Carbohydrate Polymers 94 (2013) 647–654

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

A heparin-like compound isolated from a marine crab rich in glucuronic acid 2-*O*-sulfate presents low anticoagulant activity

Giulianna P.V. Andrade[a], Marcelo A. Lima[b,c], Airton A. de Souza Junior[b,d], Jawed Fareed[e], Debra A. Hoppensteadt[e], Elizeu A. Santos[a], Suely F. Chavante[a], Fernanda W. Oliveira[a], Hugo A.O. Rocha[a], Helena B. Nader[b,*]

[a] *Departamento de Bioquímica, Universidade Federal do Rio Grande do Norte, Natal, RN, Brazil*
[b] *Departamento de Bioquímica, Universidade Federal de São Paulo, São Paulo, SP, Brazil*
[c] *Department of Structural and Chemical Biology, University of Liverpool, Crown Street, Liverpool L69 7ZB, UK*
[d] *Departamento de Biologia, Instituto Federal do Rio Grande do Norte, Natal, RN, Brazil*
[e] *Department of Pathology, Loyola Medical School, Maywood, IL, USA*

ARTICLE INFO

Article history:
Received 27 November 2012
Received in revised form 21 January 2013
Accepted 22 January 2013
Available online 29 January 2013

In honor of Prof. Carl P. Dietrich.

Keywords:
Heparin
Heparan sulfate
Nuclear magnetic resonance
Heparinases
Anticoagulant activity
Hemorrhagic effect

ABSTRACT

A natural heparin-like compound isolated from the crab *Goniopsis cruentata* was structurally characterized and its anticoagulant and hemorrhagic activities were determined. Enzymatic and nuclear magnetic resonance analysis revealed that its structure is rich in disulfated disaccharides, possessing significant amounts of 2-*O*-sulfated-β-D-glucuronic acid units. Furthermore, low amounts of trisulfated disaccharide units containing 2-*O*-sulfated-α-L-iduronic acid were detected, when compared to mammalian heparin. In addition, this heparin-like structure showed negligible *in vitro* anticoagulant activity and low bleeding potency, facts that make it a suitable candidate for the development of structure-driven, heparin based therapeutic agents with fewer undesirable effects.

1. Introduction

Heparin and low molecular weight heparins are the main anticoagulant and antithrombotic drugs currently used in medicine. Besides its well-described anticoagulant/antithrombotic actions, heparin and heparin-like molecules are known to interact with multiple proteins modulating several biological processes (Brito et al., 2008; Dreyfuss et al., 2010; Paredes-Gamero et al., 2010), however, its further clinical use is impaired by its strong anticoagulant activity and hemorrhagic complications.

Heparin and heparan sulfate share structural features, yet, they can be differentiated by the levels of glucosamine *N*-acetylation, total sulfate and glucuronic/iduronic acid ratio (Casu, Naggi, & Torri, 2010). Furthermore, heparan sulfates are ubiquitous components of all tissue-organized animal life forms (Cassaro & Dietrich, 1977; Medeiros et al., 2000; Sampaio et al., 2006; Toledo & Dietrich, 1977), whereas heparin shows a peculiar distribution in mammalian and other vertebrates, as well as in invertebrates (Nader, Lopes, Rocha, Santos, & Dietrich, 2004).

In invertebrates, heparin is found in some species of mollusk, crustacean, annelid, echinodermate, tunicate and urochordate life (Cassaro & Dietrich, 1977; Cavalcante et al., 2000; Dietrich et al., 1985; Luppi, Cesaretti, & Volpi, 2005; Medeiros et al., 2000; Pejler et al., 1987; Sampaio et al., 2006). In some invertebrates, the presence of heparin-like structures with similarities to heparin but, with some structural peculiarities, have been described (Brito et al., 2008; Chavante et al., 2000; Dietrich et al., 1999a; Nader et al., 2004). These previous studies have shown that their structures vary according to the species and that such differences reside, mainly, in the relative abundance of the different disaccharide units (Nader et al., 2004). Additionally, these heparin-like compounds show variable biological activities (Boucas et al., 2006; Brito et al., 2008; Cassaro & Dietrich, 1977; Chavante et al., 2000; Dietrich et al., 1999a; Dreyfuss et al., 2010; Medeiros et al., 2000; Santos et al., 2007). Thus, each heparin/heparin-like compound from invertebrate tissues tends to be a hitherto unknown compound with unique structural features and a potential novel therapeutic agent.

* Corresponding author at: Molecular Biology, Departamento de Bioquímica, Universidade Federal de São Paulo, Rua Três de Maio, 100, CEP 04044-020, São Paulo, SP, Brazil. Tel.: +55 11 5570 3175; fax: +55 11 5573 6407.
E-mail address: hbnader.bioq@epm.br (H.B. Nader).

0144-8617
http://dx.doi.org/10.1016/j.carbpol.2013.01.069

In the present manuscript, the isolation, structural characterization and anticoagulant activity of an unusual heparin-like compound present in tissues of the crab *Goniopsis cruentata* are described.

2. Materials and methods

2.1. Materials

Heparan sulfate from bovine pancreas and heparin from bovine intestinal mucosa were gifts from the late Dr. P. Bianchini (Opocrin Research Laboratories, Corlo, Modena, Italy). Heparin from porcine intestinal mucosa was obtained from Kin Master (Passo Fundo, RS, Brazil) and enoxaparin (low molecular weight heparin) from Sanofi-Aventis (Maison-Alford, France). Chondroitin 4- and 6-sulfate and dermatan sulfate were purchased from Seikagaku Kogyo (Tokyo, Japan). Heparinase (Heparinase I, EC 4.2.2.7), heparitinases I and II were prepared from *Flavobacterium heparinum* as previously described (Nader et al., 1990). Ethylenediamine (1,2-diaminoethane) and propylenediamine (1,3-diaminopropane) were purchased from Sigma–Aldrich Co. (Milwaukee, WI, USA). Low-Mr agarose was purchased from Bio-Rad (Richmond, CA, USA). Maxatase, a protease from *Sporobacillus*, was purchased from Biocon do Brasil Industrial Ltd. (Rio de Janeiro, RJ, Brazil).

2.2. Extraction and purification of crab heparin-like

The Animal Ethics Advisory Committee approved all experiments involving animals in accordance to the Brazilian Federal Law (11,794/2008) for the use and care of animals for scientific purposes. Adult specimens of *G. cruentata* (Latreill, 1803) were collected at Potengi river estuary (Macaíba, RN, Brazil), immediately killed and stored at −20 °C. Sulfated glycosaminoglycans were extracted after proteolysis and ion exchange fractionation. Ten kilograms of crab were ground with 2 volumes of cold 0.5 M NaCl in a blender. The pH of the mixture was adjusted to 8.0 with NaOH and Maxatase was added (3.5 mg/kg wet weight). After incubating for 24 h at 60 °C, with agitation and periodic pH adjustments, the mixture was filtered and Lewatit ion exchange resin (Bayer, SP, Brazil) was added (60 mg/kg wet weight), and the resulting mixture was agitated for 24 h at 60 °C under a layer of toluene. The suspension was again filtered and the resin retained, washed with 10 volumes of water at 60 °C and, subsequently, washed with 10 volumes of 0.5 M NaCl at room temperature. The washed resin was then suspended in 2 volumes of 1 M NaCl, agitated for 3 h and filtered again. This procedure was repeated using with 2 and 3 M NaCl. The filtrates were maintained for 24 h at 4 °C after the addition of 2 volumes of methanol and the precipitate formed was collected by centrifugation (10,000 × *g*, 20 min), dried under vacuum, suspended in distilled water, and analyzed by agarose gel electrophoresis. Fractions eluted with 2 and 3 M NaCl, which showed the presence of compounds migrating as heparin and heparan sulfate were pooled, dialyzed and dried. These compounds were further dissolved in 0.15 M NaCl and 0.5 volumes of ice-cold acetone was added to the solution under gentle agitation and maintained at 4 °C for 24 h. The precipitate was collected by centrifugation at 4 °C. This procedure was repeated successively by addition to the supernatant of 0.6, 0.7, 0.8, 0.9, 1.0 and 2.0 volumes of acetone according to the volume of the initial solution. The resulting precipitates were dialyzed, dried and analyzed. Fractions precipitated with 0.6 volumes and 0.7 volumes of acetone corresponded to 90% of the total heparin-like compound.

