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Partial and total C-6 oxidation of gelling carrageenans. Modulation of the antiviral activity with the anionic character

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ABSTRACT

The optimal conditions for the full C-6 oxidation of κ- and ι-carrageenans using (2,2,6,6-tetramethylpiperidinyl)oxy (TEMPO) in the presence of sodium hypochlorite and sodium bromide were assessed. The fully oxidized products were characterized by NMR spectroscopy. Partially oxidized products were also obtained and analyzed by chemical and spectroscopical methods. The antiviral activity of carrageenans against herpes simplex virus HSV-1 and HSV-2 determined by plaque reduction assay, was not largely affected by full oxidation of the polysaccharides, but an increase in activity was detected by partial oxidation. A specific overoxidation on C-2 of the 3,6-anhydrogalactose moiety of κ-carrageenan was identified, solved experimentally and rationalized through the application of molecular modeling.

1. Introduction

Many natural polysaccharides have been used in the food industry, as well as in other applications (Stephen & Churms, 2006). Polysaccharides from marine sources (alginates, agarans and carrageenans) are especially chosen for industrial applications due to their availability, and their (usually) regular structure (Stortz & Cerezo, 2000). Carrageenans have structures based on linear chains of alternating 3-linked β-D-galactopyranosyl residues and 4-linked α-D-galactopyranosyl or 3,6-anhydrogalactopyranosyl residues, substituted with sulfate esters in different positions. The polyanionic characteristics of carrageenans allow them to carry many proven biological activities, such as antitumor (Bondu, Deslandes, Fabre, Berthou, & Guangli, 2010; Haijin, Xiaolu, & Huashi, 2003;), anticoagulant (Carlucci et al., 1997; Wijesekara, Pangestuti, & Kim, 2011), and especially antiviral (Carlucci et al., 1997; Damonte, Matulewicz, & Cerezo, 2004; Talarico et al., 2004; Tischer et al., 2006).

Carrageenans are classified according to their idealized structure, and named by specific Greek letters. The most important gelling carrageenans (κ- and ι-) are 4-sulfated on the galactose moiety, have a 4-linked 3,6-anhydrogalactose moiety and differ only by the sulfation pattern of O-2 of this anhydro residue (sulfated in ι-, not sulfated in κ-).

A number of different chemical modifications of carrageenans have been carried out. The simplest and best known, even at an industrial level, is the alkaline treatment which converts by an intramolecular nucleophilic attack 6-sulfated α-D-galactose units into 3,6-anhydro-α-D-galactose moieties (Ciancia, Noseda, Matulewicz, & Cerezo, 1993; Navarro & Stortz, 2005). Other modifications included oversulfation (Yuan et al., 2005), phosphorylation (Yuan et al., 2005), replacement of sulfate groups by seleniate groups (Campos, Kawano, da Silva Jr., & Carvalho, 2009), *O*-maleolylation (Jiang & Guo, 2005), and *O*-succinylation (Jiang, Guo, & Chen, 2007), used to increase and/or modify the anionic properties, and thus their interactions with biological receptors.

Oversulfation, phosphorylation, and introduction of spacers terminating in carboxyl groups were thus the most common ways to increase the anionic charge of carrageenans, and then improve their biological activities. A simpler way might be the oxidation of the primary hydroxyl group of the galactose units to generate a C-6 carboxyl group (galacturonic acid). The most successful reagent for this reaction has been (2,2,6,6-tetramethylpiperidinyl)oxy or TEMPO, a stable water-soluble nitroxyl radical which can be used

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in catalytic amounts by the addition of another oxidant (usually NaOCl or commercial bleach) which regenerates the nitroxyl oxidant (Bragd, van Bekkum, & Besemer, 2004). With an adequate pH regulation to avoid oxidation by the hypochlorite, selective C-6 oxidation was achieved for different polysaccharides like cellulose (Follain, Montanari, Jeacomine, Gambarelli, & Vignon, 2008; Saito & Isogai, 2005; Xu, Dai, Sun, Wang, & Wu, 2012) and starch (Bragd, Besemer, & van Bekkum, 2001; de Nooy, Besemer, & van Bekkum, 1995; Kato, Matsuo, & Isogai, 2003; ter Haar et al., 2010; Thaburet, Merbouh, Ibert, Marsais, & Queguiner, 2001), among many others. Recently, the C-6 oxidation of agarose (Su et al., 2013), a polysaccharide related to carrageenan, was reported. The modified polysaccharides showed improved solubility properties (Chang & Robyt, 1996), metal chelating abilities (Muzzarelli, Muzzarelli, Cosani, & Terbojevich, 1999; Saito & Isogai, 2005) and the possibility of introducing new functional groups through amidation (Follain et al., 2008; Su et al., 2013) or esterification (Muzzarelli et al., 1999). Besides, the biological activity was modified by the introduction of new anionic groups (Bae, Kim, Lee, & Lee, 2011; Delattre et al., 2015; Elboutachfaiti et al., 2011; Wang et al., 2011).

Herpes simplex virus (HSV) types 1 and 2 may cause a broad range of human diseases, including oral and genital infections, keratoconjunctivitis and encephalitis, with different degrees of severity (Whitley & Roizman, 2009). As prolonged therapies with acyclovir, the most successful antiherpetic drug, have resulted in the emergence of drug-resistant variants (Piret & Boivin, 2011), the development of new compounds with different targets is required. In particular, sulfated polysaccharides like carrageenans have shown a potent anti-HSV activity (Damonte et al., 2004). Thus, the improvement of their antiviral effectiveness becomes of considerable interest.

With the aim of introducing modified materials with enhanced biological or functional properties, we have carried out a detailed study of the optimal conditions for achieving different degrees of C-6 oxidation of the κ-carrageenan from *Hypnea musciformis* (Cosenza, Navarro, Fissore, Rojas, & Stortz, 2014). We also report the study of a side reaction and its rationalization through molecular modeling, the extension of the reaction to ι-carrageenan, the chemical and spectroscopical characterization of all the products, and the assessment of the anti-herpes simplex virus (HSV) activity of fully and partly oxidized carrageenans.

