacárido K5 con un grado muy alto de sulfatación, un proceso para su preparación, nuevos intermedios altamente o-sulfatados útiles en su síntesis y composiciones farmacéuticas que contienen dichos derivados de polisacáridos K5 como ingredientes activos básicamente libres de actividad sobre la coagulación.

En particular, la invención se refiere a un proceso para la preparación sobre sulfatos de epi k5 -N, O -que comienzan con un polisacárido K5 previamente N-desacetilado, N-sulfatado y C-5 epimerizado por lo menos 20%, por O-sobre sulfatación en condiciones adecuadas y N-sulfatación subsecuente, a dichos epi k5 -N,O- sobre sulfatos de actividad antiangiogenética y antiviral y a nuevos intermedios de epi-k5-N sulfatos de bajo peso molecular.

Antecedentes de la Invención

Los glicosaminoglicanos tales como la heparina, sulfato de heparan, sulfato de dermatan, sulfato de codrotina y ácido hialurónico son biopolímeros que son extraídos industrialmente de diversos órganos animales.

En particular, la heparina, principalmente obtenida mediante la extracción de membranas de mucosa intestinal de cerdos o de los pulmones bovinos, es un copolímero polidispersado con una distribución de peso molecular de aproximadamente de 3.000 a aproximadamente 30.000 D consistente de una mezcla de cadena consistente básicamente de un ácido urónico (ácido glucurónico o ácido idurónico) y de un azúcar amino (glucosamina) enlazado por enlaces α -1 $\rightarrow$ 4 o β -1 $\rightarrow$ 4. En la heparina, la unidad urónica puede ser O-sulfatada en la posición 2 y la unidad de glucosamina es N-acetilada o N-sulfatada, 6-O-sulfatada y 3-O-sulfatada en aproximadamente 0.5% de las unidades de glucosamina presentes.

Las propiedades y la biosíntesis natural de heparina en mamíferos ha sido descrita por Lindahl et al., 1986 en

lene, D. Y Lindahl, U. (editores) "heparin. Chemical and biological properties; clinical applications", Edward Arnold, London, páginas 159-190, por Lindahl, U, Feingold D.S. and Rodén L, 1986 TIBS, 11, 221-225 y por Conrad H.E "Heparin Binding proteins", capítulo 2: estructura de los heparinoides. Academic Press, 1998. La biosíntesis de heparina ocurre comenzando con su precursor N-acetil-heparosan que consiste en una mezcla de cadena que consiste de la unidad disacárida repetitiva glucuronil- β -1 $\rightarrow$ 4-N-acetilglucosamina. Dicho precursor es sometido a modificaciones enzimáticas que hidrolizan parcialmente el grupo N-acetilo, sustituyéndolo con un grupo SO₃, epimerizado el carboxilo en la posición 5 de una parte de las unidades glucurónicas las convierte en unidades idurónicas e introduce grupos O-sulfato para obtener un producto que una vez extraído industrialmente, tiene aproximadamente el doble del número de grupo sulfato con respecto a los grupos carboxilos por unidad de disacárido. Estas modificaciones enzimáticas llevan a, por otro lado, la formación de la región pentasacárido o un enlace a la antitrombina III (ATIII), llamada pentasacárido activo, que es la estructura necesaria para el enlace de alta afinidad de la heparina al ATIII y

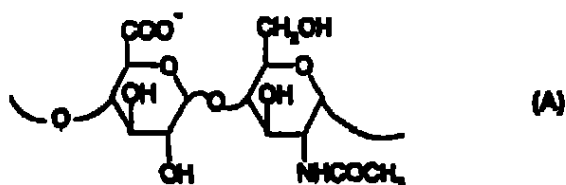
fundamentalmente para actividad anticoagulante y antitrombótica de la heparina misma. Este pentasacárido, presente al interior solamente de algunas de las cadenas que forman la heparina, contiene una unidad de glucosalina sulfatada en la posición 3 y un ácido glucurónico espaciado hacia fuera entre los disacáridos que contienen ácidos idurónicos.

En la naturaleza, la formación del pentasacárido activo se hace posible por medio de la reacción de epimerización del carboxilo de una parte de las unidades glucurónicas en unidades idurónicas llevadas a cabo por glucuronil-C5-epimerasa (epimerización-C5) y por sulfatación adecuada que también conlleva a la introducción de un grupo sulfato en el hidroxilo de la posición 3 de la glucosamina. Mas particularmente, en la naturaleza la formación del pentasacárido activo se hace posible por el hecho de que la epimerización C5 ocurre en regiones es decir porciones de cadenas, y extensivamente lo que resulta en un producto que contiene mas unidades idurónicas que aquellas glucurónicas. De echo, la heparina comercial contiene aproximadamente 70% de unidades idurónicas y 30 % de unidades glucurónicas.

A lo largo de las actividades o junto con las actividades principales anticoagulantes y antitromboticas, la heparina también ejerce actividades antilipaémicas, antiproliferativas, antivirales, antitumorosas, y antimestastaticas, pero su uso como droga se ha ocultado por los efectos colaterales debido a la acción anticoagulante que puede causar el sangrado.

ESTADO DE LA TÉCNICA

Es conocido que el polisacárido K5 capsular aislado de *Escherichia coli*, descrito por Vann W. F. Et al., en el periódico Europeo de bioquímica 1981, 116, 359-364 ("Vann 1981"), consiste en una mezcla de cadena que consiste de la unidad de disacárido repetitiva glucuronil- β -1 $\rightarrow$ 4-N-acetil glucosamina y por lo tanto muestra la misma secuencia repetitiva (A)



Del precursor N-acetil-heparosan de la heparina. El polisacárido K5 capsular, referido aquí como "polisacárido K5" o mas simplemente "K5", fue modificado químicamente por Lormeau et al. Como se describió en la patente estadounidense 5,550, 116 y por Casu et al. Como se describió en la investigación de Carbohidratos, 1994, 263, 271-284. Los K5-O-sulfatos tienen actividades antitumorales, antimetastáticas, antivirales, y en particular anti-HIV son descritos en la EP 333243 y WO 98/34958. El K5 fue también modificado químicamente y enzimáticamente para poder obtener productos que tengan el mismo tipo de actividad biológica in Vitro sobre la coagulación como aquella de la heparina como se extrajo de órganos animales (heparina extractiva)

El logro de los productos que tienen una actividad sobre la coagulación del mismo tipo que aquella de la heparina extractiva ocurre por procesos que imitan lo que ocurre en la naturaleza y visualizan la etapa clave completa de la epimerización C5 con D-glucuronil C5 epimerasa.

Los procesos descritos en la IT 1230785, WO 92/17507, WO 96/14425 y WO 97/43317 utiliza K5 como el material de partida. El K5 que se origina de la fermentación esta

sometido a N-desacetilación seguida por N-sulfatación y sobre el K5-sulfato así obtenido epimerización C5 con C5-epimerasa en solución es llevada a cabo, obteniéndose sea por una cromatografía de una solución de enzimas microsomales de la mastositoma de ratón (IT 1230 785) o de hígado de bovino (WO 92/17507, WO 96/14425, y WO 97/43317).

La D-glucuronil C5 epimerasa del hígado de bovino fue purificada por Campbell, P. et al. En J. Biol. Chem., 1994, 269/43, 26953-26958 ("Campbell 1994") quien también suministro su composición en amino ácidos y describió su uso en la solución para la transformación de un K5-N-sulfato en el producto epimerizado al 30% correspondiente, demostrando la formación del ácido idurónico por medio del método HPLC seguido por despolimerización nitrosa al disacáridos.

El documento WO 98/48006 describe la secuencia de ADN que codifica para el D-glucuronil C5 epimerasa y un D-glucuronil C5 epimerasa recombinante, obtenida de un vector de expresión recombinante que contiene dicho ADN, posteriormente purificado por Campbell et al. Como lo



mostró Jin-Ping L. Et al. en J. Biol. Chem. 2001, 276, 20069-20077 ("Jin-Ping 2001")

La secuencia completa de la epimerasa C5 fue descrita por Crawford B. E. Et al. In J. Biol.. Chem., 2001, 276(24), 21538-21543 (Crawford 2001)

El documento WO 01/72848 describe un método para la preparación de derivados N- de acetilados N-sulfatados de polisacárido K5 por lo menos 40% epimerizado de ácido idurónico con respecto al total de los ácidos urónicos, que tienen un peso molecular desde 2.000 a 30.000, que contienen desde 25 hasta 50% de cadenas de alta afinidad para ATIII y que tienen una actividad anticoagulante y antitrombótica expresada como proporción HCII/antiXa entre 1,5 a 4. Dicho documento describe la sobre sulfatación de un K5-N-sulfato, 40-60% epimerizado y muestra que el producto obtenido, cuyo ^{13}C -RMN está ilustrado, tiene un contenido de grupo sulfato por unidad de disacárido 2-3,5. Repitiendo la sobre sulfatación anteriormente mencionada en las condiciones descritas y examinando el ^{13}C -RMN se pudo tener certeza de que el producto obtenido realmente es una amina libre cuyo

contenido de 6-O-sulfato esta entre 80-95%, que de el 3-O-sulfato en el azúcar amino es 30%, pero cuyo grado de sulfatación es 3,2. También se observó que en las condiciones de sobre sulfatación descritas en la WO 01/72848 no se obtiene un grado de sulfatación de mas de 3,2 no fue obtenido. El documento US 2002/0062019 describe un proceso para la preparación de los sulfatos epik5-N, O-, activo en el control de la coagulación que tiene un grado de sulfatación entre 2,3 a 2,9 y un peso molecular entre 2.000 a 30.000, o entre 4.000 a 8.000, o entre 18.000 a 30.000. El proceso anteriormente mencionado involucra las etapas: (p-a) una O-desacetilación del polisacárido K5 y un N-sulfatación de la K5-amina resultante, (p-b) una epimerización del k5-N-sulfato, (p-c) una O- sobre sulfatación del epik5-N-sulfato, (p-d) una O- desulfatación parcial, (p-e) una 6-O-sulfatación selectiva, (p-f) una N-sulfatación del producto así obtenido ,cualquier producto obtenido luego de la terminación de una de las etapas (p-b)-(p-f) capaz de ser sometida a la despolimerización. Dicho documento describe un epik5-N, O-sulfato que tiene un peso molecular de 7.400 obtenido por las etapas anteriormente mencionadas (p-a)-(p-f) seguido por una despolimerización

nitrosa al final de la etapa (p-f), con un grado de sulfatación entre 2,3 a 2,9.

El mismo documento también describe una parte de k5 con un peso molecular de aproximadamente 5.000 que también puede ser sometida a las etapas (p-a)-(p-f).

Para poder estandarizar la terminología y convertir el texto más comprensible, en la presente descripción los términos convencionales y expresiones serán usados en singular o en plural. En particular:

- Por "K5" o "polisacárido K5" significa el polisacárido capsular de la *Escherichia coli* obtenido por fermentación, es decir, una mezcla de cadenas que consiste de unidades de disacáridos (A) contienen opcionalmente un doble enlace en el extremo no reductor como se muestra anteriormente, en cualquier caso preparado y purificado de acuerdo con los métodos descritos en la literatura, en particular de acuerdo a Vann 1981, de acuerdo con Manzoni M. Et al., Periódico de Polímeros Bioactivos Compatibles, 1996, 11, 301-311 ("Manzoni 1996") o de acuerdo con el método descrito en WO 01/72848 y en WO 02/068447; es obvio para una persona

avanzada en materia que lo que se muestra de aquí en adelante se puede aplicar a cualquier N- acetilheparosan;

- Por "epimerasa C5" significa la D-glucoronil C-5 epimerasa, extraída o recombinante, en cualquier caso preparada, aislada y purificada, en particular, como se describió en Campbell 1994, en la WO 98/48006, en la Jin-Ping L. Et al. En J. Bio Chem. 2001, 276, 20069-20077 (Jin-ping 2001) o en Crawford 2001;
- Por k5-amina que significa al menos 95% K5 N-desacetilado pero generalmente en la cual los grupos acetilos son indetectables por un aparato RMN normal;
- Por "K5-N-sulfato" que significa al menos 95% de K5 N- desacetilada y N-sulfatada, normalmente 100%, ya que los grupos N-acetilos son indetectables con un aparato de RMN normal, según se describe de aquí en adelante;

- Por "epik5" significa que el K5 y sus derivados en los cuales 20-60% de las unidades glucurónicas son C5-epimerizadas a unidades idurónicas;
- Por "epik5-N-sulfato" que significa el K5-N-sulfato en el cual 20-60% de las unidades glucurónicas C5-epimerizadas a unidades idurónicas;
- Por "epik5-amina-O-sobresulfato" que significa una epik5-amina O-sulfatada con un grado de sulfatación de al menos 3,4;
- Por "epik5-N, O-sobresulfato" que significa una epik5-amina-O-sulfatado completamente N-sulfatado, con un grado de sulfatación de al menos 4;

En adición:

- Los términos convencionales y expresiones definidos de aquí en adelante se refieren al K5 como aislado después de fermentación, generalmente con una distribución de peso molecular desde 1.500 hasta aproximadamente 50.000 con un peso molecular

promedio de 10.000-25.000, ventajosamente de 15.000-25.000;

- Los términos convencionales y expresión como se designo de la aquí anteriormente cuando son seguidos por el acrónimo "LMW" (bajo peso molecular), por ejemplo LMW-K5-Nsulfato, LMW-epik5-N-sulfato, indican productos de bajo peso molecular, obtenidos por fraccionación o por despolimerización del k5-N-sulfato consiste o es derivado de k5-N-sulfatos que tienen un peso molecular promedio entre aproximadamente 1.500 a 12.000, calculado en un producto 100% N-sulfatado;
- Los términos convencionales y de expresiones como se ha definido aquí anteriormente, cuando están seguidas por "-derivados" indican como un total tanto de derivados como de k5 nativos y aquellos de bajo peso molecular;
- Por el termino "aproximadamente" en referencia al peso molecular que significa el peso molecular medido por el viscosímetro $\pm$ el peso teórico de una

unidad de disacárido, incluyendo el peso del sodio, calculado como 461 en el caso de un epik5-N-sulfato-derivado y 806 en el caso de un epik5-N, O-sobresulfatado-derivado con un grado de sulfatación de 4,26;

- Por la expresión "especies preponderantes", que significa el compuesto que, en la mezcla constituye el LMW-epik5-N-sulfato, el LMW-epik5-amina-O-sobresulfato o el LMW-epik5-N,O-sobresulfato, es el tipo mas representado, determinado, por el pico de la curva del peso molecular medido mediante HPLC;
- A menos que se especifique de otra manera, por "grado de sulfatación" significa la $\text{SO}_3^-/\text{COO}^-$, expresada también como el numero de grupos sulfato por unidad de disacárido, medida con el método conducto métrico descrito por Casu B. Et al. En Carbohydrate Research, 1975, 39, 168-176 (Casu 1975), el mismo utilizado en la WO 01/72848;
- Por " condiciones de O-sobresulfatación" se significa una O-sulfatacion extrema llevada a cabo

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por ejemplo de acuerdo al método C descrito por B. Casu et al. En Carbohydrate Research, 1994, 263, 271-284 (Casu 1994);

- Por el termino "alquilo" que significa un alquilo lineal o ramificado, mientras que el termino "tetrabutylamonio" indica el grupo tetra-n-butylamonio.

RESUMEN DE LA INVENCION

Se ha encontrado de manera sorprendente, que a diferencia de lo que ocurre con el proceso descrito en la IT 1230785, WO 92/17507, WO 96/14425, WO97/43317, WO 01/72848 y US 2002/0062019, partiendo con un epik5-N-sulfato es posible obtener un epik5-amina-O-sobresulfato con un grado mayor de sulfatación que cada uno de los otros epik5-amina-O-sulfato descritos en la literatura, por ejemplo en la WO01/72848, por medio de preparar la sal con base orgánica terciaria o cuaternaria de dicho epik5-N-sulfato teniendo cuidado de dejar la mezcla de reacción descansar durante un periodo de tiempo de 30 a 60 minutos manteniendo el pH en aproximadamente 7 con la misma base

orgánica y luego tratando la sal obtenida con un reactivo de O-sulfatación en las condiciones de O-sobresulfatación.

