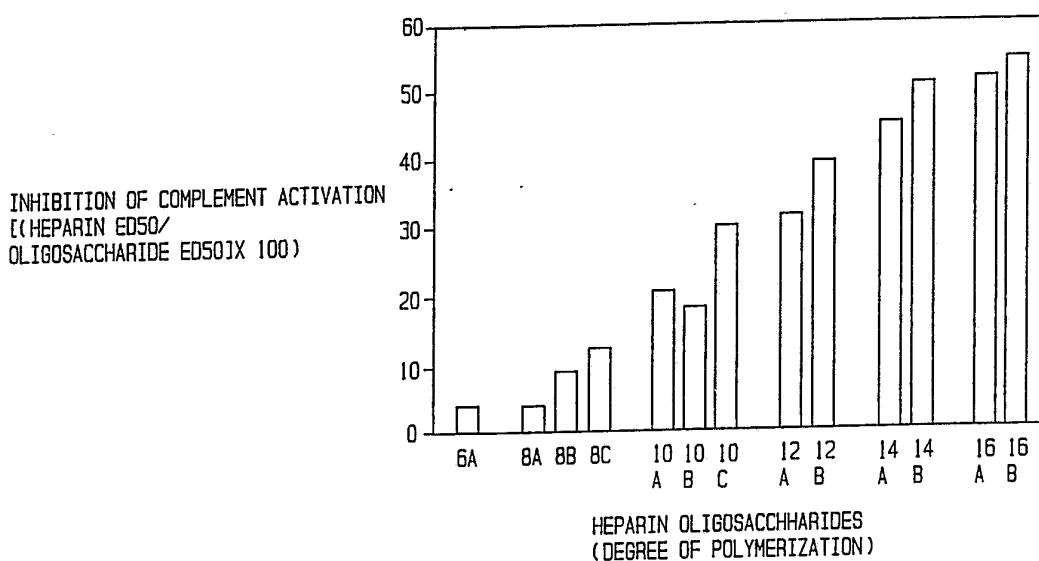




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(54) Title: OLIGOSACCHARIDE HEPARIN FRAGMENTS AS INHIBITORS OF COMPLEMENT CASCADE

**(57) Abstract**

There are disclosed oligosaccharide compounds having heparin-like anticomplement activity and reduced anticoagulant activity as compared with heparin on a weight or molar basis. The oligosaccharide compounds have at least 5 and no greater than 25 saccharide units. The oligosaccharides can have an even number of saccharide units with a terminus nonreducing sugar, or an odd number of saccharide units without a terminus nonreducing sugar. There are also disclosed anticomplement pharmaceutical compositions with reduced anticoagulant side effect activity and a process for preparing the oligosaccharide compounds.

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DescriptionOLIGOSACCHARIDE HEPARIN FRAGMENTS AS
INHIBITORS OF COMPLEMENT CASCADETechnical Field

This application is a continuation-in-part of
5 U.S. Patent Application Serial No. 717,213.

The present invention relates to compounds and
pharmaceutical compositions for inhibiting complement
cascade without undue side effects of anticoagulant
activity. The compounds are small chain fragments of the
10 larger polysaccharides heparin of from about 6 saccharide
units to about 16 saccharide units.

Background of the Invention

Heparin is a highly sulfated, polydisperse,
 α -(1 \rightarrow 4)-linked copolymer of uronic acid and glucosamine.
15 Jacques, Science 206:528-33, 1979. Heparin is synthesized
as a proteoglycan of approximately one million daltons
molecular weight. Heparin is attached to a protein core.
The protein core is removed in commercial processing, to
obtain glucosaminoglycan heparin (average molecular weight
20 10,000 to 14,000 daltons).

Heparin's major application is as an anticoagu-
lant. However, heparin has a multiplicity of other
biological activities, including: (1) the ability to
regulate angiogenesis (Folkman et al., Science, 221:719-
25 25, 1983); (2) the ability to regulate other cell growth
and proliferation processes; (3) the ability to activate
and release plasma lipoprotein lipase (Merchant et al.,
Atherosclerosis, 62:151-58, 1986); and (4) the ability to
inhibit complement cascade.

30 In 1929, Ecker and Gross first demonstrated the
capacity of heparin to regulate complement activation.
Subsequently, other investigators demonstrated multiple
sites in the classical as well as the alternative-amplifi-

cation pathways of complement at which heparin may act. Heparin's anticoagulant activity is mediated through a specific oligosaccharide sequence on the heparin polymer capable of binding antithrombin III. Heparin's structure
5 activity relationship on the complement cascade system, however, is still poorly understood.

Heparin's complement cascade inhibiting activity can be used for a variety of functions to reduce the host's immune response. Organ transplant rejection is an
10 example of an appropriate immune response which is detrimental to the individual host (i.e., mammals). Similarly, there are a variety of autoimmune diseases which result from the immune response attacking the individual's own cells. These immune and autoimmune
15 diseases represent a class of diseases which are often difficult to treat effectively. Although there are a number of drugs which can be immune suppressant, none of the currently available therapeutic agents are adequate to control or specifically inhibit complement cascade
20 activation. The inhibition of complement may be an important aspect in a variety of immune disorders. The diseases in which a substance that inhibits the complement cascade system may be therapeutically useful include paroxysmal nocturnal hemoglobulinuria, rheumatoid
25 arthritis (in which the substance might be administered directly into a joint capsule to prevent complement activation), and hereditary angioedema (in which a deficiency in complement control protein leads to an active complement consumption). Other diseases include
30 septic shock, rheumatoid arthritis and systemic lupus erythematosus.

Heparin's anticoagulant activity has been demonstrated to be associated with the presence in its structure of a specific oligosaccharide sequence for the
35 binding of antithrombin III. Once bound to heparin, antithrombin III can then inhibit a number of blood

coagulation factors, and thus prevent the coagulation of blood.

Heparin's anticomplement activity is mediated by binding to a variety of complement cascade proteins and thereby regulates both the classical and alternate cascade pathways. Heparin and heparin oligosaccharides inhibit a portion of the complement cascade by inhibiting the generation of cell-bound amplification pathway C3 convertases, C3b,Bb, C3b,Bb,P and C3b,Bb,Nef. Heparin's anticomplement activity interferes with the binding site on the C3b. Furthermore, heparin prevents the fluid phase consumption of B by D in the presence of C3b, again indicating a direct action on C3b.

Accordingly, there is a need in the art for therapeutic agents with heparin-like anticomplement activity and greatly reduced (e.g., 10% or less) of heparin's anticoagulant activity on a weight basis. This invention fulfills the need.