2. Materials and methods

2.1. Materials

The κ-carrageenan from *Hypnea musciformis* was obtained as reported elsewhere (Cosenza et al., 2014). It corresponds to the fraction isolated after extraction of the seaweed with hot water, precipitation with 0.125 M KCl, dialysis and freeze-drying. ι-Carrageenan and TEMPO were purchased from Sigma–Aldrich. Dialysis was carried out using cellulose membranes of molecular weight cut-off 3500 against distilled water. All chemical reagents and solvents were of analytical grade.

2.2. Optimization of the reaction of κ-carrageenan with TEMPO

Oxidation experiments were performed at different pHs, reaction times and quenching methods. The general method was as follows: 100 mg of κ-carrageenan (containing *ca.* 0.25 mmol of primary alcohol), 1.6 mg of TEMPO and 20 mg of NaBr were dissolved in 15 mL of water and cooled to 0 °C in an ice bath. The solution was adjusted to the expected pH by adding 0.1 M aq NaOH, or else, when a $NaHCO_3/Na_2CO_3$ buffer solution was used, the reagents were dissolved directly in 15 mL of the buffer solution. The external oxidant was prepared by diluting commercial bleach (0.77 M NaClO) with an equal volume of water and adjusting to the desired pH by the addition of 1 M aq HCl. The bleach solution was titrated by a $Na_2S_2O_3$ solution using iodine/soluble starch as end point indicator. The oxidant solution was added dropwise to the carrageenan solution during 20 min. The volume of bleach added was calculated as to keep a ratio of 1.25 eq of NaClO per eq of primary alcohol. The appropriate pH was kept constant by adding 0.1 M NaOH. Both the pH adjustment of the bleach solution and the addition of 0.1 M NaOH were not necessary when buffer solutions were used. The reaction was left for the desired length of time. At this moment, the reaction volume was divided in two halves. One was quenched by addition of 2 mL of ethanol, neutralized with 0.1 M HCl and dialyzed. The other half was quenched by the addition of 50 mg of $NaBH_4$ and left 2 h at 0 °C before dialysis. Finally, the product was isolated by freeze-drying. The set of different conditions used, together with the acronyms of the treated polysaccharides are shown in Table 1.

Table 1
Nomenclature of the polysaccharides and conditions assayed for the oxidation with TEMPO.

Acronym	Treated polysaccharide	Base	pH	Time (h)	Eq NaClO	Quencher
K-Ox$_E$-N$_{10.5}$	κ-carrageenan	NaOH	10.5	2	1.25	EtOH
K-Ox$_R$-N$_{10.5}$	κ-carrageenan	NaOH	10.5	2	1.25	$NaBH_4$
K-Ox$_R$-B$_{10.4}$	κ-carrageenan	Buffer	10.4	2	1.25	$NaBH_4$
K-Ox$_R$-B$_{10}$	κ-carrageenan	Buffer	10	2	1.25	$NaBH_4$
K-Ox$_R$-B$_{9.4}$	κ-carrageenan	Buffer	9.4	2	1.25	$NaBH_4$
K-Ox$_R$	κ-carrageenan	NaOH	10	2	1.25	$NaBH_4$
K-Ox$_R$-1h	κ-carrageenan	NaOH	10	1	1.25	$NaBH_4$
K-Ox$_R$-3h	κ-carrageenan	NaOH	10	3	1.25	$NaBH_4$
K-1/10-Ox$_R$	κ-carrageenan	NaOH	10	2	0.125	$NaBH_4$
K-1/4-Ox$_R$	κ-carrageenan	NaOH	10	2	0.313	$NaBH_4$
K-1/2-Ox$_R$	κ-carrageenan	NaOH	10	2	0.625	$NaBH_4$
S-Ox$_E$	Starch	NaOH	10.5	2	1.25	EtOH
I-Ox$_E$	ι-carrageenan	NaOH	10.5	2	1.25	EtOH
I-Ox$_E$-D	ι-carrageenan	NaOH	10.5	2	1.25	EtOH
I-Ox$_R$	ι-carrageenan	NaOH	10	2	1.25	$NaBH_4$
I-1/4-Ox$_R$	ι-carrageenan	NaOH	10	2	0.313	$NaBH_4$
I-1/2-Ox$_R$	ι-carrageenan	NaOH	10	2	0.625	$NaBH_4$

2.3. Optimal conditions for the oxidation of carrageenans with TEMPO

The optimal conditions found for κ-carrageenan were used for the total oxidation of this and other polysaccharides. Briefly, an amount of polysaccharide containing 0.25 mmol of primary alcohol, 1.6 mg of TEMPO and 20 mg NaBr were dissolved in 15 mL of water and cooled to 0 °C in an ice bath. The solution was adjusted to the pH 10 by adding 0.1 M aq NaOH. The oxidant solution, prepared as indicated above containing 1.25 eq NaClO per eq of primary alcohol was added dropwise in 20 min. The reaction mixture was left for 1–2 h, keeping the pH at 10 with 0.1 M aq NaOH. After that period, $NaBH_4$ was added and left for 2 h at 0 °C before dialysis.

2.4. Partial oxidation of polysaccharides with TEMPO

The partial oxidation was carried out using the optimal conditions (see above), but reducing the amount of oxidant added: partially oxidized κ-carrageenan was obtained by addition of 0.625, 0.313 and 0.125 eq NaClO per eq primary alcohol. For ι-carrageenan the reaction was carried out after addition of 0.625 and 0.313 eq NaClO (Table 1).

2.5. Determination of the composition

Quantification of galactose was carried out by gas–liquid chromatography (GLC) after reductive hydrolysis and acetylation as depicted elsewhere (Navarro & Stortz, 2003; Stevenson & Furneaux, 1991). GLC was performed on an HP 5890A apparatus fitted with a capillary column SP 2330 (30 m × 0.25 mm i.d., thickness 0.20 μm), equipped with a flame ionization detector operating at 240 °C. The injector temperature was 240 °C and the oven temperature started at 200 °C, rose 2 °C/min to 230 °C, and stayed at this temperature for 20 min. Nitrogen was used as the carrier gas at a head pressure of 15 psi. Aliquots were injected with a split ratio of 80:1. *Myo*-inositol was used as the internal standard. Sulfate was determined by ion chromatography (Navarro, Flores, & Stortz, 2007). Uronic acids in the oxidized polysaccharides were determined by the method of Filisetti-Cozzi and Carpita (1991), using galacturonic acid as standard. 3,6-Anhydrogalactose was determined by GLC for the original polysaccharides (Navarro & Stortz, 2003; Stevenson & Furneaux, 1991), and by the resorcinol method (Yaphe & Arsenault, 1965) using fructose as standard for the oxidized polysaccharides. In the latters the GLC area of the 3,6-anhydrogalactose peak appears underestimated due to its linkage to galacturonic acid, partly resistant to hydrolysis. Desulfation was carried out by the solvolytic microwave method of Navarro et al. (2007).

