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Structure and anti-metapneumovirus activity of sulfated galactans from the red seaweed *Cryptonemia seminervis*

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ABSTRACT

The anti-HMPV (human metapneumovirus) activity was determined for sulfated DL-hybrid galactans obtained from the red seaweed *Cryptonemia seminervis* and their depolymerized products obtained by reductive partial hydrolysis. Structural studies carried out in three homogeneous depolymerized fractions DS-1, DS-2e and DS-3 (Mw of 51.6–63.8 kDa) showed that these galactans present different chemical characteristics, as monosaccharide composition, content of sulfate groups (14.1–29.9%) and agaran:carrageenan molar ratio diads, 2.7:1 for DS-1 and DS-2e and 1:1 for DS-3. The sulfate groups are located principally on C-2 of β-D-galactopyranose and 4,6-*O*-(1′-carboxyethylidene)-β-D-galactopyranose residues and on C-6 of α-galactose residues. Sulfated DL-galactans and their depolymerized products exhibited antiviral activity at a very early stage of the viral infection cycle. All fractions, except DS-2e inhibited HMPV replication by binding to the viral particle. Besides depolymerized galactans DS-2e and DS-3 inhibited the recognition of cell receptor by HMPV and penetration to the host cell, respectively.

1. Introduction

Human metapneumovirus (HMPV) member of the family *Paramyxoviridae* is a causative agent of acute respiratory illness (Williams, Edwards, & Weinberg, 2010). First identified in 2001 (van den Hoogen et al., 2001), HMPV has been described worldwide and serological studies have revealed that HMPV seropositivity is almost universal by the age of 5 years (Collins & Crowe, 2007). Re-infection with HMPV is common and currently there is no vaccine available (Arnott et al., 2013). Other than for influenza virus, antiviral therapy for respiratory viruses has not shown satisfactory results. In this way, studies of new compounds with potential anti-HMPV activity could have clinical value. Polysaccharides extracted from seaweeds have been reported to exhibit antiviral activity against a wide spectrum of viruses including important human pathogenic agents such as Human Immunodeficiency virus (HIV), Herpes simplex virus (HSV), Vesicular stomatitis virus (VSV), Cytomegalovirus (CMV) (Cassolato et al., 2008; Duarte et al., 2004; Faria-Tischer et al., 2006; Harden, Falshaw, Carnachan, Kern, & Prichard, 2009; Haslin, Lahaye, Pellegrini, & Chermann, 2001; Matsuhiro et al., 2005; Witvrouw & De Clercq, 1997). Secondary metabolites obtained from green and brown marine algae were able to inhibit HMPV replication (Mendes et al., 2010, 2011). However, in spite of the broad-spectrum of antiviral activities reported for sulfated polysaccharides, as far as we know anti-HMPV activity has not been described for these polymers.

As previously reported red seaweeds from the genus *Cryptonemia* synthesize sulfated galactans named DL-hybrid galactans. These kind of galactans are composed by repeating units of 3-linked β-D-galactopyranosyl (A-unit) and 4-linked α-galactopyranosyl units (B-unit) where the last are present in both D- and L-enantiomeric series (Zibetti, Noseda, Cerezo, & Duarte, 2005; Zibetti et al., 2009). The DL-hybrid family of galactans from

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Cryptonemia seminervis contains the B-units as α-D- and α-L-galactose and 3,6-anhydro-α-D- and 3,6-anhydro-α-L-galactose besides minor amounts of monomethylated galactoses (6-*O*- and 2-*O*-methylgalactose) and 3,6-anhydro-2-*O*-methyl-α-L-galactose. The structural complexity of these galactans is increase by the presence of sulfate groups located at different positions, xylose and/or 4-*O*-methylgalactose as side chain and β-D-galactopyranosyl 4,6-(1′-carboxyethylidene). S2S-4 is one of the homogeneous hybrid galactans (Mw 332.4 kDa) isolated from *C. seminervis*. S2S-4 presents the A-units constituted predominantly by β-D-galactopyranosyl 2-sulfate (11.9 mol%), β-D-galactopyranosyl 2,6-sulfate (18.6 mol%), β-D-galactopyranosyl 2-sulfate 4,6-(1′-carboxyethylidene) (8.8 mol%) whereas the B-units are constituted mainly by 3,6-anhydro-α-L-galactose (7.3 mol%), 3,6-anhydro-α-L-galactose 2-sulfate (14.4 mol%) and α-L- and/or α-D-galactose 2,3,6-sulfated (8.2 mol%). From the enantiomeric analyses, the agaran and carrageenan blocks of S2S-4 are present in a molar ratio of ~1.7:1.

Previous reports have described the antiviral activity against herpes simplex virus and dengue virus of the DL-hybrid galactans obtained from *Cryptonemia crenulata* (Talarico et al., 2004, 2005) however, there are no studies reporting antiviral activity of the sulfated polysaccharides from *C. seminervis.*

The present study describes the chemical structure of the partially depolymerized products obtained from the red seaweed *C. seminervis* and reports for the first time the antiviral activity of sulfated polysaccharides against human metapneumovirus.

2. Materials and methods

2.1. Collection of specimens

Specimens of the red seaweed *C. seminervis* (C. Agardh) J. Agardh were collected at the southeastern coast of Brazil (Marataízes, Espírito Santo State). A voucher specimen was deposited at the herbarium of the Department of Botany, Federal University of Paraná (Curitiba, Brazil) with the herbarium identification code UPCB-59441. The algal material was cleaned to remove contaminants, washed with tap water, sun-dried, and milled. Whole thalli were used for polysaccharide extractions.

2.2. Extraction of polysaccharides

The milled alga was extracted with phosphate buffer pH 6.5 (1.5%, w/v) at 80 °C under mechanical stirring for 6 h. After centrifugation, the supernatant was concentrated and treated with EtOH (3:1, v/v). The precipitated material was redissolved in distilled water, dialyzed (cut-off 12–14 kDa) sequentially against distilled water, 1.0 M NaCl and distilled water, concentrated and freeze-dried. This extraction process was repeated three times and the four extracts were combined rendering the crude extract CE.

2.3. Fractionation by KCl-treatment

For KCl treatment (Cardoso, Noseda, Fujii, Zibetti, & Duarte, 2007) CE crude extract was dissolved in water (0.25%, w/v) and KCl was added to a concentration of 2.0 M under magnetic stirring for 3 h. The solution was maintained at 4 °C overnight. After centrifugation, the supernatant and precipitate were separately dialyzed (distilled water, 1.0 M NaCl and distilled water) and freeze-dried giving rise to the KCl-soluble fraction S and precipitate fraction P.

2.4. Partial depolymerization

Galactan S (1.1 g) was dissolved in distilled water (1.5%, w/v), the solution was heated to 65 °C and borane-4-methylmorpholine complex (6.6 g) and 1.5 M TFA (25 mL) were added, giving a final concentration of 0.375 M TFA (Usov & Elashvili, 1991; Zibetti et al., 2005, 2009). The solution was maintained at 65 °C for 6 h. Acid was removed by co-distillation with water and the partially depolymerized product was dialyzed (cut-off 6–8 kDa) against distilled water yielding the DS fraction.

