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1 **Glycosaminoglycan-Mimetics Obtained by Microwave-Assisted Sulfation of**  
2 **Marine Bacterium Sourced Infernan Exopolysaccharide**

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17

18 **ABSTRACT**

19 Sulfated glycosaminoglycans (GAGs) are fundamental constituents of both cell surface and  
20 extracellular matrix. By playing a key role in cell-cell and cell-matrix interactions, GAGs are  
21 involved in many physiological and pathological processes. To design GAG-mimetics with  
22 similar therapeutic potential than the natural ones, the specific structural features, amongst  
23 sulfate content, sulfation pattern and chain length should be considered. In the present study,  
24 we describe a sulfation method based on microwave radiation to obtain highly-sulfated  
25 derivatives as GAG-mimetics. The starting low-molecular weight (LMW) derivative was  
26 prepared from infernan exopolysaccharide, a highly branched naturally slightly-sulfated  
27 heteropolysaccharide synthesized by the deep-sea hydrothermal vent bacterium *Alteromonas*  
28 *infernus*. LMW highly-sulfated infernan derivatives obtained by a conventional heating  
29 sulfation have already been shown to display GAG-mimetic properties. Here, the potential of  
30 microwave-assisted sulfation *versus* conventional method to obtain GAG-mimetics was  
31 explored. Structural analysis by NMR revealed that both highly-sulfated derivatives shared  
32 similar structural features, emphasizing that microwave-assisted sulfation with twelve-fold  
33 shorter reaction time is as efficient as the classical one.

34

35 **KEYWORDS:** exopolysaccharide, microwave-assisted sulfation, semi-synthesis,  
36 glycosaminoglycan-mimetics

37

## 38 INTRODUCTION

39 Sulfated glycosaminoglycans (GAGs), such as heparin, heparan sulfate and chondroitin  
40 sulfate are highly evolutionary conserved complex anionic linear polysaccharides found in a  
41 broad range of both vertebrates and invertebrates. They are fundamental constituents of both  
42 cell surface and extracellular matrix, and through their localization they participate in many  
43 biological processes by playing a key role in cell-cell and cell-matrix interactions.<sup>1</sup> Biological  
44 effects of GAGs are mainly due to their interaction with various proteins present in their  
45 microenvironment, including chemokines, cytokines, growth factors, morphogens, enzymes  
46 and their natural inhibitors and adhesion molecules. The binding between GAGs and proteins  
47 requires specific structural features, such as sulfation pattern, conformational flexibility, chain  
48 length and counter-ions, which determine the level of affinity.<sup>2</sup> These structural requirements  
49 are essential to design compounds that mimic the bioactive function of GAGs and to fine-tune  
50 their benefit/risk ratio.<sup>3</sup> With the demand of both animal-free molecules and environmentally  
51 friendly processes, the production of GAG-mimetics from other sources than mammalian  
52 tissues is flourishing, especially to avoid a risk of contamination by harmful substances and/or  
53 unconventional pathogens.<sup>4</sup> The production of GAG-mimetics with specific biological targets  
54 can be performed by organic synthesis or chemo-enzymatic approaches.<sup>5,6</sup> Synthetic routes  
55 have however some limits because oligosaccharides longer than dodecasaccharides are not  
56 easily achieved and their specific sulfation is still a challenge<sup>7</sup>. GAG-mimetics have also been  
57 developed from natural polymers, especially polysaccharides obtained from different sources,  
58 such as plant, algae and bacteria. The use of exopolysaccharide (EPS) producing bacteria is  
59 highly advantageous over traditional polysaccharide sources. Indeed, EPS production by  
60 fermentation represents a renewable and sustainable process devoid of risks related to raw  
61 material supply. In addition, their production in bioreactors can be controlled and optimized to  
62 obtain high yields, while keeping the EPS composition and structure. Some bacteria are able to

63 synthesize polysaccharides with structures close or even apparently identical to mammalian  
64 GAGs. *Escherichia coli* serotype K5 can produce a precursor of heparin, unsulfated heparosan,  
65 whereas *E. coli* K4 produces unsulfated chondroitin.<sup>8,9</sup> However, bacterial EPSs are often  
66 unsulfated or only weakly sulfated, and their chemical oversulfation is therefore required to  
67 obtain GAG-mimetics. The use of sulfur trioxide complexes as sulfating agent in organic  
68 solvents such as formamide, *N,N*-dimethylformamide (DMF), dimethylsulfoxide or pyridine is  
69 recommended to avoid uncontrolled depolymerization usually observed with sulfonyl chloride,  
70 sulfuric acid or chlorosulfuric acid.<sup>10</sup> In comparison to the conventional heating, sulfation using  
71 microwave radiation has recently been recognized as a powerful method allowing to improve  
72 the heating regulation, reagent mixing and reaction kinetics. The time of reaction can  
73 considerably be reduced, which minimizes product degradation.<sup>11,12</sup> Therefore, microwave-  
74 assisted synthesis leads to homogeneously sulfated polysaccharides with both excellent yield  
75 and reproducibility.