Sometiendo los epik5-amina-O-sobresulfatos así obtenidos a N-sulfatación, nuevos epik5-N, O-sobresulfatos son obtenidos los cuales, a diferencia de los productos descritos en la IT 1230785, WO 9/17507, WO 96/14425, WO 97/43317, WO 01/72848 y US 200/006219, están libres de actividad sobre la coagulación y son útiles para la preparación de medicinas, particularmente composiciones farmacéuticas de actividad antiangiogénica y antiviral o de composiciones cosméticas.

Por la despolimerización de ácidos nitrosos de dichos epik5-N, O-sobresulfatos nuevos LMW-epik5-N,O-sobresulfatos son obtenidos libres de la actividad sobre la coagulación y con actividad antiangiogénica y antiviral.

Al preparar derivados de N,O-sulfato N-desacetilados de polisacáridos k5, por lo menos 40% epimerizados de ácido idurónico con respecto al total de los ácidos urónicos y que tiene bajo peso molecular de acuerdo con el método

descrito en la WO 01/72848, se logro estar en lo correcto de que la despolimerización del producto de alto peso molecular obtenido al final de la etapa N-sulfatación final del proceso puede dar resultados variables ya que esto produce generalmente algunos productos despolimerizados que muestran una actividad mucho menor, que aquella de los productos de alto peso molecular de los cuales surge, en todos los parámetros de coagulación. Se asume que esto tiene lugar debido a la degradación con ácido nitroso que influencia por la presencia de grupos sulfato. En particular los sulfatos en la posición 3 de la glucosamina resultan en productos heterogéneos, como se describió por Nagasawa et al. En Trombosis Research, 1992,65,462-467 (Nagasawa 1992).

Se ha encontrado ahora que someter un epik5-N-sulfato a despolimerización nitrosa en el cual el contenido de ácido idurónico con respecto al total de los ácidos urónicos es entre el 20 y el 60%, ventajosamente entre 40 al 60% preferiblemente alrededor del 50%, se obtienen LMW-epik5-N-sulfatos que constituyen nuevos intermediarios efectivos para la preparación de LMW-epik5-N, O-sobresulfatos que tienen un alto grado de actividad sobre diferentes parámetros biológicos, con o sin actividad

sobre parámetros de coagulación. En particular, se ha encontrado que es posible despolimerizar un epik5-N-sulfato de modo que se obtienen nuevos LMW-epik5-N-sulfatos de peso molecular promedio de aproximadamente 2.000 a aproximadamente 4.000, más particularmente LMW-epik5-N-sulfatos específicos que consisten de mezclas en las cuales el compuesto predominante es un deca-sacárido o un dodeca-sacárido o un tetradeca-sacárido. También estos LMW-epik5-N-sulfatos, no obtenibles de otra manera, tienen propiedades biológicas interesantes y son intermedios útiles para la preparación de LMW-epik5-N, O-sobresulfatos de actividad antiviral y/o antiangiogénica y es sorprendentemente libre de actividad sobre la coagulación.

Por medio de someter un LMW-epik5-N-sulfato al método anteriormente mencionado de salificación con una base orgánica terciaria o cuaternaria, teniendo cuidado de dejar que la mezcla de reacción permanezca en descanso durante un periodo de tiempo de 30 a 60 minutos manteniendo el pH aproximadamente en 7 con la misma base orgánica y luego tratando la sal obtenida con un reactivo de O-sulfatación en las condiciones de O-sobresulfatación, nuevos LMW-epik5-amina-O-sobresulfatos

son obtenidos. Por medio de someter el LMW-epik5-amina-O-sobresulfato a N-sulfatación, nuevos derivados N-sulfatados y O-sobresulfatados (LMW-epik5-N, O-sobresulfatos) son obtenidos sorprendentemente libres de actividad sobre la coagulación y de con actividad antiviral y/o antiangiogénica, útil para la preparación de composiciones farmacéuticas o cosméticas. Estos LMW-epik5-N-sulfatos son obtenidos partiendo de k5-N-sulfato con una reacción de epimerización con una c5-epimerasa recombinante aislada y purificada, inmovilizada sobre un soporte sólido, a una temperatura de aproximadamente 30°C y a un pH de aproximadamente 7 durante 12 a 24 horas en la presencia de un catión bivalente seleccionado entre calcio, magnesio, bario y manganeso y una reacción de despolimerización nitrosa subsecuente del producto epimerizado así obtenido o viceversa.

Sorprendentemente, de las observaciones hechas en el curso de la reacción de epimerización en las condiciones anteriormente dichas, es posible asumir que contrario a lo que ocurre en la naturaleza en la biosíntesis de la heparina, la C5-epimerización ordinaria y no del tipo "región" del sustrato ocurre cada 2 unidades de ácido

glucurónico lo que lleva a los epi-k5-N-sulfatos-derivados caracterizados por una unidad tetrasacárido repetitiva consistente de 2 unidades de gliucosamina separados por una unidad glucurónica y seguidos por una unidad idurónica o viceversa.

DESCRIPCIÓN DETALLADA

Así, de acuerdo con uno de sus aspectos, la presente invención suministra un proceso para la preparación de epik5-N, O-sobresulfato-derivados, caracterizados en que

- (a) un derivado k5-N-sulfato, en forma acídica, es tratado con una base orgánica terciaria o cuaternaria dejando la mezcla de reacción descansar durante un periodo de 30 a 60 minutos, manteniendo el pH de la solución en un valor de 7 por medio de la adición de la misma base orgánica terciaria o cuaternaria, y su sal es aislada con dicha base orgánica;
- (b) Dicha sal de base orgánica de dicho epik5-N-sulfato-derivado es tratada con un reactivo de O-sulfatación en las condiciones de O-sobresulfatación;

- (c) El epi-k5-amina-O-sobresulfato-derivado y tratado con un reactivo de N-sulfatación y el epik5-N, O-sobresulfato-derivado es aislado.

Generalmente, el epik5-N,O-sobresulfato-derivado es aislado en forma de sal de sodio y opcionalmente dicha sal de sodio es transformada en otra sal químicamente y farmacéuticamente aceptable.

En este contexto, el termino "químicamente aceptable" se refiere a un catión utilizable en síntesis química, tal como el sodio amonio, tetraalquilamonio (con 1 a 4 carbonos) ión o para la purificación del producto, en donde "farmacéuticamente aceptable es autoexplicatorio.

Los cationes ventajosos son aquellos derivados de metales alcalinos, metales de tierra alcalina, amonio, tetraalquilamonio (C1- C4), aluminio y zinc, los cationes preferidos son el sodio, el calcio y los iones tetrabutilamonio.

De acuerdo con una manera ventajosa del procedimiento, la etapa (a) es llevada a cabo por medio de pasar una solución de la sal de sodio del epik5-N-sulfato-derivado,

es decir del polisacárido k5, previamente N-desacetilizado, N-sulfatado, normalmente 100% y 20 a 60% C5-epimerizado y opcionalmente despolimerizado con ácido nitroso, que tiene un peso molecular promedio entre aproximadamente 1.000 a aproximadamente 25.000, ventajosamente des aproximadamente 1.500 a aproximadamente 25.000, por una resina de intercambio iónica ácida, por ejemplo del tipo IR-120H+, recolectando el eluato también incluye el agua de lavado de la resina y neutralizar el eluato con una base orgánica terciaria o cuaternaria, preferiblemente con una solución acuosa de hidróxido de tetrabuliamonio. La solución se deja descansar durante una hora, manteniendo su pH en 7 por medio de agregar la misma base y la sal así obtenida es aislada por liofilización.

En la etapa (b), la O-sobresulfatación ocurre por medio de usar un exceso de agente O-sulfatante y trabajar entre una temperatura entre 20 y 70°C durante un periodo de tiempo de hasta 24 horas en un disolvente polar aprótico.

Ventajosamente, la sal con una base orgánica terciaria o cuaternaria del epik5-N-sulfato-derivado, es decir del polisacárido k5, previamente N-desacetilizado, N-

sulfatado preferiblemente al 100%, y 20 a 60% C5-epimerizado y opcionalmente despolimerizado con ácido nitroso, que tiene un peso molecular promedio entre aproximadamente 1.000 a aproximadamente 25.000, ventajosamente desde aproximadamente 1.500 a aproximadamente 25.000 según se aisló en la etapa (a), es disuelto en dimetilformamida y tratado con 2 a 10 moles de un reactivo de O-sulfatación por cada hidroxilo libre a una temperatura entre 40 a 60° durante 10 a 20 horas. Como un reactivo de O-sulfatación es ventajosamente usado el aducto de piridina.SO₃ en una cantidad de 2,5 a 5 moles, preferiblemente 2,5 a 4 moles por hidroxilo libre por disacárido y la reacción es llevada a cabo de manera ventajosa entre 50 a 60°C, preferiblemente a 55°C, durante la noche. El producto obtenido luego de la terminación de la reacción es aislado por la adición de 0,1 a 1 volumen de agua y neutralización, preferiblemente con hidróxido de sodio, precipitación con una solución de cloruro de sodio saturada en acetona, filtración y posible ultrafiltración.

El producto así obtenido es generalmente la sal de sodio de un epik5-amina-O-sobresulfato-derivado que tiene un contenido de ácido idurónico entre 20 a 60% del total de

los ácidos urónicos, que tiene un peso molecular promedio entre aproximadamente 3.500 a aproximadamente 40.000, ventajosamente desde aproximadamente 4.500 a aproximadamente 40.000 y un grado de sulfatación de por lo menos 3,4, ventajosamente de por lo menos 3,5, mas ventajosamente de 3,55 a 4, preferiblemente entre 3,55 a 3,8. La sal de sodio así obtenida puede ser convertida en otra sal. Por medio de ejemplo un intercambio con el ión calcio puede ser llevado a cabo trabajando con membranas de ultrafiltración. En la etapa (c), el epik5-amina-O-sobresulfatado-derivado con un grado muy alto de sulfatación es N-sulfatado usando los métodos conocidos de N-sulfatación en la literatura.

En la práctica, la N-sulfatación es llevada a cabo por medio de tratar una solución acuosa que contiene el epik5-amina-O-sobresulfatado-derivado que se origina de la etapa (b) con un carbonato de sodio y un agente de N-sulfatación, por ejemplo un trialquilamina.SO₃ (C₁ -C₄.) piridina SO₃ como aductos manteniendo la mezcla entre 30 a 50°C durante 8 a 24 horas y aislando el epik5-N,O-sobresulfato-derivado deseado, por ejemplo por diafiltración. Opcionalmente la etapa de N-sulfatación es



repetida hasta que se obtiene más del 95% de la sustitución, preferiblemente completa.

Los nuevos epik5-N, O-sobresulfato-derivados así obtenidos están generalmente en su forma de sal de sodio. Dicha sal de sodio puede ser convertida en otra sal química o farmacéuticamente aceptable. Sale4s particularmente ventajosas son aquellas de metales alcalinos, de metales de tierras alcalinas, de amonio, de tetraalquilamonio con (C₁-C₄), de aluminio y zinc. Las preferidas son las sales de sodio, calcio y tetrabutilamonio.

El epik5-N-sulfato de partida sometido a la etapa (a) del proceso de la presente invención se deriva de un polisacárido k5, previamente N-desacetilizado, N-sulfatado virtualmente al 100%, y 20 a 60% C5-epimerizado, ventajosamente 40 a 60%, y opcionalmente despolimerizado con ácido nitroso, que tiene un peso molecular promedio entre aproximadamente 1.000 a aproximadamente 25.000, ventajosamente entre aproximadamente 1.500 a aproximadamente 25.000. Preferiblemente, dicho material de partida es un epi-k5-N-sulfato que tiene un peso molecular promedio entre

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10.000 y 25.000 o un LMW-epik5-N-sulfato teniendo un peso molecular de aproximadamente 1.000 a aproximadamente 12.000, ventajosamente de aproximadamente 1.000 a 10.000, preferiblemente entre aproximadamente 1.500 y aproximadamente 8.000.

Los epik5-N-sulfatos, preparados por C5-epimerización de k5-N-sulfatos, son bien conocidos en la literatura y ampliamente descritos por ejemplo en la WO 92/17507, Wo 01/72848, WO 98/14425, WO 97/43317 o US 2002/0062019. Su preparación por epimerización C-5 de la unidad glucurónica del k5-N-sulfato con D-glucuronil C5 epimerasa fue descrito en los documentos citados aquí anteriormente.

Un LMWepik5-N-sulfato que tiene un contenido de unidad idurónica de aproximadamente 20%, obtenido por N-desacetilación, N-sulfatación y C5-epimerización de una parte del k5 que tiene un peso molecular promedio de 5.000 se describió en la WO 92/17507. Sin embargo tal LMW-k5-N-sulfato contiene una cantidad considerable de grupos acetilo.

Un epik5-N-sulfato con un contenido de ácido idurónico de 40 a 60%, particularmente ventajoso como material de partida, es aquel obtenido por la epimerización de un k5-N-sulfato virtualmente libre de grupos acetilos, a su vez preparado de k5 particularmente puro, en particular que no contenga sustancias lipofílicas, descrito en la WO 02/068477. De acuerdo con una manera preferencial del procedimiento, por epimerización de un k5-N-sulfato se obtiene de un k5 libre de sustancia lipofílicas como aquellas descritas en la WO 02/068477 la epimerización C5 es llevada a cabo con una D-glucuronil C5-epimerasa que es aislada, purificada e inmovilizada sobre un soporte sólido, a un pH de aproximadamente 7, a una temperatura de aproximadamente 30°C y durante un periodo de tiempo entre 12 a 24 horas en la presencia de por lo menos ión bivalente seleccionado entre calcio, magnesio, bario y manganeso.

Los LMW-epik5-N-sulfatos que tienen un contenido mas alto de unidades idurónicas, en particular 40 a 60% preferiblemente 50 a 55%, son nuevos, y productos particularmente ventajosos como materiales de partida en la preparación de los LMW-epik5-N, O-sobresulfato-derivados.



Los LMW-epik5-N-sulfatos como se mostró anteriormente son preparados por un proceso caracterizado en que el k5-N-sulfato es sometido, en cualquier orden,

- (i) a epimerización C5 con una D-glucuronil C5-epimerasa que es aislada, purificada y en solución o inmovilizada en un soporte sólido, a un pH de aproximadamente 7, a una temperatura de aproximadamente 30°C y durante un periodo de tiempo entre 12 a 24 horas en presencia de por lo menos un ión bivalente seleccionado entre calcio, magnesio, bario y manganeso;
- (ii) A una despolimerización nitrosa opcionalmente seguida por reducción, normalmente con borohidruro de sodio.

La expresión "en cualquier orden" muestra que el proceso puede ser llevado a cabo de manera indiferente en cualquiera de las direcciones (i)-(ii), es decir en la secuencia mostrada anteriormente, lo mismo que en la dirección inversa, es decir también en la dirección (ii)-(i) sometiendo el k5-N-sulfato primeramente a la despolimerización nitrosa, opcionalmente seguido por

reducción con borohidruro de sodio, y luego a epimerización C5 en las condiciones anteriormente mencionadas. El orden preferido es el de la dirección (i)-(ii). La secuencia (ii)-(i) es utilizada preferiblemente partiendo con los LMW-k5-N sulfatos que tienen un peso molecular promedio de mas de 4.000 preferiblemente partiendo con aproximadamente 6.000. Por ejemplo, uno puede determinar la cantidad de nitrito de sodio que, partiendo con 1 g de epik5-N-sulfato, permite el logro de un LMW-epik5-N sulfato con un peso molecular de mas de 4.000, en particular de por lo menos 6.000, de modo que se obtengan intermedios útiles para la preparación de los LMWepik5-N, O-sobresulfatos. De hecho, en este caso, en la etapa (ii) el porcentaje epimerización óptima es obtenido. De acuerdo con un aspecto preferencial de la invención, el C5-epimerasa es inmovilizada sobre un soporte sólido inerte.

La C5-epimerasa, preferiblemente recombinante, aislada y purificada por ejemplo de acuerdo con Campbell 1994, WO 98/48006, Jin -Ping 2001 o Crawford 2001, es inmovilizada sobre un soporte inerte en la presencia del sustrato, es decir en la presencia k5-N-sulfato-derivado de partida o en la presencia LMW-k5-sulfato, que ventajosamente tiene

un peso molecular promedio de 4.000, preferiblemente de por lo menos 6.000. La inmovilización se lleva a cabo de acuerdo con los métodos convencionales, por ejemplo como se describió en la WO 01/72848.