2.6. Gel permeation chromatography

GPC analysis was performed with a high performance liquid chromatography system equipped with a Shimadzu refractive index detector (Model RID-10A), and a Shimadzu LC-20AT pump. Polysaccharides were analyzed using two columns connected in series: a Supelco Progel-TKS G4000 and a Waters Ultrahydrogel 250 (300 × 7.8 mm). A degassed solution of 0.05 M $NaNO_3$ prepared with ultra-pure water and containing 0.02% NaN_3 was used as solvent and eluant. 100 μl of polysaccharide solutions (5 mg/ml) were filtered through a 0.22 μm PVDF membrane (GV, Millipore) and injected into the column using a manual valve. The eluant flow rate was of 0.6 mL/min. The column was calibrated using dextran standards of different molecular weights (Sigma–Aldrich), and the molecular weight (MW) was determined at the top of each peak.

2.7. Spectroscopical methods

The NMR spectra were obtained on a Bruker Avance II 500 spectrometer at 500.13 (^{1}H) and 125.77 (^{13}C) MHz provided with a 5-mm probe, at room temperature, using *ca.* 20 mg polysaccharide in 0.6 mL of D_2O. Acetone was added as internal standard (referred to Me_4Si by calibrating the acetone methyl group to 31.1 ppm in ^{13}C, 2.22 ppm in ^{1}H). ^{1}H NMR spectra were carried out with an acquisition time of 4.36 s, a pulse angle of 30°, a pulse delay of 1 s, after accumulating 16 scans. For ^{13}C NMR spectra, the acquisition time was 1.1 s, the pulse delay was 0.1 s, and the pulse angle was 45°. Typically 30,000–36,000 scans were accumulated. The ^{1}H–^{13}C HSQC (heteronuclear simple quantum correlation) spectra was carried out with the Bruker program 'hsqcetgpsi2' with a FID size of 512 (F1) × 1024 (F2) using 24 scans and adjusting the $^1J_{C\text{-}H}$ to 144.9 Hz. FT-IR spectra were performed on a Nicolet Magna-IR 550 spectrophotometer using thin films of the polysaccharide. The range measured was 4000–400 cm^{-1}. For the analysis of the reaction products with $NaBD_4$, GLC-MS analyses were carried out on a Shimadzu QP 5050 A GC/MS apparatus working at 70 eV using similar conditions to those described for the GLC analysis, but using He as carrier gas with a split ratio of 60:1.

2.8. Molecular modeling

Energy comparisons were carried out by molecular modeling using density functional theory (DFT, see Results). Geometry optimizations for TEMPO-substituted analogs were carried out as vacuum calculations at the M06-2X/6-31+G(d,p) level, whereas those for the methylated analogs were carried out at the same level, but with inclusion of the polarizable continuum model (Tomasi, Mennucci, & Cammi, 2005) (PCM) in water. In all cases, energy determinations were made by single point calculations at the M06-2X/6-311+G(d,p) level with PCM in water. The program Gaussian 09-W was used for all these calculations (Frisch et al., 2009). The conformations with lower energy for each sugar and intermediate were searched previously with MM3 (Allinger, Yuh, & Lii, 1989).

2.9. Measurement of anti-HSV capacity of the polysaccharides

Vero (African green monkey kidney) cells were grown in minimum essential medium (MEM) supplemented with 5% bovine serum. HSV-1 strain F and HSV-2 strain G were obtained from the American Type Culture Collection (Rockville, USA). Vero cell viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) method (Denizot & Lang, 1986). The CC_{50} (cytotoxic concentration 50%) was calculated as the compound concentration required to reduce cell viability by 50%. Antiviral activity was evaluated by a plaque reduction assay. Vero cell monolayers grown in 24-well plates were infected with about 50 plaque-forming units (PFU) of virus/well in the absence or presence of various concentrations of the compounds. Plaques were counted after 48 h of incubation at 37 °C. The inhibitory concentration 50% (IC_{50}) was calculated as the compound concentration required to reduce virus plaques by 50%. All determinations were performed twice and each in duplicate.

3. Results and discussion

3.1. Full TEMPO-oxidation of carrageenans

Oxidation with TEMPO is expected to follow the mechanism (Tojo & Fernández, 2007) shown in Fig. 1. The first attempts of oxidation were carried out with the standard conditions accepted to perform better with different polysaccharides (de Nooy, Besemer, & van Bekkum, 1995; Elboutachfaiti et al., 2011) *i.e.* 1.25 equivalents of NaOCl per oxidizable C-6, catalytic amounts of TEMPO, pH = 10.5, addition of NaBr and a temperature of 0 °C. The reaction was terminated by the addition of ethanol. When this reaction was carried out with the κ-carrageenan (**K**), the ι-carrageenan (**I**) and

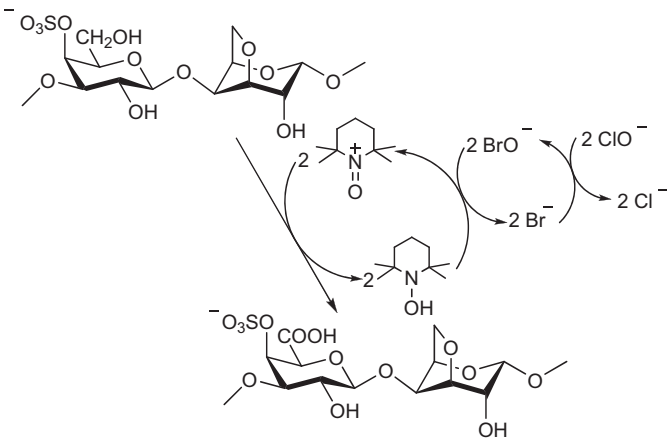

Fig. 1. General mechanism of oxidation of κ-carrageenan with TEMPO.