2.5. Fractionation of the partially depolymerized product

The DS fraction (790 mg) was submitted to anion-exchange chromatography on a DEAE-Sephacel column (30 cm × 6 cm i.d.) that was sequentially eluted (stepwise) with water and aq. NaCl solutions of increasing concentrations (0.25–4.0 M). The column eluents were analyzed for carbohydrate content by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The eluents obtained were concentrated, dialyzed and freeze-dried, giving rise to the following fractions: DS-W (eluted with water); DS-1 (eluted with 0.25 M NaCl); DS-2 (0.50 M); DS-3 (0.75 M); DS-4 (1.0 M) and DS-5 (1.5 M). The higher yield fraction DS-2, was submitted to a new chromatographic fractionation under the same conditions described above, but with a concentration range of 0.1–1.0 M NaCl with an increment of 0.1 M for each elution step, giving rise to the following fractions: DS-2W (water) and DS-2a–DS-2f (0.1–0.8 M NaCl, respectively).

2.6. Analytical methods

Total carbohydrate content was estimated by the phenol–sulfuric acid method using galactose as standard (Dubois et al., 1956). Sulfate content was determined by the turbidimetric method of Dodgson and Price (1962). Protein content was measured by the method of Lowry, Rosebrough, Farr, and Randall (1951) using bovine serum albumin as standard. The specific rotation was measured at 20 °C, using a 10-cm cell and the sodium D line (589.3 nm) on a Rudolph Autopol III automatic polarimeter.

The determination of monosaccharide composition was carried out by reductive hydrolysis (Stevenson & Furneaux, 1991) using extra reducing agent [borane·4-methylmorpholine complex (4-MMB)] before and after pre-hydrolysis and hydrolysis steps (Falshaw & Furneaux, 1994; Jol, Neiss, Penninkhof, Rudolph, & De Ruiter, 1999). The hydrolytic process was performed as described by Ferreira et al. (2012). After acetylation (Stevenson & Furneaux, 1991) the alditol acetate derivatives were analyzed by GC–MS in the same conditions described by Ferreira et al. (2012) and were identified by their typical electron-impact fragmentation profiles and GC retention times (Jansson, Kenne, Liedgren, Lindberg, & Lonngren, 1976). A double hydrolysis-reductive amination method was used to determine the absolute configuration of the monosaccharide constituents (Navarro & Stortz, 2003). Chiral 1-amino-2-propanol was used to determine the ratio of D- and L-galactose and its 6-*O*-methyl derivative, whereas the configuration of 2-*O*-methylgalactose, 3,6-anhydrogalactose and their 2-*O*-methyl derivatives were determined using chiral α-methylbenzylamine. The resulting alditol acetates derivatives were analyzed by GC–MS using the conditions described by Ferreira et al. (2012).

2.7. Desulfation

Fractions DS-1, DS-2e and DS-3 in the pyridinium salt form (Stevenson & Furneaux, 1991) were submitted to partial solvolytic desulfation (Nagasawa, Inoue, & Kamata, 1977) yielding DS-1D, DS-2eD and DS-3D, respectively.

2.8. High-pressure size-exclusion chromatography (HPSEC) analysis

HPSEC was carried out with a 1 mg/mL soln. of the polysaccharide, using a multidetection equipment with a Waters 2410 differential refractometer (RI) and a Wyatt Technology Dawn-F multi-angle laser light scattering (MALLS) detector adapted on-line. Four Waters Ultrahydrogel columns (2000, 500, 250 and 120) were connected in series and coupled to the multi-detection equipment. The eluent was 0.1 M $NaNO_2$ soln., containing NaN_3 (0.2 g/L). The d*n*/d*c* value was determined using five concentrations, between 0.2 and 1.0 mg/mL. HPSEC data were collected and analyzed by the Wyatt Technology ASTRA program. All experiments were carried out at 25 °C.

2.9. Nuclear magnetic resonance (NMR)

NMR analyses were carried out using a Bruker Advance DRX400 NMR spectrometer equipped with a 5-mm multinuclear inverse detection probe. The base frequency was 400.13 MHz for ^1H and 100.63 MHz for ^{13}C nuclei. Analyses were recorded at 70 °C. For ^{13}C NMR spectra, the samples were dissolved in D_2O (40 mg/mL). For ^1H and 2D NMR experiments, the samples were deuterium exchanged by successive freeze-drying steps in D_2O (99.9%) and then dissolved in D_2O (20–25 mg/mL). ^1H, ^{13}C and ^{13}C DEPT acquisition parameters were previously reported (Ascêncio, Orsato, França, Duarte, & Noseda, 2006) 2D ^1H,^1H COSY, and ^1H,^{13}C HSQC experiments were carried out using the pulse programs supplied with the Bruker manual. Chemical shifts are expressed relative to an internal acetone standard at 30.2 and 2.224 ppm for ^{13}C and ^1H NMR spectra, respectively.

2.10. Cell and virus

LLC-MK2 cells (*Macaca mulatta* [monkey, rhesus]) were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 3 mM L-glutamine, 50 mg/mL garamicin, 2.5 mg/mL fungizon, 0.25% sodium bicarbonate and 10% of heat-inactivated fetal bovine serum (FBS) and maintained at 37 °C in an atmosphere of 5% CO_2. All experiments were carried out using 5×10^5 cells/mL.

HMPV NL/1/00 was kindly provided by ViroNovative BV, Erasmus University Rotterdam (The Netherlands). Trypsin was added to the culture medium for a final concentration of 1 μg/mL in all the antiviral and cytotoxicity experiments.

2.11. Evaluation of cytotoxicity

The cytotoxicity assay was performed by incubating LLC-MK2 cell monolayers cultivated in 96-well microplates with two-fold serial dilutions of the sulfated galactans, in triplicate, for seven days at 34 °C. The cellular viability was further evaluated by the neutral red dye-uptake method (Borefreund and Puerner, 1985). In this experiment were determined the cytotoxic concentration for 20% (CC_{20}: concentration required to reduce the number of viable cells by 20%), and 50% (CC_{50}: concentration required to reduce the number of viable cells by 50%) of cell culture. CC_{20} was used in the antiviral and mechanism of action assays, and CC_{50} for determination of the selectivity index (SI).

2.12. Antiviral assay

LLC-MK2 cell monolayers cultivated in 48 well-microplates were treated with the sulfated galactans at the CC_{20} concentration. The wells reserved for cell and virus controls were not treated with the sulfated galactans. Immediately after, 100 μL of a HMPV suspension diluted at 10^{-1} corresponding to 1.12×10^7 copies/mL were added to treated and untreated cell cultures and incubated in a 5% CO_2 atmosphere at 34 °C for seven days. All experiments were carried out in triplicate.

After incubation the supernatant of the cell monolayers was removed and then lysated using guanidine thiocyanate buffer. The viral RNA was extracted and a real time RT-PCR for detections of HMPV genome was performed using primers that amplify a 151 bp fragment of the HMPV N gene, as previously described by Brittain-Long et al. (2008). The antiviral activity was evaluated comparing the number of copies of viral genome obtained from the supernatant of the cell cultures in the presence of the sulfated galactans with the virus control that was inoculated in the untreated cell culture.

The dose–response curve was established starting from the non-cytotoxic concentrations, and the 50% effective dose (ED_{50}) was defined as the concentration of sulfated galactans that inhibits 50% of viral replication. The selectivity index (SI) was determined as the ratio of CC_{50} to ED_{50}.

2.13. Extraction of viral RNA

Viral RNA was extracted from the cells supernatant lysate using the commercial kit Totally RNA™ (Applied Biosystems/Ambion, USA), according to the manufactory's instructions.