La reacción epimerización C-5 se lleva a cabo por medio de recircular 20 a 1.000 ml de una solución HEPES de 25 mm a un pH de aproximadamente 7 que contiene 0,001-10 g del sustrato (k5-N-sulfato o LMW-k5-N-sulfato, preferiblemente con un peso molecular de mas de 4.000, en particular de por lo menos 6.000) y un catión seleccionado entre calcio , magnesio, bario y manganeso a una concentración entre 10 y 60 mm por una columna que contiene $1,2 \times 10^7$ a 3×10^{11} cpm de la encima inmovilizada, manteniendo el pH a aproximadamente 7 a aproximadamente 30°C, a un flujo entre 30 a 220 ml/hora durante un periodo de tiempo de 12 a 24 horas ventajosamente entre 15 a 24 horas.

Preferiblemente dicha solución es recirculada a un flujo de aproximadamente 200 ml/hora durante la noche (15 a 20 horas). El producto obtenido es purificado y separado de acuerdo con métodos conocidos, por ejemplo por ultrafiltración y precipitación con etanol. El producto

así obtenido es consistente de epik5-N-sulfato (y en tal caso es disuelto en agua y sometido despolimerización) o LMW-epik5-N-sulfato (en cuyo caso este constituye el producto final). El porcentaje de epimerización, en la práctica la cantidad de unidades idurónicas con respecto a las glucurónicas, es calculado, usando ^1H -RMN de acuerdo con el método descrito en la WO 96/4425.

La reacción de despolimerización nitrosa se lleva a cabo de acuerdo con métodos conocidos por la despolimerización de la heparina, por ejemplo en el método descrito en la EP 37319, en WO 82/03627 o de acuerdo con el método por despolimerización de un k5-N-sulfato descrito en la EP 544592, pero partiendo con un k5-N-sulfato o un epik5-N-sulfato que contiene entre 0 a no mas de 10%, preferiblemente no mas del 5% de grupos acetilo. Preferiblemente, la despolimerización, llevada a cabo con nitrito de sodio y ácido clorhídrico sobre un epik5-N-sulfato virtualmente libre de grupos acetilos, es seguido por reducción in situ con borohidruro de sodio.

En la práctica, una solución acuosa fría de epik5-N-sulfato se lleva a un pH ácido (aproximadamente 2) con ácido clorhídrico y, todavía frío, tratado con nitrito de

sodio manteniendo la temperatura (aproximadamente 4°C) y el pH (aproximadamente 2) constante y luego de la terminación de la despolimerización (aproximadamente 15 a 30 minutos) la solución es neutralizada con hidróxido de sodio y tratada, todavía aproximadamente 4°C con una solución acuosa de borohidruro de sodio. Luego de la terminación de la reducción (aproximadamente 4 horas) el exceso de borohidruro de sodio es destruido con ácido clorhídrico, la solución es neutralizada con hidróxido de sodio y el producto despolimerizado (y reducido) es aislado de acuerdo con métodos conocidos, por ejemplo por precipitación directa con etanol o acetona. El producto obtenido luego de la terminación de la despolimerización puede ser un LMW-epik5-N-sulfato (en cuyo caso este constituye el producto final) o un LMW-k5-N-sulfato (y en tal caso es sometido directamente a epimerización C5 como se ha mostrado aquí, luego de aislamiento y también en solución sin ser aislado previamente), en particular cuando este tiene un peso molecular promedio de mas 4.000, preferiblemente de por lo menos 6.000, o es utilizado para preparar LMW-k5-N, O-sobresulfatado con actividad antiangiogénica y antiviral. Por medio de controlar apropiadamente la reacción de despolimerización, en particular usando cantidades

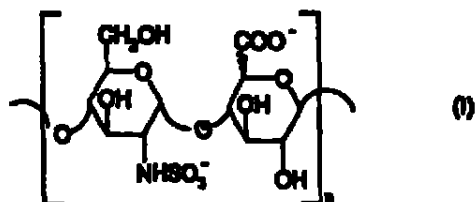
diferentes de nitrito de sodio/ácido clorhídrico, se han obtenido LMW-K5-N-sulfatos LMW-epik5-N-sulfatos que tienen un peso molecular promedio en el intervalo completo entre 1500 a aproximadamente 12.000 , ventajosamente entre aproximadamente 1500 a aproximadamente 10.000, preferiblemente entre aproximadamente 1500 a aproximadamente 7500, calculado en el espectro ^{13}C -RMN por medio de la integración de la señal atribuida al C2 del 2,5-anhidromanitol con aquel del carbón anomérico de la glucosamina al interior de la cadena del polisacárido. De acuerdo con un procedimiento de manera general, partiendo por ejemplo, 1 con g de epiK5-N-sulfato, el producto de partida es disuelto en 100 a 200 ml de agua desionizada y termoestablecido a 4 °C. Entonces una cantidad de nitrito de sodio es agregada de modo que se obtiene el peso el peso molecular promedio deseado, por ejemplo entre aproximadamente 2.000 a aproximadamente 4.000. Por lo tanto, partiendo con un epiK5-N-sulfato que tiene un peso molecular de 20.000 medido con el método HPLC equipado con una columna Biorad Biosil 250 y usando un estándar de heparina de peso molecular conocido, se requerirá la adición de 330 a 480 mg de nitrito de sodio disuelto en una solución acuosa al 0,2%. La solución contiene el epiK5-N-sulfato y el

nitrito de sodio, mantenida a 4 °C se lleva a un pH de 2 por medio de la adición de HCl al 0,1 N enfriado a 4 °C. Se le deja reaccionar bajo agitación lenta durante 20 a 40 minutos, y luego es neutralizado con NaOH al 0,1 N. El producto obtenido es llevado a temperatura ambiente y tratado con un agente reductor tal como por ejemplo el boro hidruro de sodio (250-500 mg disueltos en 50-100 ml de agua) y se deja reaccionar durante 4 a 8 horas. El exceso de boro hidruro de sodio es eliminado llevando el pH entre 5 a 5,5 con HCl al 0,1 N y dejándolo descansar durante 2 a 4 horas adicionales. Al final este es neutralizado con NaOH al 0,1 N y el producto es recuperado por precipitación con acetona y etanol después de haber sido concentrado el producto por evaporación a presión reducida.

Similarmente, la cantidad de nitrito de sodio puede ser determinada la cual, partiendo con 1 g de K5-N-sulfato o epiK5-N-sulfato, permite el logro de un LMW- K5-N-sulfato o un LMW-epiK5-N-sulfato con un peso molecular promedio entre aproximadamente 4.000 a aproximadamente 12.000, ventajosamente entre aproximadamente 4.000 a aproximadamente 7.500, y en particular de 6.000 a 7.500.

El LMW-epiK5-N-sulfato así obtenido, con un contenido de ácido idurónico entre 20 a 60%, ventajosamente entre 40 a 60%, preferiblemente entre 50 a 55% y virtualmente libre de NH_2 y grupos acetilo, tiene un peso molecular promedio de aproximadamente 1.500 a 12.000, ventajosamente desde aproximadamente 1.500 a aproximadamente 10.000, preferiblemente desde aproximadamente 1.500 a aproximadamente 7.500 y sus sales química y farmacéuticamente aceptables constituyen nuevos productos útiles de interés particular como materiales de partida en la preparación de los LMW-epiK5-N,O-sobresulfato, pero también ellos mismos son útiles como ingredientes activos de composiciones farmacéuticas o cosméticas y constituyen un aspecto adicional de la presente invención.

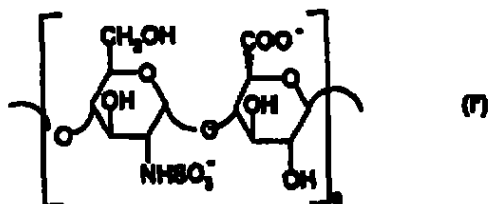
ventajosamente, los materiales de partida en la preparación de los epiK5-N,-O-sobresulfato-derivados de la presente invención son epik5-N-sulfatos-derivados que consisten de una mezcla de cadena en la cual al menos 90% de dichas cadenas tiene la fórmula I



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en la cual las unidades urónicas son el 20 al 60% consistentes de ácido idurónico, n es un entero entre 2 a 100, ventajosamente entre 3 a 100 y el catión correspondiente es química o farmacéuticamente aceptable. Más ventajosamente, dichos epiK5-N-sulfato-derivados de partida consisten de una mezcla de cadena en la cual por lo menos el 90% de dichas cadenas tiene la fórmula I en la cual las unidades urónicas son en 40 al 60% consistentes de ácido idurónico, n es un entero entre 2 a 100, ventajosamente entre 3 a 100 y el correspondiente catión es químicamente aceptable. Los materiales de partida preferidos son los LMW-epiK5-N-sulfatos como se mostró anteriormente, consistentes de una mezcla de cadena en la cual por lo menos el 90% de dichas cadenas tiene la fórmula I en la cual las unidades urónicas son en 20 al 60% comprenden ventajosamente 40 al 60%, preferiblemente 50 al 55%, de ácido idurónico, n es un entero entre 2 a 20, ventajosamente entre 3 a 15 y el correspondiente catión es químicamente aceptable.

En la práctica, dichos LMW-epiK5-N-sulfatos preferidos consisten de una mezcla de cadenas en la cual por lo menos 90% de dichas cadenas tienen la fórmula I'

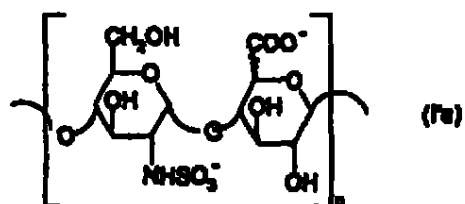


en la cual las unidades urónicas son el 20 al 60% comprendidas, ventajosamente al 40 al 60%, preferiblemente 50 al 55%, de ácido idurónico, q es un entero entre 2 a 20, ventajosamente entre 3 a 15 y el catión correspondiente es química o farmacéuticamente aceptable.

En este contexto, el término "químicamente" se refiere a un catión utilizable en la síntesis química tal como sodio, amonio, iones tetraalquilamonio(C₁-C₄), o para la purificación del producto. Los cationes ventajosos son derivados de metales alcalinos, metales de tierra alcalina, amonio, tetraalquilamonio(C₁-C₄), aluminio y zinc. Los cationes preferidos son el sodio, calcio y los iones tetrabutilamonio.

Particularmente interesante son los LMW-epiK5-N-sulfatos que consisten de una mezcla de cadenas en la cual por lo

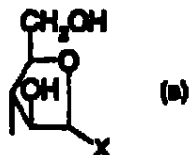
menos el 90% de dichas cadenas tiene la fórmula I' mostrada aquí anteriormente, obtenida por despolimerización nitrosa de los epiK5-N-sulfatos correspondientes mostrados anteriormente y subsecuentemente la posible reducción por ejemplo con boro hidruro de sodio. Entre estos son preferidos los LMW-epiK5-N-sulfatos que consisten de una mezcla de cadenas en la cual las especies preponderantes tienen la fórmula I'a



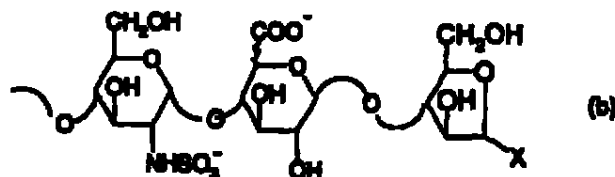
en la cual las unidades urónicas son el 60 al 40% consistentes de ácido glucurónico y 40 al 60% de ácido idurónico, p es un entero entre 4 a 8. El peso molecular promedio de estos productos es de aproximadamente 2.000 a aproximadamente 4.000 y el catión correspondiente es química o farmacéuticamente aceptable.

El origen de estos epiK5-N-sulfatos de una etapa de despolimerización nitrosa involucra, en un extremo

reductor en la mayoría de las cadenas en dicha mezcla de cadenas, la presencia de una unidad 2,5-anhidromanosa o, en el caso de la reducción con por ejemplo boro hidruro de sodio, de 2,5-anhidromanitol de estructura (a)



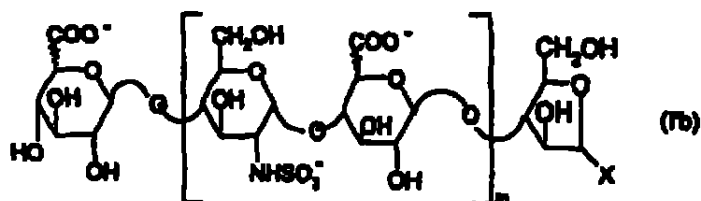
En la cual X representa un grupo formilo o un grupo hidroximetilo. Por lo tanto, el extremo reductor de la mayoría (60-70% de las cadenas) es representado realmente por la estructura (b)



en la cual X es como se definió anteriormente.

La presencia de la estructura (a) no tiene ninguna influencia en las características químicas de los epiK5-N-sulfatos y sus derivados ya que cualesquier sulfataciones llevarían a una introducción posible de uno

o dos grupos sulfatos que no moverían de manera significativa el grado de sulfatación de los derivados O-sulfatados. Sin embargo es preferible que la despolimerización nitrosa se seguida por reducción como por ejemplo con boro hidruro de sodio ya que, de acuerdo con el proceso de la presente invención, dichos LMW-epiK5-N-sulfatos están sometidos a reacciones de sulfatación y acilación cuya influencia, del radical 2,5-anhidromanosa de la estructura (a), es desconocido en el grupo formilo en el cual X representa formilo. Por otro lado la presencia de la estructura (a) no influencia la actividad Biológica de los productos, como se demostró por Ostergaard P. B. et al, en Trombosis Research, 1987, 45, 739-749 (Ostergaard 1987) para las heparinas de bajo peso molecular. Los LMW-epiK5-N-sulfato particularmente ventajosos de acuerdo con la presente invención consisten de mezclas de cadenas en las cuales las especies preponderantes son un compuesto de fórmula I'b



pl
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en la cual X es formilo o, preferiblemente hidroximetilo, m es 4, 5 o 6, el catión correspondiente es un ión química y farmacéuticamente aceptable y las unidades glucurónicas e idurónicas están presentes alternadamente partiendo con una unidad glucurónica o idurónica. En tal caso la proporción glucurónico/idurónico está entre 45/55 a 55/45, es decir aproximadamente 50/50.

El uso de la C5-epimerasa, preferiblemente recombinante, preferiblemente inmovilizada en un soporte sólido en las condiciones mostradas anteriormente por lo tanto no permite la epimerización de "regiones" de los epiK5-N-sulfato-derivados en epiK5-N-sulfatos-derivados, como ocurre en la naturaleza, pero del tipo ordinario.

Así, de acuerdo con otro de los aspectos, de la presente invención suministra el uso de C5-epimerasa aislada y purificada, para conversión del epiK5-N-sulfato-derivado en un epiK5-N-sulfato-derivado correspondiente caracterizado por una unidad tetrasacárido repetitiva consistente de dos unidades de glicosamima separadas por una unidad glucurónica y seguidas por una unidad idurónica o separadas por una unidad idurónica y seguidas por una unidad glucurónica.

Dicha epimerización ocurre óptimamente si se lleva a cabo en un epiK5-N-sulfato-derivado que tiene un peso molecular promedio desde más de 6.000 a 7.500.

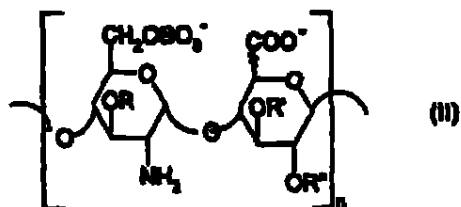
De acuerdo con la presente invención, los epiK5-N-sulfato-derivados, preferiblemente 100% N-sulfatados (en particular los epiK5-N-sulfato-derivados consistentes de mezclas de cadenas en las cuales el 90% de dichas cadenas tiene la fórmula I o I' o en las cuales las especies preponderantes tiene la fórmula I'a o I'b en la cual X es hidroximetilo), son sometidas a las etapas antes mencionadas (a) y (b), luego de la terminación de las cuales son aislados los nuevos epiK5-amina-O-sobresulfato-derivados correspondientes, en los cuales la amina no es sustituida, normalmente en forma de sal de sodio, las cuales pueden ser transformadas en otras sales química o farmacéuticamente aceptable. Sales particularmente ventajosas son aquellas de metales alcalinos, metales de tierra alcalina, amoniaco, tetraalquilamonio(C1- C4), aluminio y zinc, y entre estas las sales de sodio, calcio y tetrabutilamonio son las preferidas.

