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Selective sulfation of carrageenans and the influence of sulfate regiochemistry on anticoagulant properties

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ABSTRACT

Sulfated polysaccharides are recognized for their broad range of biological activities, including anticoagulant properties. The positions occupied by the sulfate groups are often related to the level of the inherent biological activity. Herein the naturally sulfated galactans, kappa-, iota- and theta-carrageenan, were additionally sulfated by regioselective means. The anticoagulant activity of the resulting samples was then studied using the aPTT *in vitro* assay. The influence of sulfate regiochemistry on the anticoagulant activity was evaluated. From kappa-carrageenan three rare polysaccharides were synthesized, one of them involved a synthetic route with an amphiphilic polysaccharide intermediate containing pivaloyl groups. Iota- and theta-carrageenan were utilized in a selective C6 sulfation at β-D-Gal*p* units to produce different structures comprising trisulfated diads. All the samples were characterized by NMR (1D and 2D). The resulting aPPT measurements suggested that sulfation at C2 of 3,6-anhydro-α-D-Gal*p* and C6 of β-D-Gal*p* increased the anticoagulant activity.

1. Introduction

Sulfated polysaccharides have important biological functions in mammalian cells, which are mainly related to specific cell–cell and cell–extracellular matrix interactions (Bishop, Schuksz, & Esko, 2007; Esko & Linhardt, 2009). These biological events are generally mediated by the formation of carbohydrate–protein complexes. The regiochemistry of the sulfate groups onto the carbohydrate domain have a crucial participation in the molecular recognition, which is based on the sulfate and basic amino acid contacts (Kinnunen, 2006). The anticoagulant mechanism of heparin is one of the most studied examples. Heparin can inhibit plasma proteases of coagulation cascade, including thrombin and activated factor X (Xa) through the potentiation of their natural inhibitors, such as antithrombin and heparin cofactor II. Two modes of action are postulated which involve allosteric positive modulation on serpin inhibitor and bridging mechanism, where the polysaccharide chain brings together both protease and inhibitor. The heparin minimal pentasaccharide sequence necessary for molecular interactions contains sulfate at specific positions (Capila & Linhardt, 2002; Olson, Richard, Izaguirre, Schedin-Weiss, & Gettins, 2010). In this way, heparin obtained from animal sources has been used as anticoagulant agent for more than 50 years. However, heparin based pharmaceutical products have several limitations due to well-known side effects and structural variations as a function of the source from where they are obtained. In addition, some years ago, a contamination episode of heparin with oversulfated chondroitin sulfate was associated with anaphylactoid-type reactions, hypotension and death. That event has raised concerns over the discovery of new alternatives to prepare heparin analogs from non animal tissues (Liu, Zhang, & Linhardt, 2009; Turnbull, 2011).

Chemical sulfation of polysaccharides is one of the most utilized procedures to produce heparin analogs. This type of chemical modification is reproducible and allows the employing of high amounts of polysaccharides obtained from renewable non-animal sources (Cipriani et al., 2009; Papy-Garcia et al., 2005). Regioselective sulfation approaches have been introduced to synthesize polysaccharides with defined sulfation patterns, and in association with biological activity studies, the best sulfate positioning have been identified (Groth & Wagenknecht, 2001; Peschel, Zhang, Fischer, & Groth, 2012). Therefore, such kind of study is of fundamental importance in the design of new therapeutic agents. The regioselective sulfation of polysaccharides usually needs protection/deprotection steps. Several strategies have been applied successfully with protecting groups widely used in carbohydrate chemistry and a great diversity of polysaccharides have been regioselectively sulfated, including cellulose (Richter & Klemm, 2003), curdlan (Gao et al., 1997), agaran (Zhang et al., 2010), chitin

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and chitosan (Nishimura et al., 1998; Kurita, Yoshida, & Umemura, 2010).

Several red seaweed species produce linear sulfated galactans known as carrageenans. The backbone found in carrageenans is constituted of alternating (1 → 3)-linked β-D-galactopyranose and (1 → 4)-linked α-D-galactopyranose units, of which the last are frequently in the 3,6-anhydro form (Usov, 2011). These polysaccharides are recognized for a broad spectrum of biological activities, which are dependent of the sulfation pattern (Jiao, Yu, Zhang, & Ewart, 2011; Silva et al., 2010; Talarico, Noseda, Ducatti, Duarte, & Damonte, 2011). Carrageenans are produced on a large scale because they are used as gelling–stabilizing agents and consumer products for the food and pharmaceutical industries (De Ruiter & Rudolph, 1997). Therefore, they are abundant starting materials for polysaccharide modification. Several chemical reactions have been reported in order to alter physicochemical and biological properties of carrageenans (Campo, Kawano, da Silva, & Carvalho, 2009). However, only oversulfation reactions protocols have been conducted to evaluate anticoagulant (Opoku, Qiu, & Doctor, 2006), antiviral (Yamada et al., 1997), antitumor (Yuan, Song, Li, & Li, 2011) and antioxidant (Yuan et al., 2005) properties. Thus, additional regioselective sulfation on the cyclized carrageenan backbone is considered important in the evaluation of the sulfate distribution effect on biological activities.

The goal of this study was to synthesize unusual sulfated cyclized carrageenan diads to evaluate their anticoagulant properties *in vitro*. This report describes the selective sulfation of kappa-, iota-, and theta-carrageenan and the characterization of the products through NMR analysis. Samples containing a diversity of sulfated patterns were submitted to aPTT assay to correlate the regiochemistry of sulfate groups with the anticoagulant activity.

2. Materials and methods

2.1. Materials

Kappaphycus alvarezii samples were obtained in the laboratory and cultivated in the sea of Ubatuba, São Paulo, Brazil (Paula, Pereira, & Ohno, 1999). Commercial samples of *Eucheuma denticulatum* were obtained from Gelymar S. A. (Puerto Montt, Chile). Samples of the tetrasporic phase of *Gigartina skottsbergii* were collected in Bahia Camarones, Chubut Province, Argentina (Gonçalves, Ducatti, Paranha, Duarte, & Noseda, 2005). Unfractionated heparin (UFH-202 IU/mg), anhydrous *N*,*N*-dimethylformamide, 2-methyl-2-butene, SO_3–pyridine and SO_3–Me_3N complexes, pivaloyl chloride and SDS were purchased from Sigma–Aldrich (USA). Pyridine and 4-dimethylaminopyridine (4-DMAP) were purchased from Merck (Germany). All other chemicals and reagents used were of analytical grade.