2.3. Agarose gel electrophoresis

Agarose gel electrophoresis were performed in 0.6% agarose gels (7.5 cm × 10 cm, 0.2 cm thick) prepared in three different buffers: 0.05 M 1,3-diaminopropane–acetate buffer, pH 9.0, discontinuous buffer 0.04 M barium acetate, pH 4.0/0.05 M diaminopropane–acetate, pH 9.0 or 0.06 M Tris–acetate buffer, pH 8.0, as previously described (Bianchini et al., 1980). Aliquots of the fractions (about 10 μg) were applied to the gel and subjected to electrophoresis. The gels were fixed with 0.1% cetyltrimethylammonium bromide (CETAVLON) solution for 4 h, dried and stained for 15 min with 0.1% toluidine blue in 1% acetic acid in 50% ethanol and further destained with the same solution without the dye. Quantification was carried out by densitometry at 530 nm of the toluidine blue-stained electrophoretic slide. The extinction coefficients of the GAGs were calculated using standards of chondroitin sulfate (CS), dermatan sulfate (DS) and heparan sulfate (HS). The error of the method was in the order of 5%. Identification of the sulfated GAGs was initially based on the migration of the compounds compared with those of standards.

2.4. Enzymatic degradation

Samples were incubated with different heparin lyases (heparinase I, heparitinase I and II, 2.5 mIU each) as well as with a mixture of all enzymes. The disaccharides produced by the enzymatic action were resolved on a 150 × 4.6 mm Phenosphere SAX column (Phenomenex, Torrance, CA, USA) using a NaCl gradient of 0–1 M during 30 min with a 1 mL/min flux and UV detection at 232 nm.

2.5. NMR spectroscopy

NMR spectra were recorded either using a Bruker DRX 600 with a triple resonance 5-mm probe or in an Agilent 600 MHz System with 5-mm Cold Probe. The spectra were recorded at either 60 or 25 °C with HOD suppression by pre-saturation. COSY, TOCSY and $^{1}H/^{13}C$ heteronuclear correlation (HSQC) spectra were recorded using states-time proportion phase increment for quadrature detection in the indirect dimension. All chemical shifts were relative to external trimethylsilyl-propionic acid and [^{13}C]-methanol.

2.6. In vitro anticoagulant activity

All coagulation assays (aPTT, PT, TT and HEPTEST®) were performed using a coagulometer as described earlier (Dietrich et al., 1999a) using citrated normal human plasma. All assays were performed in duplicate and repeated at least three times on different days ($n = 6$). Generation of thrombin and factor Xa was measured by amidolytic assays using the specific chromogenic substrates (Spectrozyme TH and Spectrozyme FXa, American Diagnostica Inc., Stamford, CT) according to a method previously described (Kaiser et al., 1992). All assays were performed on a fast kinetic centrifugal analyzer (ACL-300, Lexington, MA, USA).

2.7. Hemorrhagic effect

Hemorrhagic activity of the heparin-like compound was measured in a rat-tail model as previously described (Dietrich et al., 1999b). Following anesthesia with nembutal (40 mg/kg) and urethane (0.8 g/kg), scarification with a razor blade (1–2 mm deep, 5 mm long) was made 15 mm from the distal part of the rat tail (males, three months old). The tail was then immersed in isotonic NaCl, scraped with gauze, and immersed again in fresh saline to observe bleeding. Normal bleeding ranged from 30 to 60 s. Grazed tails were then immersed in saline solution containing the crab heparin-like compound or mammalian heparin (200 μg/mL) for

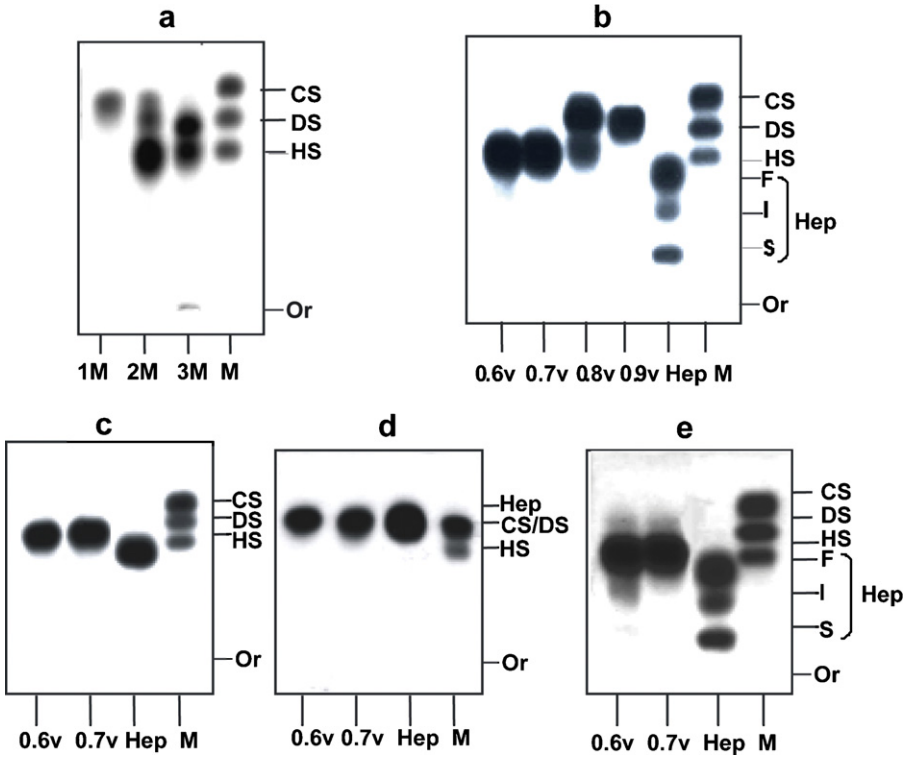

Fig. 1. Electrophoretic behavior of the crab sulfated glycosaminoglycans in agarose gels. About 5–20 μg of the glycosaminoglycans purified from crab tissues were subjected to electrophoresis in different buffers. After the run, the compounds were precipitated and the gels dried and stained with toluidine blue. (a) Fractions eluted from the ion exchange chromatography were evaluated using 1,3-diaminopropane-acetate (PDA). 1 M, 2 M and 3 M fractions were eluted from ion exchange chromatography with 1.0, 2.0 and 3.0 M of NaCl, respectively. (b) Fractions 2 M and 3 M were pooled and fractionated with acetone, and the electrophoretic mobility of the compounds evaluated using the discontinuous buffer barium acetate/PDA. Electrophoretic mobility of the heparin-like compounds precipitated with 0.6 and 0.7 volumes of acetone in different buffers: (c) PDA buffer, (d) tris-acetate and (e) discontinuous buffer barium acetate/PDA. M, mixture of standard sulfated glycosaminoglycans containing 5 μg of chondroitin sulfate (CS), dermatan sulfate (DS) and heparan sulfate (HS); Hep, porcine intestinal mucosa heparin and S, I, F, slow, intermediate and fast moving components, respectively; Or, origin.