Así, de acuerdo con otro de sus aspectos, la presente invención se refiere a nuevos epiK5-amina-O-sobresulfato-derivados y sus sales química o farmacéuticamente aceptables, obtenibles por un proceso caracterizado en que

- (a) un epiK5-N-sulfato-derivado, en forma ácida, es tratado con una base orgánica terciaria o cuaternaria, dejando que la mezcla de reacción descanse por un periodo de 30 a 60 minutos, manteniendo el pH de la solución aun valor de 7 por la adición de dicha base orgánica terciaria o cuaternaria y su sal es aislada con dicha base orgánica.
- (b) Dicha sal de base orgánica de dicho epiK5-N-sulfato-derivado es tratado con un reactivo de O-sulfatación en las condiciones de O-sobresulfatación y el epiK5-amina-O-sobresulfato-derivado es aislado.

Usando, como materiales de partida ventajoso de la etapa (a), los epiK5-N-sulfato-derivados consistentes de una mezcla de cadenas en las cuales por lo menos 90% de dichas cadenas tiene la fórmula I anteriormente dicha, en

las cuales las unidades urónicas son el 20 al 60% consistentes de ácido idurónico, n es un entero entre 3 a 100 y el catión correspondiente es química o farmacéuticamente aceptable, al final de la etapa (b) un epiK5-amina-O-sobresulfato-derivado es obtenido consistente de una mezcla de cadenas en la cual por lo menos 90% de dichas cadenas tiene la fórmula II



en la cual las unidades urónicas son el 20 al 60% consistentes de ácido idurónico, n es un entero entre 2 a 100, preferiblemente entre 3 a 100, R, R' y R'' son hidrógeno o SO₃⁻, para un grado de sulfatación de 3,4, ventajosamente de por lo menos 3,5, más ventajosamente entre 3,55 a 4, preferiblemente desde 3,55 a 3,8 y el catión correspondiente es química o farmacéuticamente aceptable.

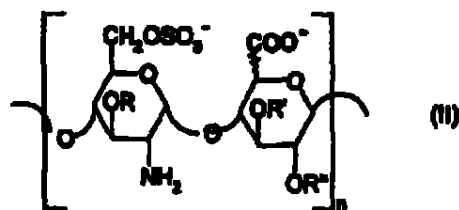
Estos epiK5-amina-O-sobresulfato-derivados con un muy alto grado de sulfatación son nuevos productos útiles

como intermedios en la preparación de sus derivados N-sulfato o N-(C₂-C₄)acilados básicamente libres de actividad sobre los parámetros de coagulación, pero que tienen otras propiedades farmacológicas interesantes.

Ventajosamente los epiK5-amina-O-sobresulfato-derivados ventajosos con un grado muy alto de sulfatación consisten desde una mezcla de cadenas en las cuales por lo menos el 90% de dichas cadenas tiene la fórmula II en la cual las unidades urónicas son 40 al 60% consistentes de ácido idurónico, n es un entero que va de 2 a 100, preferiblemente entre 3 a 100, con un peso molecular de aproximadamente 2.000 a aproximadamente 40.000, ventajosamente de aproximadamente 4.500 a aproximadamente 40.000, R es por lo menos 40% preferiblemente 50 a 80% SO₃⁻, R' y R' ' son ambos SO₃⁻ y uno es hidrógeno y el otro es 5 a 10% SO₃⁻ en ácido monosulfato glucurónico y 10 a 15% SO₃⁻ en ácido monosulfato idurónico, el grado de sulfatación es de mas de 3,4, ventajosamente de por lo menos 3,5, más ventajosamente de 3,55 a 4, preferiblemente de 3,55 a 3,8 y el catión correspondiente es químicamente aceptable.

Los epiK5-amina-O-sobresulfato-derivados preferidos con un muy alto de sulfatación son los LMW-epiK5-amina-O-sobresulfato consistentes de una mezcla de cadenas en las cuales por lo menos el 90% de dichas cadenas tienen la fórmula II en las cuales las unidades urónicas están comprendidas entre 40 al 60%, preferiblemente 50 al 55%, de ácido idurónico, R es por lo menos 40% ventajosamente 50 a 80%, preferiblemente aproximadamente 65% de SO_3^- , R' y R' ' son ambos SO_3^- o uno es hidrógeno y el otro es 5 a 10% SO_3^- en ácido glucurónico y 10 a 15% SO_3^- en ácido idurónico, n es un entero de 2 a 20, ventajosamente desde 3 a 15, con un peso molecular promedio de aproximadamente 4.000 a aproximadamente 8.000 y el catión correspondiente es químicamente aceptable.

En la práctica, dichos LMW-epiK5-amina-O-sobresulfatos preferidos consistente de una mezcla de cadenas en la cual por lo menos 90% de dichas cadenas tiene la fórmula II



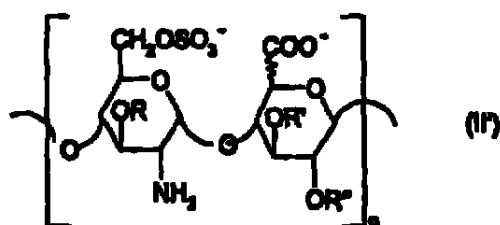
en la cual las unidades urónicas son el 20 al 60% consistentes de ácido idurónico, n es un entero entre 2 a 100, preferiblemente entre 3 a 100, R , R' y R'' son hidrógeno o SO_3^- , para un grado de sulfatación de 3,4, ventajosamente de por lo menos 3,5, más ventajosamente entre 3,55 a 4, preferiblemente desde 3,55 a 3,8 y el catión correspondiente es química o farmacéuticamente aceptable.

Estos epiK5-amina-O-sobresulfato-derivados con un muy alto grado de sulfatación son nuevos productos útiles como intermedios en la preparación de sus derivados N-sulfato o N-(C₂-C₄)acilados básicamente libres de actividad sobre los parámetros de coagulación, pero que tienen otras propiedades farmacológicas interesantes.

Ventajosamente los epiK5-amina-O-sobresulfato-derivados ventajosos con un grado muy alto de sulfatación consisten desde una mezcla de cadenas en las cuales por lo menos el 90% de dichas cadenas tiene la fórmula II en la cual las unidades urónicas son 40 al 60% consistentes de ácido idurónico, n es un entero que va de 2 a 100, preferiblemente entre 3 a 100, con un peso molecular de

aproximadamente 2.000 a aproximadamente 40.000, ventajosamente de aproximadamente 4.500 a aproximadamente 40.000, R es por lo menos 40% preferiblemente 50 a 80% SO_3^- , R' y R'' son ambos SO_3^- y uno es hidrógeno y el otro es 5 a 10% SO_3^- en ácido monosulfato glucurónico y 10 a 15% SO_3^- en ácido monosulfato idurónico, el grado de sulfatación es de mas de 3,4, ventajosamente de por lo menos 3,5, más ventajosamente de 3,55 a 4, preferiblemente de 3,55 a 3,8 y el catión correspondiente es químicamente aceptable.

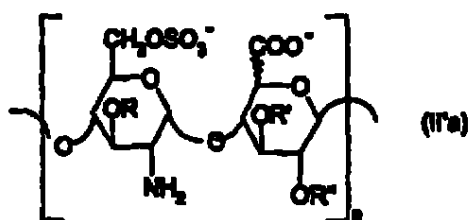
Los epiK5-amina-O-sobresulfato-derivados preferidos con un muy alto de sulfatación son los LMW-epiK5-amina-O-sobresulfato consisten de una mezcla de cadenas en las cuales por lo menos el 90% de dichas cadenas tienen la fórmula II'



En la cual las unidades urónicas son 20 a 60% consistente de ácido idurónico, q es un entero entre 2 a 20,

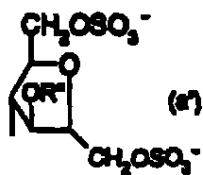
ventajosamente entre 3 a 15, R , R' y R'' son a hidrógeno o SO_3^- , para un grado de sulfatación de por lo menos 3,4, ventajosamente de por lo menos 3,5, más ventajosamente desde 3,55 a 4, preferiblemente entre 3,55 a 3,8 y el catión correspondiente es un ión química o farmacéuticamente aceptable.

Entre estos LMW-epiK5-amina-O-sobresulfatos son preferidos los que consisten de una mezcla de cadenas en la cual las especies preponderantes tienen la fórmula II'a



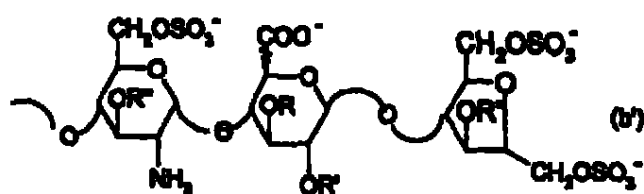
en la cual las unidades urónicas están entre el 20 al 60% consistentes de ácido idurónico, p es un entero entre 4 a 8, R , R' y R'' son como se definió anteriormente, el grado de sulfatación es de por lo menos de 3,4, ventajosamente de por lo menos 3,5, más ventajosamente de 3,55 a 4, preferiblemente de 3,55 a 3,8 y el catión correspondiente es química o

farmacéuticamente aceptable. El origen de los nuevos LMW-epiK5-amina-O-sobresulfatos de LMW-epik5-sulfatos obtenidos por despolimerización nitrosa y subsecuente reducción con por ejemplo boro hidruro de sodio, involucra, en el extremo reductor en al mayoría de las cadenas en dicha mezcla de cadenas, la presencia de una unidad 2,5-anhidromanitol sulfatada de estructura (a')

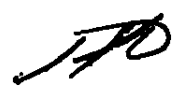


En la cual R representa hidrógeno o SO_3^- .

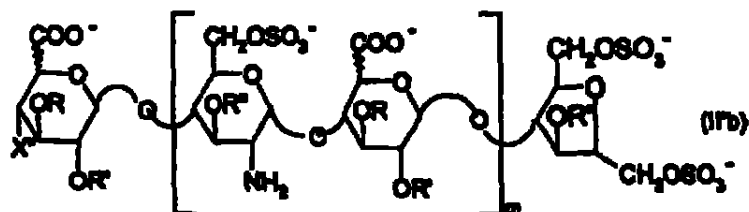
Así, el extremo reductor de la mayoría de las cadenas en dicha mezcla de cadenas está representado por la estructura (b')



en la cual la cual la unidad urónica puede ser glucurónica o idurónica.

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Entre los nuevos LMW-epiK5-amina-O-sobresulfatos mencionados anteriormente son preferidos aquellos que consisten de mezclas de cadenas en las cuales las especies preponderantes es un compuesto fórmula II'b



en la cual las unidades urónicas son el 40 al 60% consistentes de ácido idurónico, m es 4, 5 o 6, R, R' y R' ' son hidrógeno, o SO₃⁻, X'' es OH o OSO₃, para un grado de sulfatación es de por lo menos de 3,4,ventajosamente de por lo menos 3,5, más ventajosamente de por lo menos 3,55 a 4, preferiblemente entre 3,55 a 3,8, las unidades idurónicas están presentes de manera alterna, iniciando con una unidad glucorónica o idurónica, y el catión correspondiente es un ión química o farmacéuticamente aceptable.

Todos estos epiK5-amina-O-sobresulfato-derivados con un grado muy alto de sulfatación son productos nuevos que

son intermedios útiles para la preparación de los nuevos epiK5-amina-O-sobresulfato-derivados N-sustituídos y por lo tanto constituyen un aspecto adicional de la presente invención.

En particular, de acuerdo con otro de sus aspectos, se relaciona con el uso de los epiK5-amina-O-sobresulfato-derivados anteriormente mencionados con un muy alto grado de sulfatación para la preparación de nuevos epiK5-amina-O-sobresulfato-derivados N-sustituídos, en particular N-sulfatados o N-acilados.

Luego de la terminación de la etapa (c) del proceso de la presente invención, consistente de una N-sulfatación de los epiK5-amina-O-sobresulfato-derivados obtenidos al final de la etapa (b) (en particular los epiK5-amina-O-sobresulfato-derivados consistentes de mezclas de cadenas en las cuales al menos el 90% de dichas cadenas tiene la fórmula II o II' o en las cuales las especies preponderantes tiene la fórmula II'a o II'b) los epiK5-amina-O-sobresulfato-derivados son obtenidos cuyos contenidos de ácido idurónico está en 20 a 60% del total de ácidos urónicos y cuyo grado de sulfatación es de por lo menos 4, preferiblemente entre 4 a 4,6.

Así, con otro de sus aspectos, la presente invención suministra nuevos derivados de polisacárido K5 N-desacetilado, O-sulfatado y N-sulfatado, C5-epimerizado a ácido idurónico en por lo menos 20% en total de las unidades urónicas, tiene un peso molecular promedio entre aproximadamente 2.000 a aproximadamente 45.000, un grado de sulfatación de por lo menos 4, dichos derivados son básicamente inactivos bajo los parámetros de coagulación. Similarmente a lo establecido anteriormente, dichos nuevos derivados son, en su totalidad, denotados con el término general "epiK5-N,-O-sobresulfato-derivados" independientemente de su peso molecular.

En particular, el peso molecular promedio está entre aproximadamente 2.000 a aproximadamente 45.000 ya que dichos derivados se originan de un epiK5-N-sulfato obtenido por N-desacetilación y N-sulfatación de K5 por fermentación o por la despolimerización nitrosa de este último. Por medio de controlar dicha despolimerización nitrosa es posible obtener derivados de bajo peso molecular en virtualmente todos los intervalos anteriormente dichos. Sin embargo, para el uso de los derivados de la presente invención como productos

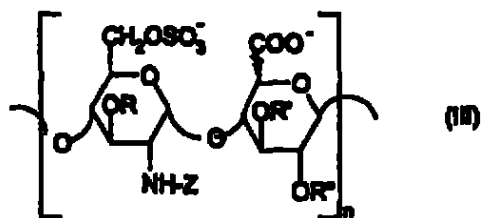
farmacéuticos o cosméticos es ventajoso preparar derivados de bajo peso molecular, con un peso molecular promedio entre aproximadamente 2.000 a aproximadamente 16.000, ventajosamente desde aproximadamente 3.500 a aproximadamente 13.000 con una distribución de peso molecular entre aproximadamente 1.000 y aproximadamente 15.000, preferiblemente entre aproximadamente 4.500 a aproximadamente 9.000, con una distribución de peso molecular desde aproximadamente 2.000 a aproximadamente 10.000 de o derivados de alto peso molecular que se originan del K5 no fraccionado, con un peso molecular promedio entre aproximadamente 20.000 a aproximadamente 45.000, con una distribución de peso molecular entre aproximadamente 2.000 a aproximadamente 70.000.

En los epiK5-N,O-sobresulfato-derivados de la presente invención el grado de sulfatación es muy alto, preferiblemente entre 4 a 4,6, el nitrógeno de la glucosalina es virtualmente 100% sulfatada. Aparte, los epiK5-N,O-sobresulfato-derivados son 100% 6-O-sulfatados y 50-80% 3-O-sulfatados en sus unidades glicosamina, 5 a 10% 3-O-monosulfatados en unidades glucurónicas, 10 a 15% O-monosulfatadas en unidades idurónicas y 2,3-di-O-sulfatadas en las unidades urónicas restantes,

considerando que el grado de sulfatación es de por lo menos 4.

Ventajosamente los epiK5-N,O sobresulfato-derivados de acuerdo con la presente invención son obtenidos por epik5-amina-O-sobresulfato-derivados a su vez preparados de epik5-N-sulfato-derivados consistentes de una mezcla de cadenas en la cuales por lo menos 90% de dichas cadenas tienen la anteriormente mencionada fórmula I, en la cual las unidades urónicas son el 20 al 60% consistentes de ácido idurónico, n es un entre 2 a 100, ventajosamente entre 3 a 100 y el catión correspondiente es química o farmacéuticamente aceptable.