76 The bacterial EPSs from terrestrial origin present a great structural diversity but this diversity  
77 is tremendously increased in the marine environment due to the enormity of the marine  
78 biosphere.<sup>13</sup> Several EPS-producing marine strains have been studied, which led to the  
79 discovery and isolation of novel macromolecules exhibiting valuable biological activities, in  
80 particular GAG-mimetic properties.<sup>14-16</sup> *Alteromonas infernus*, a deep-sea hydrothermal vent  
81 bacterium isolated in Guaymas basin in Gulf of California, produces a high-molecular weight  
82 (HMW), slightly sulfated (3% of sulfur) EPS, named infernan.<sup>17,18</sup> In our previous studies,  
83 GAG-mimetic properties of infernan resulting not only from the presence of sulfate groups but  
84 also from uronic acid residues were shown both *in vitro* and *in vivo*.<sup>19-24</sup> The addition of the  
85 native EPS into cellulose-based hydrogel supported the 3D culture of functionally competent  
86 chondrocytes able to produce type II collagen and sulfated GAGs.<sup>19</sup> In order to further enhance  
87 its GAG-mimetic properties, native infernan was depolymerized and chemically oversulfated

88 to obtain low-molecular weight (LMW) and highly sulfated derivatives, with molecular weight  
89 ranging from 8,000 to 30,000 g/mol and a sulfur content from 12 to 14 %S (w/w).<sup>20</sup> Through  
90 their physical interactions with growth factors, e.g. TGF- $\beta$ 1 and BMP-2, these highly sulfated  
91 derivatives were able to induce cellular processes essential for tissue regeneration.<sup>21-23</sup>  
92 Moreover, their presence allowed to effectively inhibit both migration and invasiveness of  
93 osteosarcoma cells *in vitro*, as well as the establishment of lung metastases *in vivo*.<sup>24</sup> LMW  
94 highly sulfated derivatives displayed also some anticoagulant properties, remaining however  
95 lower compared to LMW heparin and unfractionated heparin (2.5 and 6.5 times, respectively).<sup>25</sup>  
96 These bioactive derivatives are classically prepared in the presence of sulfur trioxide pyridine  
97 complex (SO<sub>3</sub>·py) in dry DMF and the reaction is followed for 2h at 45°C.<sup>20</sup> In this context, the  
98 objective of the present study was to prepare highly sulfated infernan derivatives as GAG  
99 mimetics using microwave radiation with similar features to those usually prepared by  
100 conventional method. By tuning the sulfation conditions, derivatives with different sulfate  
101 contents were obtained and fully characterized. The structure of the EPS derivatives with the  
102 highest sulfate content obtained by both conventional and microwave radiation sulfation  
103 methods was analyzed by NMR and compared with the LMW-infernan precursors. The  
104 obtained results clearly emphasized that microwave-assisted sulfation is a fast and efficient  
105 method to get suitable GAG-mimetic derivatives.

106

## 107 **EXPERIMENTAL SECTION**

108 **LMW EPS Derivative Production and Modification.** Infernan EPS was produced by  
109 fermentation of the marine bacterium *A. infernus* as described previously.<sup>25</sup> LMW EPS  
110 derivatives (EPS DR), with an average molecular weight of 7,800 g/mol (EPS DR<sub>7,8k</sub>) and  
111 19,000 g/mol (EPS DR<sub>19k</sub>) were obtained by the depolymerization of the native EPS using a  
112 free-radical process as previously described.<sup>20</sup> After depolymerization, polysaccharide chains

113 were reduced with sodium borohydride, purified on Chelex® resin, ultrafiltered on a 1 kD (EPS  
114 DR<sub>7,8k</sub>) or 10 kDa (EPS DR<sub>19k</sub>) cut-off membrane and finally freeze-dried. In order to obtain a  
115 homogeneous fraction with a low polydispersity, a gel filtration chromatography on a  
116 Superdex® 30 (GE Healthcare Life Sciences), using an AKTA FPLC system coupled with a  
117 refractometric detector (Gilson®), was performed in water. EPS DR fractions were pooled and  
118 freeze-dried prior to sulfation step.

119 **Sulfation by Conventional Heating.** Oversulfated EPS derivatives (EPS DRS) were  
120 obtained by a chemical sulfation of EPS DR<sub>7,8k</sub>, as described previously.<sup>20</sup> Sulfation was  
121 performed in duplicate at three different EPS/SO<sub>3</sub>·py w/w ratios: 1/5, 1/2.5 or 1/1.25. Briefly,  
122 EPS DR<sub>7,8k</sub> (20 mg) in its pyridinium salt form was firstly solubilized in extra dry DMF over  
123 molecular sieve (4 mL) for 2h at 45°C under continuous stirring and then sulfated for the next  
124 2h at 45°C in the presence of SO<sub>3</sub>·py (100, 50, or 25 mg). The final aqueous solution (pH 7)  
125 was dialyzed against water for three days before freeze-drying.

126 **Sulfation by Microwave Radiation.** Oversulfated EPS derivatives (EPS DRS-MW) were  
127 obtained in duplicate by a microwave-assisted sulfation of EPS DR<sub>7,8k</sub> using three different  
128 EPS/SO<sub>3</sub>·py w/w ratios: 1/5, 1/2.5 or 1/1.25 using a Biotage Initiator Microwave Synthesizer  
129 (Sweden). Briefly, EPS DR<sub>7,8k</sub> (25 mg) in its pyridinium salt form was firstly solubilized in  
130 extra dry DMF over molecular sieve (12.5 mL) under stirring for 2 h at 45 °C. Then, SO<sub>3</sub>·py  
131 (125, 62.5, or 31.2 mg) was added and the mixture was placed in the microwave oven turn table  
132 and exposed to 300 Watts maximum power (temperature was measured and controlled  
133 externally by an IR probe) for 10 min at 45°C. The obtained solution (pH 7) was then dialyzed  
134 against water for three days before freeze-drying. In order to determine the potential effect of  
135 microwave radiation on polysaccharides, EPS DR<sub>7,8k</sub> was also exposed to microwaves without  
136 any SO<sub>3</sub>·py addition.

137 **Physico-chemical Analyses. Monosaccharide Composition.** Monosaccharide composition  
138 was determined in triplicate according to the Kamerling *et al* method as modified by Montreuil  
139 *et al.*<sup>26,27</sup> Briefly, infernan and its LMW derivatives (EPS DR) were hydrolyzed for 4h at 100°C  
140 by 3 M MeOH/HCl with *myo*-inositol used as internal standard. After re-*N*-acetylation with  
141 acetic anhydride overnight at room temperature, the methyl glycosides were converted to their  
142 corresponding trimethylsilyl derivatives. Separation and quantification of the per-*O*-  
143 trimethylsilyl methyl glycosides were performed by gas chromatography (GC-FID, Agilent  
144 Technologies 6890N).