2 min and washed extensively with saline. The treated tails were then immersed in isotonic saline solution, and the amount of blood oozed was measured by protein content. The bleeding was observed with the use of a stereoscope. All experiments were performed at 37 °C. The bleeding was calculated as the sum of the protein values of each tube minus the amount of protein present before the exposure to the test substances. Bleeding potency was expressed as the cumulative amount of protein released from the wounds after exposure to the compounds relative to the control (absence of drug).

2.8. Other methods

Hexosamine was determined after acid hydrolysis (4 M HCl, 100 °C, 6 h) by the Rondle–Morgan procedure (Rondle & Morgan, 1955) and uronic acid by the carbazole reaction (Dische, 1947). Total sulfate was measured by a method previously described (Dodgson & Price, 1962). For molecular weight analysis, 300 μg of each sample was analyzed by GPC-HPLC on a 300 mm × 7.8 mm BioSep SEC^{TM} S-2000 LC Column. The samples were submitted to an isocratic elution (0.3 M Na_2SO_4 mobile phase) at a flow rate of 1 mL/min and UV detection at 205 nm. The column was previously calibrated with polysaccharides of known molecular weights (1.7 kDa, 4 kDa, 10 kDa, 16 kDa and 20 kDa).

2.9. Statistical analysis

The SPSS software package (release 16.0; SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The difference between the groups was evaluated using the non-parametric two-tailed Mann–Whitney *U*-test. $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Purification of the crab heparin-like compound

A natural heparin-like compound was obtained from the crab *G. cruentata* after proteolysis, ion-exchange chromatography and acetone fractionation. The fractions eluted with 2 M and 3 M NaCl were rich in compounds with electrophoretic characteristics of heparin/heparan sulfate (Fig. 1a). Consequently, these fractions were pooled and fractionated by stepwise addition of different volumes of acetone (Fig. 1b). The fractions precipitated with 0.6 and 0.7 volumes of acetone showed similar electrophoretic mobility in the diamino-propane-acetate (PDA) buffer. Hence, the purity and similarity of these fractions were further confirmed by agarose gel electrophoresis using three different buffers. In PDA buffer, these fractions presented a single band with electrophoretic mobility between heparan sulfate and dermatan sulfate (Fig. 1c). In contrast, in Tris–acetate buffer they showed electrophoretic mobility similar to heparin (Fig. 1d). Furthermore, in the discontinuous system barium/PDA, while heparin was fractionated into 3 different components (Bianchini et al., 1980), the crab heparin-like compounds showed one main component with higher migration than the heparin fast moving band (Fig. 1e). Such electrophoretic behavior in different buffers is the preliminary indication that the structural characteristics of the crab heparin-like compound (sulfation pattern and/or glucuronic and iduronic acid) are different to heparin and other sulfated GAGs. Chavante et al. (2000) isolated, from the

Table 1
Relative Hepn disaccharide composition.

Percentage (%)							
ΔUA-NAc	ΔUA-NS	ΔUA-NAc(6S)	ΔUA(2S)-NAc	ΔUA-NS(6S)	ΔUA(2S)-NS	ΔUA(2S)-NAc(6S)	ΔUA(2S)-NS(6S)
≤1	45.9	8.1	7.5	5.1	18.0	3.0	12.0

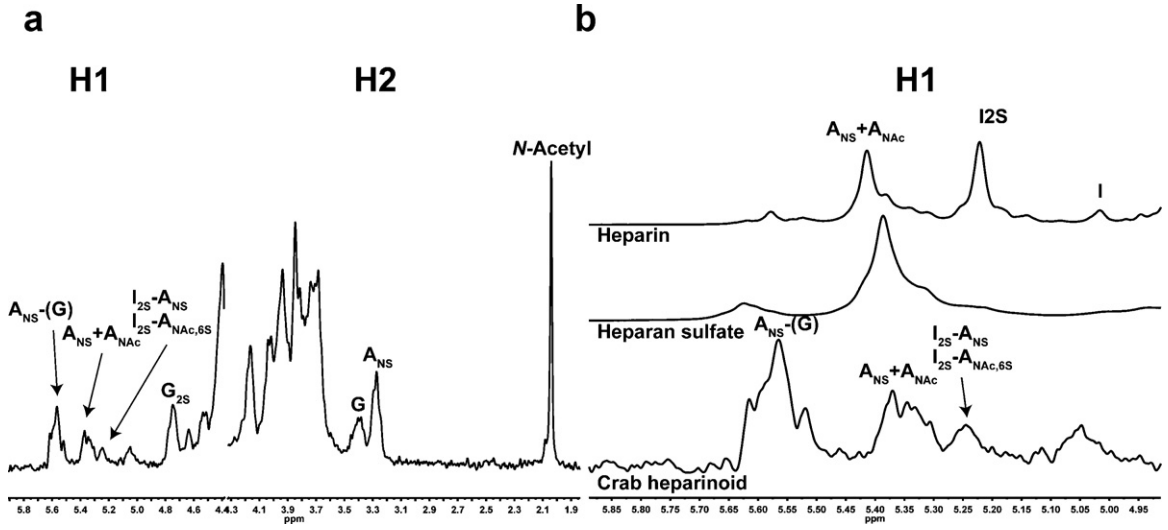

Fig. 2. ^{1}H spectra at 600 MHz of the crab heparin-like, heparan sulfate and heparin recorded at 60 °C and 25 °C respectively. (a) Crab heparin-like compound (Hepn) ^{1}H spectrum; (b) ^{1}H spectra anomeric region of crab Hepn, heparan sulfate from bovine pancreas and heparin from porcine intestinal mucosa. A_{NS}, *N*-sulfated glucosamine; A_{NAc}, *N*-acetylated glucosamine; G, glucuronic acid; G_{2S}, 2-*O*-sulfated glucuronic acid; I_{2S}, 2-*O* sulfated iduronic acid; I, iduronic acid; G, glucuronic acid.

crustacean *Artemia franciscana*, an unusual heparan sulfate with similar electrophoretic behavior to the crab compound that presented a high degree of *N*-sulfation. On the other hand, a compound from the shrimp *Penaeus brasiliensis* with similar electrophoretic behavior was isolated and characterized as a natural low molecular weight heparin (Dietrich et al., 1999a).

3.2. Chemical analysis and molecular mass

The molecular mass and the molar ratios of hexosamine, uronic acid and sulfate present in the heparin-like compounds, mammalian heparin and heparan sulfate were also analyzed. The crab compounds showed an average molecular mass (~19 kDa) similar to porcine heparin (~16 kDa) and lower when compared to bovine heparan sulfate (~25 kDa). Furthermore, both compounds showed similar molar ratio of sulfate/hexosamine of 2.1:1.0, respectively. These ratios are between the values described for heparan sulfate (sulfate/hexosamine, 1.6:1.0) (Dietrich & Nader, 1974) and heparin (sulfate/hexosamine, 2.8:1.0) (Dietrich et al., 1985). Neutral sugars were not detected. Overall, the data suggested that the two fractions corresponded to the same compound. The two fractions were pooled and hereafter is referred to as Hepn.