Table 2
Composition and molecular weight of the carrageenans and their oxidized products.

Sample	Gal:GalA:AnGal:SO_4[a]	MW (kDa)[b]
K	1.00:0.00:1.02:1.17	215
K-Ox$_E$-N$_{10.5}$	0.03:0.97:0.30:0.88	65
K-Ox$_R$-N$_{10.5}$	0.07:0.93:1.04:0.87	93
I	0.97:0.03:1.02:2.02	460
I-Ox$_E$	0.05:0.95:0.85:1.73	167
I-Ox$_E$-D	0.03:0.97:0.76:0.22	16

[a] See Section 2. Molar ratios were calculate so that Gal+GalA=1
[b] Determined by GPC considering the top of the peak

Table 3
Assignment[a] of the ^{1}H and ^{13}C NMR signals of **K**, **I** and their fully oxidized products.

	δ (ppm)							
	K		**K-Ox$_R$-N$_{10.5}$**		**I**		**I-Ox$_E$**	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
C/H	Gal 4S		GalA 4S		Gal 4S		GalA 4S	
1	4.70	102.5	4.63	102.4	4.69	102.2	4.63	102.2
2	3.65	69.4	3.60	69.5	3.66	69.1	3.64	69.3
3	4.06	78.2	4.02	79.4	4.06	76.8	4.03	77.6
4	4.91	73.7	5.17	76.2	4.95	72.0	5.24	74.2
5	3.87	74.8	4.16	74.8	3.85	74.8	4.10	78.0
6	3.86	61.2		174.2	3.85	61.7		174.0
	δ (ppm)							
	K		**K-Ox$_R$-N$_{10.5}$**		**I**		**I-Ox$_E$**	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
	3,6-AnGal		3,6-AnGal		3,6-AnGal 2S		3,6-AnGal 2S	
1	5.15	94.7	5.15	95.4	5.32	92.1	5.33	92.4
2	4.19	69.7	4.16	70.1	4.73	74.9	4.68	75.4
3	4.58	79.2	4.52	79.6	4.89	77.8	4.84	78.2
4	4.65	78.4	4.57	78.8	4.73	78.3	4.70	78.8
5	4.70	76.7	4.72	77.1	4.72	76.9	4.77	77.3
6	4.19	69.7	4.26	69.9	4.31	69.8	4.31	70.3
6′	4.10		4.11		4.14		4.15	

[a] The assignments were conferred with literature data for κ- and ι-carrageenans (Cosenza et al., 2014; Tojo & Prado, 2003; van de Velde, Knutsen, Usov, Rollema, & Cerezo, 2002).

starch (**S**) as control, after neutralization and dialysis, good yields (80–90%) of oxidized polysaccharides **K-Ox$_E$-N$_{10.5}$**, **I-Ox$_E$** and **S-Ox$_E$**, respectively were obtained. The characterization of **S-Ox$_E$** was straightforward, as it gave NMR spectra identical to that previously reported (de Nooy et al., 1995). For oxidized ι-carrageenan (**I-Ox$_E$**) both its analytical data (Table 2), and the presence of twelve ^{13}C NMR signals (Fig. 2d, Table 3) showing the presence of the diad →3)-β-D-Gal*p*A 4S-(1→4)-3,6-An-α-D-Gal 2S-(1→ indicated that the oxidation proceeded smoothly, with no major side reaction. A reduction of the molecular weight is observed, in agreement with previous results (Jiang, Drouet, Milas, & Rinaudo, 2000).

On the other hand, when the same procedure was utilized to oxidize the κ-carrageenan (**K-Ox$_E$-N$_{10.5}$**), the product showed analytical characteristics compatible with a full oxidation of the C-6 (nearly all the galactose has been converted into galacturonic acid), and some decrease in molecular weight. However, a considerable loss of 3,6-anhydrogalactose (Table 2) and an NMR spectrum much more complicated than that expected from the regular repeating structure of a fully oxidized κ-carrageenan was observed (Fig. 2b). As shown below, a secondary reaction, caused by a specific overoxidation of 3,6-anhydrogalactose occurs. In order to surmount this problem, $NaBH_4$ was added at the end of the reaction, instead of ethanol, in order to produce both the effects of quenching and reducing some oxidized by-products without affecting the carboxylic acids. In this way, the product **K-Ox$_R$-N$_{10.5}$** has been obtained (Table 2). Its NMR spectrum shows twelve main signals (Fig. 2c, Table 3), characteristic of the expected oxidized structure, with just some minor peaks, indicative of by-products.

The oxidation product of **I** (**I-Ox$_E$**), obtained without side reactions, was desulfated to generate a polysaccharide (**I-Ox$_E$-D**) whose negative charge was provided mainly by the carboxyl group (79%