Terminology

The complement abbreviations used herein are C3 for the third complement protein, C3b for the activated third complement protein, C4b for the activated fourth complement protein, B, P and D are letter components of the alternative amplification pathway of complement. Other abbreviations include EAC4b,3b for the sheep erythrocyte cellular intermediate containing surface C4b and C3b. EAC4b,3b⁵, EAC4b,3b²⁰, and EAC4b,3b¹⁰⁰ refer to cellular intermediates produced from EAC1,4b using 5 μ g, 20 μ g, and 100 μ per 1×10^9 EAC1,4b. For the saccharides, Δ UA refers to 4-deoxy- α -L-threo-hex-4-enopyronosyluronic acid (a nonreducing sugar); p refers to pyranose; GlcA refers to glucuronic acid; IdoA refers to iduronic acid; and S refers to sulfate.

Disclosure of the Invention

Briefly stated, the present invention comprises heparin oligosaccharide fractions having degrees of polymerization from about 6 to about 24 saccharide units. The oligosaccharides of the present invention can have an even number of saccharide units, wherein groups of saccharide units are disaccharides, tetrasaccharides and hexasaccharides.

The present invention also comprises heparin oligosaccharide fractions having an odd-number degree of polymerization from about 5 to about 23 saccharide units wherein the nonreducing terminus (Δ UAp) saccharide is removed. Removal of the nonreducing terminus saccharide can be accomplished by treatment of the even-numbered oligosaccharide, as described herein, with ozone at an acidic pH.

Preferably, the disaccharide group of saccharide unit is a trisulfated disaccharide such as Δ UAp2S(1 \rightarrow 4)- α -D-GlcNp2S6S (2 in Table 1 and where n=0 in Formula I). The tetrasaccharide group of saccharide unit is selected from the group consisting of pentasulfated tetrasaccharides, such as Δ UAp2S(1 \rightarrow 4)- α -D-GlcNp2S(1 \rightarrow 4)- α -L-IdoAp2S- α -D-GlcNp2S6S (4A in Table 1) and Δ UAp2S(1 \rightarrow 4)- α -D-GlcNp2S6S(1 \rightarrow 4)- β -D-GlcAp(1 \rightarrow 4)- α -D-GlcNp2S6S (4B in Table 1), and hexasulfated tetrasaccharides, such as Δ UAp2S(1 \rightarrow 4)- α -D-GlcNp2S6S(1 \rightarrow 4)- α -L-IdoAp2S(1 \rightarrow 4)- α -D-GlcNp2S6S (4C in Table 1); and the hexasaccharide group of saccharide unit is a septasulfated hexasaccharide, such as Δ UAp2S(1 \rightarrow 4)- α -D-GlcNp2S6S(1 \rightarrow 4)- α -L-IdoAp(1 \rightarrow 4)- α -D-GlcNAcp6S-(1 \rightarrow 4)- β -D-GlcAp(1 \rightarrow 4)- α -D-GlcNp2S3S6S (6C in Table 1).

An example of a trisulfated disaccharide is 4-deoxy- α -L-threo-hex-4-enopyronosyluronic acid-2-sulfate linked 1 \rightarrow 4 to α -D-2-deoxy-2-amino-gluco-pyronosyl-2,6 disulfate, wherein α and β represent the anomeric configurations of the sugars and D and L represent the absolute configuration of the sugars.

Most preferably, the hexasaccharide, octasaccharide and decasaccharide are tri-, tetra- and penta-oligomers of the heparin disaccharide (2), which is a trisulfated disaccharide, such as decasaccharide 10D (in Table 1) which has the structure (2)₅.

The process of the present invention prepared groups of saccharide units polymerized to form an oligosaccharide with an even-numbered degree of polymerization of from about 6 to about 24 saccharide units. The invention process further comprises the step of removing the terminus nonreducing sugar to form an odd-numbered degree of polymerization saccharide with from about 5 to about 23 saccharide units. The oligosaccharides are highly sulfated. For example, when the percentage sulfur is calculated on a percentage molecular weight basis using the sodium salt, the oligosaccharides have at least 14% sulfur.

The oligosaccharides of the present invention have strong anticomplement activity, to being equipotent with heparin on a molar basis. The oligosaccharides of the present invention also have reduced anticoagulant activity, defined as less than 10% of heparin's anticoagulant activity on a weight basis.

Brief Description of the Drawings

Figure 1 shows the strong anion-exchange-HPLC of a heparin-oligosaccharide mixture prepared by heparinase-catalyzed depolymerization of heparin to 30% reaction completion. Absorbance at 232 nm of 1 mg (A) and 4 mg (B) of heparin-oligosaccharide sample eluting from the column are plotted against elution time in minutes. The peaks collected from the column are labeled to identify the sample (sample number corresponds to degree of polymerization).

Figure 2 shows the effect of heparin and structurally characterized heparin-oligosaccharides on the alternate-amplification pathway of complement. A dose

response curve is shown for heparin (○); septasulfated hexasaccharide-6A (◆), hexasulfated tetrasaccharide-4C (▲), pentasulfated tetrasaccharide-4B (□), and trisulfated disaccharide-2 (●).

5 Figure 3 shows the inhibition of lysis of EAC4b,3b¹⁰⁰ by heparin-oligosaccharides. Activity is expressed as [(ED50 heparin/ED50 heparin-oligosaccharide) X 100]. ED50 is measured on a molar basis using an average molecular weight for heparin of 14,000.

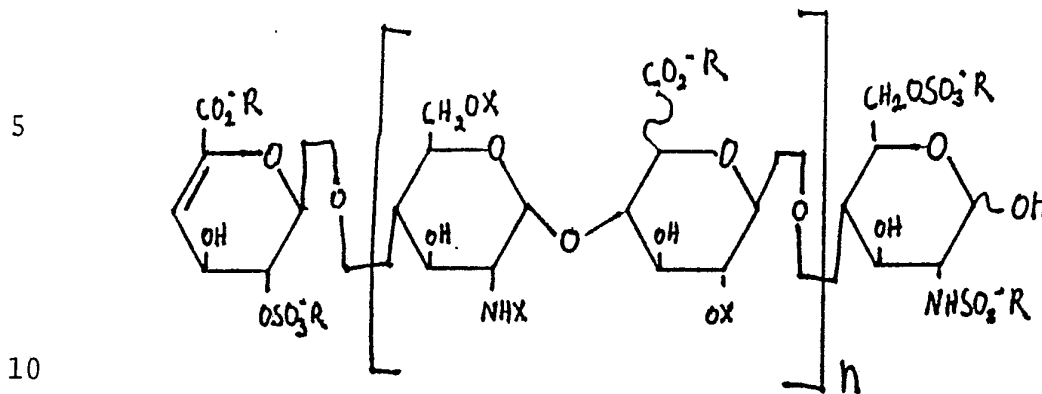
10 Figure 4 depicts the inhibition of the generation of amplification pathway C3 convertase on EAC4b,3b²⁰ by 4 μg of heparin and homogeneous structurally defined heparin-oligosaccharides. The heparin oligosaccharides 6A, 6B, 6C, 8A, 8B, 8C, 8D, 10A, 10B, 10C, and 10D are
15 defined in Table 1 herein.