2.14. Real-Time RT-PCR

Reverse transcription was performed in a 10 μL reaction mixture containing 5 μL of extracted viral RNA and 360 nM of the antisense primer, using ImProm-II Reverse Transcriptase (Promega, Madison, WI, USA). Real-time PCR assays were performed in a Step-One Real Time PCR System (Applied Biosystems, Carlsbad, CA, USA) and consisted of 2 min at 50 °C and 10 min at 95 °C, followed by 45 cycles of 10 s at 95 °C and 1 min at 60 °C. Amplification was carried out in 24 μL reaction volumes, including 5 μL of cDNA, 900 nM of sense primer and 300 nM of antisense primer, and 12 μL of Maxima qPCR SYBR Green (Fermentas, Canada). The quantification of HMPV RNA was performed using a standard curve generated by the Ct (Threshold cycle) values obtained from serial 10-fold dilutions of *in vitro* transcripts. Each dilution was quantified using the Quant-IT™ RNA Assay Kit (Invitrogen, Carlsbad, CA, USA), which allows us to correlate the amount of RNA, *i.e.* the number of genome copies, with the Ct of each dilution. The results were analyzed in triplicate, and the average number of copies of the viral RNA was calculated.

2.15. Virucidal assay

One hundred microliters of a HMPV suspension diluted at 10^{-1} were added to either 900 μL of the sulfated galactans at CC_{20} concentration or DMEM-Eagle (control), according to Chen, Griffith, Lucia, and Hsiung (1988). All mixtures were incubated at 37 °C for 2 h. After this period, the mixtures were diluted to 10^{-2} and immediately inoculated in LLC-MK2 cell monolayers grown in 48-well plates, which were incubated for seven days at 34 °C in atmosphere of 5% CO_2. After incubation the supernatant of the cell monolayers was removed, and then lysated using guanidine thiocyanate buffer for RNA extraction and real time RT-PCR as previously described (Mendes et al., 2011).

2.16. Cellular receptors assay

The sulfated galactans at CC_{20} concentration were added to LLC-MK2 cell monolayers, incubated at 4 °C for 1 h, washed three times with cold DMEM-Eagle and 100 μL of a HMPV suspension diluted at 10^{-1} were added to treated and untreated cell culture followed by incubation at 34 °C for seven days. After incubation, the supernatant

of the cell monolayers was removed and lysated using guanidine thiocyanate buffer for RNA extraction and real time RT-PCR as previously described (Mendes et al., 2011).

2.17. Cell entry assay

LLC-MK2 cell monolayers were inoculated with 100 μL of a HMPV suspension diluted at 10^{-1} and incubated for 1 h at 4 °C. After absorption, the monolayers were washed, treated with the sulfated galactans at CC_{20} concentration, followed by incubation for 1 h at 37 °C. Afterwards, the monolayers were washed, DMEM-Eagle was added and the cells were incubated at 34 °C for seven days in an atmosphere of 5% CO_2. After incubation the supernatant of the cell monolayers was removed and lysated using guanidine thiocyanate buffer for RNA extraction and real time RT-PCR as previously described (Mendes et al., 2011).

2.18. Intracellular assay

LLC-MK2 cell monolayers were inoculated with 100 μL of a HMPV suspension diluted at 10^{-1} and incubated at 37 °C for 2 h. After incubation, the cell monolayers were washed and the sulfated galactans added at CC_{20} concentration. Then, the cells were incubated at 37 °C for 10 h. After incubation the supernatant of cell monolayers was removed and the cells were washed with culture medium and lysated using guanidine thiocyanate buffer for RNA extraction and real time RT-PCR for HMPV as previously described (Mendes et al., 2011).

3. Results and discussion

3.1. Extraction, partial depolymerization and fractionation of the polysaccharides obtained from C. seminervis

The milled red seaweed *C. seminervis* was extracted four times with phosphate buffer (pH 6.5) at 80 °C. The crude extracts obtained from each extraction were pooled and treated with 2 M KCl yielding the soluble fraction S (95.0%, w/w) and the insoluble fraction P. Yield, analysis, specific rotation and monosaccharide composition of P and S are shown in Tables 1 and 2, respectively. P fraction presents significant amounts of proteins (15.6%) and low percentage of sulfate groups (10.4%). The monosaccharide composition together with the specific rotation (+54.5°) are indicative of the presence of carrageenan structures. This result is similar to those previously reported for the 2 M KCl precipitate fractions obtained from *C. seminervis* (Zibetti et al., 2009). The sulfated fraction S (19.1% SO_3Na) contains galactose and 3,6-anhydrogalactose as major sugar constituents besides methylated galactose derivatives, xylose and glucose. Fraction S was submitted to partial reductive hydrolysis to afford the partially depolymerized galactans DS (76.5% yield). DS was submitted to anion exchange chromatography on a DEAE-Sephacel column giving rise to three high yield fractions (DS-1, DS-2 and DS-3, ~80% of total recovery). HPSEC-MALLS-RID analyses showed that DS-1 and DS-3 are homogeneous with a Mw of 51.6 and 63.8 kDa, respectively. DS-2 was refractionated using the same column above mentioned originating five fractions (DS-2a-2e). HPSEC-MALLS-RID analyses of the major fractions DS-2e, Ds-2c and DS-2d (93% of total recovery) showed that only the former one presents a symmetrical profile indicative of its homogeneity (Mw of 60.1 kDa). The partially depolymerized sulfated galactans DS-1, DS-2e and DS-3 (14.1, 23.0 and 29.9% of sulfate, respectively) are principally constituted by galactose (60.3–85.6 mol%) and 3,6-anhydrogalactose (6.8–15.5 mol%) besides minor amounts of 3,6-anhydro-2-*O*-methylgalactose, 2-, 6-, 3-, and 4-*O*-methylgalactose. Moreover DS-1 contains xylose (6.0 mol%) and glucose (3.7 mol%) (Tables 1 and 2). The presence of glucose originates from floridean starch, a neutral polysaccharide that co-elutes with the sulfated galactan (Faria-Tischer et al., 2006). Enantiomeric analyses carried out with DS-1, DS-2e and DS-3 showed the presence of both D- and L-forms of galactose, 3,6-anhydrogalactose and its 2-*O*-methyl derivatives besides the D-forms of 2- and 6-*O*-methylgalactose. Considering the enantiomeric form of these units and that 3-, 4-*O*-methylgalactose and xylose are constituent of side chains (Zibetti et al., 2009), the agaran and carrageenan structures are present in a molar ratio of 2.7:1 for DS-1 and DS-2e and 1:1 for DS-3. The negative specific rotations are in agreement with the presence of agaran structures.

3.2. Desulfation

Solvolytic desulfation of DS-1, DS-2e and DS-3 removed 90, 99 and 94% of the sulfate groups giving rise to DS-1D, DS-2eD and DS-3D (73.0, 58.0 and 67.0% of yields, respectively). Monosaccharide compositions of the desulfated fractions (not shown) were very similar to those of the correspondent sulfated fractions showing that the solvolytic treatment did not promote modifications on sugar composition.

3.3. NMR analyses

The ^{13}C and ^{1}H NMR assignments were determined by using spectral data from the sulfated and desulfated samples, HSQC, DEPT, COSY and literature data. The ^{13}C NMR spectra of DS-1, DS-2e and DS-3 are shown in Fig. 1 whereas the spectra of the desulfated fractions DS-2eD and DS-3D and the DEPT spectrum of DS-1D are shown in Fig. 2. The HSQC spectra correlations of DS-1, DS-2e and DS-3 and their corresponding desulfated products are shown in Figure S1 and S2.