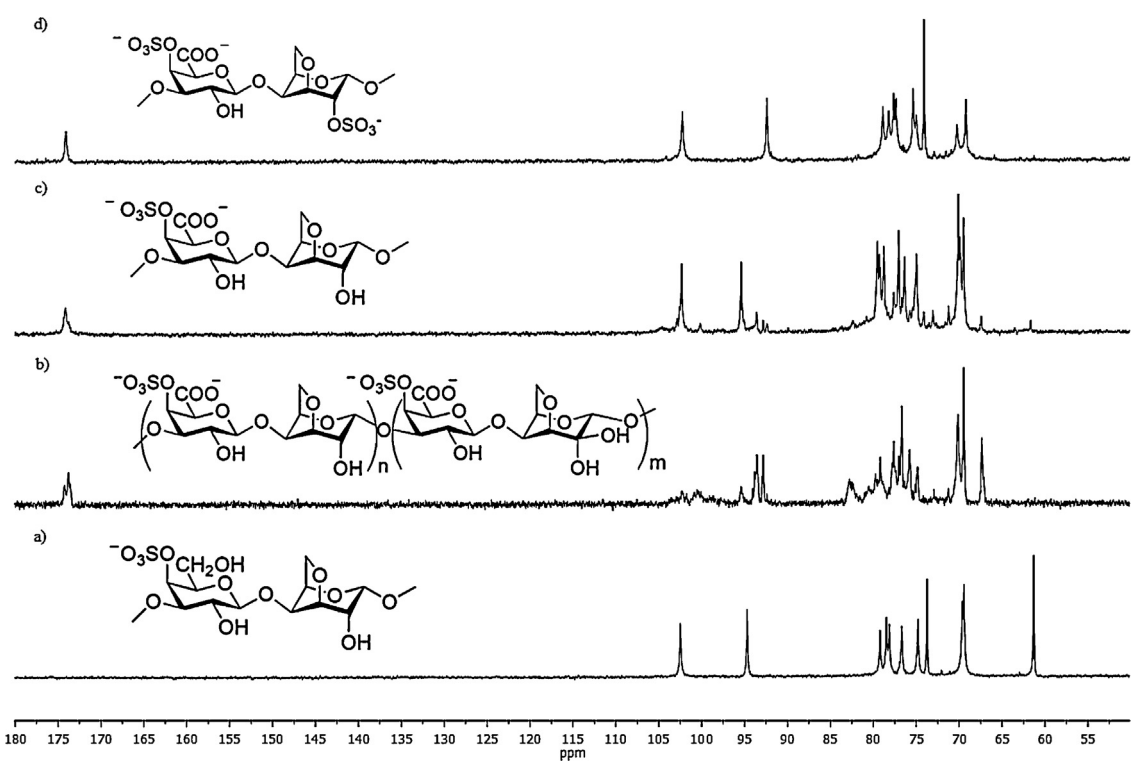

Fig. 2. 125 MHz ^{13}C-NMR spectra in D_2O of (a) **K**, (b) **K-Ox$_E$-N$_{10.5}$**, (c) **K-Ox$_R$-N$_{10.5}$**, and (d) **I-Ox$_E$**.

of the original sulfate groups were removed). It is worth noting that the desulfation reaction caused a marked depolymerization of this polysaccharide.

3.2. Study of the side reaction in the TEMPO-oxidation of κ-carrageenan

As explained above, the ^{13}C NMR spectrum of **K-Ox$_E$-N$_{10.5}$** showed more than the twelve signals expected to appear in a fully-C-6 oxidized polysaccharide. Total oxidation of C-6 occurred, as galactose was nearly absent in the product hydrolyzate and because no signal for a free C-6 (around 61 ppm in the ^{13}C NMR spectrum) was observed.

Literature shows examples of possible side reactions occurring in TEMPO oxidations (de Nooy, Besemer, van Bekkum, van Dijk, & Smit, 1996; Tojo & Fernández, 2007). Halogenation and lactonization at neutralization were discarded as they are not compatible with the structure. Oxidation at a secondary hydroxyl group appears to be the most likely side reaction. It has already been reported to occur in the TEMPO oxidation of inulin (de Nooy, Besemer, & van Bekkum, 1994), pullulan (de Nooy, Besemer, van Bekkum, van Dijk, & Smit, 1996), and agarose (Su et al., 2013). However, there was no additional carbonyl group found. The FT-IR spectrum of **K-Ox$_E$-N$_{10.5}$** shows a signal at 1621 cm^{-1} consistent with the carboxylate group of galacturonic acid, but no other carbonyl signal. The carbonyl region in the ^{13}C NMR spectrum shows two signals around 174 ppm that could be assigned to the carboxyl groups, but no additional signal. Nevertheless, the HSQC spectrum of **K-Ox$_E$-N$_{10.5}$** showed two additional signals in the anomeric region, that disappeared after borohydride reduction (**K-Ox$_R$-N$_{10.5}$**, Fig. 2c). This agrees with the generation of a ketone (or aldehyde) by oxidation. All the evidence points to C-2 of the 3,6-anhydrogalactose moiety. In a previous work of cationization of agarose (Prado, Matulewicz, Bonelli, & Cukierman, 2011), it has already been shown that O-2 of 3,6-AnGal could be about as reactive for an electrophilic attack as O-6 of the galactose moiety. A specific oxidation of this secondary position can also explain the lack of side reactions for the oxidation of ι-carrageenan, which has a sulfate group on O-2, as well as the specificity for the reaction on other polysaccharides not containing 3,6-anhydrogalactose. The lack of signals for a ketone observed for **K-Ox$_E$-N$_{10.5}$** can be easily explained by taking into account that the generated ketone will be present in a strained bridged bicycle like that of 3,6-AnGal, and thus, in aqueous solution, it will alleviate this strain by converting into a hydrate, keeping the sp^3 hybridation for C-2. This also explains the presence of a ^{13}C NMR signal for the hydrated C atom at 92.8 ppm (Fig. 2b). The presence of two anomeric signals for GalA (at *ca.* 102–100 ppm) and two for the carboxyl group (at *ca.* 174 ppm) can be explained in terms of the effect of the neighboring 3,6-AnGal unit and its oxidation product on the GalA unit.

Thus, the overoxidation reaction occurring for κ-carrageenan does not occur at random, but specifically over C-2 of the 3,6-AnGal moieties. Some 3,6-AnGal moieties remain intact due to an insufficient amount of oxidant. The nature of the side reaction was confirmed by reduction with NaBD$_4$, where a specific mass increase was observed in the mass spectrum of the 3,6-AnGal fragments containing C-2, whereas no other shift was found in other locations (*e.g.* C-2 of the small amounts of galactose which were not oxidized).

Reduction of **K-Ox$_E$-N$_{10.5}$** with NaBH$_4$ restores the secondary alcohol at C-2 without affecting the carboxyl at C-6. Although two different epimers might be expected after this reduction step (3,6-AnGal and 3,6-AnTal), reduction, hydrolysis and derivatization shows the presence of only one peak for the 3,6-anhydrohexose derivative, with a retention time identical to that of 3,6-AnGal. Moreover, the NMR spectra of **K-Ox$_R$-N$_{10.5}$** show no evidence of two different epimers, in spite of the expected chemical shift difference between the anomeric carbons of 3,6-AnGal and 3,6-AnTal. These facts suggest that the hydride is reacting specifically from the top face, yielding back 3,6-AnGal. de Nooy et al. (1996) have already reported stereoselectivity when reducing with NaBH$_4$ the overoxidized C-2 and C-3 of glucose units in pullulan.