Figure 5 shows the inhibition of lysis of EAC4b,3b¹⁰⁰ by various commercial heparins and heparin-oligosaccharides (shown in Table 1) plotted against anticoagulant activity. Anticoagulant activity was
20 measured by anti-factor IIa amidolytic assay relative to a standard porcine heparin (H1) on a weight basis, as shown in Table 2. Inhibition of complement activity is expressed as [(ED50 heparin/ED50 heparin-oligosaccharide) X 100] on a weight basis. Porcine heparins (■ H1-H6),
25 bovine heparins (□ H7,H8), low molecular weight heparin (● H9) and heparin-oligosaccharides (◆ 2+16b) are identified by the adjacent number.

Detailed Description of the Invention

30 The partial enzymatic depolymerization of heparin produced a controlled and reproducible distribution of heparin-oligosaccharides of the formula:

Formula I



wherein R is a metallic or non-metallic cation or hydrogen for a free acid, X is H or SO₃R, Y is H, COC₁₋₆ alkyl or SO₃R, and n is an integer from about 1 to about 11.
 15 Preferably, Y is COCH₃, n is 4 and R is hydrogen, sodium or potassium.

Heparin oligosaccharides having a degree of polymerization of 6 units to 24 units and formed by polymerizing 2, 4 or 6 saccharide unit complexes were
 20 found to have complement inhibiting activity potency comparable to native heparin on a weight basis and very little or no anticoagulant activity.

At 30% reaction completion, the heparin-oligosaccharide mixture contained the maximum number in
 25 concentration of heparin-oligosaccharides with degrees of polymerization between 10 and 24 saccharide units, wherein all the heparin-oligosaccharides contained an even number of saccharide units. The oligosaccharide mixture is fractionated on the basis of charge using strong anion
 30 exchange-HPLC (as shown in Figure 1) to obtain purified heparin-oligosaccharide samples of a defined degree of polymerization and containing a single major component. The elution order from the HPLC column was dependent on the degree of polymerization (i.e., disaccharide followed
 35 by tetrasaccharide, followed by hexasaccharide, etc.), and within each size group, elution order was dependent on degree of sulfation (i.e., tetrasaccharide 4A and 4B in

Table 1 having 5 sulfates, followed by tetrasaccharide 4C having 6 sulfates). The refractionation of the oligosaccharide components produced heparin-oligosaccharides of sufficient purity to obtain a compositional analysis as shown in Table 1 below.

Table 1
Structure of Heparin Oligosaccharides Having Degree of Polymerization from 6 to 10

Heparin Oligosaccharide	Oligosaccharide ^{a, b} Composition	Mol. Wt.	Number of Sulfate Groups
6A	<u>2, 4A</u>	1893	8
6B	<u>2, 4B</u>	1893	8
6D ^C	<u>2, 4C</u>	1995	9
8A	<u>2, 2, 4A</u>	2456	11
8B	<u>2, 2, 4B</u>	2456	11
8C	<u>2, 6C</u>	2498	10
8D ^C	<u>2, 2, 4C</u>	2660	12
10A	<u>2, 2, 2, 4A</u>	3223	14
10B	<u>2, 2, 2, 4B</u>	3223	14
10C	<u>2, 2, 6C</u>	3163	13
10D ^C	<u>2, 2, 2, 4C</u>	3325	15

- 25 a. No sequence is implied by the order of the fundamental oligosaccharide components.
- b. The structures of the fundamental oligosacchrides making up each transient oligosaccharide are:
2, trisulfated disaccharide, Δ UAp2S(1→4)- α -D-GlcNp2S6S;
4A, pentasulfated tetrasaccharide, Δ UAp2S(1→4)- α -D-GlcNp2S(1→4)- α -D-GlcNp2S6S;
4B, pentasulfated tetrasaccharide, Δ UAp2S(1→4)- α -D-GlcNp2S6S(1→4)- β -D-GlcAp(1→4)- α -D-GlcNp2S6S;
4C, hexasulfated tetrasaccharide, Δ UAp2S(1→4)- α -D-GlcNp2S6S(1→4)- α -L-IdoAp2S(1→4)- α -D-GlcNp2S6S;
6C, septasulfated hexasaccharide, Δ UAp2S(1→4)- α -D-GlcNp2S6S(1→4)- α -L-IdoAp(1→4)- α -D-GlcNAcp6S-(1→4)- β -D-GlcAp(1→4)- α -D-GlcNp2S3S6S.

c. The oligosaccharide composition of this heparin-oligosaccharide can only be arranged in one way since $4C = \underline{2,2}$ thus this oligosaccharide's sequence is established.

5 Only one sequence is possible for hexasaccharide 6D, octasaccharide 8D, and decasaccharide 10D, since their compositional analysis indicated that they were simply oligomers of heparin disaccharide 2.

The compounds of the present invention are
10 characterized by the capacity to inhibit complement activation, while having little or no anticoagulant activity, as shown in Table 2.

Oligosaccharides with degrees of polymerization from about 2 to about 24 saccharide units can be prepared
15 by partial depolymerization of heparin using heparinase. The degree of depolymerization (i.e., percent reaction completion) can be varied from about 1% to about 100% to optimize the production of an oligosaccharide having a particular size or degree of polymerization. Following
20 partial or complete depolymerization, the oligosaccharide mixture is fractionated using a strong anion exchange HPLC column or other suitable techniques, such as polyacrylamide gel electrophoresis (See, Rice et al., Biochem J., 244:515-22 (1987)). Refractionation by one or a combination of these techniques results in a highly purified
25 oligosaccharide. Oligosaccharides of greater than six saccharide units (i.e., molecular weight greater than 1000 daltons) is desalted by exhaustive dialysis against deionized water using controlled pore dialysis membranes.

30

35

Table 2

Anticoagulant activity of heparin-oligosaccharides.