The ^{13}C NMR spectra of DS-1, DS-2e and DS-3 showed signals at 63.0 and 63.1 ppm corresponding to C-1 of 3,6-anhydrogalactitol units besides the C-6 signals of the same units at 73.0 ppm, all of them inverted in the ^{13}C-DEPT experiment. The presence of these signals are in agreement with the partial reductive hydrolysis conditions used in the present work, which split some of the 3,6-anhydrogalactosidic linkages giving products containing 3,6-anhydrogalactitol residues as terminal units (Usov & Elashvili, 1991; Zibetti et al., 2005, 2009). The HSQC spectra (Figure S2A, S2C and S2E) showed correlations at 86.3/4.29 and 85.5/4.33 ppm corresponding to C-4/H-4 of 3,6-anhydrogalactitol residues substituted on C-4 by β-D-galactose 2-sulfate and β-D-galactose residues, respectively. In the HSQC spectra of the desulfated fractions (DS-1D, DS-2eD and DS-3D, Figure S2B, S2D and S2F, respectively) the disappearance of the former correlation agrees with the removal of C-2 sulfate groups. Additionally the C-1 signals at 63.0 ppm (DS-1) and 63.1 ppm (DS-2e and DS-3) correlate with its geminal protons at 3.71 and 3.72 ppm, respectively and were attributed to 3,6-anhydrogalactitol units linked to pyruvylated β-D-galactose units (either bearing or not bearing sulfate groups on C-2) (Gonçalves, Ducatti, Duarte, & Noseda, 2002; Usov & Elashvili, 1991). Some of the chemical shift assignments of 3,6-anhydrogalactitol units are shown in Table S1.

The DEPT NMR spectra of DS-1, DS-2e and DS-3 (not shown) presented inverted signals at 67.1–67.3, 66.9–67.3 and 66.9–67.5 ppm, respectively, attributed to C-6 of C-6 substituted galactose residues. The intensity of these signals increases from DS-1 to DS-3 as showed in their ^{13}C NMR spectra (Fig. 1). In the ^{13}C NMR spectra of the corresponding desulfated fractions DS-1D (not shown), DS-2eD and DS-3D these signals disappeared (DS-2eD) or decreased (DS-1D and DS-3) indicating that they correspond principally to 6-sulfated galactose residues (Fig. 2). In the HSQC spectra (Figure S2) the correlations at 67.1/4.21, 67.1/4.34 (DS-1), 67.3/4.22, 67.3/4.30 (DS-2e) and 67.2/4.22, 66.9/4.35 ppm (DS-3)

Table 1
Yield, analyses and specific rotation of the galactans obtained from *C. seminervis*.

Fractions[a]	Yield (%)	Carbohydrate (%)	SO_3Na (%)	Protein (%)	$[\alpha]_D^{25}$ (°)	*Mw* (kDa)
P	5.0	43.0	10.4	15.6	+54.5	n.d.
S	95.0	52.5	19.1	2.6	−18.5	n.d.
DS	76.5	57.9	20.8	1.5	−15.5	n.d.
DS-w	2.7	96.7	n.d.	0.7	+112.5	n.d.
DS-1	29.3	66.3	14.1	0.5	−34.0	51.6
DS-2	35.0	n.d.	25.1	0.5	−44.0	n.d.
DS-3	15.4	53.3	29.9	–	−12.0	63.8
DS-4	9.0	48.5	31.7	0.6	+25.0	n.d.
DS-5	5.8	47.7	38.5	0.8	+36.0	n.d.
DS-2a	2.0	n.d.	n.d.	n.d.	n.d.	n.d.
DS-2b	5.0	n.d.	n.d.	n.d.	n.d.	n.d.
DS-2c	30.0	53.0	n.d.	–	−42.0	n.d.
DS-2d	36.0	51.2	n.d.	–	−43.0	n.d.
DS-2e	27.0	65.0	23.0	–	−40.0	60.1
S2S-4	28.0	52.5	26.6	–	−19.0	332.4

n.d., not determined; –, not detected.
[a] Fractions are defined in the text.

were attributed to C-6 sulfated galactose residues considering that in the HSQC spectra of the desulfated fractions these correlations were not present, whereas the correlations at 67.3/4.29 (DS-1D), 67.3/4.28 (DS-2e) and 67.2/4.29 ppm (DS-3D) were ascribed to C-6 of substituted galactoses and/or to residual C-6 sulfate groups. The ^{13}C NMR spectra of the depolymerized sulfated galactans showed C-6 non-substituted signals of α-galactose and β-galactose at 61.3 ppm (minor) and 61.0 ppm (major), respectively (Fig. 1). After desulfation the C-6 signal of α-galactose compared to that of β-galactose presented higher intensity in all homogeneous depolymerized galactans (compare Figs. 1 and 2). These results indicate that the sulfate groups are principally (or totally) located on α-L-galactose and/or α-D-galactose residues and that the content of α-L-galactose 6-sulfate and/or α-D-galactose 6-sulfate increases from DS-1 to DS-3. Additionally the DEPT spectra of the sulfated fractions showed inverted signals corresponding to C-6 of 4,6-*O*-(1′-carboxyethylidene)-β-D-galactopyranose residues (64.6 and 64.8 ppm) and C-6 of anhydrogalactosyl units at 68.9 (DS-1), 69.3 and 68.9 ppm (DS-3). The inverted signals at 65.2 and 65.3 ppm were ascribed to C-5 of xylosyl units. DS-1 spectrum showed an inverted signal at 71.4 ppm attributed to C-6 of 6-*O*-methylgalactose. In the methoxyl region (~56–61 ppm) ^{13}C NMR spectra (Fig. 1) showed signals of low intensity, in agreement with the presence of naturally methoxylated galactosyl units (14.5, 6.6 and 8.1 mol% for DS-1, DS-2e and DS-3, respectively, Table 2). Using HSQC spectra these signals were partially assigned. In the HSQC spectra of DS-1 (Figure S2A) and DS-3D (Figure S2F) correlations at 58.6/3.41 and 58.4/3.41 ppm, respectively, were attributed to methoxylation on C-6 of galactose residues, whereas the correlation for the methoxyl ether on C-2 of 3,6-anhydro-α-L-galactose units was observed in the HSQC spectra of DS-1 (58.3/3.51 ppm) and DS-2e (58.5/3.53 ppm) (Lahaye, Yaphe, Viet, & Rocha, 1989). Correlations at 61.3/3.52 (DS-1), 61.4/3.54 (DS-2e) and 61.4/3.52 ppm (DS-3) were assigned to methoxyl on C-4 of D-galactose units. 4-*O*-Methylgalactose units belonging to L-series has been described as side chains for some agarans obtained from *Gracilaria* genus as *G. tikvahaie* (Craigie & Jurgens, 1989) and *G. cervicormis* (Freile-Pelegrin and Murano, 2005), whereas *C. seminervis* presents 4-*O*-methyl-D-galactose as branching units (Zibetti et al., 2009). 2-*O*-Methylgalactose was also detected by the correlations at 57.4/3.50, 57.7/3.52 and 57.5/3.52 ppm for DS-1, DS-2e and DS-3, respectively (Ferreira et al., 2012; Navarro & Stortz, 2008). In

Table 2
Monosaccharide and enantiomeric compositions of the galactan fractions obtained from *C. seminervis*.