In order to rationalize why C-6 is the preferred oxidation site, followed by C-2 of the 3,6-AnGal units, whereas none is observed in other hexose positions like C-2 or C-4, we carried out a theoretical study through a molecular modeling approach. The mechanism of the TEMPO oxidation consists of a nucleophilic attack of the O atom over the N atom, to give an intermediate which will later lose the H attached to the C atom (Tojo & Fernández, 2007) (Fig. 3a). As the TEMPO reagent contains four methyl groups adjacent to the N atom, this intermediate is subject to considerable sterical hindrance, causing a selectivity toward primary, less hindered alcohols (Tojo & Fernández, 2007). To confirm this suggestion, intermediates with the TEMPO attached to O-2 of 4-*O*-Me-3,6-An-α-D-Gal-(1 → OMe), and to O-2, O-4, and O-6 of 3-*O*-Me-β-D-Gal(1 → OMe) have been studied searching for those which give the lowest DFT energy for each combination of exocyclic groups. In a second approach, which has no influence of steric factors, the nucleophilicity of the different OH groups measured through reaction with a methyl cation (Fig. 3b) was also studied. This method has already shown success for determining the regioselectivity of glycosidation reactions for some sugar derivatives (Colombo, Rúveda, & Stortz, 2011).

Table 4 shows the outcome of the calculations of both ΔE for each of the four candidates. When analyzing ΔE_1 (which contains steric and electronic effects) the reaction toward the intermediates substituted at O-6 of Gal and O-2 of 3,6-AnGal shows the lower energies, whereas the other two intermediates show higher energies. This indicates a lower steric hindrance of these two positions, and agrees with the reported oxidation behavior. C-6 in galactose corresponds to a primary alcohol, and O-2 in 3,6-AnGal is in axial position with no close neighboring substituent (O-3 is axial and involved in the bicycle whereas although O-1 is equatorial, the exoanomeric effect drives its substituent away from O-2). O-2 and O-4 in galactose showed considerable more hindering.

This rationalization helps to explain why in **K-Ox$_E$-N$_{10.5}$**, O-2 of 3,6-AnGal can be a preferred site for oxidation than other secondary alcohols. However, it does not explain why the primary alcohol is still the preferred oxidation site. As shown below, when low amounts of NaClO are used, there is practically no

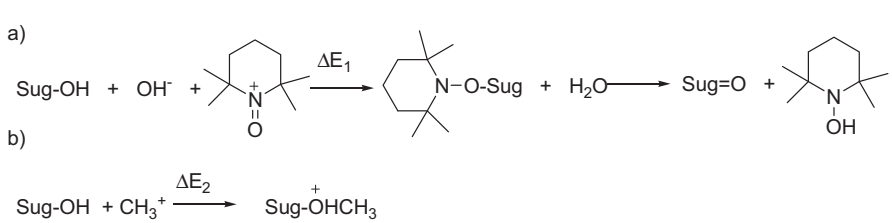

Fig. 3. (a) Characteristic steps of the reaction between the –OH of a sugar (Sug) and TEMPO. The first step involves an energy step named ΔE_1 (b) Simplified reaction for estimating nucleophilicity; it involves an energy difference ΔE_2.

Table 4
Values of reaction energies ΔE_1 and ΔE_2 determined by DFT calculations for substitution/oxidation in different positions of κ-carrageenan.

Position	ΔE_1[a] (kcal/mol)	ΔE_2[b] (kcal/mol)
O-2 of AnGal	−37.36	−66.48
O-2 of Gal	−33.82	−66.35
O-4 of Gal	−34.69	−72.82
O-6 Of Gal	−37.79	−74.88

[a] Determined at M06-2X/6-311+G(d,p) level with PCM in water over geometries determined as vacuum calculations at the M06-2X/6-31+G(d,p) level.
[b] Determined at M06-2X/6-311+G(d,p) level with PCM in water over geometries determined as calculations at the M06-2X/6-31+G(d,p) level with PCM in water.

overoxidation, showing selectivity toward O-6. This selectivity can better be explained by looking at "pure" nucleophilic factors (Colombo et al., 2011) (*i.e.* not considering sterical hindrance) as estimated by ΔE_2 (Table 4, Fig. 3b). This approach shows clearly O-6 of the Gal moiety as the most nucleophilic site, and thus it can explain why it is the preferred site for oxidation.

3.3. Optimization of the TEMPO oxidation

The TEMPO oxidation as carried out in the original conditions generated a decrease in molecular weight (Table 2), which was already reported as a side reaction of the oxidation (Jiang et al., 2000; Saito, Yanagisawa, & Isogai, 2005). In order to control the different variables acting on the reaction, including the overoxidation (see above), the influence of several factors were assessed, working with the κ-carrageenan **K**. Table 1 shows the acronyms and conditions of the different experiments, and Table 5 shows the results in terms of molar ratios, molecular weights and degree of overoxidation. The latter parameter was obtained from a semiquantitative determination of the HSQC integrals of the 3,6-AnGal anomeric signals on a non-reduced aliquot. These signals are expected to have similar $^1J_{C\text{-}H}$ values; thus, their integrals can be compared (Heikinnen, Toikka, Karhunen, & Kilpeläinen, 2003). The use of a buffered solution instead of hand-controlling the pH, as suggested by Xu et al. (2012), showed less overoxidation, but more depolymerization. A prior reduction of the reducing end does not alter the results, confirming that alkaline peeling from the reducing end is not the origin of depolymerization. The pH balance is expected to be part of a delicate equilibrium: a higher pH diminishes the rate of the reaction, whereas a lower pH (*ca.* 8.5) enables the primary oxidant (NaOCl) to act instead of TEMPO (de Nooy et al., 1995). The experiment with buffers of pHs 10 and 9.4 showed results similar to that of the 10.4 buffer, with less overoxidation, but with larger depolymerization. Hand-control of the pH at a value of *ca.* 10 yielded results similar to those working with a pH 10.4 buffer, *i.e.* larger molecular weights, although overoxidation was diminished. Those results suggest that a pH range of 9.5–10 is more likely to work with over-oxidizable products like carrageenans.