	<u>Heparin Oligosaccharide</u>	<u>aPTT</u>	ACTIVITY ^a (units/ μ mole)	
			<u>Antithrombin III mediated Anti-factor IIa</u>	<u>Anti-factor Xa</u>
5				
10	<u>2</u>	<1	<1	<0.1
	<u>4A</u>	2	<1	<0.1
	<u>4B</u>	2	<2	0.1
	<u>4C</u>	2	<2	3
15	<u>6A</u>	nd ^b	<2	<2
	<u>6B</u>	nd	<2	4
	<u>6C</u>	26	11	26
	<u>6D</u>	11	<2	4
20	<u>8A</u>	16	<3	118
	<u>8B</u>	21	<3	25
	<u>8C</u>	nd	<3	29
	<u>8D</u>	22	<3	32
25	<u>10A</u>	37	<3	nd
	<u>10B</u>	45	<3	116
	<u>10C</u>	nd	<3	61
30	<u>10D</u>	54	<3	136
	Heparin	2,338	2,338	2,338

a. Activity is determined based on a standard curve using heparin (Mr avg 14,000) with an activity of 167 units/mg.

35 b. nd, not determined

The compounds of the present invention are obtained by depolymerizing heparin with the lyase enzyme, heparinase. The mixture of oligosaccharides obtained by the heparinase digestion have the desired anticomplement activity and have significantly reduced or no anticoagulant activity as compared with native heparin on a weight or molar basis. The oligosaccharides that are produced by heparinase digestion and subsequent purification have from about 6 to about 24 saccharide units and contain an even number of saccharide units. Preferably, the oligosaccharids have 16 to 24 saccharide units. Most preferably, the oligosaccharide is a 24 saccharide oligosaccharide.

The compounds of the present invention also include a complimentary series of oligosaccharides having an odd number of saccharide units, from about 5 to 23 saccharide units, and prepared by treating the even-numbered oligosaccharide of the present invention with ozone under acidic conditions to remove the terminus nonreducing sugar.

The following examples illustrate the synthesis of the oligosaccharides and their anticomplement and anticoagulant activity. The examples are offered by way of illustration and not limitation.

25

EXAMPLE 1

Heparin-oligosaccharides were prepared with heparinase (0.25 unit of purified enzyme as 50 units/mg) or as commercial enzyme (0.23 units/mg). Heparinase was added to 300 mg of heparin (from a commercial source, such as porcine mucosal or bovine lung heparin) in 37.5 ml of 250 mM sodium acetate, 2.5 mM calcium acetate solution at pH 7.0. The mixture was incubated at 30°C for 40 hours. Aliquots were removed periodically and the absorbance was measured at 232 nm after diluting the sample 100-fold in 0.03 N hydrochloric acid. The reaction was terminated

after approximately 30% completion by heating the reaction mixture to 100°C for one minute. The product was frozen at -70°C, freeze-dried and reconstituted with distilled water to a volume of 4 mL. The reconstituted sample was then desalted on Sephadex G-10, collected, freeze-dried and reconstituted with 6 mL of distilled water to a final concentration of 50 mg/mL.

The partial enzymatic depolymerization of heparin produced a controlled and reproducible distribution of heparin-oligosaccharides at 30% reaction completion, the mixture contained the maximum number and concentration of heparin-oligosaccharides with degrees of polymerization between about 10 and about 16 saccharide units. This mixture also contained heparin-oligosaccharides with degrees of polymerization of 2 to 10 saccharide units and degrees of polymerization of 16 to 24 saccharide units in sufficient quantities to lead to their purification. If heparin-oligosaccharides of degrees of polymerization of 2 to 10 saccharide units are required, it is desirable to terminate the reaction at between 50 and 75% completion. If heparin-oligosaccharides of degrees of polymerization of 16 to 24 saccharide units are required, it is desirable to terminate the reaction at 10 to 20% completion.

25

EXAMPLE 2

This example illustrates the fractionation of heparin-oligosaccharides. The heparin-oligosaccharide mixture (20 mg) was injected into a strong anion exchange HPLC semi-preparative column, pre-equilibrated with 0.2 M sodium chloride at pH 3.5 at a flow rate of 1.5 mL/min. A linear salt gradient was begun immediately using sodium chloride at pH 3.5 at 1.5 mL/min. flow rate. Fractions from each peak were combined, freeze-dried, reconstituted with 4 mL of distilled water and desalted either by Sephadex G-10 gel permeation chromatography on a 3 cm x 45

cm column at a flow rate of 3 mL/min. (samples with degree of polymerization of less than 6), or by dialysis against 3 x 1000 volumes of deionized water (samples with degree of polymerization greater than 6). The samples were
5 freeze-dried and stored at -70°C.

The results of the fractionation on the basis of charge was the obtaining of purified heparin-oligosaccharide samples of a defined degree of polymerization and containing a single major component. Elution order from
10 the anion exchange-HPLC column, as shown in Figure 1, was dependent on the degree of polymerization (i.e., disaccharide (2) followed by tetrasaccharides, followed by hexasaccharides, etc.). Within each size group, elution order was dependent on degree of sulfation (i.e.,
15 tetrasaccharide 4A and 4B having five sulfates followed by tetrasaccharide 4C having six sulfates). This method of fractionation is suitable to prepare purified heparin-oligosaccharides with degrees of polymerization between 2 and 24 saccharide units.

20

EXAMPLE 3

This example illustrates the quantitation and analysis of the heparin-oligosaccharides. Freeze-dried
25 samples were dissolved in distilled water to a volume of 1 mL and aliquots of each were removed, added to 0.03 N hydrochloric acid and the absorbance was measured at 232 nm. The concentration of each sample was then estimated based upon its molecular weight. Molecular weight was
30 calculated either directly from its chemical structure or estimated from its degree of polymerization as measured by gel permeation HPLC (See, Sharath et al., Immunopharmacology 9:73-83 (1985)) and a molar absorptivity value of $5.2 \times 10^3 \text{ M}^{-1} \text{ cm}$.

35 The more concentrated samples were further diluted until all the samples were at approximately the same concentration (1 mg/mL). Quantitation of the 1 mg/mL

stock solutions relied on uronic acid determination by carbazole assay against a standard curve constructed using the heparin from which they were prepared. The compositional analysis is shown in Table 1 herein.