En tal caso, lo nuevos epiK5-N,O-sobresulfato-derivados consistentes de las mezclas de cadenas en las cuales por lo menos 90% de dichas cadenas tienen la fórmula III



En la cual las unidades urónicas son 20 a 60% consistentes de ácido idurónico, n es un entero entre 2 a 100, preferiblemente entre 3 a 100, R , R' y R'' son hidrógeno o SO_3^- , Z es SO_3^- el grado de sulfatación es de por lo menos 4, preferiblemente entre 4 a 4,6 y el catión correspondiente es química o farmacéuticamente aceptable.

Dichos cationes son ventajosamente aquellos de metales alcalinos, metales de tierra alcalina, amonio, tetraalquilamonio con uno a cuatro carbonos, aluminio y zinc y entre estos preferiblemente las sales de sodio, calcio y tetrabutylamonio.

Entre los anteriormente dichos nuevos epiK5-amina-N,O-sobresulfato-derivados, aquellos consistentes de mezclas de cadenas en la cuales por lo menos 90% de dichas cadenas tiene la anteriormente dicha fórmula 3 en la cual R es SO_3^- en 50% a 80%, preferiblemente en aproximadamente 65% de dichas cadenas y el grado de sulfatación es de por lo menos 4, ventajosamente están entre 4 a 4,6, preferiblemente de 4 a 4,3.

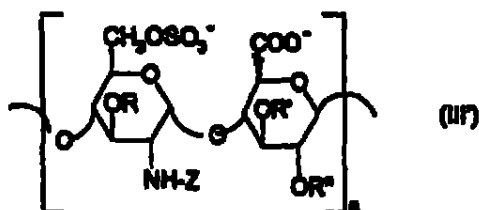
Los epiK5-N,O-sobresulfato-derivados ventajosos con un muy alto grado de sulfatación son consistentes de una

mezcla de cadena en las cuales por lo menos 90% de dichas cadenas tienen la fórmula III, en la cual z es SO_3^- las unidades urónicas son 40 a 60% consistentes de ácido idurónico, n es un entero entre 2 a 100, preferiblemente de 3 a 100, con un peso molecular promedio entre aproximadamente 2.000 a aproximadamente 45.000, ventajosamente desde aproximadamente 4.500 a aproximadamente 45.000, R es por lo menos 40%, preferiblemente 50 a 80% SO_3^- . R' y R'' son ambos SO_3^- o uno es hidrógeno y el otro es 5 a 10% SO_3^- en ácido monosulfatoglucurónico y 10 a 15% SO_3^- en ácido monosulfato idurónico, al grado de sulfatación es de por lo menos 4, desde 4 a 4,6 y el catión correspondiente es química o farmacéuticamente aceptable.

Los-epiK5-amina-O-sobresulfatados-derivados N-sustituídos preferidos son los LMW-epiK5-amina-O-sobresulfatados consistentes de una mezcla de cadenas en las cuales por lo menos 90% de dichas cadenas tienen la fórmula III en la cual las unidades urónicas son 40 a 60% comprendidas, preferiblemente 50 a 55%, de ácido idurónico, R es por lo menos 40%, ventajosamente 50 a 80%, preferiblemente aproximadamente 65% SO_3^- , R' y R'' son ambos SO_3^- o uno es hidrógeno y el otro es 5 a 10% SO_3^- en ácido glucorónico y

10 a 15% SO_3^- en ácido idurónico, Z es 100% SO_3^- o acilo con 2 a 4 carbonos, n es un entero entre 2 a 20, preferiblemente 3 a 15, con un peso molecular promedio entre aproximadamente 4.000 a aproximadamente 8.500 y el correspondiente catión es química o farmacéuticamente aceptable.

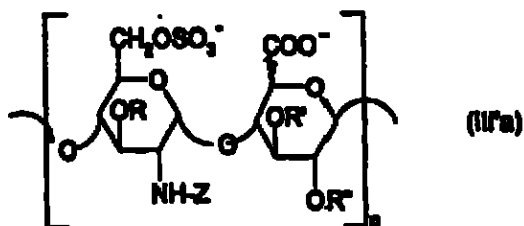
En la práctica, dichos epik5-N,O-sulfalto-derivados preferidos con un muy alto grado de sulfatación consisten de una mezcla de cadenas en las cuales por lo menos 90% de dichas cadenas tienen la fórmula III'



En la cual las unidades urónicas son 20 a 60% consistentes de ácido idurónico, q es un entero entre 2 a 20, ventajosamente de 3 a 15, R, R' y R'' representa hidrógeno o un grupo SO_3^- Z es SO_3^- , para un grado de sulfatación de por lo menos 4, preferiblemente entre 4 a 4,6 y el catión correspondiente es un ión química o farmacéuticamente aceptable.

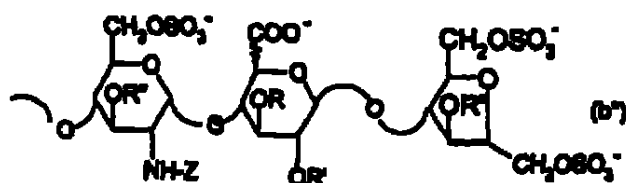
Particularmente interesante son las mezclas de cadenas de fórmula III' en las cuales las unidades urónicas son 40 a 60% comprendidas, preferiblemente 50 a 55%, ácido idurónico, R es por lo menos 40%, ventajosamente 50 a 80%, preferiblemente aproximadamente 65% SO_3^- , R' y R'' son ambos SO_3^- o uno es hidrógeno y el otro es 5 a 10 % SO_3^- en ácido glucurónico y 10 a 15 % SO_3^- en ácido idurónico, n es un entero de 2 a 20, ventajosamente de 3 a 15, con un peso molecular de aproximadamente 2.000 a aproximadamente 16.000, ventajosamente desde aproximadamente 3.500 a aproximadamente 13.000, preferiblemente desde aproximadamente 4.500 a aproximadamente 9.000 y el catión correspondiente es química y farmacéuticamente aceptable.

Entre estos LMW-EPIK5-N,O-sobresulfatos, son ventajosos aquellos consistentes de una mezcla de cadena en la cual las especies preponderantes tienen la fórmula III'a



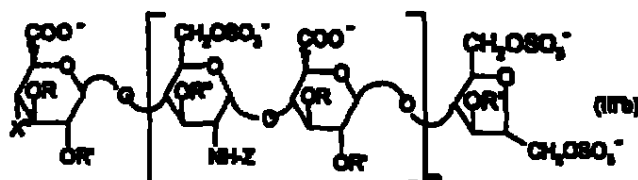
En la cual las unidades urónicas son 20 a 60% consistentes de ácido idurónico, p es un entero de 4 a 8, Z es SO_3^- , R , R' y R'' son hidrógeno o SO_3^- , para un grado de sulfatación de por lo menos 4, preferiblemente 4 a 4,6 y el catión correspondiente es química o farmacéuticamente aceptable. El origen de los nuevos LMW-EPIK5-N,O-sobresulfatos obtenidos de LMW-EPIK5-sulfatos obtenidos por despolimerización nitrosa y reducción subsecuente con, por ejemplo, borohidruro de sodio involucra, en el extremo reductor de la mayoría de las cadenas en dicha mezcla de cadenas, la presencia de una unidad 2,5-anhidromanitol sulfatada de estructura (a') como se mostró anteriormente, en la cual R'' representa hidrógeno o SO_3^- .

Así, el extremo reductor de la mayoría de las cadenas en dicha mezcla de cadena es representada por la estructura (b'')



En la cual Z representa SO_3^- y la unidad urónica puede ser glucurónica o idurónica.

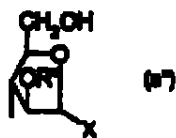
Entre los nuevos LMW-epiK5-N,O-Sobresulfatos anteriormente mencionados, son preferidos aquellos consistentes de mezclas en las cuales las especies preponderantes es un compuesto de fórmula III'b



En la cual R, R' y R'' son hidrógeno o SO_3^- , Z es SO_3^- , X'' es OH o OSO_3^- , m es un 4, 5 o 6, para un grado de sulfatación de por lo menos 4 a 4,6, las unidades urónicas están presentes alternadamente, iniciando con una unidad glucurónica o idurónica, y el catión correspondiente es un ión química o farmacéuticamente aceptable. Dichos cationes son ventajosamente aquellos de metales alcalinos, metales de tierra alcalina, amonio, tetraalquilamonio con uno a cuatro carbonos, aluminio y zinc y, entre estos, preferiblemente los iones de sodio, calcio y tetrabutilamonio.

Si un epik5 es usado como partida epik5 derivado del proceso de la presente invención, es decir un polisacárido K5, previamente N-desacetilado, N-sulfatado normalmente 100%, y 20 a 60% C5-epimerizado y no es depolimerizado, luego de la terminación de la etapa (c) un epik5-N,O-sobresulfato es aislado el cual puede ser sometido a despolimerización nitrosa y posible, reducción subsecuente con , por ejemplo, boro hidruro de sodio para obtener el correspondiente LMW-EPIK5-N,O-sobresulfato que tienen el mismo grado de sulfatación. En particular, los LMW-EPIK5-N,O-Sobresulfatos son obtenidos consistentes de una mezcla de cadenas en las cuales por lo menos 90% de dichas cadenas tienen la fórmula III' o III'a, en la cual las unidades urónicas son 20 a 60% consistentes de ácido idurónico, q, R, R' R'' y Z tiene , los significados definidos anteriormente, para un grado de sulfatación de por lo menos 4, preferiblemente entre 4 a 4,6 y el catión correspondiente es un ión química o farmacéuticamente aceptable. En tal caso, el origen de estos LMW-EPIK5-N,O-sobresulfatos a partir de un reacción de despolimerización y posible, reducción subsecuente con, por ejemplo, borohidruro de sodio involucra, en el extremo reductor de la mayoría de las cadenas en dicha

mezcla de cadenas, la presencia de un 2,5-anhidromano de estructura (a'')



En la cual X es formilo o hidroximetilo y R'' representa hidrógeno o SO₃⁻.

Los nuevos epik5-N,O-sobresulfato-derivados, especialmente en su forma de sal, son productos altamente aniónicos capaces de capturar los radicales libres y son utilizables en la industria cosmética como ayudantes contra la perdida de cabello o para preparar cremas "anti envejecimiento" y , en la industria farmacéutica, como productos para el tratamiento de la dermatitis. Aparte los epik5-N,O-sobresulfato-derivados de la presente invención, en particular los LMW-EPIK5-N,O-sobresulfatos poseen actividad anti angiogenética y antiviral y por lo tanto constituyen ingredientes activos para la preparación de medicinas.

Así, de acuerdo con uno de sus aspectos adicionales, la presente invención suministra composiciones farmacéuticas incluyendo, como uno de sus ingredientes activos, una cantidad farmacológicamente activa de un epik5-N,O-sobresulfato-derivado como se mostró anteriormente o de una de sus sales farmacéuticamente aceptables, en mezcla con un excipiente farmacéutico.

En las composiciones farmacéuticas de la presente invención para administración oral, subcutánea, intravenosa, transdérmica o tópica, los ingredientes activos son administrados preferiblemente en la forma de unidades de dosificación, en mezcla con excipientes o vehículos farmacéuticos clásicos. La posología puede variar ampliamente dependiendo de la edad, peso, y la condición de salud del paciente. Esta posología incluye la administración de una dosis de 1 a 1.000 mg, ventajosamente desde 10 a 750 mg preferiblemente entre 250 a 500 mg de una a tres veces al día por administración intravenosa, subcutánea, oral, transdérmica o tópica. Las composiciones farmacéuticas de la presente invención son formuladas con los excipientes clásicos adecuados para las diferentes maneras de administración. Particularmente ventajosos son las

fórmulaciones en la forma de cremas, ungüentos, linimentos, geles, espumas, bálsamos, supositorios vaginales, supositorios, soluciones o suspensiones adecuadas para administración local.

Ventajosamente, las composiciones de la presente invención incluyen como uno de sus ingredientes activos, un epik5-N,O-sobresulfato-derivado obtenible partiendo de un epik5-derivado de acuerdo con las etapas (a), (b) y (c) del proceso descrito anteriormente, o partiendo con un epik5 no despolimerizado, de acuerdo con las etapas (a), (b) y (c) del proceso descrito anteriormente, con posibles despolimerización nitrosa subsecuente después de la etapa (c) o uno de sus sales farmacéuticamente aceptables, en mezcla con un excipiente farmacéutico. Ventajosamente, dicho epik5-N,O-sobresulfato-derivado consiste de una mezcla de cadenas en las cuales por lo menos 901% de dichas cadenas tienen la fórmula 3 o III' o en la cual las especies preponderantes de un compuesto es un compuesto de fórmula III' a o III'b . el ingrediente activo preferido es un LMW-EPIK5-N,O sobresulfato que tiene un grado de sulfatación de por lo menos 4, preferiblemente entre 4 a 4,6 , ventajosamente tiene un peso molecular promedio entre aproximadamente 3.500 a

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aproximadamente 11.000, más ventajosamente entre aproximadamente 3.500 a aproximadamente 5,200 y es básicamente libre de grupos N-acetil.

Finalmente, de acuerdo con otro de sus aspectos, la presente invención suministra una composición cosmética que incluye un cantidad efectiva de un epik5-N,O-sobresulfato-derivado o unos de sus sales farmacéuticamente aceptables, en mezcla con un excipiente cosmético.

Una sal seleccionada del grupo que consiste de sales de sodio, potasio, calcio, magnesio, aluminio y zinc de los epik5-N,O-sobresulfato-derivados, en particular aquellos consistentes de mezclas de cadenas en las cuales por lo menos 90% de dichas cadenas tiene la fórmula III o III' o en las cuales las especies preponderantes tienen la fórmula III'a o III'b, constituyen un ingrediente activo efectivo en las composiciones farmacéuticas o cosméticas de la presente invención.

Los siguientes ejemplos ilustran la invención sin embargo limitarla.

PREPARACIÓN I

Preparación del polisacárido K5 a partir de la *Escherichia coli*

En la primera fermentación se lleva a cabo en un frasco Erlenmeyer usando el siguiente medio:

Comida de soya libre de grasa	2	g/l
K ₂ HPO ₄	9.7	g/l
KH ₂ PO ₄	2	g/l
MgCl ₂	0,11	g/l
Citrato de sodio	0,5	g/l
Sulfato de amonio	1	g/l
Glucosa	2	g/l
Agua fresca	1000	g/l
pH= 7,3		

El medio es esterilizado a 120° C durante 20 minutos. La glucosa es preparada separadamente en forma de solución la cual es esterilizada a 120°C durante 30 minutos y agregada estérilmente al medio. El frasco Erlenmeyer es inoculado con una suspensión de células *E.coli* Bi 8337/41 (O10: K5:H4) que se originan de una cepa mantenida en

agar de soya triptico, e incubada a 37°C durante 24 horas bajo agitación controlada (160 rpm, carrera de 6 cm). El crecimiento bacterial es medido por medio de contar las células usando un microscopio. En una operación subsecuente, un fermentador de 14 l Chemap-Braun quen contienen el mismo medio anterior es inoculado con 0,1% con el cultivo del frasco Erlenmeyer según lo anterior y la fermentación es llevada a cabo por la reacción de un vvm, (vvm= volumen de aire por volumen de líquido por minuto) una agitación de 400 rpm y temperatura a 37°C durante 18 horas. Durante la fermentación se ha medido el pH, el oxígeno, el residuo de glucosa, el polisacárido K5 producido y el crecimiento bacterial. Al final de la fermentación la temperatura es llevada a 80°C durante 10 minutos. Las células son separadas del medio por medio de centrifugación a 10.000 rpm y el sobrenadante es ultrafiltrado usando un modulo SS 316 (MTS) ajustado con una membrana PEF con corte nominal de 800 a 10.000 D para reducir el volumen de 1/5. El polisacárido K5 es entonces precipitado por la adición de cuatro volúmenes de acetona a 4°C y se deja asentar durante la noche a 4°C. Finalmente es recuperado por centrifugación a 10.000 rpm durante 20 minutos, o filtración. La desproteínización del sólido obtenido es llevada a cabo usando una proteasa

tipo 2 de *Aspergillus orizae* en un amortiguador de NaCl al 0,1 M y EDTA al 0,15M a un pH 8 que contiene SDS (0,5% de sulfato dodecil de sodio) (10mg/l o de filtrado) a 37°C durante 90 minutos. La solución obtenida es ultrafiltrada sobre el modelo SS 316 con membrana de un corte nominal de 10.000 D con dos extracciones con NaCl al 1M y lavado con agua hasta la desaparición de la absorbancia en el ultra filtrado. El polisacárido K5 es entonces precipitado con acetona y una producción de 850 mg por litro de fermentadores obtenido. La pureza del polisacárido obtenido es medida por medio de la determinación de los ácidos urónicos (método carbazol), portón y carbón 13 NMR, UV y contenido de proteína. La pureza es más del 80%.