Fractions[a]	Monosaccharide (mol%)													
	G[b]			AG		2MAG		2MG		6MG		3MG	3/4MG	4MG	Xyl	Glc
	D[c]		L[d]	D	L	D	L	D		D					D	D
P		64.1			10.3		–		2.9		4.7		–		6.2	11.8
S	45.0		17.4	2.1	13.9	0.1	1.7	2.1		3.3			3.5		4.9	6.0
DS	37.7		20.9	1.0	15.7	0.3	1.8	3.0		3.3		0.9		3.5	5.3	6.6
DS-w		20.6			8.3		–		–		9.4		–		9.7	52.0
DS-1	45.9		14.4	1.2	14.3	0.1	2.0	2.7		3.7		1.0		5.0	6.0	3.7
DS-2	56.2		18.9		10.5		1.4		2.4	2.6			4.5		3.5	tr.
DS-3	68.4		17.2	0.8	6.0	0.6	1.6	1.2		1.5		0.3		1.0	1.4	tr.
DS-4	88.6		1.1	3.1	2.0		0.2	1.0		1.7			–	–	1.5	tr.
DS-5	81.0		8.5	1.6	0.4	1.0	1.6		0.5	1.2			–	–	2.0	2.2
DS-2a		35.9			13.1		–		–		–		–	–	23.2	27.8
DS-2b		58.3			10.3		–		2.3		2.0		5.7		9.0	12.4
DS-2c	51.3		19.4	0.5	11.5	0.2	1.3	3.7		1.7			6.4		2.3	1.7
DS-2d	51.0		21.1	0.5	12.4	0.3	1.9	3.2		1.3			4.2		4.1	tr.
DS-2e	54.5		19.9	1.0	13.5	0.2	1.0	2.5		1.6		0.6		2.6	2.6	tr.
DS-2f		80.9			5.6		–		–		7.7		–		2.9	2.9
S2S-4	63.5		10.1	–	18.3	–	1.0	1.5		2.8			tr.		2.8	tr.

–, not detected; n.d., not determined; tr., for glucose percentages lower than 1.0% are considered as trace amounts.
[a] Fractions are defined in the text.
[b] G, AG, 2MG correspond to galactose, 3,6-anhydrogalactose and 2-*O*-methylgalactose, respectively, *etc.*
[c] Percentages of D-galactose determined after enantiomeric analyses.
[d] Percentages of L-galactose determined after enantiomeric analyses.

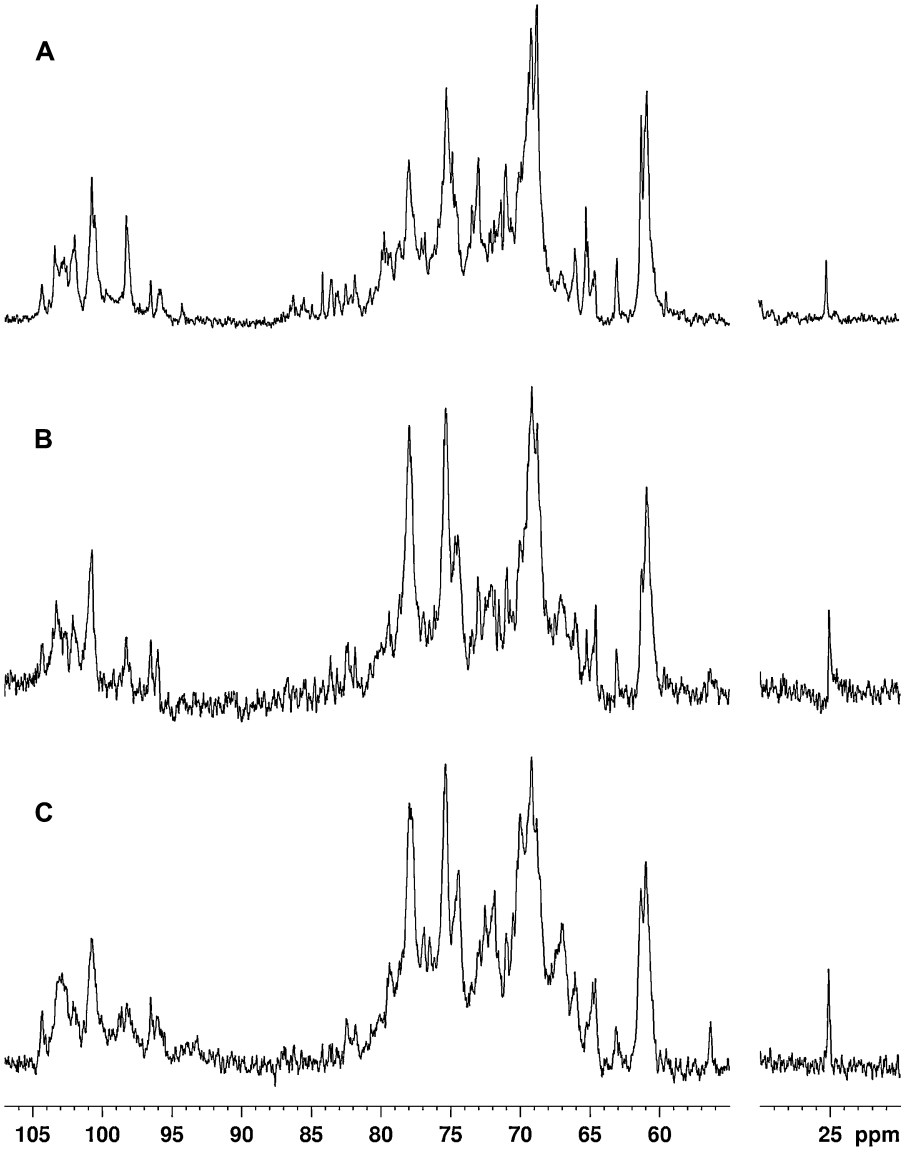

Fig. 1. ^{13}C NMR spectra of partially depolymerized galactans DS-1 (A), DS-2e (B) and DS-3 (C).

HSQC spectra the correlations at 56.3/3.46, 3.50 (DS-1), 56.5/3.48 (DS-2e) and 56.4/3.45 ppm (DS-3D) were attributed to methyl ether hydrogens located on C-3 of galactose residues, whereas those of the carbon at ~56 ppm with hydrogens at 3.12, 3.09 (DS-2e), 3.38 (DS-2eD) and 3.13 ppm (DS-3) remain unassigned.

In the ^{13}C NMR spectra, the signals at 25.1 and 175.0 ppm were attributed to the methyl and carboxyl carbons of pyruvic acid ketal (Garegg, Lindberg, & Kvarnström, 1979). From HSQC spectra the correlations of methyl group (25.1/1.49), C-6/H-6,H-6′ (64.6/4.05, 3.98) and C-5/H-5 (66.1/3.61 ppm) confirm the presence of pyruvylated β-D-galactopyranose residues (Lahaye et al., 1989; Gonçalves et al., 2002). Table 3 shows the NMR chemical assignments of the principal diads and/or constituent units of the partially depolymerized galactans before and after solvolytic desulfation. The ^{13}C NMR spectra of DS-1, DS-2e and DS-3 (Fig. 1) showed a complex anomeric region with at least nine signals. The prominent and broad signal centered at ~100.7 ppm corresponds to the overlapping of the C-1 signal of β-D-galactose 2-sulfate (C-2 at 78.0 ppm) pyruvylated or not and α-L-galactose residues (sulfated or not). From HSQC spectra (Figure S1) it was possible to determine the C-1/H-1 signals of the above mentioned β- and α-L-galactosyl units constituting the agaran diad G2S(P) → L(6S) (Knutsen's nomenclature, Knutsen, Myslabodski, Larsen, & Usov, 1994). The ^{13}C NMR spectra of DS-1, DS-2e and DS-3 contain signals at 103.4, 103.2, 103.2, and 103.4, 103.2, 103.0 ppm, respectively correspondent to β-D-galactose residues constituting the agaran diad G(6M)(6R)(P) → L(6S).