5

EXAMPLE 4

This example illustrates the activity of heparin and heparin-oligosaccharides to inhibit generation of the
10 alternative-amplification pathway C3 convertase. The method described in Weiler et al., J. Exp. Med. 147:409-421, 1978; Weiler, Immunopharmacology 6:245-255, 1983; and Sharath et al., Immunopharmacology 9:73-83, 1985, was used. Briefly, inhibition was examined in experiments
15 that used EAC4b,3b which were prepared to have high, intermediate, and low amounts of C3 on the cell surface. EAC4b,3b¹⁰⁰, EAC4b,3b²⁰, and EAC4b,3b⁵ were produced using a C3 input of 100 μ g, 20 μ g, and 5 μ g per 10⁹ EAC4b,2a cellular intermediates, respectively. Complement assay
20 buffer (DGVB⁺⁺) 100 μ L, alone was added to tubes used for the reagent blank, no inhibition, and 100% lysis; or complement assay buffer containing a heparin or heparin-oligosaccharide solution was added to the remaining tubes. At time zero, 100 μ l of complement assay buffer containing
25 a suspension of 1 x 10⁷ EAC4b,3b, an excess amount of P and D (100 ng P), and an amount of B needed to lyse the noninhibited tubes at an average of 1 hemolytic event per cell (0.03 ng B for EAC4b,3b¹⁰⁰, 0.15 ng B for EAC4b,3b²⁰, and 0.50 ng B for EAC4b,3b⁵) was added to each tube. The
30 tubes used for the reagent blank contained no B. The mixtures were incubated for 30 minutes at 30°C with shaking. Then, 300 μ L of a 1:20 dilution of rat serum in 40 mM EDTA was added to each tube and incubation was continued with shaking for 60 minutes at 37°C. Saline
35 (1.5 mL) was then added to each tube, except the 100% tube which was lysed with 1.5 mL of water in place of the saline. Finally, the contents of the tubes were mixed,

centrifuged and the absorbance of the supernatant was measured at 414 nm.

The percent lysis, the average number of hemolytic sites per cell (Z), and the percent inhibition were then calculated. Data are expressed as either percent inhibition or as 100 times the ED50 (the amount of heparin resulting in 50% inhibition) for heparin divided by the ED50 for heparin-oligosaccharide. Concentration of heparin and heparin-oligosaccharides are expressed as micrograms per 1×10^7 cellular intermediates. The volume of the reaction tubes during convertase generation varied between 0.2 mL and 0.3 mL; the number of cellular intermediates was held at 1×10^7 per tube. The amount of lysis (Z) seen in the nonhibited tube was relatively independent of the volume of buffer present in the tube during convertase generation. Similarly, the amount of inhibition of convertase generation was independent of the volume of buffer present in the tube during convertase generation.

The results of this experiment demonstrate that heparin's inhibitory activity was reduced as C3b loading was increased. In this experiment, the amount of B input was increased as the concentration of C3b on the cellular intermediate was decreased in order to maintain an average of 1 hemolytic event per cell (Z). The experiments determined whether the B input alone might play a role in determining the degree of inhibition of heparin to interfere with the assembly of effective convertase on EAC4b,3b²⁰ in the presence of low, moderate and high concentrations of B. The amount of B present in the cellular intermediates did not have any affect on the ability of heparin or heparin-oligosaccharide to inhibit lysis.

The results of the experiment in Figure 2 illustrate the regulation of complement activation by heparin-oligosaccharides using a disaccharide, tetrasaccharides 4A, 4B, and 4C, and a hexasaccharide 6C of the

sequences defined in Table 1 herein. The oligosaccharide samples exhibited low activity for complement activation giving linear dose response curves from 1 to 8 μg per 10^7 cellular intermediates (Figure 2). Heparin-oligosaccharides of higher degrees of polymerization, from 6 to 16 saccharide units, having molecular weights ranging from 1893 to 5320, were then tested for activity. The results were compared with native heparin's activity on a weight basis. A high molecular weight heparin-oligosaccharide (16B, molecular weight 5320) was 54% as potent as native heparin on a molar basis and 130% on a weight basis.

Heparin oligosaccharides having as many as 24 saccharide units were prepared according to the methods described herein. The oligosaccharides had greatly reduced anticoagulant activity (defined as less than 10% of native heparin's anticoagulant activity on a weight basis). The heparin oligosaccharides were tested for the ability to regulate complement activation as described herein. An oligosaccharide with 24 saccharide units was equipotent with heparin on a molar basis.

This example also illustrates the relationship of oligosaccharide structure to complement regulatory activity. Eleven pure heparin-oligosaccharides of defined compositions ranging in size from hexasaccharide through decasaccharide (Figure 4) were examined. These homogeneous heparin-oligosaccharides displayed less anticoagulant activity and virtually identical complement activation inhibitory activity as compared to their less purified counterparts.

EXAMPLE 5

This example illustrates the anticoagulant activity of each heparin and heparin-oligosaccharide fragment. Anticoagulant activity was determined by aPTT (activated thromboplastin time) (Linhardt et al., J. Biol. Chem. 257:7310-13, 1982). Antithrombin III-mediated anti-factor IIa and antifactor Xa activity activity was

measured using purified plasma proteins as described in Linhardt et al. and in Table 2. As shown in Figure 5, five porcine heparins showed activities ranging from 90% to 107% of standard porcine heparin (H1) on a weight basis. Two bovine heparins had 106% and 124% of standard porcine heparin's activity and the low molecular weight heparin had 100% of standard porcine heparin's activity on complement activation.

The anticoagulant activity of the heparin-oligosaccharides and commercial heparins using activated partial thromboplastin time and antithrombin III-mediated anti-factor IIa assays gave similar results, with the anti-factor IIa assay demonstrating slightly lower values (Table 2). The anticoagulant activity of heparin-oligosaccharides and commercial heparins measured by anti-factor IIa assays are plotted against their ability to inhibit complement activation in Figure 5. The porcine heparins, bovine heparins, and low molecular heparins cluster, showing only minor differences in activities. By contrast, the heparin-oligosaccharides showed a slight increase in anticoagulant activity with a marked increase in complement-regulating activity as the degree of polymerization increased.

EXAMPLE 6

This example illustrates a process for the removal of the Δ UAp saccharide unit from the nonreducing end of heparinase (EC 4.2.2.7) -prepared heparin oligosaccharides having an even number of saccharide units. This process produces a complementary set of heparin oligosaccharides with an odd number of saccharide units. A heparin oligosaccharide of from 6 to 24 saccharide units is dissolved at 100 μ g/mL in 0.03 N hydrochloric acid. Ozone gas is bubbled through this solution until the solution absorbance at 232 nm is eliminated. The solution pH is adjusted to pH 7.0 and the low molecular weight

fragments of the Δ UAp saccharide unit are removed by gel permeation chromatography on a Sephadex 6-15 column. The heparin oligosaccharide having an odd number of saccharide units is recovered and quantitated using an uronic acid
5 acid.

Although the foregoing invention has been described, in part, by way of illustration and example for the purposes of clarity and understanding, it will be
10 appreciated that certain changes or modifications will be practiced without deviating from the spirit and scope of the invention.