2.2. Extraction and preparation of carrageenan samples

2.2.1. Sample 1

The polysaccharides from red algae *K. alvarezii* were extracted as described by Gonçalves et al. (2005). The extracted material (10.0 g) was first dissolved in H_2O (4.0 L) and then KCl (29.8 g) was added portionwise. The resulting solution was stirred at room temperature for 3 h, kept to rest for 16 h at 4 °C, and then centrifuged (8000 rpm, 20 min, 20 °C). The precipitated material was dialyzed (cut-off 12–14 kDa) first against 1 M NaCl solution and then distillated H_2O. The dialyzed solution was lyophilised to give Sample 1 (7.0 g).

2.2.2. Sample 2

To a cooled stirred solution of Sample 1 (400 mg) in H_2O (40 mL), $NaBH_4$ (60 mg) was added in one portion. The resulting solution was stirred at room temperature for 12 h, diluted with AcOH (0.2 mL), stirred for an additional 10 min, dialyzed first against 1 M NaCl solution and then distillated H_2O. After lyophilization, a portion of the reduced material (200 mg) was dissolved in DMF (40 mL), and then SO_3–pyridine complex (2.0 g), 2-methyl-2-butene (4.0 mL) and Et_3N (0.3 mL) were added. The resulting mixture was stirred for 1 h at 20 °C, then diluted with 1 M $NaHCO_3$ solution (10 mL), stirred for an additional 10 min, treated with EtOH (160 mL), and centrifuged (8000 rpm, 20 min). The precipitated material was dissolved in 0.025 M phosphate buffer pH 7.0 (10 mL), dialyzed first against 1 M NaCl solution and then distillated H_2O. The dialyzed solution was lyophilised to give Sample 2 (215 mg).

2.2.3. Sample 3

Sample 1 was reduced with $NaBH_4$ as described for the preparation of Sample 2. The reduced polysaccharide (500 mg) was dissolved in H_2O (40 mL), and then treated with Dowex 50 × 8 triethylammonium form (5 g). The resulting mixture was stirred for 16 h at room temperature, filtered and washed thoroughly with H_2O. The filtrate was dialyzed against H_2O, and lyophilised. A portion of the resulting material (200 mg) was dissolved in DMF (40 mL), and then mixed with SO_3–pyridine complex (2.0 g), 2-methyl-2-butene (4.0 mL). The resulting mixture was stirred for 7 h at 20 °C, then diluted with 1 M $NaHCO_3$ solution (10 mL), stirred for an additional 10 min, treated with EtOH (160 mL), and centrifuged (8000 rpm, 20 min). The precipitated material was dissolved in 0.025 M phosphate buffer pH 7.0 (10 mL), dialyzed first against 1 M NaCl solution and then distillated H_2O. The dialyzed solution was lyophilised to give Sample 3 (295 mg).

2.2.4. Sample 4

Sample 1 was reduced with $NaBH_4$ as described for the preparation of Sample 2. The reduced material (200 mg) was dissolved in DMF (40 mL), and then SDS (0.4 g), 4-DMAP (0.17 g), pyridine (6.4 mL), and PivCl (8.7 mL) were added. The resulting mixture was stirred for 17 h at 20 °C, cooled in an ice bath, treated with EtOH (165 mL), and centrifuged (8000 rpm, 20 min). The precipitated material was dissolved in 0.025 M phosphate buffer pH 7.0 (10 mL), dialyzed first against 1 M NaCl solution and then distillated H_2O. The dialyzed solution was lyophilised to give Sample 4 (205 mg).

2.2.5. Sample 5

Sample 4 (100 mg) was dissolved in DMF (20 mL), and then SO_3–pyridine complex (1.46 g), 2-methyl-2-butene (2.0 mL) and Et_3N (0.3 mL) were added. The resulting mixture was stirred for 17 h at 20 °C, cooled in an ice bath, treated with EtOH (65 mL), and centrifuged (8000 rpm, 20 min). The precipitated material was dissolved in 0.025 M phosphate buffer pH 7.0 (10 mL), dialyzed first against 1 M NaCl solution and then distillated H_2O, and lyophilised. The resulting material (112 mg) was dissolved in 1 M NaOH solution (20 mL), and stirred for 4 h at 20 °C. The solution was neutralized to pH 7.0 with 37% HCl, dialyzed against distillated H_2O, and lyophilised to give Sample 5 (95 mg).

2.2.6. Sample 6

Kappa-carrageenan was desulfated according to Nagasawa, Inoue, and Kamata (1977) method. To a stirred solution of Sample 1 (200 mg) in H_2O (40 mL), Dowex 50 × $8H^+$ form (2 g) was added in one portion. The resulting mixture was stirred for 45 min at 20 °C, filtered and washed thoroughly with H_2O. The filtrate was neutralized to pH 7.0 with pyridine, and then lyophilised. A portion of the resulting material (200 mg) was dissolved in a mixture of DMSO (53.4 mL), MeOH (6.0 mL) and pyridine (0.6 mL). The resulting solution was heated at 100 °C for 4 h, cooled to room temperature, and

then dialyzed exhaustively with distillated H_2O. The dialyzed solution was lyophilised to give Sample 6 (114 mg).

2.2.7. Samples 7 and 8

A mixture of the dried milled red seaweed *E. denticulatum* (5.0 g) and H_2O (0.5 L) was stirred at 80 °C for 4 h, and then centrifuged (8000 rpm, 20 min, 20 °C). To the resulting supernatant (0.5 L), EtOH (1.5 L) was added portionwise, while the mixture was stirred with a glass rod. The precipitated polysaccharide was removed with the glass rod, dissolved in H_2O, and dialyzed against 1 M NaCl solution and then distillated H_2O. The resulting solution was lyophilised to give Sample 7 (2.8 g). Sample 7 (2.0 g) was dissolved in H_2O (1.5 L), and then $NaBH_4$ (200 mg) was added. The resulting solution was stirred at room temperature for 12 h, diluted with 3 M NaOH solution (0.5 L), stirred for an additional 10 min, and then $NaBH_4$ (100 mg) was added in one portion. The resulting solution was heated at 80 °C for 30 min, cooled to room temperature, and neutralized to pH 7.0 with 37% HCl. The neutral solution was concentrated under vacuum, dialyzed with distillated H_2O, and lyophilised to give Sample 8 (1.7 g) (Viana, Noseda, Duarte, & Cerezo, 2004).