From HSQC spectra (Figure S1), the anomeric correlations at 98.3/5.15 (DS-1), 98.0/5.16, 98.3/5.19 (DS-2e) and 98.2/5.13, 98.6/5.18 ppm (DS-3) ppm were attributed to 3,6-α-L-anhydrogalactosyl units and their 2-*O*-methyl derivatives. Additionally, DS-1 spectrum showed correlation at 98.3/5.24 ppm corresponding to C-1/H-1 of 3,6-anhydro-α-L-galactosyl units constituting the diad GP → LA. The correlations at 97.9/5.24 and 97.8/5.24 ppm present in HSQC spectra of DS-1D and DS-2eD, DS-3D (Figure S1) showed that in the DS-2e and DS-3 galactans LA units are constituents of the diad G2SP → LA. Additionally, the signal at 96.5 ppm indicates that the 3,6-anhydro α-L-galactosyl units are also present as its 2-*O*-sulfated derivative.

Together with the agaran diads the ^{13}C NMR spectra of DS-1, DS-2e and DS-3 also showed signals corresponding to carrageenan diads, in agreement with the presence of α-D-galactosyl

Table 3
Chemical shift (ppm) assignments of NMR spectra of the sulfated (DS-1, DS-2e and DS-3) and desulfated (DS-1D, DS-2eD and DS-3D) partially depolymerized galactans.

Diads and/or Units[a]	Fraction											
	DS-1		DS-2e		DS-3		DS-1D		DS-2eD		DS-3D	
	A-Unit C-1/H-1	B-Unit C-1/H-1	A-Unit C-1/H-1	B-Unit C-1/H-1	A-Unit C-1/H-1	B-Unit C-1/H-1	A-Unit C-1/H-1	B-Unit C-1/H-1	A-Unit C-1/H-1	B-Unit C-1/H-1	A-Unit C-1/H-1	B-Unit C-1/H-1
G(6M)(6R)→ LA(2M)	102.0/4.60 102.7/4.51	98.3/5.15	102.0/4.62 102.7/4.54	98.0/5.16	102.1/4.61	98.2/5.13	102.1/4.60	97.9/5.15	101.9/4.59	97.8/5.15	102.0/4.60	97.8/5.16
G(P)2S→ LA			100.9/4.66	98.3/5.19	100.7/4.75	98.6/5.18						
LA2S		96.5/5.30		96.5/5.30		96.5/5.30						
GP→ LA	102.0/4.60 102.7/4.51	98.3/5.24					102.1/4.60	97.9/5.24	101.9/4.59	97.8/5.24	102.0/4.60	97.8/5.24
G2S(P)→ L(6S)	100.7/4.75 100.8/4.51 100.9/4.61	100.8/5.29 100.8/5.35	100.7/4.75 100.9/4.66	100.7/5.31	100.7/4.75 100.9/4.68	100.8/5.30						
G(6M)(6R)(P)→ L(6S)	103.2/4.46 103.4/4.63	100.8/5.29 100.8/5.35	103.2/4.48 103.2/4.64	100.7/5.31	103.2/4.48 103.0/4.71 103.4/4.66	100.8/5.30						
G(6M)(6R)(P)→ L							103.1/4.70 103.2/4.65	100.5/5.30	103.2/n.d.	100.5/5.30	103.2/4.70 103.2/4.60	
G→ DS	104.3/4.65	95.9/5.41 96.5/5.30	104.2/4.68	96.0/5.39 96.5/5.30	104.3/4.65	96.1/5.40 96.3/5.36 96.5/5.30 96.3/5.30						
G2S P→ D(S)					100.7/4.74	96.1/5.40 96.3/5.36 96.5/5.30 96.3/5.30						
G→ D	104.3/4.65	95.8/5.15			104.3/4.65	95.7/5.17	104.3/4.60	95.7/5.16	104.3/n.d.	95.7/5.15	104.4/4.60	95.7/5.16
GP→ D											104.0/n.d.	94.7/5.32
G2S(6M) (6R)→ DA	100.7/4.74 100.9/4.61	94.3/5.20										
DA2S						93.6/5.49						
G(6M)(6R)→ DA	102.0/4.60 102.7/4.51	94.1/5.09					102.1/4.60	94.1/5.09			102.0/4.60	94.3/5.09

n.d., not determined.

[a] G(6M)(6R) corresponds to G, G6M and/or G6R, LA(2M) corresponds to LA and/or LA2M, *etc.*

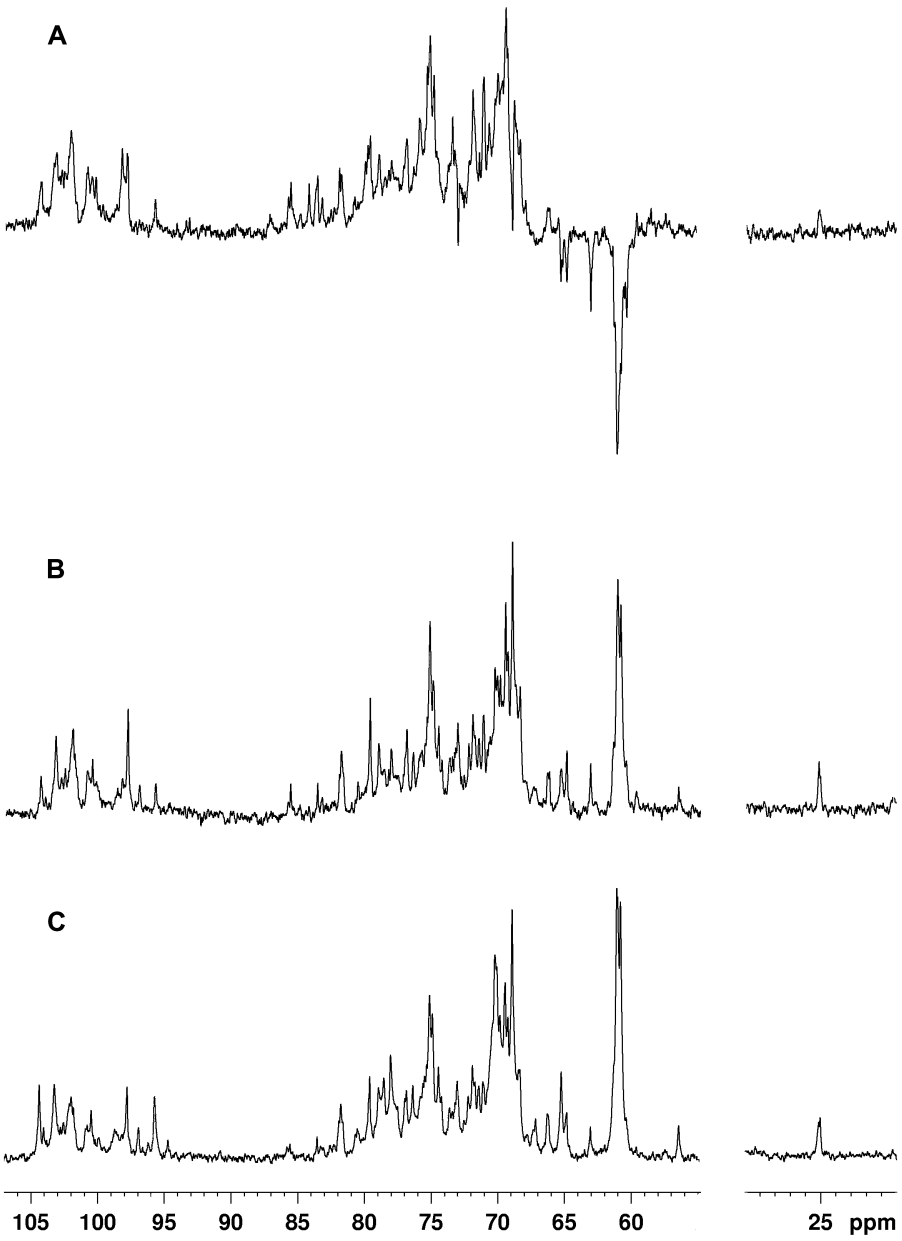

Fig. 2. DEPT NMR spectrum of partially depolymerized galactan DS-1D (A) and ^{13}C NMR spectra of partially depolymerized galactans DS-2eD (B) and DS-3D (C).