145 *Molecular Weight and Polydispersity Index.* The weight-average molecular weight ( $M_w$ )  
146 was determined by high-performance size-exclusion chromatography (HPSEC) coupled with  
147 multi-angle light scattering (MALS, Dawn Heleos-II, Wyatt Technology) and differential  
148 refractive index (RI) (Optilab Wyatt technology) detectors. HPSEC system was composed of a  
149 Prominence Shimadzu HPLC system, a PL aquagel-OH mixed, 8  $\mu$ m (Varian) guard column  
150 ( $U$  7.5mm  $\times$   $L$  50 mm), and a PL aquagel-OH mixed (Varian) separation column. Samples (in  
151 duplicate) were eluted with 0.1 M ammonium acetate at 1 mL/min flow rate. The mean  
152 molecular weight was calculated using a refractive index increment  $dn/dc$  of 0.145 mL/g. To  
153 estimate the heterogeneity of measured sizes of the native EPS and its derivatives, the  
154 polydispersity index ( $I_p$ ) was calculated by the  $M_w/M_n$  ratio, where  $M_n$  is the number average  
155 molar mass.

156 *Elemental Analysis and degree of sulfation.* Elemental analysis was performed in duplicate  
157 using a FlashSmart V CHNS instrument (ThermoFischer) to determine the mean carbon (%C)  
158 and sulfur (%S) contents of the samples. The degree of sulfation (DS) was calculated using  
159 mean %C and %S values following the equation (1):

$$DS = \frac{\%S}{\%C} \times \frac{awC}{awS} \times nC \quad (1)$$



160 where  $awC$  and  $awS$  are the atomic weights of carbon (C) and sulfur (S), respectively, and  $nC$   
161 is the number of carbon atoms per nonasaccharide (54 C) or octasaccharide (48 C) repeating  
162 units.

163 *ATR-FTIR Spectroscopy.* Infrared spectra of native EPS and its derivatives were recorded  
164 with a FTIR VERTEX 70 spectrometer (Bruker) in ATR mode in the range 4000-500  $\text{cm}^{-1}$ .

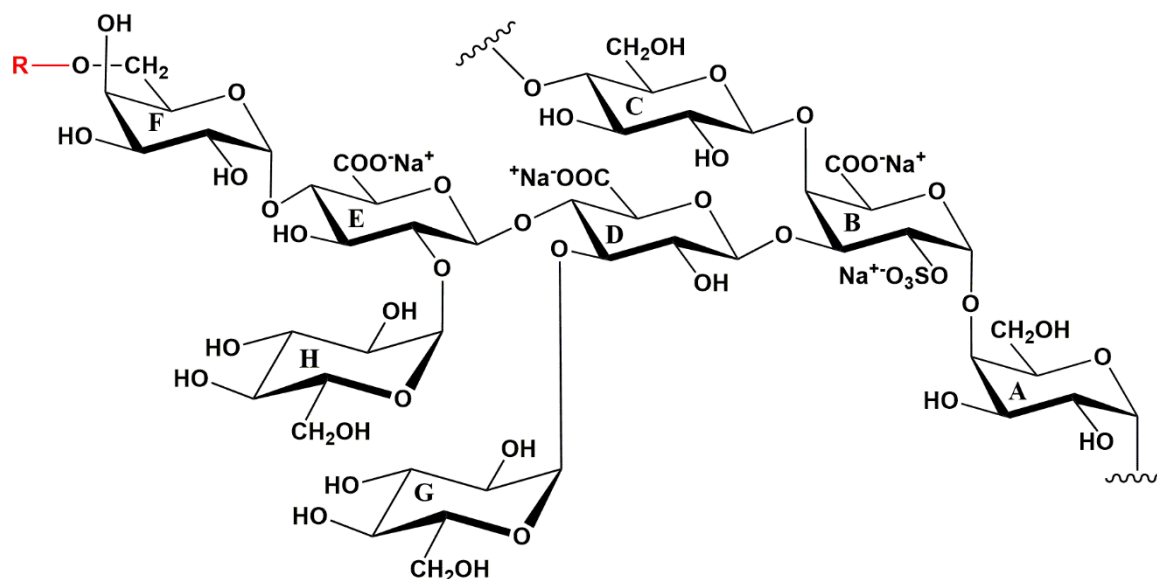
165 *NMR Analysis.* NMR spectra were recorded on a Bruker (Billerica, MA, USA) Avance-III  
166 HD ( $^1\text{H}$ : 400 MHz,  $^{13}\text{C}$ : 100 MHz) or on a Bruker Avance-III ( $^1\text{H}$ : 600 MHz,  $^{13}\text{C}$ : 150 MHz)  
167 instrument – the latter equipped with a cryo-probe – in  $\text{D}_2\text{O}$  (acetone as internal standard,  $^1\text{H}$ :  
168  $(\text{CH}_3)_2\text{CO}$  at  $\delta$  2.22 ppm;  $^{13}\text{C}$ :  $(\text{CH}_3)_2\text{CO}$  at  $\delta$  31.5 ppm). Data were processed using the data  
169 analysis packages integrated with Bruker TopSpin<sup>®</sup> 4.0.5 software.  $^1\text{H}$ ,  $^{13}\text{C}$ -HSQC and  $^1\text{H}$ ,  $^{13}\text{C}$ -  
170 HMBC experiments were measured in the  $^1\text{H}$ -detected mode via single quantum coherence with  
171 proton decoupling in the  $^{13}\text{C}$  domain, using data sets of  $2048 \times 256$  points and typically 80  
172 increments (160 for HMBC).