2.2.8. Sample 9

Sample 8 (200 mg) was dissolved in DMF (40 mL), and then SO_3–pyridine complex (2.92 g), 2-methyl-2-butene (6.0 mL) and Et_3N (0.6 mL) were added. The resulting mixture was stirred for 1 h at 35 °C, cooled in an ice bath, treated with EtOH (140 mL), and centrifuged (8000 rpm, 20 min). The precipitated material was dissolved in 0.025 M phosphate buffer pH 7.0 (10 mL), dialyzed first against 1 M NaCl solution and then distillated H_2O. The dialyzed solution was lyophilised to give Sample 9 (212 g).

2.2.9. Samples 10 and 11

A mixture of the dried milled red seaweed *G. skottsbergii* (15 g) and H_2O (1.0 L) was stirred at 70 °C for 4 h, and then centrifuged (8000 rpm, 20 min, 20 °C). To the resulting supernatant (1.0 L), EtOH (3.0 L) was added portionwise, while the mixture was stirred with a glass rod. The precipitated polysaccharide was removed with the glass rod, dissolved in H_2O, dialyzed against distillated H_2O, and lyophilised to give a polysaccharide extract (7.95 g). The precipitated polysaccharide (7.95 g) was first dissolved in H_2O (3.2 L) and then KCl (190.8 g) was added portionwise. The resulting solution was stirred at room temperature for 4 h, kept to rest for 16 h at 4 °C, and then centrifuged (8000 rpm, 20 min, 20 °C) (Matulewicz, Ciancia, Noseda, & Cerezo, 1989). The precipitated material was dialyzed first against 1 M NaCl solution and then distillated H_2O. The dialyzed solution was lyophilised to give Sample 10 (6.6 g,). Sample 10 (3.0 g) was dissolved in H_2O (2.25 L), and then $NaBH_4$ (300 mg) was added. The resulting solution was stirred at room temperature for 12 h, diluted with 3 M NaOH solution (0.75 L), stirred for an additional 10 min, and then $NaBH_4$ (150 mg) was added in one portion. The resulting solution was heated at 80 °C for 28 h, cooled to room temperature, and neutralized to pH 7.0 with 37% HCl. The neutral solution was concentrated under vacuum, dialyzed with distillated H_2O, and lyophilised to give Sample 11 (1.3 g) (Ciancia, Noseda, Matulewicz, & Cerezo, 1993).

2.2.10. Sample 12

Sample 11 (300 mg) was dissolved in H_2O (24 mL), and then treated with Dowex 50 × 8 triethylammonium form (3 g). The resulting mixture was stirred for 16 h at room temperature, filtered and washed thoroughly with H_2O. The filtrate was dialyzed against H_2O, and lyophilised. A portion of the resulting material (100 mg) was dissolved in DMF (20 mL), and then SO_3–pyridine complex (1.0 g), 2-methyl-2-butene (2.0 mL) and Et_3N (0.15 mL) were added. The resulting mixture was stirred for 1 h at 20 °C, cooled in an ice bath, treated with EtOH (65 mL), and centrifuged (8000 rpm, 20 min). The precipitated material was dissolved in 0.025 M phosphate buffer pH 7.0 (10 mL), dialyzed first against 1 M NaCl solution and then distillated H_2O. The dialyzed solution was lyophilised to give Sample 12 (63 mg).

2.3. Analytical methods

The sulfate content was determined by the turbidimetric method of Dodgson and Price (1962) and the degree of sulfation (DS) was calculated according Whistler and Spencer (1964).

Molecular weights were determinate on a Waters High-Performance Size-Exclusion Chromatography (HPSEC) apparatus coupled to a differential refractometer (RI). The chromatographic separation was achieved with four Waters Ultrahydrogel columns (2000, 500, 250 and 120) connected in series. Eluition was carried out with 0.1 M $NaNO_2$ solution containing NaN_3 (0.2 g/L), with a flow rate of 0.5 mL/min. HPSEC data were collected and analyzed by the Wyatt Technology ASTRA program (Ferreira et al., 2012). A series of different molecular weight sulfated galactans isolated previously in our lab were used as standards (Colodi, 2011).

1D and 2D NMR spectra were acquired on a Bruker Avance DRX400 NMR spectrometer equipped with a 5 mm wide bore probe, operating at 400.13 and 100.63 MHz for ^1H and ^{13}C, respectively. Samples were deuterium exchanged by successive lyophilization steps in D_2O. The experiments were carried out using the pulse programs supplied with Brucker manual. The chemical shifts were measured relative to internal acetone (δ = 2.208 ppm for ^1H and δ = 32.69 ppm for ^{13}C) (Velde, Pereira, & Rollema, 2004). The data were analyzed using the Brucker Topspin 2.1 software.

2.4. Anticoagulant activity assay

The activated partial thromboplastin time (aPTT) test was determined with a kit HemosIL® (Instrumentation Laboratory Company, Bedford, MA, USA), in a COAG-A-MATE XM coagulometer (Organon Teknika Corporation, Durhan, NC), using a pool of normal human plasma. Plasma (90 μL) was incubated at 37 °C with saline, heparin, or polysaccharides samples (10 μL) and rabbit cephalin (100 μL). After 2 min, 0.025 M $CaCl_2$ (100 μL) was added, and the clotting time measured. For each group (n = 2), mean aPTT ± standard error of the mean (SEM) was determined. The concentration required to doubling the aPTT of saline ($CaPTT_2$) was calculated from linear regression analysis.