3.3. Enzymatic analysis

Some structural characteristics of the Hepn were examined with enzymes purified from *F. heparinum*. Heparitinase I, which acts upon *N*-acetyl or *N*-sulfate glucosamine-glucuronic acid linkages (Desai, Wang, & Linhardt, 1993; Dietrich et al., 1999a; Silva & Dietrich, 1974), degraded less than 5% of the

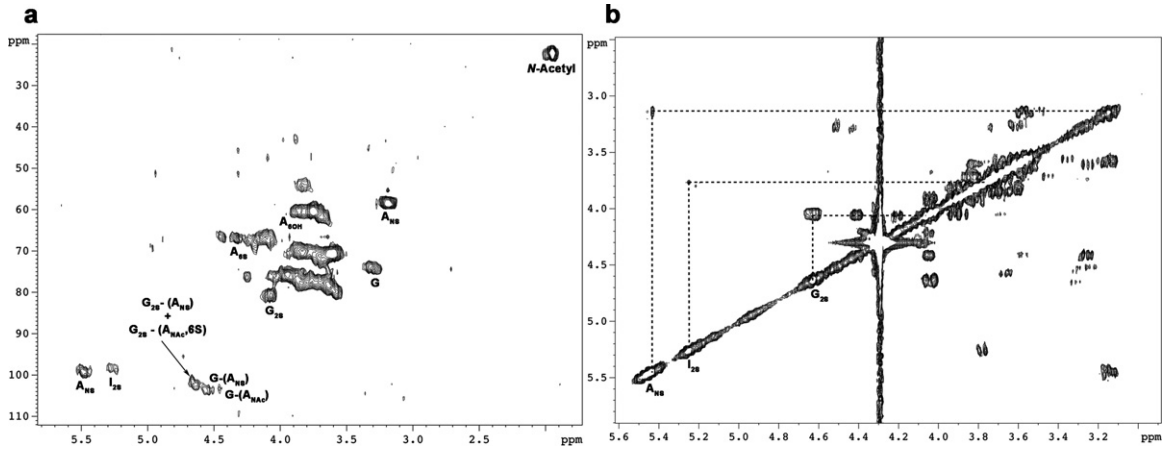

Fig. 3. ^{1}H/^{13}C HSQC (b) and ^{1}H/^{1}H COSY (b) spectra of the crab heparin-like compound recorded at 60 °C. A_{NS}, *N*-sulfated glucosamine; A_{NAc}, *N*-acetylated glucosamine; G, glucuronic acid; G_{2S}, 2-*O*-sulfated glucuronic acid; I_{2S}, 2-*O* sulfated iduronic acid.

Table 2
^{1}H chemical shifts for the heparin-like compound of the crab *G. cruentata*.

Unit	^{1}H chemical shifts[a]										
	Uronic acid					Glucosamine					
	H1	H2	H3	H4	H5	H1	H2	H3	H4	H5	H6
[a](β-GlcA)→(α-D-GlcNS-6S) (I)→(A)	4.60	3.31	3.71	3.88	3.79	5.57	*3.18*	3.65	3.85	4.04	*4.14*
[b]β-GlcA→α-D-GlcNS-6S	4.58	3.36	3.82	3.84	3.78	5.56	*3.27*	3.67	3.70	3.96	*4.37/4.17*
[a](β-GlcA)→(α-D-GlcNS) (I′)→(A′)	4.58	3.28	3.70	3.88	3.79	5.57	*3.18*	3.65	3.65	3.75	3.80
[b]β-GlcA→α-D-GlcNS	4.55	3.41	3.83	3.90	3.83	5.58	*3.27*	3.68	3.68	3.79	3.81/3.83
[a](β-GlcA-2S)→(α-D-GlcNS) (H)→(B)	4.67	*4.12*	~4.03	~3.97	–	5.55	*3.24*	3.62	3.86	3.72	3.92
[c]β-GlcA-2S→α-D-GlcNS	4.69	*4.12*	3.99	3.83	–	5.45	*3.23*	3.75	3.64	3.93	3.88
[a](β-GlcA-2S)→(α-D-GlcNS-6S)(H′)→(B′)	4.70	*4.12*	~4.03	~3.97	–	5.55	*3.24*	3.62	3.86	4.04	*4.14*
[b]β-GlcA-2S→α-D-GlcNS-6S	4.74	*4.14*	3.98	3.85	3.88	5.44	–	–	–	–	–
[a](α-IdoA-2S)→(α-D-GlcNS-6S) (F)→(C)	5.20	*4.28*	4.22	4.08	5.01	5.48	*3.19*	–	–	–	*~4.40*
[d]α-IdoA-2S→α-D-GlcNS-6S	5.23	*4.37*	4.22	4.14	4.82	5.42	*3.31*	3.69	3.79	4.05	*4.42/4.30*
[a](β-GlcA-2S)→(α-D-GlcNAc-6S)(G)→(D)	4.73	*4.08*	–	–	–	5.32	3.83	3.92	3.77	4.15	*4.25*
[e]β-GlcA-2S→α-D-GlcNAc-6S	4.76	*4.14*	3.92	–	–	5.42/5.32	4.03/3.93	3.85	3.57	4.04	*4.48/4.22*
[e](β-GlcA)→(α-D-GlcNAc-6S) (G′)→(D′) [f]β-GlcA→α-D-GlcNAc	4.52	3.34	3.65	3.78	3.82	5.32	3.80	–	–	4.15	*4.47*
	4.64	3.41	3.66	3.75	–	5.35	3.91	3.75	3.70	3.99	–
[a](β-GlcA)→(α-D-GlcNAc) (J)→(E)	4.49	3.34	3.65	3.78	3.82	5.28	3.85	3.98	3.48	3.90	3.89
[b]β-GlcA→α-D-GlcNAc	4.48	3.37	3.69	3.78	3.78	5.36	3.89	3.86	3.64	3.82	3.84/3.84

Values in italic type indicate positions bearing a sulfate ester. A to F, spin systems.
[a] Data presented in this paper.
[b] Casu et al. (1994).
[c] Yamada et al. (1995).
[d] Yates et al. (1996).
[e] Guerrini et al. (2005).
[f] Yamada et al. (1999).

crab heparin-like product, producing few *N*-acetylated and *N*-sulfated disaccharides linked to non-sulfated glucuronic acid (ΔUGlcA-GlcNS and ΔUGlcA-GlcNAc). On the other hand, heparinase degraded the crab compound producing the same types of products obtained from the mammalian heparin, although in different proportions. This enzyme acts upon glycosidic linkages containing α-D-glucosamine-*N*-sulfate linked to α-L-iduronic acid-2-sulfate and does not act when the uronic acid is glucuronic acid 2-*O* or 3-*O* sulfate, or when the glucosamine is *N*-acetylated (Nader et al., 1999). Thus, an important difference between mammalian heparin and the crab polymer is its lower level of trisulfated disaccharides. In addition, the high levels of hexa- and tetra-saccharides present indicated oligosaccharide blocks that are resistant to the action of heparinase. Furthermore, heparitinase II which acts upon glucosaminido-glucuronic acid linkages where the *N*-acetyl or *N*-sulfate glucosamine is preferentially

Table 3
^{13}C chemical shifts for the heparin-like compound of the crab *G. cruentata*.