173

## 174 RESULTS AND DISCUSSION

175 **Native infernan and its LMW derivatives (EPS DR).** Native infernan produced by *A.*  
176 *infernus* is a highly branched anionic heteropolysaccharide with a monosulfated nonasaccharide  
177 repeating unit firstly described in 2004 by Roger *et al.* (Figure 1).<sup>18</sup> Infernan main chain is  
178 composed of only three residues, glucose (Glc), galacturonic acid (GalA) and galactose (Gal),  
179 which are covalently linked in the sequence:  $\rightarrow 4$ )- $\beta$ -D-Glcp-(1 $\rightarrow$ 4)- $\alpha$ -D-GalpA-(1 $\rightarrow$ 4)- $\alpha$ -D-  
180 Galp-(1 $\rightarrow$ . GalA residue of the main chain is substituted at *O*-2 by one sulfate group and at *O*-  
181 3 by a short side chain composed of two glucuronic acids (GlcA), Gal and Glc units linked  
182 through the sequence:  $\beta$ -D-Glcp-(1 $\rightarrow$ 6)- $\alpha$ -D-Galp-(1 $\rightarrow$ 4)- $\beta$ -D-GlcpA-(1 $\rightarrow$ 4)- $\beta$ -D-GlcpA-(1 $\rightarrow$ .  
183 In addition, each GlcA residue is substituted by a terminal Glc. Recently, a novel disulfated  
184 octasaccharide repeating unit was described after mass spectrometry analysis performed on

185 LMW infernan derivatives obtained after enzymatic depolymerization.<sup>28</sup> In this new repeating  
186 unit, the terminal Glc of the side chain initially linked to Gal residue at C-6 was replaced by a



187 sulfate group (Figure 1). It appeared therefore that during EPS biosynthesis two types of  
188 repeating unit could be assembled.

189

190 **Figure 1.** The two types of infernan repeating unit: a monosulfated nonasaccharide repeating  
191 unit ( $R = \beta\text{-Glc}$ )<sup>18</sup> and a disulfated octasaccharide repeating unit ( $R = \text{SO}_3^-\text{Na}^+$ )<sup>28</sup>

192

193 In order to prepare GAG-mimetics, native infernan of high-molecular weight (2,000,000 g/mol)  
194 was firstly depolymerized using a free-radical process to prepare LMW derivatives (EPS DR).

195 Two derivatives of two different molecular weights were obtained, EPS DR<sub>7,8k</sub> of 7,800 g/mol  
196 and EPS DR<sub>19k</sub> of 19,000 g/mol. As shown in Table 1, both derivatives presented similar

197 monosaccharide composition as starting EPS, suggesting that the depolymerization process had  
198 no major impact on the polysaccharide structure. Elemental analysis performed on the native

199 EPS and its LMW derivatives revealed the DS close to 2 assuming both a nonasaccharide and  
200 an octasaccharide repeating units (Table 1).

201

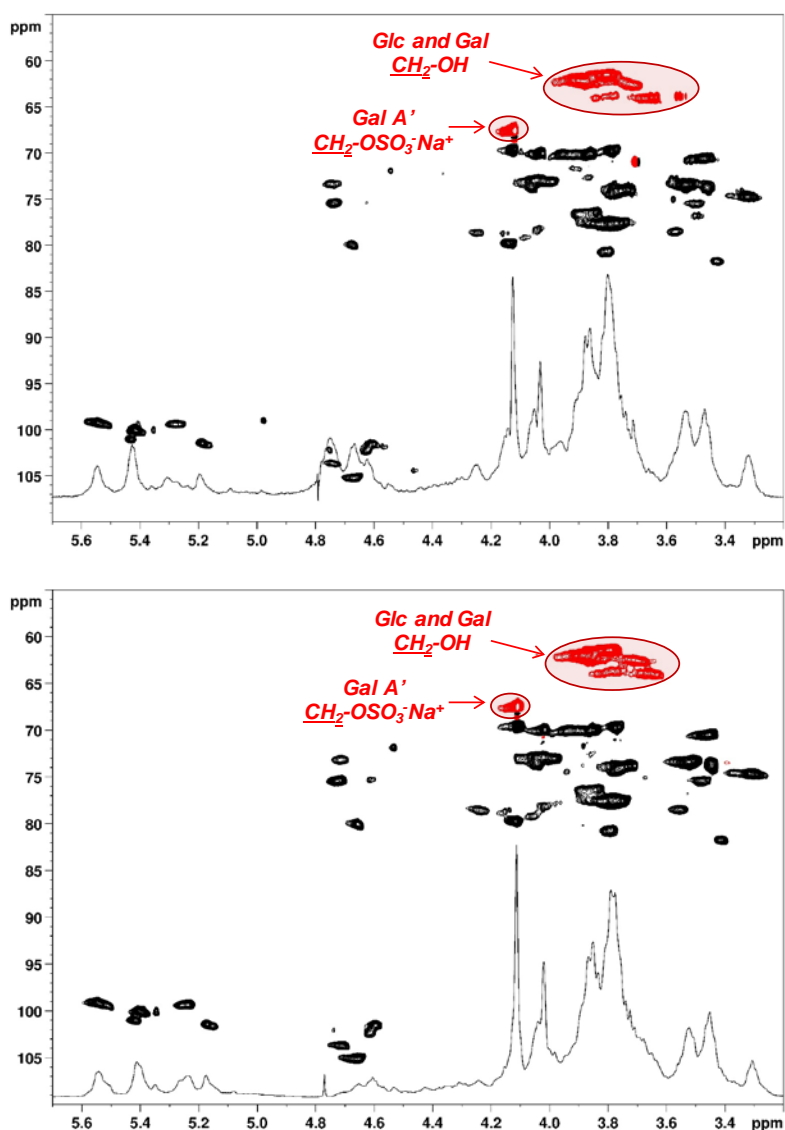
202 **Table 1.** Monosaccharide composition (w/w%), weight average molecular weight, Mw (g/mol),  
 203 carbon (%C) and sulfur (%S) contents and degree of sulfation (DS) of the native HMW EPS  
 204 and its LMW derivatives, EPS DR<sub>19k</sub> and EPS DR<sub>7,8k</sub>.