El polisacárido obtenido está compuesto de dos partes de diferente peso molecular, respectivamente 30.000 y 5.000 D según emerge de la determinación por el HPLC usando una columna farmacia 75 HR y una parte única con un tiempo de retención de aproximadamente 9 minutos usando dos columnas seriadas de Bio-sil SEC250 (Bio Rad) y Na_2SO_4 como fase móvil a temperatura ambiente y un flujo de 0,5 ml/minuto. La medida es llevada a cabo contra una curva

estándar obtenida con partes de heparina de peso molecular conocido.

El espectro ^1H - RMN del K5 purificado así obtenido muestra diferentes señales atribuibles a metilos de sustancias lipofilicas.

PREPARACIÓN II

Purificación de K5

En 100 ml de una solución acuosa saturada de cloruro de sodio y termo establecida a 4° se disuelve 1 g de K5 obtenido al final de la preparación 1 y a la solución así obtenida se agregan tres volúmenes de isopropanol frío. La concentración salina de la solución se lleva a 3M por la adición de una cantidad calculada de una solución de cloruro de sodio saturado y la solución obtenida es dejada en un ambiente frío (aproximadamente 4°) durante la noche. El precipitado que se forma es separado por centrifugación a 10.000 RPM durante 20 minutos y la pureza del producto es verificada por diálisis durante la noche y examen subsecuente del espectro ^1H -RMN, del cual las señales en la región por debajo de 1,5 ppm deben

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estar ausentes. Opcionalmente, la operación de disolución en agua saturada con NaCl y precipitación con isopropanol es repetida. El precipitado es disuelto en agua y ultrafiltrado en una membrana miniplaca milipor de 10.000 de corte hasta la desaparición de las sales. Así un K5 que tiene una pureza de por lo menos 99% es obtenido de aquellos espectros ^1H -RMN sin trazas de impurezas glipofílicas resultantes en la región por debajo de 1,5 ppm.

PREPARACIÓN III

Preparación de un K5-N-sulfato

(i) N-desacetilación

Diez gramos de polisacárido K5 puro preparado según se describió en la preparación 2 son disueltos en 1.000 ml de hidróxido de sodio al 2 y la solución así preparada es dejada a 60°C durante 24 horas. La solución es llevada a temperatura ambiente luego a un pH neutro (pH7) con ácido clorhídrico al 6N.

(ii) N-sulfatación

A una solución que contiene el K5 desacetilado, mantenido a 40°C, se agregan 16 g de carbonato de sodio y después en unas cuatro horas, 16 g de piridina.SO₃⁻. Al final de la reacción, después de 24 horas, la solución es llevada a temperatura ambiente, luego el pH entre 6,5 a 7 con una solución al 5% de ácido clorhídrico. El producto es purificado a partir de sales por diafiltración usando una membrana enrollada helicoidalmente de 1000 D (cartucho miliporo preestablecido). El proceso es terminado cuando la conductividad del permeato es menos de 1000µS, preferiblemente menos de 100 µS. la intradiálisis es reducida hasta una concentración del 10% del polisacárido obtenido usando lo mismo en el sistema de diálisis de concentración. La solución concentrada es secada por liofilización. Luego del análisis del espectro ¹³C-RMN residuos -N-acetilo o NH₂ no aparecen.

PREPARACIÓN IV

LMW-K5-N-sulfato

El producto obtenido como se describió en el ejemplo 1 etapas (i) y (ii) de la WO 02/068477 es despolimerizado

por el método de degradación con ácido nitroso y reducción subsecuente del aldehído que se forma. Uno continua disolviendo 1 g de K5-N-sulfato en 200 ml de agua destilada y agregándolo con 480 mg de nitrito de sodio disuelto en 240 ml de agua destilada. La solución es entonces llevada a 4°C y el pH a 2 con HCL al 0,1 N y mantenida durante 30 minutos. Al final de la reacción la dilución es llevada a un pH 7 NaOH al 0,1 M y entonces a temperatura ambiente. La solución es entonces agregada con 450 mg de NaBH₄ y dejada reaccionar durante 4 horas. El exceso de NaBH₄ es eliminado con HCL llevando el pH a 5 o 6. el producto, neutralizado con NaOH al 0,1 M, es recuperado por precipitación con tres volúmenes de acetona a 4°C filtrado con un embudo de filtración y secado a 40°C en un horno al vacío. 9000 mg de LMW-K5-N-sulfato son obtenidos con un peso molecular promedio de aproximadamente 2.000, consistente de una mezcla de cadenas en las cuales las especies preponderantes son un compuesto de fórmula I'b en la cual m es 4 y las unidades urónicas son aquellas de ácido glucurónico.

EJEMPLO 1

LMW-epk5-N-sulfato. Secuencia (i) (ii)

(i) Epimerización al epik5-N-sulfato

Diez gramos de K5-N-sulfato obtenido como se describió en el ejemplo 1, etapas (i) y (ii), de la WO 02/068477, de aquellos espectro ^1H -RMN, señales concernientes a grupos acetilo o NH_2 no aparecen, son disueltos en 600 ml de un amortiguador HEPES de 25 mM a pH7, que contiene CaCl_2 a una concentración de 50 ml y la solución así obtenida se hace recircular por una columna de 50 ml llenada con resina cefarosa 4B que contiene 5 g de C5- epimerasa recombinante (WO 96/14425) inmovilizada como se describió en el ejemplo 1 de la WO 01/72848. La reacción se lleva a cabo a 30° a un pH7 con un flujo de 200 ml/h durante 24 horas. El producto obtenido es purificado por ultrafiltración y precipitación con metanol. Así un epik5-N-sulfato es obtenido cuyo contenido de ácido idurónico es 54%.

(ii) Despolimerización del epik5-N-sulfato.

A una solución de 1 g del producto así obtenido, en 25 ml de agua destilada, se agregan 230 mg de nitrito de sodio disueltos en 115 ml de agua destilada. La solución es entonces llevada a 4°C y el pH a 2 con HCl al 0,1 N y

mantenida allí durante 30 minutos. Al final de la reacción la solución es llevada a temperatura ambiente y el pH a 7 con NaOH al 0,1 M. se le agrega a la solución 450 mg de NaBH₄ y se deja reaccionar durante 4 horas. El producto recuperado por precipitación con tres volúmenes de acetona a 4°C, filtración con un embudo de filtrado y secado a 40°C en un horno al vacío. 900 mg de LMW-epik5-N-sulfato son obtenidos con un contenido de ácido idurónico del 54% y una distribución de peso molecular entre 1.000 a 4.000, medido con el método HPLC.

EJEMPLO 2

LMW-epik5-N-sulfato. Secuencia (ii) (i)

(ii) Despolimerización del K5-N-sulfato

2 g de K5-N-sulfato obtenido según se describió en el ejemplo 1 etapas (i) y (ii) de la WO 02/068477, es despolimerizado según se describió en la preparación 1, usando 100 mg de nitrito de sodio y 300 mg de borohidruro de sodio. 1,8 g de LMW-K5-N-sulfato son obtenidos con un peso molecular promedio de 5.000.

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(i) Epimerización del LMW-K5-N-sulfato

1 g de LMW-K5-N-sulfato obtenido en la etapa (ii) anterior es tratado como se describió en la etapa (i) del ejemplo 1. un producto epimerizado es obtenido con una proporción ácido idurónico/ ácido glucurónico de 44/46 contrab una proporción de 0/100 del producto de partida, con una distribución de peso molecular entre 2.000 a 10.000 y con un peso molecular promedio de 5.000 D. el producto, calculado midiendo el contenido de ácidos urónicos contra un estándar con el método carbazol (Bitter y Muir, Anal. Biochem. 1971, 39,88-92) es del 90%.

EJEMPLO 3.

LMW-epik5-N-sulfato. Secuencia (i) (ii)

Epimerización de K5-N-sulfato.

Una cantidad de 2 g de K5 N-sulfato, obtenida según se describió en el ejemplo 1, etapas (i) y (ii), de la WO 02/068477, es disuelto en 120 ml de un amortiguador HEPES 25 mM, pH7 que contiene 50mM de CaCl₂. La solución

obtenida se hace recircular por una columna de 50 ml llenada con la resina que contiene la enzima inmovilizada obtenida como se describió en la WO 96/14425. Esta operación es llevada a cabo a 30°C con un flujo de 200 ml/h durante 24 horas. El producto obtenido es purificado por ultrafiltración sobre una membrana de 1.000 D y se pasa sobre una columna de intercambio iónico IR 120 H+, neutralizando el eluato con NaOH al 1N. la muestra es recuperada por precipitación con etanol o acetona. Un producto epimerizado es obtenido con una proporción de ácido idurónico/ ácido glucurónico de 55 /45 contra la proporción de 0/100 del producto de partida. El porcentaje de epimerización fue calculado con 1H-RMN de acuerdo con el método descrito en la WO 96/14425. el producto, calculado por la medición del contenido de ácidos urónicos contra un estándar por el método carbazol (Bitter y Muir Anal. Biochem. .39, 88-92-1971) es 90%.

(ii) Despolimerización del epi-K5-N-sulfato

Un gramo del producto obtenido en la etapa (a) es despolimerizado por el método de degradación con ácido nitroso y reducción subsecuente del aldehído que se forma. En particular uno continua disolviendo el producto

en 25 ml de agua destilada y agregándole 230 mg de de nitrito de sodio disuelto en 115 ml de agua destilada. La solución es entonces llevada a 4°C y el pH a 2 con HCL al 0,1 N y mantenida durante 30 minutos. Al final de la reacción la solución es llevada a temperatura ambiente y el pH a 7 con NaOH al 0,1 M. la solución se le agrega 450 mg de NaBH₄ y se deja reaccionar durante 4 horas. El producto es recuperado por precipitación con tres volúmenes de acetona a 4°C, filtración con un embudo de filtrado y secado a 40°C en un horno al vacío. 900 mg de LMW-epik5-N-sulfato son obtenidos con una distribución de peso molecular medida con el método HPLC que varia entre 1.000 a 4.000 y con un contenido de unidad glucurónica de 45% y un contenido de unidad hidrónica de 55%.

EJEMPLO 4

EPIK5-N,O-sobresulfato

(a) sal de tetrabutilamonio del epik5-N-sulfato

Una solución de 40 ml de agua de 400 mg de epik5-N-sulfato, según se obtuvo al final de la etapa (i) del ejemplo 1, es termoestablecida a 4°C y luego pasada sobre

una resina de intercambio iónico IR 120⁺ preacondicionada con agua a 4°C. El eluato obtenido, consistente de 100 ml de una solución a pH 1,94, es neutralizado con una solución al 15% de hidróxido de tetrabutilamonio y dejada a temperatura ambiente durante una hora, manteniendo el pH en 7 por medio de agregarle 15% de hidróxido de tetrabutilamonio y finalmente es liofilizada. Así 850 mg de sal de tetrabutilamonio de epik5-N-sulfato son obtenidos.

(b) Epi-k5-amina-O-sobresulfato

Una solución que contiene 805 mg de la sal ha sido obtenida en 30 ml de dimetilformamida se calienta a 55°C y se trata con 30 ml de dimetilformamida que contiene 2,26 g del aducto de piridina. SO₃. La reacción a 55° es continuada durante la noche y entonces se agregan 60 ml de agua a la mezcla. Después de neutralizar con NaOH al ¹N, el producto es precipitado con tres volúmenes de acetona saturada con NaCl y se establece a 4°C durante la noche. El precipitado es recuperado por filtración sobre Duch G4 y luego ultrafiltrado con un sistema milipore mil D TFF y secado a presión reducida. 550 mg de epi-K5-amina-O-sobresulfatado don obtenidos los cuales tienen un

contenido de ácido idurónico del 54%, glucosalina-6-O-sulfato de 100% de glucosalina 3-O-sulfato de 60%, de ácido glucurónico monosulfato de 10%, de monosulfato de ácido idurónico de 15%, el resto de unidades urónicas son di sulfatadas, con un grado de sulfatación de 3,55 medido con el método conducto métrico de acuerdo con Casu et al. 1975.

(C) EpiK5-amina-O-sobresulfatado-N- sulfato

A una solución de 250 mg de la epi-k5-amina-o-Sobresulfatada obtenida en la etapa (b) en 15 ml de agua se agregaron 400mg de carbonato de sodio, luego a la mezcla así obtenida se agrego 400 mg de piridina. SO_3 como aducto en una forma sólida un poco en un tiempo en 4 horas. La mezcla de reacción es mantenida a 55°C durante la noche, y luego se detiene llevándola a un pH de 7 con HCl al 0,1 M. después de ultra filtración sobre una membrana 1.000 D se agregan tres volúmenes de acetona saturada con cloruro de sodio y el precipitado es recuperado por centrifugación a 5.000 RPM durante 5 minutos. Así 244 mg de epiK5-N,O -sobresulfato Son obtenidos cuyo grado de sulfatación, medido con el método conducto métrico de acuerdo con Casu et al. 1975, es

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4,25. por el análisis del espectro ^1H -RMN resulta que el epiK5-N,O-sobresulfato así obtenido tiene un contenido de ácido idurónico del 54%, 6-O-sulfato del 100%, N-sulfato del 100%, glucosalina 3-O-sulfato de 60%, ha sido glucurónico monosulfato del 10%, monosulfato de ácido idurónico del 15% y el resto de las unidades urónicas siendo di sulfatadas. Del espectro ^1H -RMN se calcula entonces un grado de sulfatación de 4,35 el cual, considerando los márgenes de error de los métodos, corresponde al grado de sulfatación del epiK5-amina-O-sobresulfatado obtenido luego de la terminación de la etapa (b), 100% N-sulfatada. Por lo tanto se asume que más allá de un cierto porcentaje de grupo sulfato, la naturaleza aniónica fuerte del producto puede llevar a una subestimación del grado de sulfatación determinado con el método conductométrico.

REIVINDICACIONES

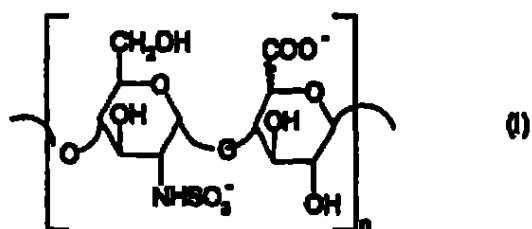
1. Un proceso para la preparación de un epiK5-N,O-sobre sulfato-derivado, caracterizado en que
 - (a) un epiK5-N-sulfato-derivado que tiene un peso molecular promedio de entre aproximadamente 1.500 a aproximadamente 25.000, en forma acidita, es tratado con base orgánica terciaria o cuaternaria, dejando la mezcla de reacción descansar durante un periodo de tiempo de 30 a 60 minutos a un pH de aproximadamente 7 y su sal es aislada con dicha base orgánica;
 - (b) dicha sal de base orgánica de dicho epiK5-N-sulfato-derivado es tratada con un reactivo de O-sulfatación en las condiciones de O-sobresulfatación;
 - (c) una sal de la base orgánica terciaria o cuaternaria del epiK5-amina-O-sobresulfato-derivado así obtenido es tratada con un reactivo de N-sulfatación y el epiK5-N,O-sobresulfato-derivado así obtenido es aislado.