Unit	^{13}C chemical shifts[a]										
	Uronic acid					Glucosamine					
	C1	C2	C3	C4	C5	C1	C2	C3	C4	C5	C6
[a](β-GlcA)→α-D-GlcNS-6S (I)→(A)	105.0	76.2	80.1	79.8	80.1	102.0	*61.2*	73.1	81.2	70.0	*69.0*
[b]β-GlcA→α-D-GlcNS-6S	105.5	76.4	79.5	79.8	80.3	100.8	*61.1*	73.1	80.6	72.3	*69.5*
[a](β-GlcA)→(α-D-GlcNS) (I)→(A′)	105.0	76.2	80.1	79.8	80.1	101.5	*61.2*	73.1	82.1	74.5	63.2
[b]β-GlcA→α-D-GlcNS	105.1	75.6	78.7	78.8	79.3	99.9	*60.5*	72.4	80.7	73.3	62.4
[a](β-GlcA-2S)→(α-D-GlcNS) (H)→(B/B′)	103.8	*83.0*	77.8	77.8	–	101.8	*61.0*	73.0	72.0	70.0	62.0
[a](β-GlcA-2S)→(α-D-GlcNS-6S)(H)→(B/B′)	103.8	*83.0*	–	–	–	101.8	*61.0*	73.0	72.0	74.2	*69.0*
[b]β-GlcA-2S→α-D-GlcNS-6S	102.8	*82.3*	77.3	79.1	79.5	100.9	–	–	–	–	–
[a](α-IdoA-2S)→(α-D-GlcNS-6S) (F)→(C)	101.8	*78.8*	72.0	79.5	71.8	100.5	*~61*	–	–	–	*70.1*
[d]α-IdoA-2S→α-D-GlcNS-6S	102.1	*78.9*	72.1	79.0	72.3	99.5	*60.7*	72.5	78.8	72.0	*69.2*
[a](β-GlcA-2S)→(α-D-GlcNAc-6S) (G)→(D)	103.8	*82.8*	–	–	–	100.8	56.4	–	–	–	*71.7*
[e]β-GlcA-2S→α-D-GlcNAc-6S	103.0	*82.4*	77.4	78.8/79.5	79.2/79.5	99.8	56.0	72.1	80.5	72.0	*68.8*
[a](β-GlcA)→(α-D-GlcNAc-6S) (G′)→(D′)	105.5	76.2	79.0	78.9	79.7	100.8	56.8	–	–	69.8	*70.0*
[a](β-GlcA)→(α-D-GlcNAc) (J)→(E)	105.5	76.2	79.0	78.9	79.7	101.7	56.7	77.9	73.2	72.1	63.0
[b]β-GlcA→α-D-GlcNAc	105.2	76.3	78.9	79.1	79.1	99.6	56.1	73.5	81.1	72.0	62.2

Values in italic type indicate positions bearing a sulfate ester. A to F, Spin systems.
[a] Data presented in this paper.
[b] Casu et al. (1994).
[c] Yamada et al. (1995).
[d] Yates et al. (1996).
[e] Guerrini et al. (2005).

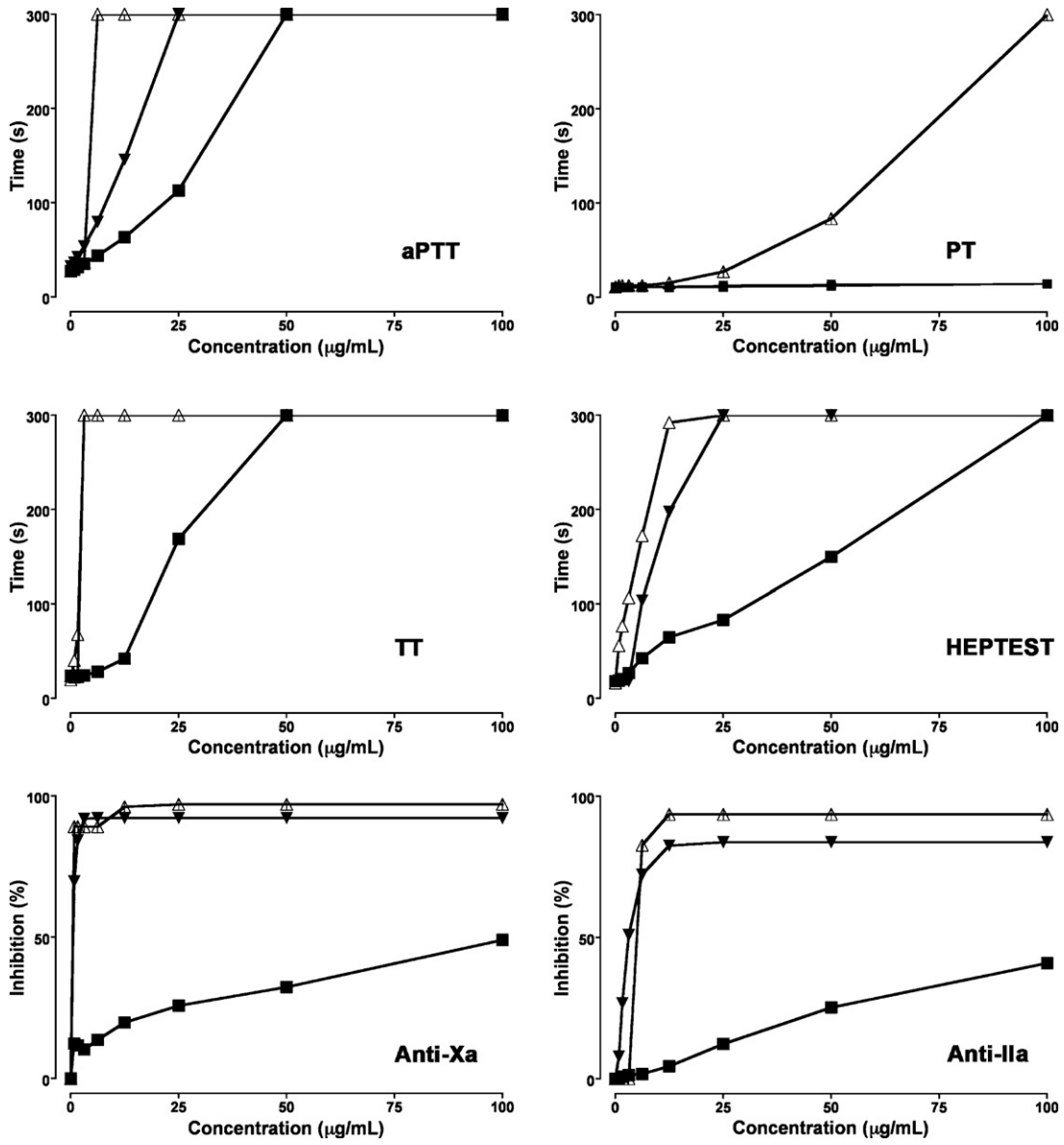

Fig. 4. Effect of the crab heparin-like compound on coagulation using different *in vitro* assays. (■), crab Hepn; (Δ),UFH; (▼), enoxaparin (low molecular weight heparin). Results in aPPT and anti-IIa are statistically different for the 3 compounds ($P < 0.5$). Significant differences ($P < 0.5$) in PT, TT, HEPTEST and anti-Xa between Hepn and heparin, as well as Hepn and enoxaparin.

sulfated at the C-6 position (Dietrich et al., 1999a; Nader et al., 1990), produced mainly disulfated disaccharides (ΔUA-GlcNS,6S/ ΔUA,2S-GlcNAc,6S/ ΔUA,2S-GlcNS). Since the use of individual enzymes led to the formation of oligosaccharides, a mixture of all three lyases was then used to ascertain the total disaccharide composition of the Hepn (Table 1). This result contrasts with data obtained for heparin, where about 80% all of disaccharides are the ΔUA,2S-GlcNS,6S, as well as for heparan sulfates where around 50–60% of all disaccharides are ΔUA-GlcNAc/ΔUA-GlcNS (Dietrich et al., 1998; Zhang, Xie, Liu, Liu, & Linhardt, 2009).