Table 5
Composition and molecular weight of κ-carrageenan oxidized in different conditions.

Sample	Gal:GalA:AnGal:SO_4[a]	MW (kDa)[b]	% 3,6-AnGal oxidized (before reduction)[c]
K-Ox$_R$-N$_{10.5}$	0.07:0.93:1.04:0.87	93	71
K-Ox$_R$-B$_{10.4}$	0.13:0.87:0.75:1.03	52	58
K-Ox$_R$-B$_{10}$	0.07:0.93:0.86:1.11	35	25
K-Ox$_R$-B$_{9.4}$	0.16:0.84:0.71:0.82	55	11
K-Ox$_R$	0.13:0.87:0.91:0.95	91	28
K-Ox$_R$-1h	0.15:0.85:1.02:0.92	92	17
K-Ox$_R$-3h	0.09:0.91:0.87:0.97	60	36

[a] See Section 2. Molar ratios were calculate so that Gal + GalA = 1.
[b] Determined by GPC considering the top of the peak.
[c] Calculated by NMR form an aliquot that was quenched with ethanol.

It has been shown that larger reaction times (3 h) lead to an increased depolymerization, whereas optimal results were obtained by working for 1–2 h (Table 5).

3.4. Partial oxidation

With those optimal conditions in hand, the possibility of obtaining products with different degrees of oxidation was evaluated. Table 6 shows the results of submitting polysaccharide **K** to oxidation with decreasing proportions of NaClO. In this way, polymers with different degree of C-6 oxidation were obtained. No overoxidation occurs when limiting amounts of primary oxidant are added. No straight proportionality between the amount of oxidant and the degree of oxidation was observed: when half the amount of bleach was used (0.625 eq), almost two thirds of the galactose moieties were oxidized (**K-1/2-Ox$_R$**). On the other hand, when half of that oxidant (**K-1/4-Ox$_R$**) was used, the amount of galactose oxidized was less than 20%, and with 0.125 eq (**K-1/10-Ox$_R$**) there was no oxidation at all (Table 6). One possible explanation would be an incomplete oxidation of C-6 to aldehyde (Huerta-Angeles et al., 2012), which, on work-up ($NaBH_4$) restores the original galactose. The ^{13}C-NMR spectra of partially oxidized products (not shown) follow the expected behavior: the spectrum of **K-1/10-Ox$_R$** is identical to that of **K**. In **K-1/4-Ox$_R$**, some peaks indicate a small degree of oxidation although the carboxyl signal is not observed. In

Table 6
Composition, molecular weight and overoxidation of the carrageenans after partial and total oxidation.

Sample	Gal:GalA:AnGal:SO_4[a]	MW (kDa)[b]	% 3,6-AnGal oxidized (before reduction)[c]
K	1.00:0.00:1.02:1.17	215	
K-1/10-Ox$_R$	1.00:0.00:0.76:0.78	207	0
K-1/4-Ox$_R$	0.82:0.18:0.92:1.14	141	0
K-1/2-Ox$_R$	0.33:0.67:0.81:1.08	163	4
K-Ox$_R$	0.13:0.87:0.91:0.95	91	28
I	0.97:0.03:1.02:2.02	460	
I-1/4-Ox$_R$	0.87:0.13:0.81:1.62	445	0
I-1/2-Ox$_R$	0.52:0.48:1.00:1.62	324	0
I-Ox$_R$	0.07:0.93:1.00:1.76	216	0

[a] See Section 2. Molar ratios were calculate so that Gal + GalA = 1.
[b] Determined by GPC considering the top of the peak.
[c] Calculated by NMR form an aliquot that was quenched with ethanol.

Table 7
Anti HSV-1 and HSV-2 activities, and selectivity indexes (SI) of carrageenans and their oxidized derivatives.[a]

Sample	MW[b] (kDa)	HSV-1		HSV-2	
		IC_{50} (μg/mL)	SI	IC_{50} (μg/mL)	SI
K	215	13.8 ± 1.3	>72.5	11.0 ± 0.7	>90.9
K-1/4-Ox$_R$	141	4.2 ± 1.1	>238.1	13.5 ± 1.8	>74.1
K-1/2-Ox$_R$	163	1.7 ± 0.6	>588.2	0.98 ± 0.4	>1020.4
K-Ox$_R$-1h	92	7.9 ± 1.2	>126.6	9.0 ± 0.6	>111.1
K-Ox$_R$-3h	60	5.5 ± 0.3	>181.8	13.9 ± 1.4	>71.9
K-Ox$_R$-B$_{10}$	35	30.2 ± 7.3	>33.1	34.3 ± 6.8	>29.1
I	460	0.67 ± 0.07	>1492.5	0.43 ± 0.03	>2325.6
I-1/4-Ox$_R$	445	0.66 ± 0.04	>1515.1	0.54 ± 0.01	>1851.8
I-1/2-Ox$_R$	324	0.4 ± 0.1	>2500	0.40 ± 0.01	>2500.0
I-Ox$_R$	216	0.9 ± 0.2	>1111.1	0.8 ± 0.2	>1250.0
I-Ox$_E$	167	0.83 ± 0.01	>1240.8	1.1 ± 0.2	>909.1
I-Ox$_E$-D	16	>200	Inactive	>200	Inactive

[a] SI = CC_{50}/IC_{50}. CC_{50} is the concentration which reduces the number of viable cells to the 50% after 48 h of incubation. IC_{50} is the compound concentration required to reduce virus plaques by 50%. The CC_{50} was >1000 μg/mL for all the compounds assayed.
[b] Determined by GPC considering the top of the peak.