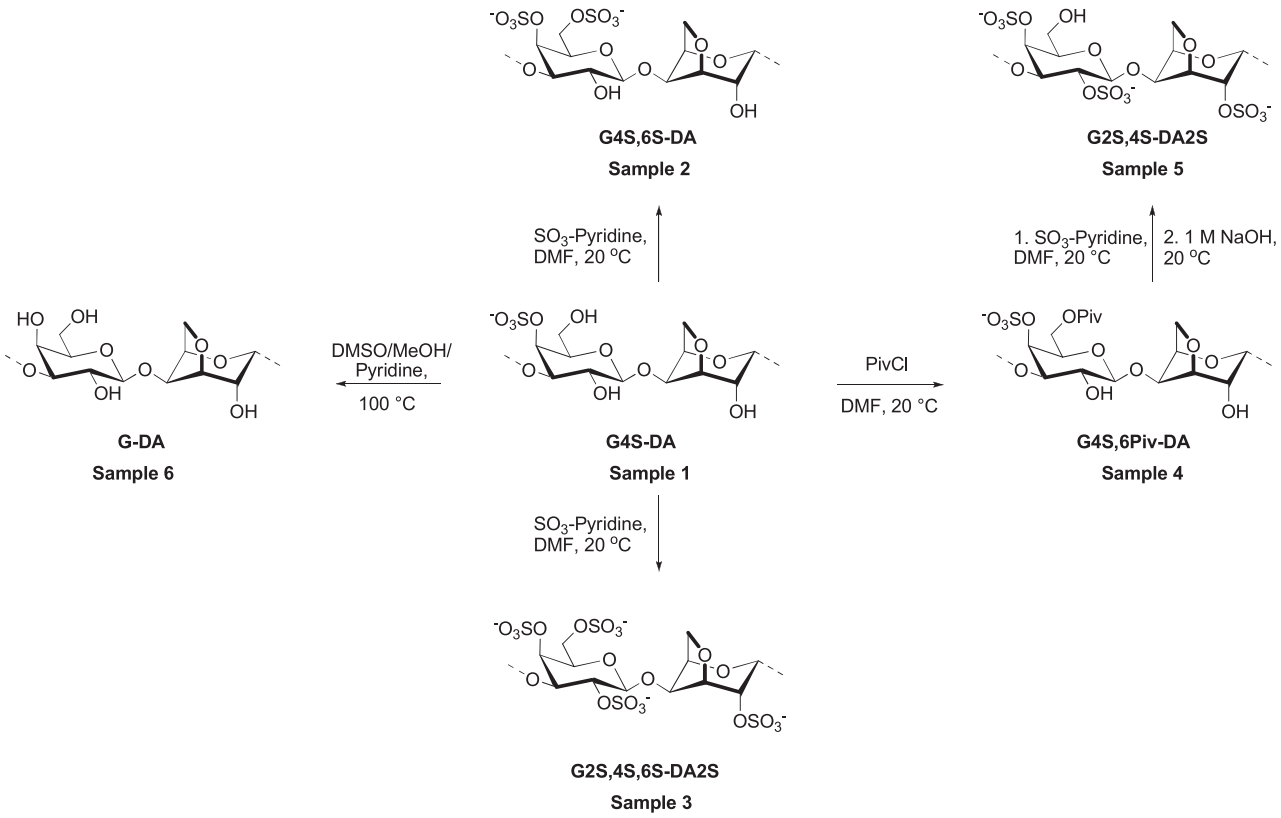
3. Results and discussion

3.1. Chemical sulfation and desulfation of kappa-carrageenan

The polysaccharides selected to be employed in the selective sulfation reactions were kappa- (G4S-DA),[1] iota- (G4S-DA2S) and theta-carrageenan (G2S-DA2S). These polymers exhibit different sulfation patterns. Therefore the sulfation strategy was planned in order to utilize the sulfate groups in cyclized carrageenan backbone (G-DA) as natural protecting groups and to explore the higher reactivity of primary hydroxyl groups on C6 of β-D-Gal*p* units to synthetize unusual sulfated diads.

The kappa-carrageenan (Sample 1) was obtained from red algae *Kapphaphycus alvarezii* (see Materials and methods) and characterized by ^1H and ^{13}C NMR analysis (Van de Velde, Knutsen,

[1] The letter code refers to the nomenclature developed by Knutsen, Myslabodski, Larsen, and Usov (1994). G refers to the β-D-Gal*p* and DA to the 3,6-anhydro-α-D-Gal*p* units. The numbers refer to the carbon atom attached to the sulfate (S) group in each residue.



Scheme 1. Selective sulfation and desulfation of kappa-carrageenan (Sample 1). The structures represent the target diads synthesized and do not reflect the strict composition of the samples.

Table 1
Optimization of the reaction conditions for the selective sulfation of kappa-carrageenan (Sample 1).

Entry	Sulfation agent	Time (h)	5.35[a]	5.29[a]	3.80[a]	3.65[a]
1	–	–	–	0.1	3.5	1.2
2[b]	SO_3–pyridine	7	1.0[d]	–	–	–
3[c]	SO_3-Me_3N	24	–	0.1	3.0	1.1
4[c]	SO_3–pyridine	1	0.1	0.3	0.7	1.2
5[c]	SO_3–pyridine	8	0.1	0.4	0.6	1.4

[a] Relative intensity to the signal at 5.09 ppm. Integrations were estimated by ^{1}H NMR analysis.
[b] Reaction performed with 50 mg of kappa-carrageenan (triethylammonium salt form), 500 mg sulfation agent, 2-methyl-2-butene (1.0 mL), in 10 mL of DMF at 20 °C.
[c] Reactions performed with 50 mg of kappa-carrageenan (sodium salt form), 500 mg sulfation agent, 2-methyl-2-butene (1 mL), Et_3N (75 μL) in 10 mL of DMF at 20 °C.
[d] Only the signal at 5.35 ppm was detected.