3.4. NMR spectroscopy

The ^{1}H NMR spectrum for Hepn is shown in Fig. 2a. The signal at 5.22 ppm from H-1 of 2-*O*-sulfated iduronic acid was found in the crab heparin-like product. The other anomeric protons showed two main regions from 5.28 to 5.57 ppm and 4.5 to 5.2 ppm that correspond to anomeric protons of the hexosamine and uronic acid residues, respectively (Yates et al., 1996). The signal at 2.04 ppm due to the acetyl groups was prominent in the Hepn. Fig. 2b shows the anomeric region for porcine heparin, bovine heparan sulfate and the crab Hepn. It is clear that the same anomeric signals present in heparin and heparan sulfate are present in Hepn. Nevertheless, as in the enzymatic degradation results, they are present in different relative proportions.

Two-dimensional assignment techniques of COSY, TOCSY, NOESY and HSQC were used to trace the spin systems from the heparin-like structure. The HSQC and COSY spectra of the heparin-like compound are shown in Fig. 3. The chemical shifts in Tables 2 and 3 were based on the interpretations of these spectra. The distribution of the spin systems can be detected by the presence of more than one H/C correlation signal in the anomeric region of the HSQC spectrum (Fig. 3a). Thus, several spin systems were traced (see Tables 2 and 3). Signals at 4.60/105.0 ppm and at 4.49–4.52/105.5 ppm indicate glucuronic acid residues linked to *N*-sulfated and *N*-acetylated glucosamines, respectively; signals at 4.70/103.1 and at 4.73/104.0 ppm indicate 2-*O*-sulfated glucuronic acid residues linked to *N*-sulfated and *N*-acetylated glucosamines,

respectively, and the signal at 5.2/101.8 ppm indicates 2-*O*-sulfated IdoA linked to *N*-sulfated glucosamine (Guerrini, Naggi, Guglieri, Santarsiero, & Torri, 2005; Yates et al., 1996). Traceable spin systems are also shown on the homonuclear proton–proton COSY spectrum exhibiting the spin–spin coupled protons (Fig. 3b).

The crab heparin-like compound exhibited NMR spectra containing similar characteristics to mammalian heparan sulfate, including a high intensity signal attributed to the acetyl groups and high intensity signals of H-1 from glucuronic acid residues. Additionally, the signals attributed to the anomeric proton of 2-*O*-sulfated iduronic acid residues (5.2 ppm), common to mammalian heparin (Casu et al., 1994; Mulloy et al., 1994; Yates et al., 1996), were also detected, yet, these 2-*O*-sulfated iduronic acid residues are linked either to *N*-sulfated, 6-hydroxyl or *N*-acetylated, 6-*O*-sulfated glucosamine (Fig. 2b). Furthermore, chemical shifts at 4.7–4.73 ppm attributed to the 2-*O*-sulfated glucuronic acid were also present. According to the literature, low amounts of 2-*O*-sulfated glucuronic acid residues are found in natural glycosaminoglycans, but this residue is usually not detectable in unfractionated heparins (Guerrini et al., 2005; Yamada, Murakami, Tsuda, Yoshida, & Sugahara, 1995). To the best of our knowledge, this is the first report of a 2-*O*-sulfated glucuronic acid-rich heparin-like compound.

The NMR signals attributed to *N,3,6*-trisulfated glucosamine residue, a typical marker of the pentasaccharide sequence of the active site of heparin and heparan sulfates for antithrombin binding (Casu et al., 1996; Kusche, Torri, Casu, & Lindahl, 1990; Lindahl et al., 2005; Sie et al., 1988), were not detected in the spectra of the crab heparin-like compound, suggesting that such compound is likely to exhibit reduced anticoagulant activity when compared to heparin.

3.5. *In vitro* anticoagulant assays

In vitro anticoagulant activity of the crab heparin-like compound is shown in Fig. 4. The Hepn exhibited an anticoagulant activity around 33 IU/mg using the activated partial thromboplastin time (aPTT) assay. This method shows that the compound, when compared to heparin (193 IU/mg), is at least 5 times less potent in preventing *in vitro* clot formation by the intrinsic pathway. The crab compound had no effect in the extrinsic pathway, confirmed by the prothrombin time (PT), dramatically contrasting with mammalian heparin. Hepn has no effect on the ability of thrombin to degrade fibrinogen measured by the thrombin time (TT), contrasting once more the effect observed with heparin. The HEPTEST test measures the action of the compounds upon heparin cofactor II. The results indicated that Hepn is at least 12 times less potent than heparin in this assay. Using biochemically defined conditions on a fast kinetic centrifugal analyzer, the effects of the heparin-like, heparin and enoxaparin on thrombin and factor Xa generation were also investigated. It is evident that the heparin-like compound is much less potent in the direct generation of thrombin and factor Xa when compared to heparin and enoxaparin. Consequently, these results indicated that Hepn from *G. cruentata* is a less potent anticoagulant agent than mammalian heparin and LMW-heparin (Fig. 4). This lower anticoagulant activity can be related to its lower degree of sulfation, in particular, the lack of *N,3,6*-trisulfated glucosamine residues, a typical marker of the pentasaccharide sequence active for antithrombin (Lindahl et al., 2005).

3.6. Hemorrhagic effect

Crab Hepn was also tested as a possible inhibitor of hemostasis. The crab compound, like heparin, also disrupted the normal control of bleeding. Nevertheless, the extent of bleeding in the animals exposed to the crab compound was less pronounced than the heparin treatment (Fig. 5). This could be related to the lower content of critical sulfation at the C-6 position of the glucosamine. Studies conducted with disaccharides derived from heparin, heparan sulfate and chondroitin sulfate showed that a sulfate at the C-6 position of the glucosamine is crucial for the antihemostatic activity (Dietrich, Tersariol, Da Silva, Bianchini, & Nader, 1991). The findings that other sulfated disaccharides, with the same sulfate/hexosamine/uronic acid ratios bearing a sulfate at a different position (C-2) or with a different glycosidic linkage (1–3), were inactive as inhibitors of hemostasis indicating that a specific structure is needed for such an effect (Dietrich et al., 1991). On the other hand, this inhibitory activity does not seem to be related to the anticoagulant activity of the compounds (Boucas et al., 2012) since structures with no anticoagulant activity are potent antihemostatic agents (Nader, Tersariol, & Dietrich, 1989).