	Monosaccharide composition (w/w%)				%C	%S	DS*	DS**	Mw (g/mol)	Ip Mw/Mn
	Gal	Glc	GalA	GlcA						
	Infernan EPS	10.4	18.5	7.6						
EPS DR <sub>19k</sub>	9.7	18.9	10.4	14.7	30.3	3.0	2.0	1.8	19,000 ± 0,400	1.9
EPS DR <sub>7,8k</sub>	9.8	18.7	10.3	14.8	30.4	2.7	1.8	1.6	7,800 ± 1,200	1.5

205 Gal, galactose; Glc, glucose; GalA, galacturonic acid; GlcA, glucuronic acid; %C, mean carbon content and  
 206 %S, mean sulfur content measured by elemental analysis; DS\*, degree of sulfation assuming a  
 207 nonasaccharide repeating unit; DS\*\*, degree of sulfation assuming an octasaccharide repeating unit. Mw,  
 208 weight average molecular weight; Ip (Mw/Mn), polydispersity index.

209 These two derivatives were then analyzed by NMR to reveal their fine structures, and in  
 210 particular to establish the presence of the monosulfated nonasaccharide and/or the disulfated  
 211 octasaccharide repeating units. <sup>1</sup>H-NMR and a set of 2D-NMR <sup>1</sup>H,<sup>1</sup>H-homonuclear (COSY,  
 212 TOCSY, NOESY) and <sup>1</sup>H,<sup>13</sup>C-heteronuclear spectra (DEPT-HSQC, HSQC-TOCSY, HMBC)  
 213 were measured for the EPS DR samples in D<sub>2</sub>O. Their comparison revealed no significant  
 214 differences between the two samples (Figure 2 and Figure S1). Chemical shift data were in  
 215 agreement with those previously reported in 2004 by Roger *et al.*,<sup>18</sup> apart for the Gal F unit  
 216 (Figure 1). Indeed, in the <sup>1</sup>H,<sup>13</sup>C-DEPT-HSQC spectra the presence of CH<sub>2</sub> signals in two  
 217 different regions could be detected. The most crowded one at δ<sub>H/C</sub> 3.50-4.00/60-65 ppm hosts  
 218 the methylene signals of Glc and Gal residues carrying a free hydroxyl at C-6, in agreement  
 219 with Roger *et al.* (2004).<sup>18</sup> The signal at δ<sub>H/C</sub> 4.13/67.5 ppm could be assigned to the CH<sub>2</sub>O-  
 220 moiety of 6-*O*-sulfated Gal F unit, in agreement with NMR data previously reported for the  
 221 synthetic α-D-Gal6Sp-(1→4)-β-D-GlcpA disaccharide (F-E fragment).<sup>29</sup> This assignment was  
 222 in disagreement with Roger *et al.* (2004), that attributed such signal to a *O*-glycosylated (with

223 a  $\beta$ -Glc residue) rather than 6-*O*-sulfated Gal F unit, in spite of the absence of any n.O.e.  
224 between  $\beta$ -Glc and Gal F residues<sup>18</sup> as well as of any correlation between the signal at  $\delta_{H/C}$   
225 4.13/67.5 ppm and anomeric signals in the  $^1H, ^{13}C$ -HMBC spectrum (Figure S2). NMR data  
226 together with the DS values determined for both derivatives, indicating the presence of 2 sulfate  
227 groups per repeating unit (Table 1), suggested that a revision of the structure of the repeating  
228 unit of infernan should be done. Indeed, a disulfated octasaccharide, in agreement with our  
229 recent mass spectrometry data on LMW infernan derivatives,<sup>28</sup> rather than a monosulfated  
230 nonasaccharide<sup>18</sup> should only be considered.



231 **Figure 2.**  $^1\text{H}$ - and  $^1\text{H},^{13}\text{C}$ -DEPT-HSQC NMR spectra (400 MHz,  $\text{D}_2\text{O}$ , 298K, zoom) of EPS  
232 DR<sub>19k</sub> (top) and EPS DR<sub>7,8k</sub> (bottom).

233

234 **Conventional versus microwave-assisted sulfation of EPS DR.** In the next step, the LMW  
235 derivative of the lowest molecular weight, namely EPS DR<sub>7,8k</sub>, was selected to explore the  
236 potential of microwave-assisted sulfation to obtain a highly sulfated compound similar to  
237 GAGs, such as LMW heparin. Firstly, the effect of microwave radiation on the polysaccharide  
238 structural integrity was assessed by exposing the derivative to the radiation without the  $\text{SO}_3\cdot\text{py}$   
239 addition. Weight average molecular weight was similar before and after microwave exposure  
240 suggesting that radiation had no destructive effect on the polysaccharide structure (Table 2).  
241 Sulfation was then performed in the presence of  $\text{SO}_3\cdot\text{py}$  complex using microwave radiation,  
242 in parallel to the conventional heating method.<sup>20</sup> By tuning EPS/ $\text{SO}_3\cdot\text{py}$  ratio, the sulfur content,  
243 corresponding to the amount of added sulfate groups, could be modulated (Table 2). Highly  
244 sulfated derivatives were obtained with the highest EPS/ $\text{SO}_3\cdot\text{py}$  ratio (1/5) using both  
245 conventional heating (EPS DRS<sub>HS</sub>) and microwave radiation (EPS DRS-MW<sub>HS</sub>). The amount  
246 of sulfur was above 10%, which corresponds to a ~32% of sulfate groups, similarly to the  
247 amount typically found in sulfated GAGs. A two-fold decrease in the sulfur content was  
248 measured for the derivatives sulfated using the EPS/ $\text{SO}_3\cdot\text{py}$  ratio of 1/2.5. The lowest  
249 EPS/ $\text{SO}_3\cdot\text{py}$  ratio (1/1.25) applied was not sufficient to sulfate the derivative, as the sulfur  
250 content was comparable to the derivative before sulfation. In addition, a molecular weight  
251 increase was observed with the increase of added sulfate groups, suggesting that the backbone  
252 of the EPS derivatives was not degraded during the reaction (Table 2), in contrast to other  
253 widely used sulfation reagents such as chlorosulfonic acid or sulfuric acid.<sup>12</sup> The polydispersity  
254 index (Mw/Mn ratio) only slightly increased with the sulfur content.