2. El proceso de acuerdo con la reivindicación 1 caracterizado en que dicho epiK5-N,O-sobresulfato- derivado es aislado en forma de sal de sodio y opcionalmente transformado en otra sal química o farmacéuticamente aceptable.
3. El proceso de acuerdo con una cualquiera de las reivindicaciones 1 y 2, caracterizado en que, en la etapa (a) el hidróxido de tetrabutilamonio es usado como una base orgánica.
4. El proceso de acuerdo con una cualquiera de las reivindicaciones 1 a 3, caracterizado en que, en la etapa (b) la O-sobresulfatación es llevada a cabo en dimetilformamida usando 2 a 4 moles del reactivo de O-sulfatación por OH disponible por disacárido a una temperatura entre 40 ma 60°C durante 15 a 20 horas.
5. . El proceso de acuerdo con una cualquiera de las reivindicaciones 1 a 4, caracterizado en que, un epiK5-N-sulfato-derivado es usado como material de partida que tiene un peso molecular promedio

entre aproximadamente 1.000 a aproximadamente 25.000

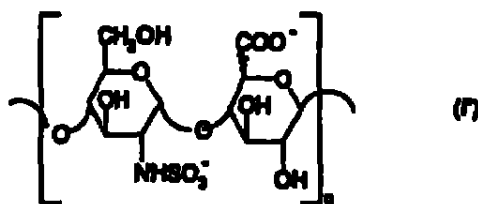
6. El proceso de acuerdo con una cualquiera de las reivindicación 5, caracterizado en que, dicho epiK5-N-sulfato-derivado de partida es 40 a 60% C5- epimerizado
7. El proceso de acuerdo con una cualquiera de las reivindicaciones 1 a 6, caracterizado en que, dicho epiK5-N-sulfato-derivado de partida tiene un peso molecular promedio de aproximadamente 1.500 a aproximadamente 25.000.
8. El proceso de acuerdo con la reivindicación 7, caracterizado en que, dicho epiK5-N-sulfato-derivado de partida tiene un peso molecular promedio entre 10.000 a 25.000.
9. El proceso de acuerdo con una cualquiera de las reivindicaciones 1 a 6, caracterizado en que, dicho material de partida tiene un peso molecular promedio de aproximadamente 1.000 a aproximadamente 12.000

10. El proceso de acuerdo con la reivindicación 9, caracterizado en que, dicho material de partida tiene un peso molecular promedio desde aproximadamente 1.500 a aproximadamente 8.000.
11. El proceso de acuerdo con la reivindicación 5, caracterizado en que, un epiK5-N-sulafato-derivado es usado como material de partida consistente de una mezcla de cadenas en las cuales por lo menos 90% de dichas cadenas tiene la fórmula I



en la cual las unidades urónicas son 20 a 60% consistentes de ácido idurónico, n es un entero de 2 a 100 y el catión correspondiente es química o farmacéuticamente aceptable.

12. El proceso de acuerdo con la reivindicación 11, caracterizado en que, dicho material de partida consiste de una mezcla de cadenas en las cuales por lo menos 90% de dichas cadenas tiene la fórmula I en la cual las unidades urónicas son 40 al 60% consistente de ácido idurónico.
13. El proceso de acuerdo con una cualquiera de las reivindicaciones 11 y 12 caracterizado en que, en la fórmula I, n representa un entero de 3 a 100.
14. El proceso de acuerdo con la reivindicación 5, caracterizado en que, dicho material de partida consiste de una mezcla de cadenas en las cuales por lo menos 90% de dichas cadenas tiene la fórmula I'

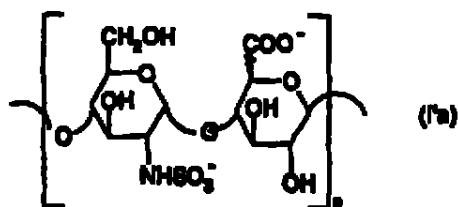


en la cual las unidades urónicas son 20 a 60% consistentes de ácido idurónico, q es un estero

entre 2 a 20, y el catión correspondiente es química o farmacéuticamente aceptable.

15. El proceso de acuerdo con la reivindicación 14, caracterizado en que dicho material de partida consiste de una mezcla de cadenas en las cuales por lo menos 90% de dichas cadenas tiene la fórmula I', en la cual n es un estero de 3 a 15.

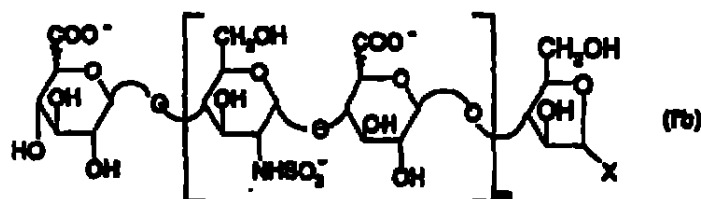
16. El proceso de acuerdo con la reivindicación 5, caracterizado en que dicho material de partida consiste de una mezcla de cadenas en las cuales las especies preponderantes tienen la fórmula I' a



en la cual las unidades urónicas son 60-40% consistentes de ácido glucurónico y 40% a 60% de ácido idurónico, p es un entero entre 4 a 8 y el

cación correspondiente es química o farmacéuticamente aceptable.

17. el proceso de acuerdo con la reivindicación 16, caracterizado en que el peso molecular promedio de dicho material de partida es desde aproximadamente 2.000 a aproximadamente 4.000.
18. El proceso de acuerdo con una cualquiera de las reivindicaciones 16 y 17, caracterizado en que, dicho material de partida consiste de una mezcla de cadenas en las cuales las especies preponderantes tienen la fórmula I'b



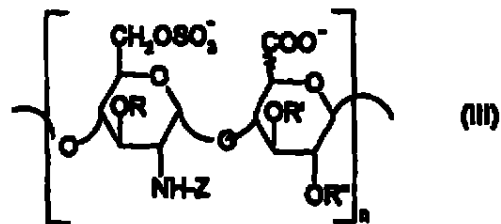
en la cual X es hidroximetilo, m es 4, 5 o 6 y las unidades glucurónicas e idurónicas están presentes alternadamente, empezando con una unidad glucurónica o idurónica.

19. El proceso de acuerdo con una cualquiera de las reivindicaciones 5 a 18, caracterizado en que dicho material de partida viene de la N-descetilación y de la una N-sulfatación de un K5 que es básicamente libre de sustancias lipofilicas.
20. Un epiK5-N, O-sobresulfato-derivado obtenible de acuerdo con el proceso de las reivindicaciones 1 a 19.
21. Un epiK5-N, O-sobresulfato-derivado que tiene un contenido de ácido idurónico entre el 20 al 60%, un peso molecular promedio de entre aproximadamente 2.000 a aproximadamente 45.000 y un grado de sulfatación de por lo menos 4, o una de sus sales química o farmacéuticamente aceptable, dicho derivado es básicamente inactivo sobre parámetros de coagulación.
22. Un epiK5-N,O-sobresulfato-derivado de acuerdo con la reivindicación 21, cuyo peso molecular promedio está entre 15.000 y aproximadamente 45.000.

23. Un epiK5-N,O-sobresulfato-derivado de acuerdo con la reivindicación 21 cuyo peso molecular promedio está entre aproximadamente 4.500 y aproximadamente 8.500.
24. Un epiK5-N,O-sobresulfato-derivado de acuerdo con una cualquiera de las reivindicaciones 21 a 23, caracterizado en que dicho grado de sulfatación está entre 4 a 4,6.
25. Un epiK5-N,O-sobresulfato-derivado de acuerdo con una cualquiera de las reivindicaciones 21 a 24, caracterizado en que es 100% 6-O-sulfatado y 50-80% 3-O-sulfatado en sus unidades de gliucosomina, 5 a 10% O-monosulfatado en las unidades glucurónicas, 10 a 15% 3-O-monosulfatado en las unidades idurónicas y 2,3-di-O-sulfatado en las unidades urónicas restantes.
26. Un epiK5-N,O-sobresulfato-derivado de acuerdo con una cualquiera de la s reivindicaciones 21 a 25, caracterizado en que este consiste de una

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mezcla de cadenas en las cuales por lo menos 90% de dichas cadenas tienen la fórmula III

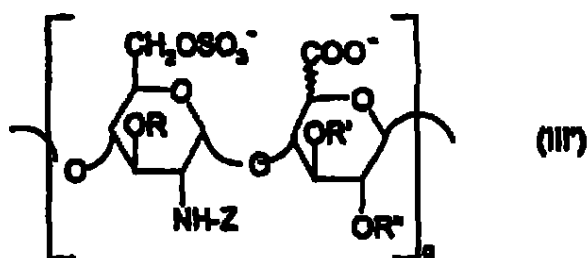


en la cual las unidades urónicas son 20 a 60% consistente de ácido idurónico, R, R' , R'' representan hidrógeno o SO₃⁻, R es SO₃⁻ en por lo menos 40% de dicha mezcla de cadenas, Z es un grupo SO₃⁻, n es un entero de 2 a 100, el grado de sulfatación es de por lo menos 45 y el catión correspondiente es química o farmacéuticamente aceptable.

27. Un epiK5-N,O-sobresulfato-derivado de acuerdo con la reivindicación 26 caracterizado en que este consiste de una mezcla de cadenas en las cuales por lo menos 90% de dichas cadenas tiene la fórmula III, en las cuales las unidades urónicas son 40 a 60% ácido idurónico.

28. Un epiK5-N,O-sobresulfato-derivado de acuerdo con una cualquiera de las reivindicaciones 26 y 27, caracterizado en que este consiste de una mezcla de cadenas en las cuales por lo menos 90% de dichas cadenas tiene la fórmula III, en las cuales n es un entero de 3 a 100.

29. un epiK5-N,O-sobresulfato-derivado de acuerdo con una cualquiera de las reivindicaciones 26 a 28, caracterizado en que es un LMW-epiK5-N, O-sobresulfato consistente de una mezcla de cadenas en las cuales por lo menos 90% de dichas cadenas tienen la fórmula III'



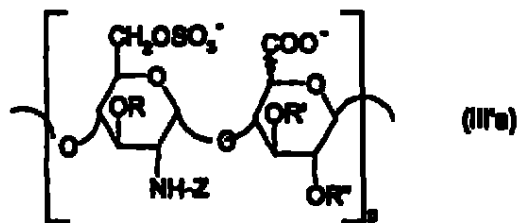
en la cual las unidades urónicas son 20 a 60% consistentes de ácido idurónico, q es un entero entre 2 a 20, R, R' y R'' representan hidrógeno o un grupo SO₃-, Z es SO₃-, para un grado de

sulfatación de por lo menos 4 , entre 4 a 4,6, y el catión correspondiente es un ión química o farmacéuticamente aceptable.

30. Un LMW epiK5-N, O-sobresulfato de acuerdo con la reivindicación 29, caracterizado en que este consiste de una mezcla de cadenas en las cuales por lo menos 90% de dichas cadenas tiene la fórmula III' en la cual q es un entero entre 3 a 15.
31. Un LMW epiK5-N, O-sobresulfato de acuerdo con la reivindicación 30, caracterizado en que este consiste de una mezcla de cadenas en las cuales por lo menos 90% de dichas cadenas tienen la fórmula III' en la cual las unidades urónicas son 40 a 60% consistentes de ácido idurónico.
32. Un LMW epiK5-N, O-sobresulfato de acuerdo con la reivindicación 31 caracterizado en que su contenido de ácido idurónico es 50 a 55%.
33. Un LMW epiK5-N, O-sobresulfato de acuerdo con una cualquiera de las reivindicaciones de la 29 a la

- 32, caracterizado en que este consiste de una mezcla de cadenas en las cuales por lo menos 90% de dichas cadenas tienen la fórmula III' en la cual R es por lo menos 40% SO_3^- , R' y R'' son ambos SO_3^- y uno es hidrógeno y el otro es 5 a 10% SO_3^- en ácido glucorónico y 10 a 15% SO_3^- en ácido idurónico.
34. Un LMW epiK5-N,O-sobresulfato de acuerdo con la reivindicación 33 caracterizado en que tiene un peso molecular promedio entre aproximadamente 2.000 a aproximadamente 16.000.
35. Un LMW epiK5-N,O-sobresulfato de acuerdo con la reivindicación 34 caracterizado en que este tiene un peso molecular de aproximadamente 4.500 a aproximadamente 9.000.
36. Un LMW epiK5-N,O-sobresulfato de acuerdo con una cualquiera de las reivindicaciones de la 33 a la 35, caracterizado en que este consiste de una mezcla de cadena en la cual por lo menos 90% de dichas cadenas tiene la fórmula III' en la cual R es 50 a 80% SO_3^- .

37. Un LMW epiK5-N,O-sobresulfato de acuerdo con una cualquiera de las reivindicaciones de la 32 a la 36, caracterizado en que este consiste de una mezcla de cadenas en las cuales las especies preponderantes tienen la fórmula III'a

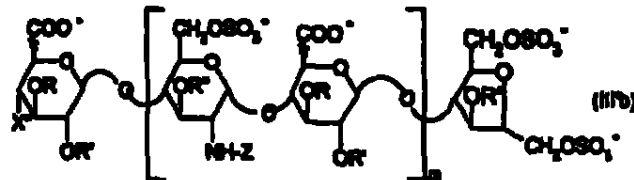


En la cual las unidades urónicas son 20 a 60% consistentes de ácido idurónico, p es un entero de 4 a 8, Z es SO_3^- , R, R' y R'' son hidrógeno o un LMW epiK5-N,O-sobresulfato de acuerdo con, para un grado de sulfatación de 4 a 4,6 y el catión correspondiente es química o farmacéuticamente aceptable.

38. Un LMW epiK5-N,O-sobresulfato de acuerdo con una cualquiera de las reivindicaciones de la 33 a la 37, caracterizado en que este consiste de una

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mezcla de cadenas en las cuales las especies preponderantes tienen la fórmula III'b

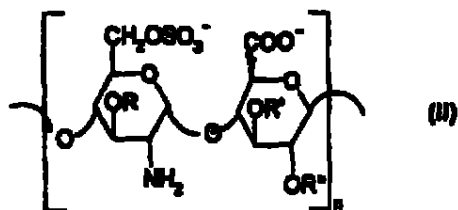


En la cual R, R' y R' 'son hidrógeno o SO₃⁻, Z es SO₃⁻, X'' es OH o OSO₃⁻, m es 4, 5 o 6 para un grado de sulfatación entre 4 a 4,6 , las unidades glucurónicas e idurónicas están presentes alternadamente, empezando por una unidad glucurónica o idurónica, y el catión correspondiente es un ión química o farmacéuticamente aceptable

39. Un epiK5-N, O-sobresulfato-derivado de acuerdo con una cualquiera de la reivindicaciones 20 a 38, caracterizado en que dicha sal química o farmacéuticamente aceptable o catión es la sal o catión de un metal alcalino, metal de tierra alcalina, amonio, tetralquilamonio con (C₁-C₄), aluminio y zinc.

40. Un epiK5-N, O-sobre sulfato-derivado de acuerdo con la reivindicación 39, caracterizado en que dicha sal o catión química o farmacéuticamente aceptable es la sal o catión de sodio, calcio o tetrabutilamonio.
41. Un epiK5-amina-O-sobre sulfato-derivado una de sus sales química o farmacéuticamente aceptables, obtenibles de acuerdo con las etapas (a) y (b) de la reivindicación 1, aislado en forma de sal de sodio y, opcionalmente, transformado en otra sal química o farmacéuticamente aceptable.
42. Un epiK5-amina-O-sobre sulfato derivado cuyo contenido de ácido idurónico es 20 a 60% del total de los ácidos urónicos, que tiene un peso molecular promedio entre aproximadamente 2.000 a aproximadamente 40.000 y un grado de sulfatación de por lo menos 3,4, o una de sus sales química o farmacéuticamente aceptables.
43. Un epiK5-amina-O-sobre sulfato-derivado de acuerdo con la reivindicación 42 caracterizado en

que dicho epiK5-amina-O-sobre sulfato-derivado consiste de una mezcla de cadenas en las cuales por lo menos 90% de dichas cadenas tienen la fórmula II



En la cual las unidades urónicas son 20 a 60% consistentes de ácido idurónico, n es un entero de 2 a 100, R, R' y R'' son hidrógeno o SO₃, el grado de sulfatación es de por lo menos de 3,4 y el catión correspondiente es química o farmacéuticamente aceptable.