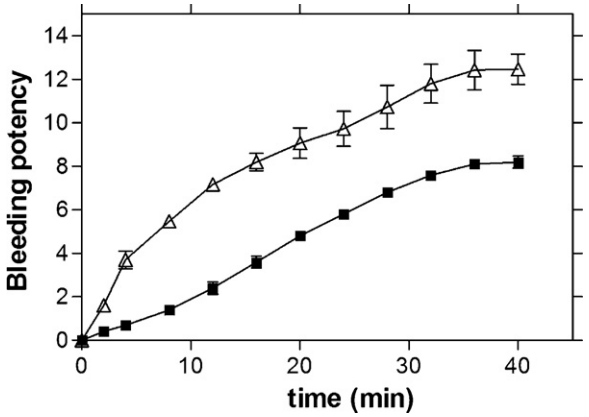

Fig. 5. Bleeding activity of the crab heparin-like compound and porcine intestinal mucosa heparin. (■), Hepn; (△), mammalian heparin. The results for the two compounds are statistically different ($P < 0.5$).

4. Conclusion

In summary, our results indicate that the heparin-like compound isolated from the crab *G. cruentata* present intermediate structure between heparin and heparan sulfate. In addition, the crab heparin-like is rich in 2-*O*-sulfated glucuronic acid residues, possesses low levels of trisulfated disaccharides and lacks the defined pentasaccharide structure related to the antithrombin binding site. To the best of our knowledge, this is the first study characterizing a natural 2-*O*-sulfated glucuronic acid-rich heparin-like glycosaminoglycan using chemical, enzymatic and spectroscopic analyses. Furthermore, concerning some pharmacological activities, it has been demonstrated that, the crab compound showed negligible *in vitro* anticoagulant activity and lower bleeding effect compared to mammalian heparin. Consequently, its unusual structural features, insignificant *in vitro* anticoagulant activity and lower bleeding risk, make this compound a suitable candidate for the development structure-driven heparin based therapeutic agents with less undesirable effects.

Acknowledgments

This work was supported by grants from FAPESP (Fundação de Amparo a Pesquisa do Estado de São Paulo), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior). The authors thank Dr. Ana Paula Valente for the use of the NMR equipment located at the Centro Nacional de Ressonância Nuclear Magnética (UFRJ, Rio de Janeiro, Brazil). NMR data were also obtained at the Advanced Magnetic Resonance Imaging and

Spectroscopy (AMRIS) facility in the McKnight Brain Institute of the University of Florida and supported through the National High Magnetic Field Laboratory.

References

Bianchini, P., Nader, H. B., Takahashi, H. K., Osima, B., Straus, A. H., & Dietrich, C. P. (1980). Fractionation and identification of heparin and other acidic mucopolysaccharide by a new discontinuous eletroforetic method. *Journal of Chromatography*, *196*, 455–462.

Boucas, R. I., Jarrouge-Boucas, T. R., Lima, M. A., Trindade, E. S., Moraes, F. A., Cavalheiro, R. P., et al. (2012). Glycosaminoglycan backbone is not required for the modulation of hemostasis: Effect of different heparin derivatives and non-glycosaminoglycan analogs. *Matrix Biology*, *31*(5), 308–316.

Boucas, R. I., Sampaio, L. O., Andrade, G. P. V., Lopes, C. C., Nascimento, F. D., Tersariol, I. L. S., et al. (2006). Heparin and heparin derivatives and their effect on hemostasis. In H. Verli (Ed.), *Insights into carbohydrate structure and biological function* (pp. 25–50). Kerala, India: Transworld Research Network.

Brito, A. S., Arimateia, D. S., Souza, L. R., Lima, M. A., Santos, V. O., Medeiros, V. P., et al. (2008). Anti-inflammatory properties of a heparin-like glycosaminoglycan with reduced anti-coagulant activity isolated from a marine shrimp. *Bioorganic and Medicinal Chemistry*, *16*(21), 9588–9595.

Cassaro, C. M., & Dietrich, C. P. (1977). Distribution of sulfated mucopolysaccharides in invertebrates. *Journal of Biological Chemistry*, *252*(7), 2254–2261.

Casu, B., Grazioli, G., Razi, N., Guerrini, M., Naggi, A., Torri, G., et al. (1994). Heparin-like compounds prepared by chemical modification of capsular polysaccharide from *E. coli* K5. *Carbohydrate Research*, *263*(2), 271–284.

Casu, B., Guerrini, M., Naggi, A., Torri, G., De-Ambrosi, L., Boveri, G., et al. (1996). Characterization of sulfation patterns of beef and pig mucosal heparins by nuclear magnetic resonance spectroscopy. *Arzneimittel-Forschung*, *46*(5), 472–477.

Casu, B., Naggi, A., & Torri, G. (2010). Heparin-derived heparan sulfate mimics to modulate heparan sulfate–protein interaction in inflammation and cancer. *Matrix Biology*, *29*(6), 442–452.

Cavalcante, M. C., Allodi, S., Valente, A. P., Straus, A. H., Takahashi, H. K., Mourao, P. A., et al. (2000). Occurrence of heparin in the invertebrate styela plicata (Tunicata) is restricted to cell layers facing the outside environment. An ancient role in defense? *Journal of Biological Chemistry*, *275*(46), 36186–36189.

Chavante, S. F., Santos, E. A., Oliveira, F. W., Guerrini, M., Torri, G., Casu, B., et al. (2000). A novel heparan sulphate with high degree of N-sulphation and high heparin cofactor-II activity from the brine shrimp *Artemia franciscana*. *International Journal of Biological Macromolecules*, *27*(1), 49–57.

Desai, U. R., Wang, H. M., & Linhardt, R. J. (1993). Specificity studies on the heparin lyases from *Flavobacterium heparinum*. *Biochemistry*, *32*(32), 8140–8145.

Dietrich, C. P., de Paiva, J. F., Moraes, C. T., Takahashi, H. K., Porcionatto, M. A., & Nader, H. B. (1985). Isolation and characterization of a heparin with high anticoagulant activity from *Anomalocardia brasiliana*. *Biochimica et Biophysica Acta*, *843*(1-2), 1–7.

Dietrich, C. P., & Nader, H. B. (1974). Fractionation and properties of four heparitin sulfates from beef lung tissue. Isolation and partial characterization of a hemogeneous species of heparitin sulfate. *Biochimica et Biophysica Acta*, *343*(1), 34–44.

Dietrich, C. P., Paiva, J. F., Castro, R. A., Chavante, S. F., Jeske, W., Fareed, J., et al. (1999). Structural features and anticoagulant activities of a novel natural low molecular weight heparin from the shrimp *Penaeus brasiliensis*. *Biochimica et Biophysica Acta*, *1428*(2-3), 273–283.

Dietrich, C. P., Shinjo, S. K., Moraes, F. A., Castro, R. A., Mendes, A., Gouvea, T. C., et al. (1999). Structural features and bleeding activity of commercial low molecular weight heparins: Neutralization by ATP and protamine. *Seminars in Thrombosis and Hemostasis*, *25*(Suppl 3), 43–50.

Dietrich, C. P., Tersariol, I. L., Da Silva, R. G., Bianchini, P., & Nader, H. B. (1991). Dependence of the C-6 sulfate of the glucosamine moiety and 1–4 glycosidic linkage of heparin disaccharides for production of hemorrhage: Reversal of the antihemostatic activity of heparin and their fragments by adenosine triphosphate and myosin. *Seminars in Thrombosis and Hemostasis*, *17*(Suppl 1), 65–73.

Dietrich, C. P., Tersariol, I. L., Toma, L., Moraes, C. T., Porcionatto, M. A., Oliveira, F. W., et al. (1998). Structure of heparan sulfate: Identification of variable and constant oligosaccharide domains in eight heparan sulfates of different origins. *Cell and Molecular Biology (Noisy-le-grand)*, *44*(3), 417–429.