255

256 Table 2. Characterization of LMW EPS derivatives sulfated by either conventional heating  
 257 (EPS DRS) or microwave radiation (EPS DRS-MW) at three different EPS/SO<sub>3</sub>·py w/w ratios.

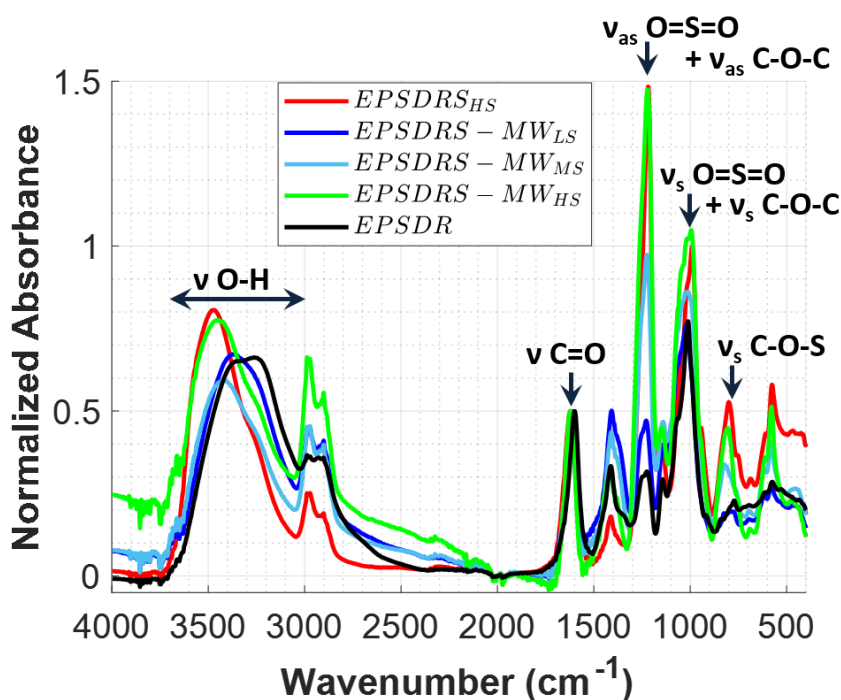
EPS derivative	T (°C)	t (min)	EPS/SO <sub>3</sub> ·py	%S	Mw (g/mol)	<i>Ip</i> Mw/Mn
EPS DR-MW	45	120	-	2.5	6,900 ± 0,600	1.3
EPS DRS <sub>HS</sub>	45	120	1/5	12.2	17,200 ± 3,400	1.6
EPS DRS <sub>MS</sub>	45	120	1/2.5	6.1	8,000 ± 0,200	1.7
EPS DRS <sub>LS</sub>	45	120	1/1.25	2.6	6,800 ± 0,200	1.5
EPS DRS-MW <sub>HS</sub>	45	10	1/5	13.7	12,600 ± 0,800	1.5
EPS DRS-MW <sub>MS</sub>	45	10	1/2.5	7.1	11,700 ± 0,500	1.4
EPS DRS-MW <sub>LS</sub>	45	10	1/1.25	3.1	7,700 ± 1,200	1.3

258 HS, high sulfate; MS, medium sulfate; LS, low sulfate; %S, mean sulfur content measured by elemental  
 259 analysis; Mw, weight average molecular weight; *Ip* (Mw/Mn), polydispersity index.

260

261 Sulfated EPS derivatives obtained by conventional and microwave-assisted sulfation methods  
 262 were analyzed by ATR-FTIR and compared to EPS DR<sub>7,8k</sub> before sulfation (Figure 3 and S3).  
 263 The raw spectra (Figure S3) were normalized with respect to the C=O stretching band at 1650  
 264 cm<sup>-1</sup> to better visualize the differences in the sulfur content between the samples (Figure 3).  
 265 Indeed, carboxylic acid groups are not involved in sulfation reaction and their band intensity  
 266 could be considered constant. On normalized ATR-FTIR spectra (Figure 3), the broad band  
 267 corresponding to O-H at 3600-3000 cm<sup>-1</sup> was considerably narrowed for both EPS DRS<sub>HS</sub> and  
 268 EPS DRS-MW<sub>HS</sub> samples having the highest sulfur content, due to the esterification of the  
 269 hydroxyl groups, when compared to EPS DR<sub>7,8k</sub> or EPS DRS<sub>LS, MS</sub> with low and medium sulfur  
 270 contents. The strong O=S=O asymmetric stretching band at 1262 cm<sup>-1</sup> clearly increased with  
 271 increasing sulfur content. For both highly sulfated derivatives, EPS DRS<sub>HS</sub> and EPS DRS-  
 272 MW<sub>HS</sub>, prepared by the two sulfation methods, the intensity of O=S=O band was comparable.  
 273 Similar results were observed for C-O-S symmetric vibration band at 816 cm<sup>-1</sup>. An important

274 absorption band was observed for all derivatives at 1100-1010  $\text{cm}^{-1}$  attributed to the C-O  
 275 stretching vibration of pyranosyl rings.