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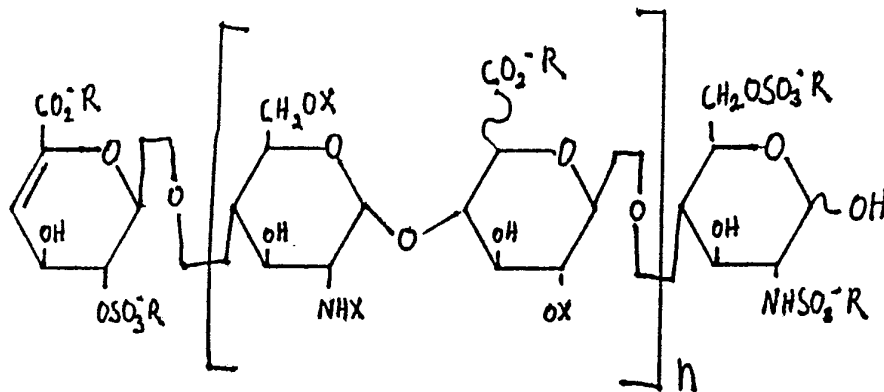
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Claims

1. An oligosaccharide of the formula:



wherein R is a metallic or nonmetallic cation, X is SO_3R , and n is an integer from 5 to 11,

wherein said oligosaccharide has heparin-like anticomplement activity,

wherein said oligosaccharide has reduced anticoagulant activity as compared with heparin, and

wherein the sodium salt of the oligosaccharide contains at least 14% sulfur.

2. The oligosaccharide of claim 1 wherein R is selected from the group consisting of Na, K and Li^2 .

3. A pharmaceutical composition comprising an oligosaccharide according to claims 1 or 2; and

an acceptable pharmaceutical carrier for parenteral administration.

4. A process for preparing an oligosaccharide with heparin-like anticomplement activity and reduced anticoagulant activity as compared with heparin, comprising:

partially depolymerizing a heparin with the enzyme heparinase to obtain a polysaccharide mixture;

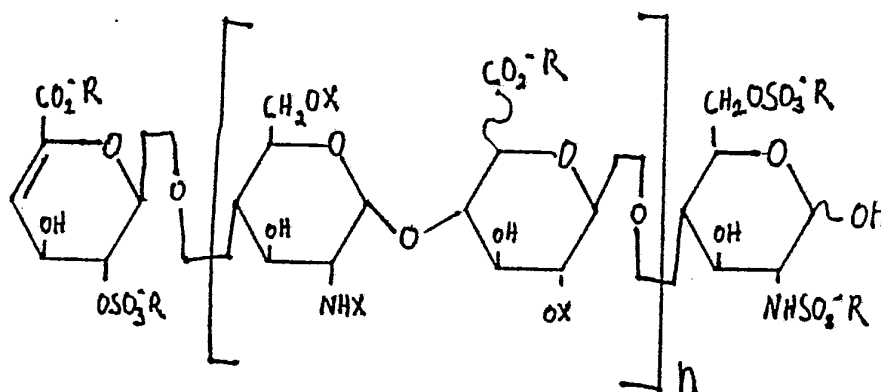
fractionating the polysaccharide mixture by an anion exchange column and collecting the separated fractions;

treating the separated polysaccharide fractions with heparinase to form the disaccharide, tetrasaccharides and hexasaccharide identified in Table 1 as 2, 4A, 4B, 4C, and 6C; and

polymerizing the disaccharide, tetrasaccharides or hexasaccharide to form an oligosaccharide with an even-numbered degree of polymerization to from 10 to 24 saccharide units.

5. The process of claim 4, further comprising removing the terminus nonreducing sugar by exposing the oligosaccharide with an even-numbered degree of polymerization to ozone in an acidic environment, to form an odd-numbered oligosaccharide.

6. An odd-numbered oligosaccharide of the formula:



wherein R is H or α metallic or nonmetallic cation, X is H or SO_2R , X is H, COCH_{1-6} alkyl or SO_3R , and n is an integer from 1 to 11,

wherein said oligosaccharide has heparin-like anticomplement activity, and

wherein said oligosaccharide has reduced anticoagulant activity as compared with heparin.

7. The odd-numbered oligosaccharide of claim 6 wherein R is selected from the group consisting of H, Na, K and Li.

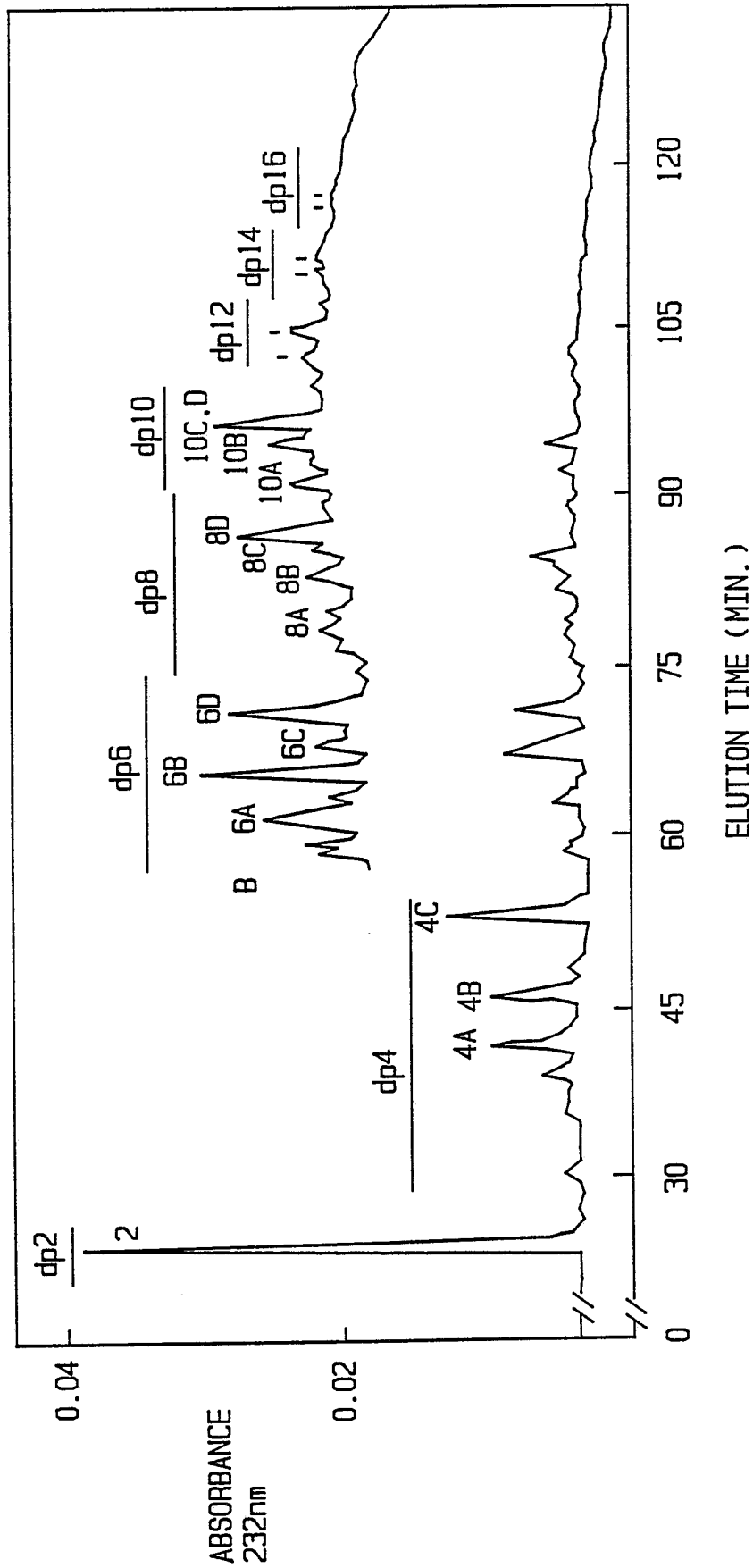
8. The odd-numbered oligosaccharide of claim 6 wherein Y is COCH_3 .