Usov, Rollema, & Cerezo, 2002). This polysaccharide was selected as a suitable substrate for an initial optimization study involving the selective C6 sulfation to produce G4S,6S-DA diad (Sample 2 – Scheme 1). The reactions were monitored by ^{1}H NMR analysis (Fig. S1) through the signals at 5.09, 3.80 and 3.65 ppm, which correspond, respectively, to H1 of DA and H5/H6/H6′ (overlapped signals) and H2 of G4S unit (Usov, 1984). The decrease of H5/H6 signal intensity accompanied by no significant losses of H1 and H2 signals indicated a selective sulfation on C6 carbon of β-D-Gal*p* units. We started the reaction optimization with kappa-carrageenan in the triethylammonium salt form to increase the polysaccharide solubility in DMF (Table 1, entry 2). The ^{1}H NMR analysis showed the disappearance of the signals at 5.09, 3.80 and 3.65 ppm and the appearance of a signal at 5.35 ppm, assigned to H1 of DA2S units linked to G2S,4S,6S units. Together, these results indicated that the sulfation occurred at all the polysaccharide free hydroxyl groups. At this stage, the use of kappa-carrageenan in the sodium salt form was envisaged to reduce the sulfation rate. This decision was based on reports describing the chemical structural determinations of galactans, in which partial methylation reactions occur in polar aprotic solvents using the polysaccharide in the sodium/potassium salt form (Stevenson & Furneaux, 1991). In addition, we decided to use SO_3-Me_3N, known as a less reactive sulfating agent than SO_3–pyridine complex (Gilbert, 1962). The reaction was performed (entry 3) and the results showed no evidence of sulfation on the polymer backbone. We changed again to the stronger sulfating agent SO_3–pyridine (entries 4 and 5), and it was observed that this reactant led mainly to the selective C6 sulfation independently of the reaction time (1 or 8 h). We observed in the ^{1}H NMR spectrum the preservation of signals at 5.09 and 3.65 ppm and a decrease of signal intensity at 3.80 ppm. Signals at 5.29 and 5.35 ppm were also observed and they were assigned to H1 of DA2S units linked to G4S or G4S,6S and to G2S,4S,6S units, respectively. Considering all the results involving the selective C6 sulfation of kappa-carrageenan, the main factor to reach the selective or total sulfation reactions was the salt form of substrate (Table 1, entries 2 and 5).

With the above information in hands, we performed the selective sulfation of Sample 1 in a major scale (200 mg) to produce Sample 2. NMR analyses were used to characterize the new synthesized G4S,6S-DA diad (Table 2). C6 sulfation was observed in the ^{13}C NMR spectrum. The C6 signal of G4S unit at 63.5 ppm was shifted to 70.1 ppm (α-shift) after sulfation, while the C5 signal was upfield shifted from 77.0 to 74.6 ppm (β-shift). In addition, the ^{13}C NMR chemical shifts of G4S,6S-DA were similar to those previously reported by Liao et al. (1996). HSQC analysis showed a minor C2 sulfation in both units of the polysaccharide. Thus, ^{1}H NMR in

Table 2
NMR assignments of target diads produced after chemical modifications.

[a]Chemical shifts (ppm) $^{13}C/^{1}H$														
Sample	Unit	1[b]	2	3	4	5	6	Unit	1[b]	2	3	4	5	6
2	G4S,6S	104.7 4.65	71.5 3.60	80.6 3.99	75.9 4.84	74.6 4.02	70.1 4.21 4.26	DA	97.2 5.09	71.8 4.11	81.4 4.51	81.0 4.64	78.9 4.71	71.3 4.05
3	G2S,4S,6S	102.8 4.83	79.0 4.37	76.7 4.15	75.7 4.98	74.5 4.05	70.0 4.26	DA2S	96.0 5.35	75.8 4.69	79.8 4.85	80.9 4.66	79.3 4.73	71.6 4.12 4.22
4[c]	G4S,6Piv	104.5 4.62	71.5 3.60	80.7 3.95	75.9 4.82	74.3 3.99	66.1 4.30	DA	97.1 5.09	71.9 4.10	81.3 4.50	80.7 4.56	78.7 4.63	71.5 4.04 4.17
5	G2S,4S	101.7 4.77	78.9 4.36	76.9 4.12	75.7 4.97	76.6 3.82	63.3 3.82	DA2S	95.8 5.34	nd[d]	79.1 4.87	nd	nd	71.8 4.12 4.26
9	G4S,6S	105.0 4.67	71.3 3.62	78.7 4.03	74.0 4.93	74.7 4.02	70.2 4.26	DA2S	94.0 5.28	77.0 4.68	80.3 4.83	81.5 4.63	79.2 4.77	71.7 4.11 4.26
11	G2S	102.6 4.76	79.7 4.37	81.5 3.91	69.8 4.14	76.9 3.71	63.2 3.77	DA2S	97.5 5.29	76.9 4.60	79.4 4.75	79.7 4.70	79.0 4.66	72.1 4.14
12	G2S,6S	103.2 4.79	79.7 4.35	81.4 3.91	69.4 4.17	74.5 3.92	69.0 4.18	DA2S	97.7 5.29	77.0 4.60	nd	nd	nd	71.9 4.18

[a] Chemical shifts from 2D NMR experiments.
[b] Numbers refer to carbons or protons at galactosyl and 3,6-anhydro galactosyl units.
[c] The chemical shifts for pivaloyl groups were ($^{13}C/^{1}H$): $COC(\underline{CH_3})_3$ = 28.8/1.20 ppm; $CO\underline{C}(CH_3)_3$ = 41.0 ppm; $\underline{C}OC(CH_3)_3$ = 183.5 ppm.
[d] Not determined.

Table 3
Diads composition, degree of sulfation and M_w of native and modified samples.

Sample	Dyads (%)[a]	DS	M_w (kDa)
1	G4S-DA (92) G4S-DA2S (8)	1.1	210
2	G4S,6S-DA (55) G4S,6S-DA2S/G4S-DA2S (18) G4S-DA (17) G2S,4S,6S-DA2S/G2S,4S-DA2S (10)	2.0	98
3	G2S,4S,6S-DA2S (81) G4S,6S-DA2S (19)	3.8	74
5	G2S,4S-DA2S (40) G2S,4S,6S-DA2S (54) G4S,6S-DA2S/G4S-DA2S (6)	3.2	89
6	G-DA (78) G4S-DA (22)	0.1	44
8	G4S-DA2S (>95)	2.1	790
9	G4S,6S-DA2S (73) G4S-DA2S (27)	2.4	250
11	G2S-DA2S (>95)	2.1	120
12	G2S-DA2S (67) G2S,6S-DA2S (33)	2.6	43

[a] Diads were identified by HSQC experiment and then estimated by ^{1}H NMR analysis.

association with 2D analyses was used to quantify and identify minor diads (Table 3). In this way it has been noted that the native kappa-carrageenan together with other diads, such as G4S,6S-DA are the main constituents in Sample 2.