276  
 277 **Figure 3.** ATR-FTIR normalized spectra with respect to the C=O band at 1650  $\text{cm}^{-1}$  of slightly  
 278 sulfated EPS DR<sub>7,8k</sub> before sulfation, highly sulfated EPS DRS<sub>HS</sub> obtained by conventional  
 279 sulfation method and EPS DRS-MW<sub>LS, MS, HS</sub> prepared using microwave radiation. HS = high  
 280 sulfate, MS = medium sulfate, LS = low sulfate, MW = Microwave.

281  
 282 By taking into account the physico-chemical characterizations, it appeared clearly that similarly  
 283 sulfated derivatives were obtained using both conventional and microwave-assisted sulfation  
 284 methods. However, the main advantage of the microwave-assisted sulfation is the reaction time  
 285 that can be considerably decreased (10 min instead of 120 min). To confirm that both methods  
 286 lead to similar derivatives, the structures of the EPS derivatives with the highest sulfate  
 287 contents, EPS-DRS<sub>HS</sub> and EPS DRS-MW<sub>HS</sub>, were further analyzed by NMR to get some  
 288 insights into their sulfation pattern. A comparison of their  $^1\text{H}$ ,  $^{13}\text{C}$ -DEPT-HSQC with respect to  
 289 EPS DR<sub>7,8k</sub> clearly confirmed an extensive sulfation for both samples, as indicated by a marked

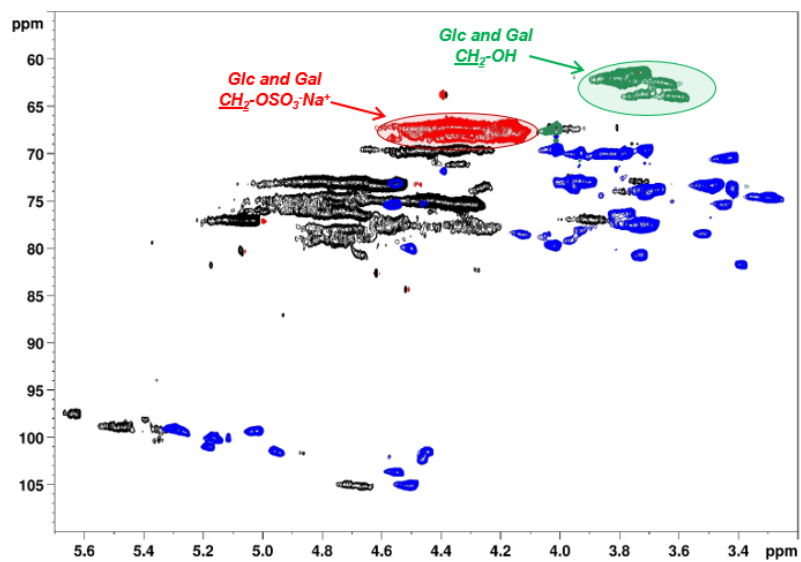
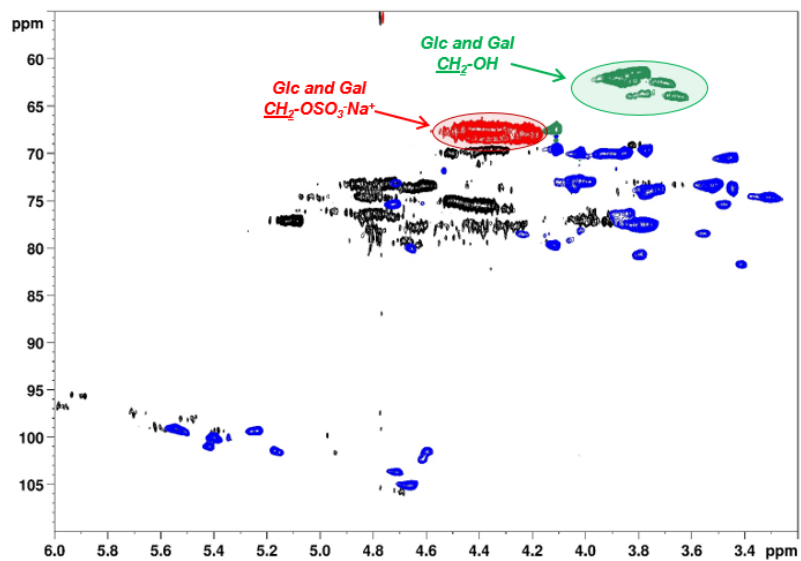
290  $^1\text{H}$  downfield shift for several CH and  $\text{CH}_2$  signals (Figure 4).<sup>30</sup> Their quite broad shape  
291 suggested that the obtained polysaccharides have a heterogeneous structure. Indeed, the only  
292 insight into their sulfation pattern could be inferred from the absence of any signal assignable  
293 to unsulfated  $\text{CH}_2$  moieties. This indicated that a quantitative degree of sulfation at the primary  
294 hydroxyl of Gal and Glc residues of both polysaccharide samples was achieved. This is not  
295 unexpected, due to the generally higher reactivity of primary *vs.* secondary alcohols.  
296 Furthermore, a comparison between the  $^1\text{H},^{13}\text{C}$ -DEPT-HSQC NMR spectra of EPS-DRS<sub>HS</sub> and  
297 EPS DRS-MW<sub>HS</sub> samples revealed no significant differences (Figures S4-S6). This confirmed  
298 that conventional and microwave-assisted sulfation reactions proceeded similarly on EPS  
299 DR<sub>7,8k</sub>. From elemental analysis, the DS of 14 and 18 per octasaccharide repeating unit were  
300 determined, respectively, for EPS-DRS<sub>HS</sub> (12.2 %S, 15.3 %C) and EPS DRS-MW<sub>HS</sub> (13.7 %S,  
301 13.6 %C). By considering that (i) the highest DS theoretically possible is 21, (ii) two sulfated  
302 positions are already present in native infernan and (iii) all the four primary hydroxyl groups (2  
303 on the backbone and 2 on the side chain) result sulfated as evidenced by NMR (Figure 5), 8 and  
304 12 sulfate groups remain, respectively, in EPS-DRS<sub>HS</sub> and EPS DRS-MW<sub>HS</sub>. Although, the  
305 accessibility of secondary hydroxyl groups on the side chains (11 groups available) during  
306 sulfation seems higher in contrast to the backbone (4 groups available), the  $^1\text{H},^{13}\text{C}$ -DEPT-  
307 HSQC spectra suggesting a heterogeneous structure for both derivatives do not indicate a high  
308 regioselectivity for sulfate group insertion on side chain *vs.* backbone secondary positions.