9. The odd-numbered oligosaccharide of claim 6 wherein n is an integer from about 4 to about 10.

10. A pharmaceutical composition comprising an odd-numbered oligosaccharide according to any of claims 6-9, and

a pharmaceutically acceptable carrier or diluent.

FIG. 1



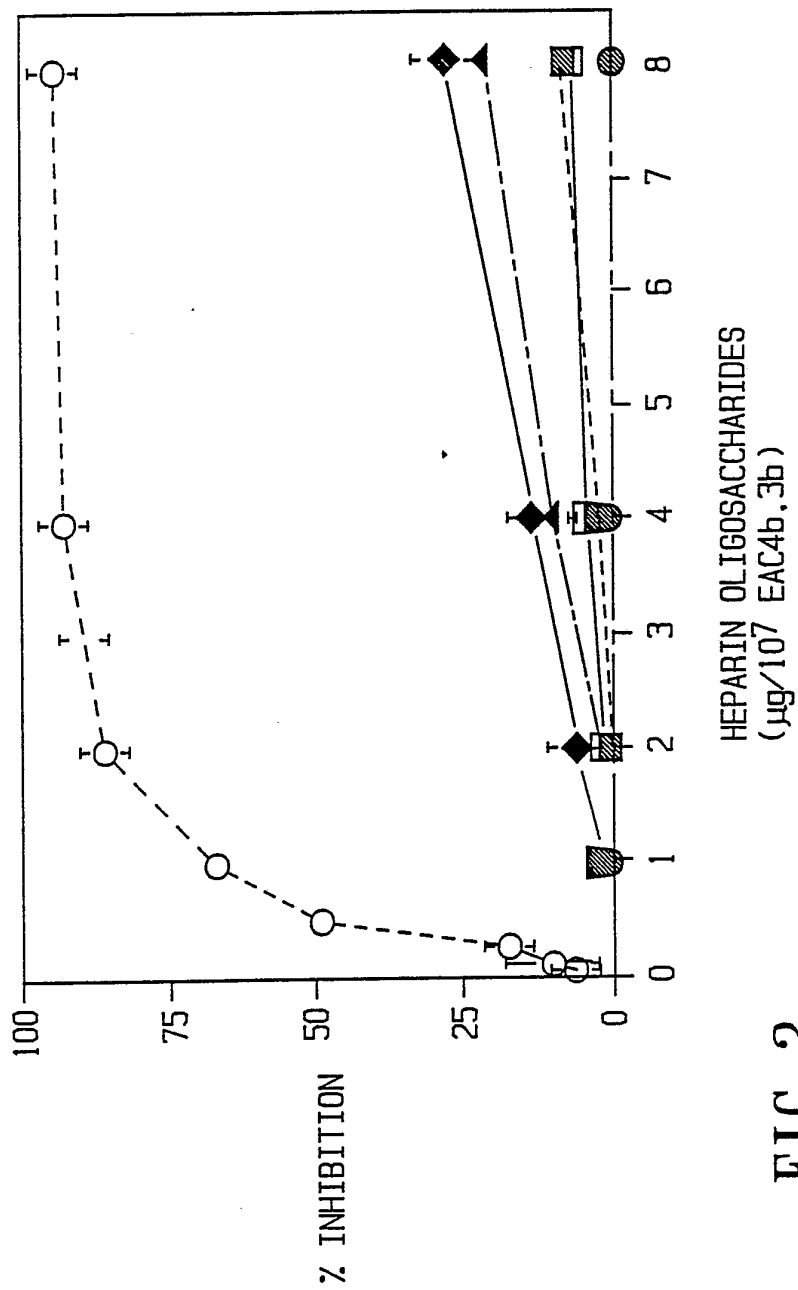
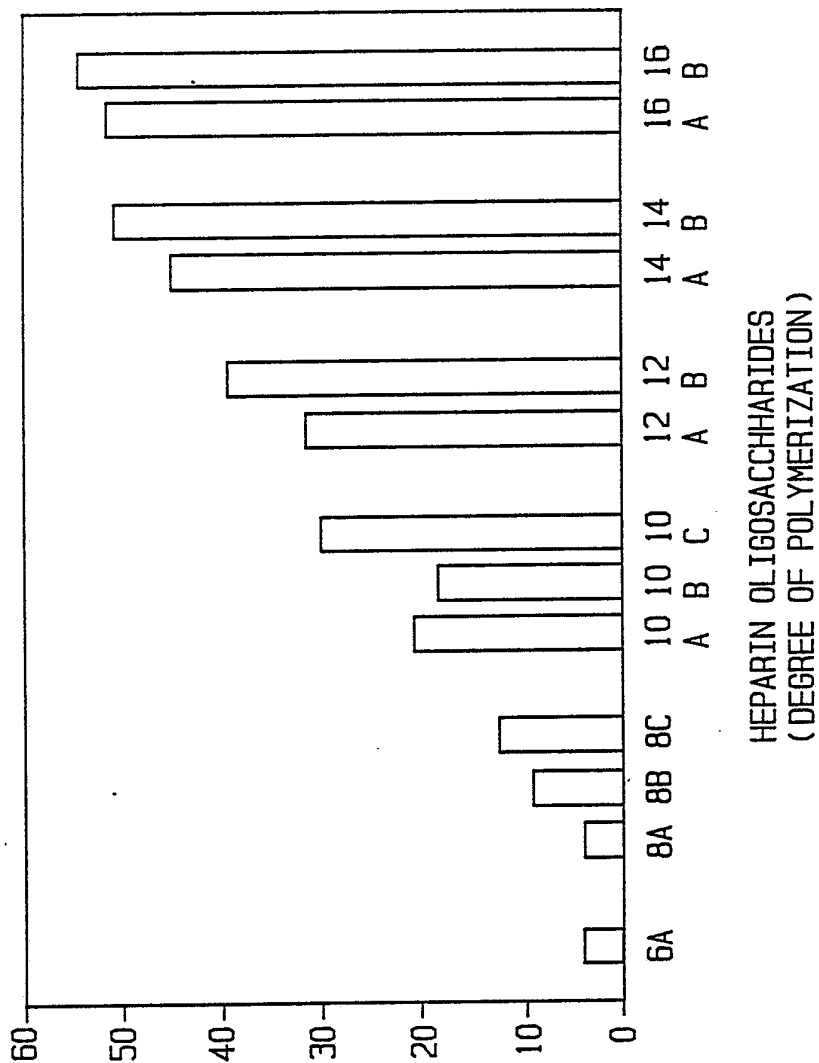