Sample 3 was produced in a larger scale (200 mg) through oversulfation of Sample 1 according to conditions found in Table 1 (entry 2). The degree of sulfation (DS) was high (3.8) and, together with NMR analysis, indicated that the initial polysaccharide was completely sulfated to produce mainly G2S,4S,6S-DA2S and a small portion of G4S,6S-DA2S diads (Table 3). The ^{13}C NMR spectrum showed similar signals to those reported in literature (Thành, Yasunaga, Takano, Urakawa, & Kajiwara, 2001) for oversulfated carrageenan (Table 2). The chemical sulfation of polysaccharides usually promotes partial depolymerization, reducing the M_w of the resulting polymer (Petit et al., 2004; Zhang et al., 2010). The reactions to produce Sample 2 and 3 were performed with 2-methyl-2-butene and triethylamine in order to reduce depolymerization (Papy-Garcia et al., 2005). However, we have observed that carrageenan backbones were partially cleaved and the products present lower M_w than the precursor polysaccharide (Table 3). As previously reported in oligosaccharide production studies from agarans and carrageenans (Ducatti, Colodi, Gonçalves, Duarte, & Noseda, 2011; Yang et al., 2009), the higher lability of 3,6-anhydro-α-galactosidic bonds could be the origin of the depolymerization process.

We were also interested in preparing the G2S,4S-DA2S diad (Sample 5) from kappa-carrageenan (Scheme 1). For this purpose, we planned a synthetic route that included the selective protection of C6 hydroxyl of G4S units with pivaloyl group (Piv) before the sulfation step. Therefore, after some experimentation (Table S1 and Fig. S2), the best conditions for C6 pivaloylation (entries 4–6) gave yields between 43% and 49%, as estimated by ^{1}H NMR analysis. The major reactivity of primary hydroxyls associated with the steric effect of pivaloyl groups were possibly the reasons of the observed regioselectivity. Sample 1 was modified using PivCl, 4-DMAP as catalysts and SDS as additive in DMF. The pivaloyl substitution was confirmed through the correlations between the methyl hydrogens (1.28 ppm) and the quaternary (41.0 ppm) and carboxylic (183.5 ppm) carbon signals in the HMBC spectrum. The ^{13}C DEPT NMR spectrum showed the C6 signal of G4S,6Piv unit at 66.1 ppm. HSQC analysis, together with the comparison with kappa-carrageenan chemical shifts, allowed the complete assignment of G4S,6Piv-DA diad (Table 2). Along with the esterified diad, G4S-DA units were observed as major constituent (63%) in Sample 4, as expected considering the reaction optimization (Table S1). It is worth noting that the esterified diad constitutes a valuable polymer, since amphiphilic polysaccharides have attracted wide interest due to their use in nanotechnology areas such as drug

delivery and surface coating (Durand, Marie, Rotureau, Leonard, & Dellacherie, 2004; Guyomard, Nysten, Muller, & Glinel, 2006; Lu, Zhang, & Liu, 2008). The conversion of Sample 4 into 5 proceeded as depicted in Scheme 1. Sulfation using SO_3–pyridine complex produced an intermediate, which was subsequently submitted to the pivaloyl group removal with 1 M NaOH to give Sample 5. The target G2S,4S-DA2S diad was confirmed by NMR analysis (Table 2). The ^{1}H NMR spectrum showed at 3.82 and 5.38 ppm signals corresponding to H5/H6/H6′ of β-D-Gal*p* and H1 of DA2S units, respectively. Together with G2S,4S-DA2S diad, it was also possible to estimate the amount of oversulfated G2S,4S,6S-DA2S and G4S,6S-DA2S/G4S-DA2S diads (Table 3).

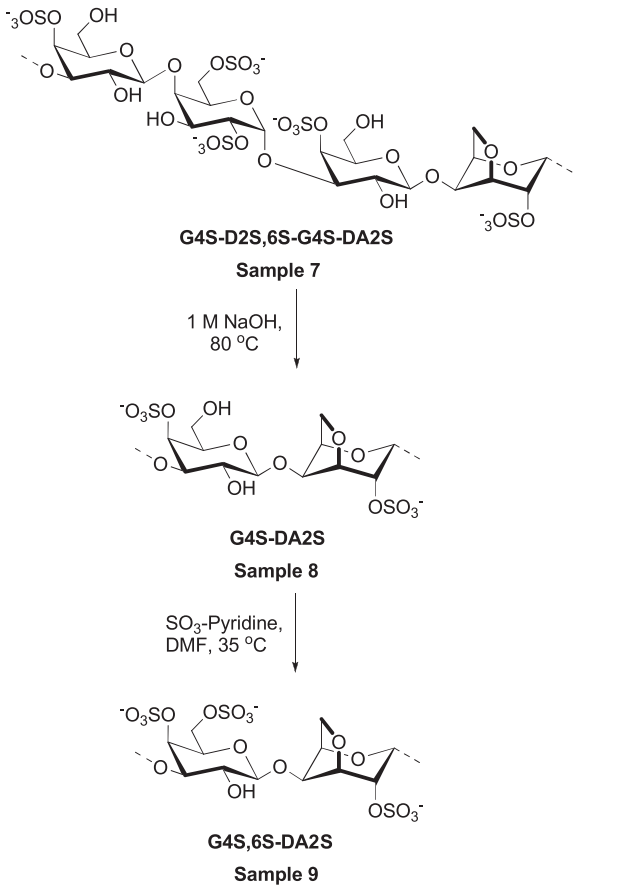
In order to evaluate the anticoagulant activity promoted by the non-sulfated carrageenan backbone, we submitted kappa-carrageenan to a desulfation process, giving rise to Sample 6 (Scheme 1). While several approaches have been reported for the desulfation of carbohydrates (Kolender & Matulewicz, 2004; Miller & Blunt, 1998; Takano, Kanda, Hayashi, Yoshida, & Hara, 1995), in this work, we selected the Nagasawa, Inoue, and Kamata (1977) method. Sample 6 showed a decrease in DS (1.1–0.1) together with a reduction in the molecular weight (Table 3). The ^{13}C and ^{1}H NMR spectra were similar to those reported for beta-carrageenan (G-DA) (Kolender & Matulewicz, 2004) indicating that this diad is the main constituent of Sample 6 (Table 3).