Dische, Z. A. (1947). A new specific color reaction of hexuronic acid. *Journal of Biological Chemistry*, *167*, 189–193.

Dodgson, K. S., & Price, R. G. (1962). A note on the determination of the ester sulphate content of sulphated polysaccharides. *Biochemical Journal*, *84*, 106–110.

Dreyfuss, J. L., Regatieri, C. V., Lima, M. A., Paredes-Gamero, E. J., Brito, A. S., Chavante, S. F., et al. (2010). A heparin mimetic isolated from a marine shrimp suppresses neovascularization. *Journal of Thrombosis and Haemostasis*, *8*(8), 1828–1837.

Guerrini, M., Naggi, A., Guglieri, S., Santarsiero, R., & Torri, G. (2005). Complex glycosaminoglycans: Profiling substitution patterns by two-dimensional nuclear magnetic resonance spectroscopy. *Analytical Biochemistry*, *337*(1), 35–47.

Kaiser, B., Fareed, J., Hoppensteadt, D., Birdsong, B., Walenga, J. M., & Markwardt, F. (1992). Influence of recombinant hirudin and unfractionated heparin on thrombin and factor Xa generation in extrinsic and intrinsic activated systems. *Thrombosis Research*, *65*(2), 157–164.

Kusche, M., Torri, G., Casu, B., & Lindahl, U. (1990). Biosynthesis of heparin. Availability of glucosaminyl 3-O-sulfation sites. *Journal of Biological Chemistry*, *265*(13), 7292–7300.

Lindahl, U., Li, J. P., Kusche-Gullberg, M., Salmivirta, M., Alaranta, S., Veromaa, T., et al. (2005). Generation of "neoheparin" from *E. coli* K5 capsular polysaccharide. *Journal of Medicinal Chemistry*, *48*(2), 349–352.

Luppi, E., Cesaretti, M., & Volpi, N. (2005). Purification and characterization of heparin from the Italian clam Callista chione. *Biomacromolecules*, *6*(3), 1672–1678.

Medeiros, G. F., Mendes, A., Castro, R. A., Bau, E. C., Nader, H. B., & Dietrich, C. P. (2000). Distribution of sulfated glycosaminoglycans in the animal kingdom: Widespread occurrence of heparin-like compounds in invertebrates. *Biochimica et Biophysica Acta*, *1475*(3), 287–294.

Mulloy, B., Forster, M. J., Jones, C., Drake, A. F., Johnson, E. A., & Davies, D. B. (1994). The effect of variation of substitution on the solution conformation of heparin: A spectroscopic and molecular modelling study. *Carbohydrate Research*, *255*, 1–26.

Nader, H. B., Kobayashi, E. Y., Chavante, S. F., Tersariol, I. L., Castro, R. A., Shinjo, S. K., et al. (1999). New insights on the specificity of heparin and heparan sulfate lyases from *Flavobacterium heparinum* revealed by the use of synthetic derivatives of K5 polysaccharide from *E. coli* and 2-O-desulfated heparin. *Glycoconjugate Journal*, *16*(6), 265–270.

Nader, H. B., Lopes, C. C., Rocha, H. A., Santos, E. A., & Dietrich, C. P. (2004). Heparins and heparinoids: Occurrence, structure and mechanism of antithrombotic and hemorrhagic activities. *Current Pharmaceutical Design*, *10*(9), 951–966.

Nader, H. B., Porcionatto, M. A., Tersariol, I. L., Pinhal, M. A., Oliveira, F. W., Moraes, C. T., et al. (1990). Purification and substrate specificity of heparitinase I and heparitinase II from *Flavobacterium heparinum*. Analyses of the heparin and heparan sulfate degradation products by 13C NMR spectroscopy. *Journal of Biological Chemistry*, *265*(28), 16807–16813.

Nader, H. B., Tersariol, I. L., & Dietrich, C. P. (1989). Antihemostatic activity of heparin disaccharides and oligosaccharides obtained by chemical and enzymatic fragmentation: Reversal of the hemorrhagic activity by ATP and myosin. *Thrombosis Research*, *54*(3), 207–214.

Paredes-Gamero, E. J., Medeiros, V. P., Farias, E. H., Justo, G. Z., Trindade, E. S., Andrade-Lopes, A. L., et al. (2010). Heparin induces rat aorta relaxation via integrin-dependent activation of muscarinic M3 receptors. *Hypertension*, *56*(4), 713–721.

Pejler, G., Danielsson, A., Bjork, I., Lindahl, U., Nader, H. B., & Dietrich, C. P. (1987). Structure and antithrombin-binding properties of heparin isolated from the clams *Anomalocardia brasiliana* and *Tivela mactroides*. *Journal of Biological Chemistry*, *262*(24), 11413–11421.

Rondle, C. J., & Morgan, W. T. (1955). The determination of glucosamine and galactosamine. *Biochemical Journal*, *61*(4), 586–589.

Sampaio, L. O., Tersariol, I. L. S., Lopes, C. C., Bouças, R. I., Nascimento, F. D., Rocha, H. A., et al. (2006). Heparin and heparan sulfates. Structure distribution and protein interactions. In H. Verli (Ed.), *Insights into carbohydrate structure and biological function* (pp. 1–24). Kerala, India: Transworld Research Network.

Santos, J. C., Mesquita, J. M., Belmiro, C. L., da Silveira, C. B., Viskov, C., Mourier, P. A., et al. (2007). Isolation and characterization of a heparin with low antithrombin activity from the body of *Styela plicata* (Chordata-Tunicata). Distinct effects on venous and arterial models of thrombosis. *Thrombosis Research*, *121*(2), 213–223.

Sie, P., Petitou, M., Lormeau, J. C., Dupouy, D., Boneu, B., & Choay, J. (1988). Studies on the structural requirements of heparin for the catalysis of thrombin inhibition by heparin cofactor II. *Biochimica et Biophysica Acta*, *966*(2), 188–195.

Silva, M. E., & Dietrich, C. P. (1974). Isolation and partial characterization of three induced enzymes from *Flavobacterium heparinum* involved in the degradation of heparin and heparitin sulfates. *Biochemical and Biophysical Research Communications*, *56*(4), 965–972.

Toledo, O. M., & Dietrich, C. P. (1977). Tissue specific distribution of sulfated mucopolysaccharides in mammals. *Biochimica et Biophysica Acta*, *498*(1), 114–122.

Yamada, S., Murakami, T., Tsuda, H., Yoshida, K., & Sugahara, K. (1995). Isolation of the porcine heparin tetrasaccharides with glucuronate 2-O-sulfate. Heparinase cleaves glucuronate 2-O-sulfate-containing disaccharides in highly sulfated blocks in heparin. *Journal of Biological Chemistry*, *270*(15), 8696–8705.

Yates, E. A., Santini, F., Guerrini, M., Naggi, A., Torri, G., & Casu, B. (1996). 1H and 13C NMR spectral assignments of the major sequences of twelve systematically modified heparin derivatives. *Carbohydrate Research*, *294*, 15–27.

Zhang, Z., Xie, J., Liu, H., Liu, J., & Linhardt, R. J. (2009). Quantification of heparan sulfate disaccharides using ion-pairing reversed-phase microflow high-performance liquid chromatography with electrospray ionization trap mass spectrometry. *Analytical Chemistry*, *81*(11), 4349–4355.