units determined by enantiomeric analyses. In the HSQC spectra of DS-1, DS-2e and DS-3 the anomeric signals at 104.3/4.65, 104.2/4.68 and 104.3/4.65 ppm, respectively were attributed to β-D-galactosyl units linked to sulfated α-D-galactosyl units (95.9/5.41, 96.5/5.30 ppm, DS-1), (96.0/5.39, 96.5/5.30 ppm, DS-2e), (96.1/5.40, 96.3/5.36, 96.5/5.30, 96.3/5.30 ppm, DS-3) as well as to non-sulfated α-D-galactosyl units (95.8/5.15 and 95.7/5.17 ppm in DS-1 and DS-3 spectra, respectively). Additionally, the HSQC spectrum of DS-1 showed correlations at 94.3/5.20 and 94.1/5.09 ppm attributed to 3,6-anhydro α-D-galactosyl units linked to β-D-galactose 2-sulfate (substituted or not on C-6) and β-D-galactose (substituted or not on C-6), respectively, whereas only DS-3 spectrum presents anomeric signals at 93.6/5.49 ppm attributed to 3,6-anhydro-α-D-galactosyl 2-sulfate.

In the HSQC of the desulfated galactans the α-anomeric correlations at 95.7/5.16 (DS-1D and DS-3D) and 95.7/5.15 (DS-2eD) were ascribed to α-D-galactosyl units linked to β-D-galactosyl units (104.3 or 104.4 ppm) (Usov, Yarotsky, & Shashkov, 1980). Differently from the other two fractions, the HSQC spectrum of DS-3D showed signals of C-1/H-1 at 94.7/5.32 ppm corresponding to α-D-galactosyl units constituting the diad GP (104.0 ppm) → D. The upfield chemical shift (−1 ppm) due to pyruvate ketal substitution on β-D-galactosyl units agree with that previously reported (Falshaw & Furneaux, 1995; Miller & Blunt, 2000). The ^{13}C NMR spectrum of DS-3D showed a decrease of the C-5 and C-6 intensity signals of the pyruvylated galactose residues when compared to the spectrum of DS-3 (Figs. 1 and 2). The unexpected partial depyruvylation probably occurred during the RMN analyses and/or during desulfation process. The increase of the signal of non substituted C-4 of β-D-galactosyl units (65.2 ppm, no DEPT inverted) present in the DS-3D spectrum was ascribed to partial remove of pyruvate ketal and not to the presence of β-D-galactose 4-sulfated in the parent fraction. This is supported by the absence of correlations in the HSQC spectrum of DS-3 corresponding to C-4 (71.5–73.4/4.83–4.98 ppm) of 4-sulfated β-D-galactosyl units (Araújo et al., 2013). Therefore in

Table 4
Cytotoxicity and antiviral activity of sulfated galactans from *C. seminervis*.

Fractions[a]	CC_{20}[b] (μg/mL)	Inhibition CC_{20} (%)	ED_{50}[c] (μg/mL)	SI[d]
S	25	99.9	1.2	>170.9
DS	100	87.4	81.2	>2.3
DS-1	100	95.4	48.4	>4.1
DS-2e	200	78.4	130.1	>1.5
DS-3	100	99.8	8.3	>24.1
S2S-4	200	99.9	9.0	>22.2
Ribavirin[e]	50	99.9	1.6	>119.0

[a] Fractions are defined in the text.
[b] Cytotoxic concentration 20%: concentration required to reduce the number of viable cells by 20%.
[c] Effective dose 50%: concentration of sulfated galactans that inhibits 50% of viral replication.
[d] Selectivity index (CC_{50}/ED_{50}). CC_{50} (cytotoxic concentration 50%): concentration required to reduce the number of viable cells by 50%. CC_{50} values were >200 μg/mL for all compounds.
[e] Standard compound.

the HSQC spectrum of DS-3D, β-D-galactosyl units (C-1–C-2/H-1–H2, C-3 and C-4–C-6/H-4–H6,6′ at 104.4/4.66, 70.2/3.38, 78.5 and 65.2/4.16, 74.9/3.70, 60.7/3.85, 3.75 ppm) linked to α-D-galactosyl units (C-1–C-4/H-1–H-4, C-5, and C-6/H6,6′at 95.7/5.16, 68.9/3.95, 70.4/4.25, 78.0/3.77, 70.1 and 61.0/3.92, 3.78 ppm) represent an important diad and showed that in the parent fraction significant amounts of pyruvylated β-D-galactose 2-sulfate are linked to α-D-galactose (sulfated) residues. Furthermore, DS-1D and DS-3D spectra presented α-anomeric correlations at 94.1/5.09 and 94.3/5.09 ppm ascribed to 3,6-anhydro-α-D-galactose residues constituting the diad G(6M)(6R) → DA (Usov & Shashkov, 1985).

Therefore the results of chemical and spectroscopic analyses carried out with DS-1, DS-2e and DS-3 show that these partially depolymerized galactans are pyruvylated, present differences in the content and location of sulfate groups, kind of diads and in the molar ratio of agaran to carrageenan diads. These results are in accordance with previous report that showed that *C. seminervis* produces pyruvylated galactans composed by agaran and carrageenan structures, namely DL-hybrid galactans (Zibetti et al., 2009). Considering the yields and the homogeneous profiles obtained by HPSEC-MALLS-RID of the partially depolymerized galactans DS-1, DS2e and DS-3, these fractions were selected to evaluate the antiviral activity against human metapneumovirus together with S, DS and S2S-4 fractions. The later corresponds to a homogeneous DL-hybrid galactan obtained from *C. seminervis*, as previously described (Zibetti et al., 2009). The yield, chemical analyses, specific rotation and monosaccharide composition of S2S-4 are shown in Tables 1 and 2, respectively.

3.4. Cytotoxicity and antiviral activity

The concentrations in which at least 80% of cells in culture remained viable (CC_{20}) were established for each sulfated galactan. These values (25–200 μg/mL) were used to evaluate the antiviral activity as shown in Table 4. It was not possible to determine the CC_{50} values, since the percentage of viable cells at the highest concentration tested (200 μg/mL) was higher than 50% for all fractions (Table 4). These results corroborate data available in literature on the subject showing that sulfated polysaccharides, in general, have low toxicity in cell cultures (Cassolato et al., 2008; Duarte, Noseda, Cardoso, Túlio, & Cerezo, 2001; Duarte, Noseda, Noseda, et al., 2001; Duarte et al., 2004; Ghosh et al., 2009; Romanos et al., 2002).

The evaluation of antiviral activity was carried out by the comparison of the number of genomic RNA copies obtained in the presence of the sulfated galactan fractions and that obtained from the viral control. Inhibition rates of 99.9% were observed for the crude 2 M KCl soluble fraction S and DL-hybrid galactan S2S-4. The depolymerized fraction DS inhibited 87.4% of HMPV replication, while the homogeneous subfractions of DS obtained by anion exchange chromatography DS-1, DS-2e and DS-3 showed 95.4, 78.4 and 99.8% of inhibition, respectively.