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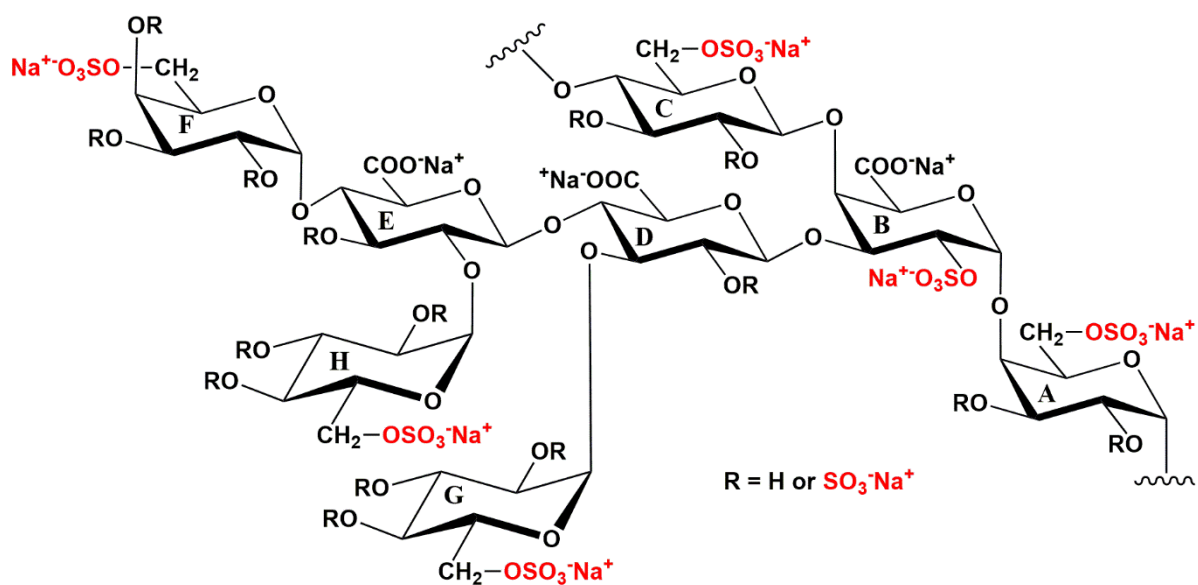


312

313 **Figure 4.** Superimposition of  $^1\text{H},^{13}\text{C}$ -DEPT-HSQC NMR spectra (400 MHz,  $\text{D}_2\text{O}$ , 298 K,  
 314 zoom) of EPS DR<sub>7,8k</sub> (blue/green) and EPS DRS-MW<sub>HS</sub> (black/red; top) or EPS DRS<sub>HS</sub>  
 315 (black/red; bottom).

316

317



318

319 **Figure 5.** The postulated structure for the octasaccharide repeating unit of infernan highly-  
 320 sulfated derivatives EPS DRS<sub>HS</sub> and EPS DRS-MW<sub>HS</sub>.

321

## 322 CONCLUSIONS

323 The present study explored a microwave-assisted sulfation method applied to LMW derivative  
 324 prepared from infernan EPS to obtain sulfated GAG-mimetics. The new method was compared  
 325 to the conventional one that we classically use to prepare highly sulfated polysaccharides  
 326 displaying biological properties similar to GAGs. For both methods, the same sulfation  
 327 conditions, including EPS/SO<sub>3</sub>·py ratios and temperature were applied, except for the reaction  
 328 time, which was twelve-fold shorter for the microwave-assisted sulfation (10 min) in  
 329 comparison to the classical one (120 min). Derivatives obtained in both cases displayed very  
 330 similar physico-chemical characteristics in terms of sulfur content and molecular weight  
 331 distribution. Moreover, by tuning EPS/SO<sub>3</sub>·py ratio, the sulfur content was increased to reach  
 332 the amount known for sulfated GAGs (EPS/SO<sub>3</sub>·py 1/5 w/w ratio). In order to get further insight  
 333 into their fine structure, and in particular the sulfation pattern, LMW derivatives displaying the  
 334 highest sulfur content prepared by both methods were analyzed by NMR. An extensive sulfation  
 335 was evidenced for both samples, with a slightly higher degree for the one obtained through the

336 microwave-assisted reaction. Both derivatives showed a highly heterogeneous structure  
337 showing all four primary hydroxyls substituted by sulfate groups. Remaining sulfates are likely  
338 randomly distributed on secondary hydroxyl groups between inernan side chains and its  
339 backbone. NMR analysis confirmed that the microwave-assisted sulfation with considerably  
340 shorter reaction time, leads to a highly-sulfated derivative sharing similar structural features  
341 with the one obtained by the classical sulfation method.

342

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346

347 **Author Contributions**

348 The manuscript was written through contributions of all authors. All authors have given  
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350

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360

361 **Notes**

362 The authors declare no competing financial interest.

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