3.2. Chemical sulfation of iota-carrageenan

A polysaccharide fraction was obtained from the red alga *E. denticulatum* through water extraction to give Sample 7 (see Materials and methods). ^{1}H NMR analysis presented anomeric signals at 5.30 and 5.55 ppm corresponding to H1 of DA2S and D2S,6S respectively (Van de Velde et al., 2002). These data indicated the presence of an iota/nu-hybrid carrageenan, as expected, considering the red alga species here utilized (Anderson, Dolan, & Rees, 1973). Sample 7 was then submitted to alkaline treatment, according to known kinetic parameters (Viana et al., 2004), to convert all the α-units into the 3,6-anhydro form (Scheme 2). The ^{1}H NMR spectrum of the alkali-treated polysaccharide (Sample 8) showed only one signal at the α-anomeric region (5.30 ppm), indicating the complete cyclization of D2S,6S to DA2S units. Sample 8 (iota-carrageenan) was then employed to extend the variety of the unusual sulfation patterns on cyclized carrageenan backbone through the preparation of G4S,6S-DA2S diad. After the reaction optimization (Table S2), we reached the selective C6 sulfation of G4S unit (70%) using iota-carrageenan in the sodium salt form with SO_3–pyridine complex in DMF at 35 °C for 1 h (Table S2, entry 5). Interestingly, the increase in the reaction time promoted the C2 sulfation of β-D-Gal*p* units, which was detected due to the appearance of H1 signal of DA2S linked to G2S,4S,6S unit at 5.35 ppm in the ^{1}H NMR spectrum (Table S2, entries 6–7 and Fig. S3). After the upscaled production of Sample 9, the target trisulfated diad G4S,6S-DA2S was characterized by NMR analysis (Table 2). The ^{13}C NMR chemical shifts were similar to those reported previously (Thành et al., 2001). By analyzing Sample 9 HSQC spectrum, only one correlation in the β-anomeric region was detected (105.1/4.60 ppm), which was assigned to C1/H1 of β-D-Gal*p* units. This result suggests the absence of sulfate groups on C2 of G4S units and the presence of G4S,6S-DA2S and G4S-DA2S diads as the main constituents of Sample 9 (Table 3).

3.3. Chemical sulfation of theta-carrageenan

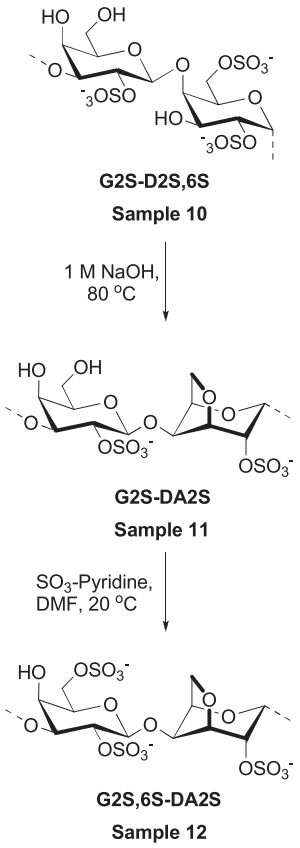
In order to obtain a galactan having theta-carrageenan substitution pattern (G2S-DA2S), we submitted lambda-carrageenan (G2S-D2S,6S, Sample 10), extracted from the red alga *G. skottsbergii*, to alkaline treatment (Ciancia et al., 1993) with 1 M NaOH at



Scheme 2. Preparation and sulfation of iota-carrageenan (Sample 8). The structures represent the target diads synthesized and do not reflect the strict composition of the samples.

80 °C (Scheme 3). The complete conversion of D2S,6S units to DA2S was monitored by ^{1}H NMR spectroscopy observing the appearance of a signal at 5.30 ppm attributed to H1 of DA2S. As previously described (Falshaw & Furneaux, 1994), there is a possibility of interchanged ^{13}C NMR assignments for theta-carrageenan. Thus, the complete assignment of G2S-DA2S diad (Sample 11) was performed using HSQC and HMBC NMR analysis (Table 2). Sample 11 was then employed for an optimization study to achieve the selective C6 sulfation of theta-carrageenan (Table S3 and Fig. S4). Similar conditions used for selective sulfation of iota- and kappa-carrageenan were inefficient and the polysaccharide modification was only reached in a moderate yield (33%), after converting the initial carrageenan into the triethylammonium salt form. Theta-carrageenan was selectively sulfated in a higher scale (100 mg) to produce Sample 12. The C6 signal of G2S,6S unit was observed in the ^{13}C NMR spectrum at 69.0 ppm, and the assignment of G2S,6S-DA2S diad was performed using HSQC analysis together with the comparison with previously described chemical shifts for theta-carrageenan (Table 2). The absence of any correlation in the HSQC spectrum at 4.82–4.97/74.0–75.9 ppm region excludes C4 sulfation of β-D-Gal*p* units. Therefore, the diads identified in Sample 12 were G2S,6S-DA2S and G2S-DA2S (Table 3).

By observing the C6 sulfation rate for the three carrageenans used as substrate, it was noted that the relative yields were remarkably different, mainly for theta-carrageenan. For kappa- and iota-carrageenans, higher C6 sulfation rates were obtained using the starting material in the less reactive sodium form. On the other hand, for theta-carrageenan, it was necessary the use of the more



Scheme 3. Preparation and sulfation of theta-carrageenan (Sample 11). The structures represent the target diads synthesized and do not reflect the strict composition of the samples.

reactive triethylammonium form to achieve a moderate C6 sulfation. These results suggest that the original sulfate regiochemistry into the polymer backbone influences the selective C6 sulfation (Table 3).