In order to determine the ED_{50} values and the selectivity indexes (CC_{50}/ED_{50}) LLC-MK2 cells were, separately, treated with different concentrations of each sulfated galactan. Figure S3 shows diagrams correlating percentages of inhibition and the genome equivalents measured by real time RT-PCR of the virus in the absence and the presence of different concentrations of the sulfated galactan fractions. These results show that the sulfated galactans present a dose-dependent response against HMPV, since viral inhibition increases with the polysaccharide concentration. The correlation between cytotoxicity and anti-HMPV activity is shown in Table 4.

The S fraction showed inhibitory activity at lower concentrations than the other sulfated galactan fractions with ED_{50} of 1.2 μg/mL. The selectivity index (SI) of this fraction was >170.9, being higher than ribavirin, the standard compound indicated for the treatment of HMPV infection (SI > 119.0). Based on these results the S galactan can be considered a highly selective anti-HMPV compound.

Galactan S2S-4 and the depolymerized galactan DS-3 showed ED_{50} values of 9.0 and 8.3 μg/mL, respectively, with similar selectivity indexes (>22.2 and >24.1, respectively). Differently, the pool of depolymerized galactans DS and its subfractions (DS-1 and DS-2e) displayed lower anti-HMPV activity when compared to the above-mentioned fractions, with ED_{50} of 81.2, 48.4 and 130.1 μg/mL, respectively.

Both the degree of sulfation and location of the sulfate groups play an important factor in the antiviral activity of the sulfated polysaccharides (Damonte, Matulewicz, & Cerezo, 2004). The partial depolymerization of the sulfated galactans present in S (19.1% of sulfate) originated the pool of products DS (20.8% of sulfate) with a substantial retention of the sulfate groups (Table 1). Accordingly, the higher anti-HMPV activity presented by S when compared with those of its depolymerized products (DS, DS-1, Ds-2e and DS-3) could be associated at least in part with the higher molecular weight of the former. This result is in agreement with a good inhibitory activity against HMPV presented by the DL-hybrid galactan S2S-4 with a high molecular weight (Mw of 332.0 kDa, Table 1). Besides the content and location of sulfate groups and molecular weight, the ability of the sulfated polysaccharides to inhibit viral biosynthesis has been associated also with other features such as constituent sugars, molecular conformation and stereochemistry (Damonte et al., 2004; Duarte et al., 2004).

3.5. Mechanisms of action

Aiming to establish the stage of the virus replication cycle at which the sulfated galactans obtained from *C. seminervis* in its native and partially depolymerized forms exert their anti-HMPV activity four different mechanisms were evaluated: virucidal, interaction with cellular receptors, inhibition of the viral penetration and the activity in intracellular events (post-penetration) and the results are shown in Table 5. All fractions, except DS-2e showed virucidal activity. The homogeneous sulfated galactan S2S-4 and the depolymerized sulfated galactan DS-1 presented the highest virucidal activity with 99.9 and 99.1% of inhibition, respectively. A good activity was observed for the depolymerized DS-3 fraction (86.9% of inhibition), while S and DS crude fractions showed 67.9 and 56.9% of inhibition, respectively.

Few reports have associated the antiviral activity of sulfated polysaccharides with direct virus inactivation (Harden et al., 2009). Studies using lambda-carrageenans against HSV showed virucidal activity and this antiviral mechanism of action was correlated with the increase of the *in vivo* activity (Carlucci, Scolaro, Noseda, Cerezo,

Table 5
Mechanisms of action of sulfated galactans from *C. seminervis*.

Fractions[a]	Mechanism of action [b]			
	Virucidal	Receptor	Penetration	Intracellular
S[c]	67.9	16.6	0	0
DS[c]	56.9	0	0	0
DS-1[c]	99.1	0	0	0
DS-2e[c]	0	68.4	0	0
DS-3[c]	86.9	0	72.7	0
S2S-4[c]	99.9	0	0	0
Ribavirin[d]	0	0	0	99.9

[a] Fractions are defined in the text.
[b] Results in percentage of inhibition.
[c] Sulfated galactans at the CC20 (see Table 4).
[d] Standard compound at the CC20 (see Table 4).

& Damonte, 2004). Virucidal activity has also been reported for a sulfated galactofucan and a fucan obtained from *Undaria pinnatifida* and *Splachnidium rugosum*, respectively, and for sulfated galactans isolated from *Gigartina atropurpurea* and *Ploclamium cartilagineum* (Harden et al., 2009). The virucidal activity of sulfated polysaccharides has been associated with the formation of a stable virion-sulfated polysaccharide complex where the sites on the viral envelope required for virus attachment to host cells are shielded by these polysulfates (Damonte et al., 2004; Harden et al., 2009).

Cell receptors assay showed that DS-2e and S fractions presented 68.4 and 16.6% of inhibition for HMPV replication, respectively, whereas the other fractions did not present this activity. In this assay, the sulfated galactans were removed prior to infection. The fact that DS-2e and S fractions retain activity under these conditions suggests that they interfered with the adsorption of the virus to target cell shielding and/or interacting with the cell receptor (Harden et al., 2009).

Viral penetration assay showed that only the depolymerized DS-3 fraction was able to inhibit HMPV replication (72.7% of inhibition). DS-3 presents different structural peculiarities when compared to the other sulfated galactans investigated in this work, such as a lower 3,6-anhydrogalactose amount (6.8 mol%) and higher percentage of sulfate groups (29.9%). Additionally DS-3 presents a higher content of α-galactose C-6 sulfate units when compared with DS-1 and DS-2e as showed by their ^{13}C NMR spectra (Fig. 1). The low content of 3,6-anhydrogalactosyl units decreases the hydrophobic character (Talarico, Noseda, Ducatti, Duarte, & Damonte, 2011), whereas the sulfate groups together with the carboxyl group of pyruvic acid ketal increase the ionic character of DS-3, when compared with the other galactans investigated in this work. DS-3 was able to inhibit HMPV by two different mechanisms: virucidal activity and inhibition of virus penetration (Table 5). The HMPV particles present three surface glycoproteins: fusion protein F, the attachment protein G and protein SH (Feuillet, Lina, Rosa-Calatrava, & Boivin, 2012; van den Hoogen et al., 2004). Considering that the former protein is not involved in the adsorption step but is required for viral fusion and penetration into the host cell we tentatively propose that DS-3 galactan interacts (directly and/or indirectly) with the HMPV fusion protein F. None of the sulfated galactan fractions tested presented intracellular activity.

Therefore, the anti-HMPV activity of the sulfated and pyruvylated DL-hybrid galactans obtained from *C. seminervis* and its depolymerized homogeneous products occurred early in the replicative cycle, which is characteristic of sulfated polysaccharides with antiviral activity (Baba, Snoeck, Pauwels, & De Clercq, 1988; Bouhlal et al., 2011). The sulfated galactans studied in the present work were able to preclude different steps of HMPV replicative cycle. Treatments using drugs with pleiotropic mechanisms of action are important since they can decrease the possibility of development of resistant virus strains when compared with compounds that present only one target in the viral life cycle.

In conclusion, the anti-HMPV activity displayed by the DL-hybrid galactans from *C. seminervis* and their depolymerized products represent a new class of compounds for further *in vivo* studies, aiming to improve our knowledge about the therapeutic potential of sulfated polysaccharides as antiviral agents for respiratory diseases. Currently, the drugs used for treatment of viral respiratory diseases are administrated as aerosols (Collins & Melero, 2011). In this way, the use of compounds with low cytotoxicity, as the sulfated polysaccharides, could be a potential alternative. Considering the promising results obtained in the present work, studies related with the anti-HPMV activity of other sulfated polysaccharides and their modified products are in progress.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.09.026.

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