FIG. 2



INHIBITION OF COMPLEMENT ACTIVATION
[(HEPARIN ED50/
OLIGOSACCHARIDE ED50]X 100)

FIG. 3

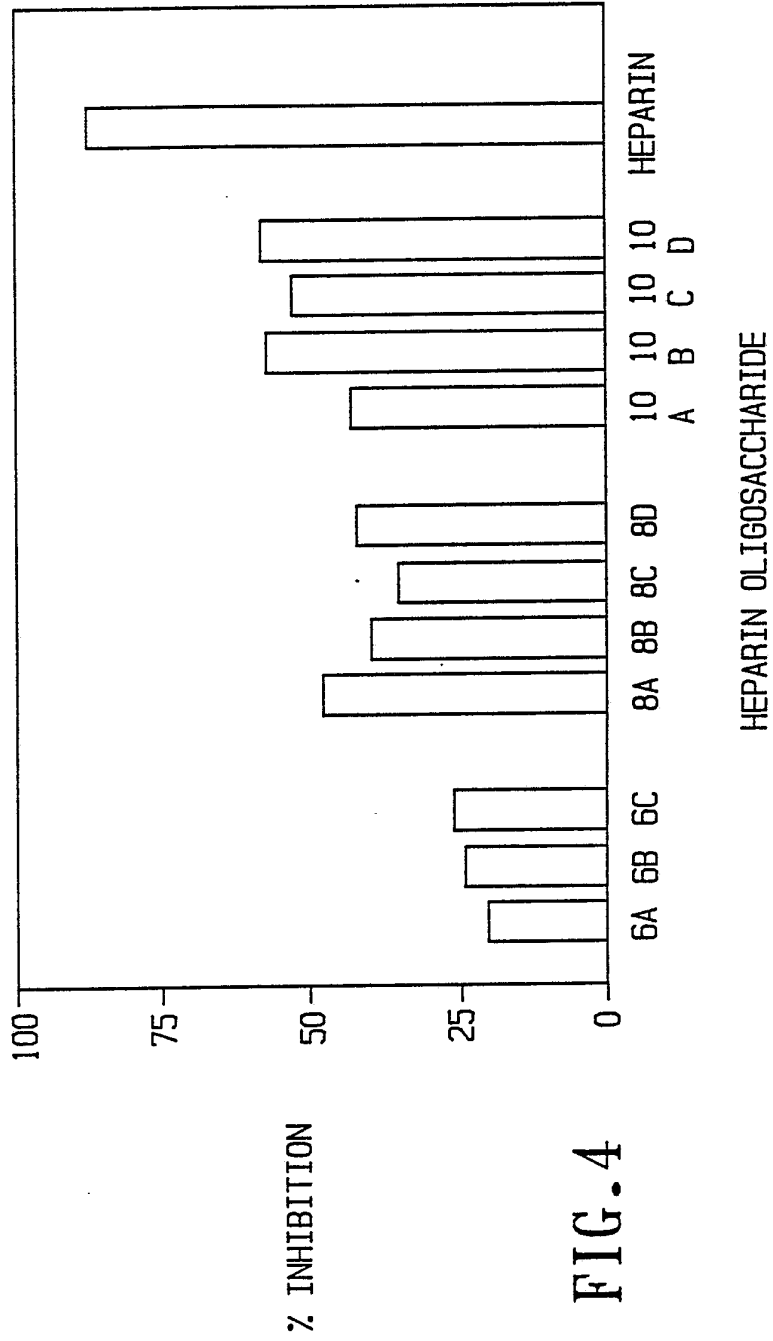



FIG. 4

% INHIBITION

HEPARIN OLIGOSACCHARIDE

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/03312

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC ⁵ : C 08 B 37/10; A 61 K 31/725, C 12 P 19/26		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
IPC ⁵	C 08 B, C 07 H, A 61 K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
P, X	US, A, 4847338 (LINHARDT et al.) 11 July 1989, see the whole document --	1-3
P, X	The Journal of Biological Chemistry, vol. 263, no. 26, 15 September 1988, the American Society for Biochemistry and Molecular Biology (US), R.J. Linhardt et al.: "Homogeneous, structurally defined heparin-oligosaccharides with low anticoagulant activity inhibit the generation of the amplification pathway C3 convertase in vitro", pages 13090-13096, see the whole article --	1-5, 10
X	Analytical Biochemistry, vol. 150, no. 2, 1 November 1985, Academic Press, Inc. (San Diego, US), K.G. Rice et al.: "High-performance liquid chromatographic separation of heparin-derived oligosaccharides", ./.	1-5
<p>⁹ Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
27th November 1989		10. 01. 90
International Searching Authority		Signature of Authorized Officer
EUROPEAN PATENT OFFICE		 L. ROSSI

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	pages 325-331, see page 327, figure 1 --	
A	The Biochemical Journal, vol. 244, 1987, The Biochemical Society (London, GB), K.G. Rice et al.: "Fractionation of heparin-derived oligosaccharides by gradient polyacrylamide-gel electrophoresis", pages 515-522 cited in the application -----	1

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

US 8903312

SA 30428

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 22/12/89. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 4847338	11-07-89	None	