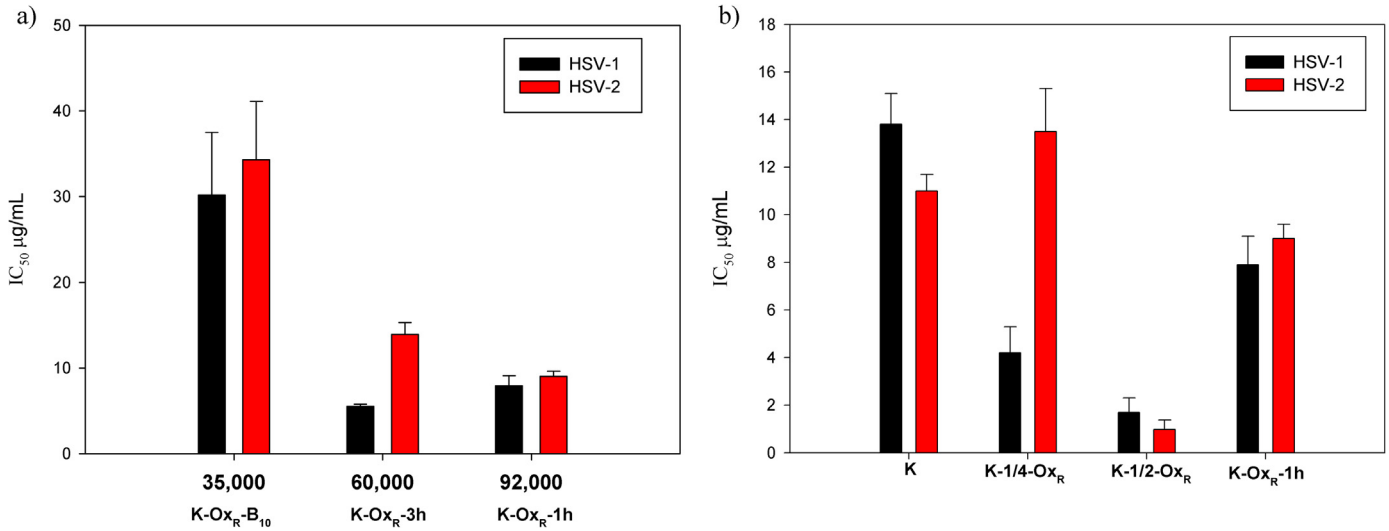

Fig. 4. (a) Relationship of the IC_{50} for HSV-1 and HSV-2 with the molecular weight for fully oxidized derivatives of **K**. (b) Relationship between the IC_{50} for HSV-1 and HSV-2 with the degree of oxidation of **K**.

K-1/2-Ox$_R$ it is possible to observe a decrease in the intensity on the C-6 of galactose and the appearance of the carboxyl group signal.

The partial oxidation reaction was extended to the ι-carrageenan **I**. Products with different degrees of oxidation were also obtained. In this case, the products of reaction with half and a quarter of the amount of primary oxidant, are less oxidized than stoichiometrically expected, suggesting than in **I-1/2-Ox$_R$** part of the C-6 was also oxidized only to aldehyde.

The decrease of the molecular weight, in both **K** and **I** oxidation products, was found to be directly proportional to the amount of primary oxidant added for the products, confirming that depolymerization is a side effect of the oxidation process in alkaline medium (Jiang et al., 2000; Saito et al., 2005).

3.5. Assessment of the anti-HSV activity of oxidized carrageenans

The antiviral activities of **K**, **I** and different oxidized derivatives were assessed against the viruses HSV-1 and HSV-2, as well as the cytotoxicity. Oxidized fractions with distinct features were chosen for these antiviral assays. Results are shown in Table 7 and Fig. 4.

Original and oxidized carrageenans did not show cytotoxicity for Vero cells up to the maximum tested concentration; thus, the CC_{50} was considered >1000 μg/mL for all compounds. Fully oxidized κ-carrageenan (**K-Ox$_R$-1h**) has a level of antiviral activity of the same order as that of the parent polysaccharide, with small variations in IC_{50} values with other compounds obtained with different oxidation conditions, which lead to different degrees of depolymerization (Table 7). Fig. 4a shows the decreasing trend with molecular weight for the inhibitory activity, at least with HSV-2. On the other hand, partially oxidized κ-carrageenans show a tendency to increase their anti-HSV properties: a ten-fold increase in inhibitory activity is observed when half of the amount of NaClO was used (**K-1/2-Ox$_R$**, Table 7 and Fig. 4b). Meanwhile, the anti-HSV activity of the carrageenan less oxidized (**K-1/4-Ox$_R$**) was only three-fold increased, suggesting that there is an optimal GalA/Gal ratio in κ-carrageenans for improvement on antiviral activity. With respect to ι-carrageenan, very active in native state, either partial or total oxidation does not affect significantly its antiviral properties (Table 7).

These results confirm that the negative charge density is not the only factor to assess the antiviral activity (Damonte et al., 2004; Kolender, Pujol, Damonte, Cerezo, & Matulewicz, 1998). Some polysaccharides increase their activity by oversulfation or introduction of carboxyl groups, but others do not show so straightforward results. Full C-6 sulfation of κ-carrageenan showed a decrease in inhibitory activity against HSV (Damonte et al., 2004; Kolender et al., 1998). This work shows that complete introduction of carboxyl groups at this position increase slightly this activity, which is further increased by leaving intact some primary alcohol groups. Although some molecular weight effects cannot be discarded, conformational factors originated in non-bonded interactions (either ionic, dipolar or van der Waals) facilitating or disrupting the interaction of the polysaccharides with the virus should be responsible for these subtle differences.

4. Conclusion

Both κ- and ι-carrageenans can be fully oxidized using TEMPO in the presence of sodium hypochlorite and sodium bromide to regular polysaccharides which exhibit increased anionic charge and appreciable antiviral activity. Best yields and qualities are obtained by working in unbuffered solutions at pH = 10, with NaOH as base, for 1 to 2 h. By regulating the amount of primary oxidant (NaOCl), partially oxidized carrageenans can be obtained, giving rise to products with a considerable increase (up to one order of magnitude) in antiviral activity against HSV-1 and HSV-2. Although for products like κ-carrageenans the oxidation reaction is not totally specific for C-6, the secondary reaction can be averted by a work-up procedure where the over-oxidized site is reduced back to the original secondary alcohol group.

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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.2015.04.030

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