3.4. Anticoagulant activity assay

The anticoagulant activity of all samples was determined by the activated partial thromboplastin time (aPTT) *in vitro* assay (Table 4), which measures the general anticoagulant effect where all the plasma proteins are included. All the samples demonstrated a dose dependent prolongation of aPTT at the concentration range of 10–50 μg/mL, except the desulfated carrageenan (Sample 6). Several molecular requirements are claimed to explain the biological activities of sulfated polysaccharides, including sulfation degree and pattern, monosaccharide composition and glycosidic bonds,

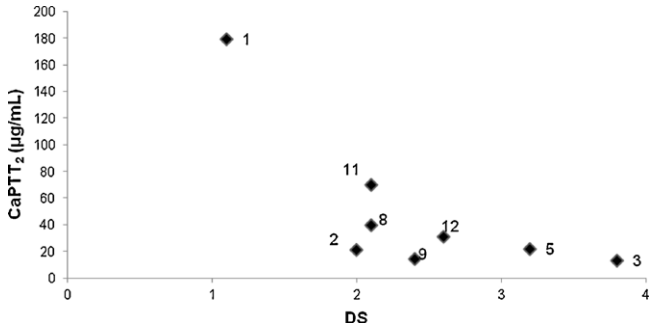


Fig. 1. Dependence of the degree of sulfation (DS) and sample concentration required to double aPTT of saline ($CaPTT_2$).

molecular size, and influence of branching residues (Pomin, 2009; Pomin & Mourão, 2008). In our work, all the samples had the same polysaccharide backbone with differences on molecular weight and sulfation degree and pattern. From Table 4 we observed that all the samples derived from sulfation reactions showed better activity than the native ones. Sample 3 (DS = 3.8) presented the highest anticoagulant activity, while Samples 6 (DS = 0.1) and 1 (DS = 1.0) showed the lowest ones. Together, these data indicated that the high degree of sulfation could be at the origin of the anticoagulant effect.

Samples 8 and 9 showed at higher doses (100 and 150 μg/mL) lower coagulation times than the negative control (saline solution), which could indicate a procoagulant effect. Fonseca et al. (2008) have reported this type of effect for sulfated galactans extracted from red algae *Gelidium crinale* and *Botryocladia occidentalis*. The authors suggest that slight differences in the proportions and/or distribution of sulfated residues along the galactan backbone are responsible for the mentioned behavior. In addition, it has been reported that sulfated polysaccharides can induce procoagulant activity through contact activation with plasma proteases (Renné, 2012). Besides the increase of polysaccharide backbone molecular size positively correlates with the induction of contact system activation in plasma (Corretge & Nigretto, 1990; Silverberg & Diehl, 1987). Thus, the high molecular weight and/or differences in the sulfation degree or sulfation pattern along the polysaccharide backbone of Samples 8 and 9 could be the reason of the procoagulant effect.

In order to compare the dependence of the anticoagulant activity on the sulfate regiochemistry of the studied carrageenans, concentration required to double the saline aPTT ($CaPTT_2$) were plotted against the degree of sulfation. Fig. 1 showed that an increased DS has a beneficial effect on anticoagulant activity as observed for CaPTT2 values of Samples 3 (13 μg/mL) and 1 (179 μg/mL). However, Sample 3 (DS = 3.8) gave a $CaPTT_2$ value comparable to that of Sample 9 (DS = 2.4; $CaPTT_2$ = 14 μg/mL), while Sample 5 (DS = 3.2) showed a $CaPTT_2$ value (21 μg/mL) equal to

Table 4
Anticoagulant activity determination using the activated partial thromboplastin time (aPTT) assay.

Sample	Polysaccharide concentration (μg/mL)[a]						
	10	20	30	40	50	100	150
1	34.4 ± 0.3	33.8 ± 0.4	35.8 ± 0.1	36.9 ± 0.2	44.9 ± 2.2	52.6 ± 0.9	56.7 ± 1.6
2	45.7 ± 2.5	55.7 ± 0.9	76.0 ± 0.9	85.9 ± 0.7	112.5 ± 1.0	>300	>300
3	52.5 ± 0.8	76.7 ± 2.3	101.5 ± 6.0	133.1 ± 5.0	165.5 ± 1.3	>300	>300
5	41.5 ± 0.7	58.7 ± 0.9	82.4 ± 1.8	107.8 ± 4.1	138.3 ± 1.2	165.1 ± 1.2	>300
6	31.4 ± 0.6	30.4 ± 0.3	30.3 ± 0.2	30.1 ± 0.1	30.3 ± 0.2	30.7 ± 0.1	34.7 ± 1.0
8	46.2 ± 2.2	53.6 ± 2.3	56.3 ± 0.3	63.1 ± 2.4	65.7 ± 0.5	20.9 ± 1.0	19.8 ± 0.7
9	52.7 ± 1.1	73.7 ± 0.3	81.7 ± 1.2	117.3 ± 1.3	148.4 ± 0.2	19.2 ± 0.1	19.5 ± 0.2
11	34.5 ± 0.3	39.0 ± 0.8	43.2 ± 1.1	49.3 ± 0.1	54.7 ± 1.3	80.3 ± 1.3	93.0 ± 0.9
12	38.2 ± 0.7	48.7 ± 0.2	54.2 ± 1.2	65.8 ± 2.2	86.7 ± 1.8	147.8 ± 1.1	>300

[a] Results are expressed as mean times (s) ± SEM. Saline was used as negative (30.8 s) and heparin (10 μg/mL; >300 s) as positive controls.

that of Sample 2 (DS = 2.0). These data suggest that not only the high density but also the position of sulfate groups in the cyclized carrageenan backbone is important for the anticoagulant behavior. The great number of diads at different proportions in each sample (Table 3) could hinder the influence of sulfate substitution on the biological results. However, comparisons between fractions containing minor number of diads with higher degree of purity indicated a tendency. Comparison of the results obtained from Samples 1, 8 and 11, containing more regularly sulfated carrageenans, indicated that C2 sulfation at DA unit decreased the $CaPTT_2$ value, contributing to anticoagulant activity. In addition, C4 sulfation at β-D-Gal*p* units seems to have a better effect than at C2 (compare Samples 8–11). Selective C6 sulfation of β-D-Gal*p* units increased the activity, as observed when comparing Samples 8–9 and 11–12.

4. Conclusions

In conclusion, we have reported the chemical regioselective increase of the sulfate content in carrageenans. The semisynthetic methods here described were effective in the production of rare sulfated diads based on cyclized carrageenan backbone, which are hardly found in nature. The great diversity of sulfate patterns synthesized offers a wide range of opportunities for studies and applications in several areas of glycobiology. Here, a preliminary anticoagulant assay indicated that sulfation at C2 of 3,6-anhydro-α-D-Gal*p* and C6 of β-D-Gal*p* have beneficial effect on biological activity *in vitro*. Further studies are needed to improve our understanding of sulfated carrageenan anticoagulant mechanism and to determine the potential of their use in anticoagulant therapy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2012.08.034.

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