44. Un epiK5-amina-O-sobre sulfatado-derivado de acuerdo con la reivindicación 43, caracterizado en que, en la fórmula II n represente un entero de 3 a 100

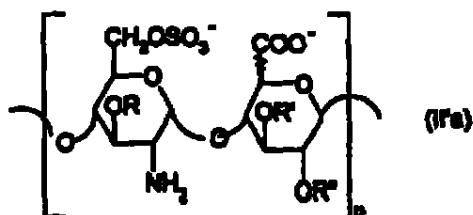
45. Un epiK5-amina-O-sobre sulfato-derivado de acuerdo con cualquiera de las reivindicaciones de

la 42 a la 44, caracterizado en que este consiste de una mezcla de cadenas en las cuales por lo menos 90% de dichas cadenas tienen la fórmula II en la cual las unidades urónicas son 40-60% consistentes de ácido idurónico, con un peso molecular promedio de aproximadamente 2.000 a aproximadamente 40.000, R es por lo menos 40% SO_3^- , R' y R'' son ambos SO_3^- o uno es hidrógeno y el otro es 5 a 10% SO_3^- en monosulfato de ácido glucurónico y 10 a 15% de SO_3^- en mono sulfato de ácido idurónico.

46. Un epiK5-amina-O-sobre sulfato-derivado de acuerdo con una cualquiera de las reivindicaciones de la 42 a la 45, caracterizado en que este es un LMW-epiK5-amina-O-sobre sulfato que consiste de una mezcla de cadenas en las cuales por lo menos 90% de dichas cadenas tienen la fórmula II en la cual las unidades urónicas son 40 a 60% consistentes de ácido idurónico, R es por lo menos 40% SO_3^- , R' y R'' son ambos SO_3^- o uno es hidrógeno y el otro es 5 a 10% SO_3^- en

ácido glucurónico y 10 a 15% SO_3^- en ácido idurónico, n es un entero de 3 a 15, con un peso molecular promedio de aproximadamente 4.000 a aproximadamente 8.000 y el catión correspondiente es químicamente o farmacéuticamente aceptable.

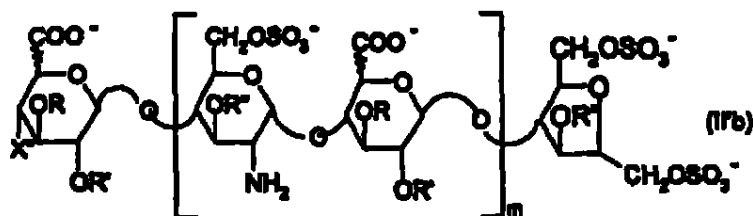
- 47 Un epiK5-amina-O-sobre sulfato-derivado de acuerdo con una cualquiera de las reivindicaciones de la 42 a la 46, caracterizado en que este es un LMWepicaK5-amina-O-sobre sulfato que consiste de una mezcla de cadenas en las cuales las especies preponderantes tienen la fórmula II'a.



En la cual las unidades urónicas son 20 a 60% consistentes de ácido idurónico, p es un entero entre 4 a 8, R, R' y R'' son hidrógeno o SO_3 , el grado de sulfatación es de por lo menos de 3,4.

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48. Un LMW-piK5-amina-O-sobre sulfato de acuerdo con la reivindicación 47 caracterizado en que el se consiste de una mezcla de cadenas en las cuales las especies preponderantes es un compuesto de fórmula II'b



En la cual las unidades urónicas son 40 a 60% consistentes de ácido idurónico, m es 4, 5 o 6, R, R' y R'' son hidrógeno o SO₃⁻ X'' es OH o OSO₃⁻ para un grado de sulfatación de por lo menos de 3,4 las unidades idurónicas están presentes alternadamente, empezando con una unidad glucurónica o idurónica.

49. Un LMW-epiK5-N-sulfato virtualmente libre de NH₂ y grupos N-acetilo, que tiene un contenido de ácido idurónico entre 20 a 60% y un peso molecular promedio de aproximadamente 1.500 a

aproximadamente 12.000, o una de sus sales química o farmacéuticamente aceptables.

50. Un LMW-epiK5-N-sulfato de acuerdo con la reivindicación 49, caracterizado en que el contenido de ácido idurónico está entre el 40 al 60% y el peso molecular promedio es de aproximadamente de 1.500 a aproximadamente 10.000.

51. Un LMW-epiK5-N-sulfato de acuerdo con la reivindicación 49, caracterizado en que el contenido de ácido idurónico es 50 a 55% y el peso molecular promedio es de aproximadamente 1.500 a aproximadamente 7.500.

52. Un LMW-epiK5-N-sulfato de acuerdo con una cualquiera de las reivindicaciones 49 a 51, obtenido por un proceso caracterizado en que un K5-N-sulfato es sometido, en cualquier orden,

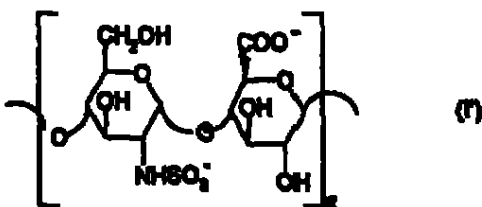
(i) a epimerización C5 con un D-glucuronil C5-epimerasa aislada, purificada y en solución o

inmovilizada sobre un soporte sólido, a un pH de aproximadamente 7, a una temperatura aproximada a 30% y durante un periodo de tiempo de 12 a 24 horas en la presencia de por lo menos un ión bivalente seleccionada entre calcio, magnesio, bario y manganeso; y

(ii) a despolimerización nitrosa opcionalmente seguida por reducción.

53. Un LMW-epiK5-N-sulfato de acuerdo con la reivindicación 52, caracterizado en que, en la etapa (ii) la despolimerización es seguida por reducción con borohidruro de sodio.

54. Un LMW-epiK5-N-sulfato de acuerdo con la reivindicación 53, caracterizado en que este consiste de una mezcla de cadenas en las cuales el 90% de dichas cadenas tiene la fórmula I'



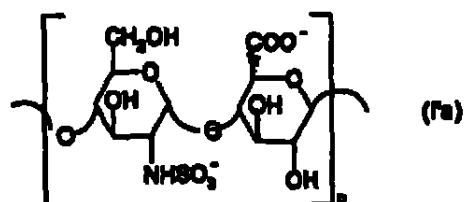
En al cual las unidades urónicas son 20 al 60% consistentes de ácido idurónico q es un entero entre 2 a 20 y el catión correspondiente es química o farmacéuticamente aceptable.

55. Un LMW-epiK5-N-sulfato de acuerdo con la reivindicación 54, caracterizado en que caracterizado en que este consiste de una mezcla de cadenas en las cuales el 90% de dichas cadenas tiene la fórmula I' en la cual las unidades urónicas son 40 a 60% de ácido idurónico.

56. Un LMW-epiK5-N-sulfato de acuerdo con una cualquiera de las reivindicaciones 54 y 55, caracterizado en que este consiste de una mezcla de cadenas en las cuales el 90% de dichas cadenas tiene la fórmula I' en la cual n es un entero de 3 a 15.

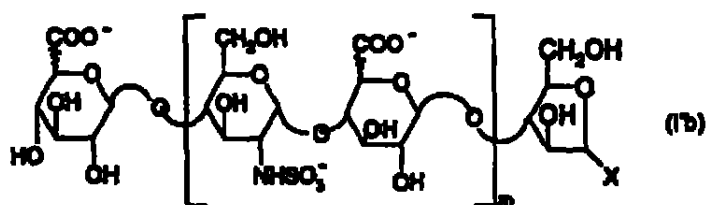
57. Un LMW-epiK5-N-sulfato de acuerdo con una cualquiera de las reivindicaciones 54 y 55, caracterizado en que este consiste de una mezcla

de cadenas en las cuales las especies preponderantes tienen la fórmula I'a



En la cual las unidades urónicas son 60 a 40 consistentes de ácido glucurónico y 40 a 60% de ácido idurónico, p es un entero entre el 4 y a 8 y el catión correspondiente es química o farmacéuticamente aceptable.

58. Un LMW-epiK5-N-sulfato de acuerdo con una cualquiera de las reivindicaciones de la 55 ala 57, caracterizado en que este consiste de una mezcla de cadenas en las cuales las especies preponderantes tienen la fórmula I'B



En la cual X es hidroxilometilo, m es 4,5 o 6, el catión correspondiente es un ión química o farmacéutica mete aceptable y las unidades glucurónicas e idurónicas están presentes alternadamente, iniciando con una unidad glucurónica o idurónica.

59. Un LMW-epiK5-N-sulfato de acuerdo con una cualquiera de las reivindicaciones desde la 49 ala 58, caracterizado en que dicha sal o catión es seleccionada entre aquellos de metales alcalinos, metales de tierra alcalina, amonio, tetraalquilamonio (C₁-C₄) aluminio y zinc.
60. Un LMW-epiK5-N-sulfato de acuerdo con la reivindicaciones 59, caracterizado en que dicha sal o cationes seleccionado entre aquellos de sodio, calcio, y tetrabutylamonio.
61. Un proceso para la preparación de Un LMW-epiK5-N-sulfato caracterizado en que un K5-N-sulfato es sometido en cualquier orden,

- (i) a epimerización C5 con una D-glucuronil C5 epimerasa aislada purificada y en solución o inmovilizada sobre un soporte sólido, a un pH aproximadamente 7 a una temperatura de aproximadamente 30°C y durante un periodo de tiempo de 12 a 24 horas en al presencia de por lo menos un ión bivalente seleccionado entre calcio, magnesio, bario y manganeso; y
- (ii) a despolimerización nitrosa opcionalmente seguida por reducción.

62. El proceso de acuerdo con la reivindicación 61, caracterizado en que este es llevado a cabo en el orden (i)-(ii)

63. El proceso de acuerdo con la reivindicación 61, caracterizado en que este es llevado a cabo en el orden (ii)-(i).

64. El proceso de acuerdo con la reivindicación 63 caracterizado en que el producto obtenido luego de la terminación de la despolimerización es un LMW-K5-N-sulfato que es directamente sometido a epimerización C5.

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65. El proceso de acuerdo con la reivindicación 64, caracterizado en que dicho LMW-K5-N-sulfato tiene un peso molecular promedio de mas de 4.000.
66. Una composición farmacéutica que incluye, como uno de sus ingredientes activos una cantidad farmacológicamente activa de un epiK5-N-, O-sobre sulfato-derivado de acuerdo con una cualquiera de las reivindicaciones 20 a 40 en mezcla con un excipiente farmacéutico.
67. Una composición cosmética que incluye una cantidad defectiva de un epiK5-N, O-sobresulfato-derivado de acuerdo con una cualquiera de las reivindicaciones desde la 20 a la 40 en mezcla con un excipiente cosmético.
68. El uso de una C5 epimerasa aislada y purificada para la conversión de un K5-N-sulfato derivado en un epiK5-N-sulfato derivado correspondiente caracterizado por una unidad tetrasacárido repetitiva consistente de dos unidades gliucosamina separadas por una unidad glucurónica

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y seguida por una unidad idurónica o separadas por una unidad idurónica y seguida por una unidad glucurónica.

69. El uso de acuerdo con la reivindicación 68, caracterizado en que dicho K5-N-sulfato derivado tiene un peso molecular promedio de más de 4.000.

70. El uso de acuerdo con la reivindicación 69, caracterizado en que dicho peso molecular promedio esta entre 6.000 a 7.500.

RESUMEN

Se describe un nuevo método para la sobresulfatación de epiK5-N-sulfato para obtener un epiK5-amina-O-sobresulfato con un grado de sulfatación muy alto que, por N-sulfatación subsecuente, suministra nuevos derivados de epiK5-N,O-sobresulfato con un grado de sulfatación de por lo menos 4, básicamente libre de actividad sobre los parámetros de coagulación y útil en el campo de los cosméticos y farmacéutico. También se han descrito nuevos epiK5-N-sulfatos de bajo peso molecular útiles como intermedios en la preparación de derivados deLMW-epiK5-N,O-sobresulfato de bajo peso molecular.

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 900176089-2

RECIBO OFICIAL DE CAJA : 04 - 99,403
FECHA : DICIEMBRE 27 DE 2004

SUPERINDUSTRIA Y COMERCIO
Radicacion : 04129789 00000000
Fecha (HFB): 2004-12-29 18:00:21
Folios: 170
Trmite : 811 PCT-PATENT 379 FASE HRCI 411 PRESENTAC
Dependencia: 2020 DIVISION DE NUEVAS CREACIONES

***** CONSIGNACION *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	Vr. PAGO
CAVELIER ABOGADOS	CONSIGNACION	BANCO POPULAR	050-00110-6	32758702	27/12/2004	21,068,000.00

***** CONCEPTO *****

CANT.	RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1	50005-01-01 SOLICITUDES	1 TRAMITES DE SOL. DE PATENTE DE IN	422,000.00
		TOTAL :	\$ 422,000.00

SOM: CUATROCIENTOS VEINTIDOS MIL PESOS

RESPONSABLE : 

RECIBO DE CAJA APLICADO AL EXPEDIENTE No. _____

colo14594 841 0033

171 172

REQUISITOS NECESARIOS PARA PRESENTACION Y AGILIZACION DEL
TRAMITE

	USUARIO	RADICADOR
PATENTE DE INVENCION	()	()
MODELO DE UTILIDAD	()	()
- Indicación de que se solicita la concesión de una patente.	()	()
- Datos de identificación del solicitante o de la persona que se presenta la solicitud.	()	()
- Descripción de la invención.	()	()
- Dibujos de ser estos pertinentes.	()	()
- Comprobante de Pago de las tasas establecidas.	()	()
COMPLETA	()	()
INCOMPLETA	()	()
DISEÑO INDUSTRIAL ()		
- Indicación de que se solicita el registro de Diseño Industrial	()	()
- Datos de identificación del solicitante o de la persona que presenta la solicitud.	()	()
- Representación gráfica o fotográfica del Diseño Industrial o muestra del Material que incorpora el diseño.	()	()
Comprobante de pago de las tasas establecidas	()	()
COMPLETA ()	()	()
INCOMPLETA	()	()
ESQUEMA DE TRAZADO ()		
- Indicación de que solicita el registro de un Esquema de trazado.	()	()
- Datos de identificación del solicitante o de la persona que presenta La solicitud.	()	()
Representación gráfica del esquema de trazado	()	()
Comprobante de pago de las tasas establecidas.	()	()
COMPLETA ()	()	()
INCOMPLETA	()	()

Firma Responsable:



Fecha:

2020
.Bogotá D C

Doctor(a)
EMILIO FERRERO
Apoderado(a)
GLYCORES 2000 S. r. l.

Oficio N° **11019**

ASUNTO:

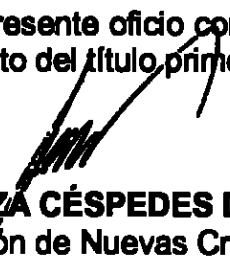
Radicación N°	04-129789
Solicitud internacional	PCT/IB03/02338
Fecha inicio fase nacional	18/01/2005
Trámite	011
Evento	379
Actuación	430
Folios	001

Como resultado del examen de forma, realizado con fundamento en el artículo 38 de la Decisión 486 de la Comisión de la Comunidad Andina, artículo 27 del Tratado de Cooperación en Materia de Patentes (PCT), regla 51 bis del Reglamento y Circular Única de la Superintendencia de Industria y Comercio, se le comunica que:

- La solicitud no contiene copia del documento en que consta la cesión del derecho de patente del inventor al solicitante o a su causante (Art. 26-k) Decisión 486).
- La solicitud no contiene el poder debidamente otorgado, con el lleno de requisitos exigidos por los artículos 63 y subsiguientes del Código de Procedimiento Civil. Adicionalmente deberá presentar el documento que acredita la existencia y representación legal de la sociedad solicitante, cuando éste fuere una persona jurídica.

En cumplimiento de lo dispuesto por el artículo 39 de la Decisión 486, se le requiere para que dentro del término de dos meses siguientes a la fecha de notificación del presente oficio, complete los requisitos anteriormente enunciados. En caso de no dar cumplimiento al presente requerimiento, la solicitud se considerará abandonada y perderá su prelación.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única N° 10 de 2001.


ALIX CARMENZA CÉSPEDES DE VERGEL
Jefe de la División de Nuevas Creaciones.

El anterior requerimiento fue notificado por fijación en lista de fecha,
21 ENE. 2005

202044

Sede Centro: Carrera 13 No. 27-00 Pisos 2, 5, 7 y 10
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