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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.¹ 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.² 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.³ 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.⁴ The production of GAG-mimetics with specific biological targets
54 can be performed by organic synthesis or chemo-enzymatic approaches.^{5,6} Synthetic routes
55 have however some limits because oligosaccharides longer than dodecasaccharides are not
56 easily achieved and their specific sulfation is still a challenge⁷. 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.^{8,9} 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.¹⁰ 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.^{11,12} 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.¹³ 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.¹⁴⁻¹⁶ *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.^{17,18} 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*.¹⁹⁻²⁴ 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.¹⁹ 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).²⁰ Through
90 their physical interactions with growth factors, e.g. TGF- β 1 and BMP-2, these highly sulfated
91 derivatives were able to induce cellular processes essential for tissue regeneration.²¹⁻²³
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*.²⁴ 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).²⁵
96 These bioactive derivatives are classically prepared in the presence of sulfur trioxide pyridine
97 complex (SO₃·py) in dry DMF and the reaction is followed for 2h at 45°C.²⁰ 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.²⁵ LMW EPS
110 derivatives (EPS DR), with an average molecular weight of 7,800 g/mol (EPS DR_{7,8k}) and
111 19,000 g/mol (EPS DR_{19k}) were obtained by the depolymerization of the native EPS using a
112 free-radical process as previously described.²⁰ After depolymerization, polysaccharide chains

113 were reduced with sodium borohydride, purified on Chelex® resin, ultrafiltered on a 1 kD (EPS
114 DR_{7,8k}) or 10 kDa (EPS DR_{19k}) 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_{7,8k}, as described previously.²⁰ Sulfation was
121 performed in duplicate at three different EPS/SO₃·py w/w ratios: 1/5, 1/2.5 or 1/1.25. Briefly,
122 EPS DR_{7,8k} (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 SO₃·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_{7,8k} using three different
128 EPS/SO₃·py w/w ratios: 1/5, 1/2.5 or 1/1.25 using a Biotage Initiator Microwave Synthesizer
129 (Sweden). Briefly, EPS DR_{7,8k} (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, SO₃·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_{7,8k} was also exposed to microwaves without
136 any SO₃·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.*^{26,27} 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 μ 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 cm^{-1} .

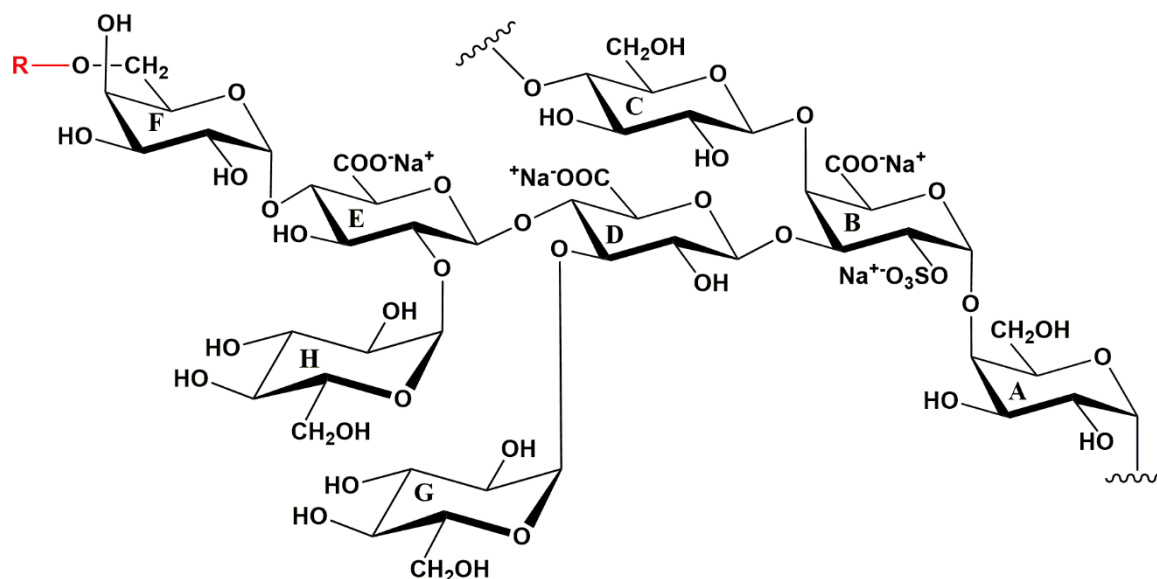
165 *NMR Analysis.* NMR spectra were recorded on a Bruker (Billerica, MA, USA) Avance-III
166 HD (^1H : 400 MHz, ^{13}C : 100 MHz) or on a Bruker Avance-III (^1H : 600 MHz, ^{13}C : 150 MHz)
167 instrument – the latter equipped with a cryo-probe – in D_2O (acetone as internal standard, ^1H :
168 $(\text{CH}_3)_2\text{CO}$ at δ 2.22 ppm; ^{13}C : $(\text{CH}_3)_2\text{CO}$ at δ 31.5 ppm). Data were processed using the data
169 analysis packages integrated with Bruker TopSpin[®] 4.0.5 software. ^1H , ^{13}C -HSQC and ^1H , ^{13}C -
170 HMBC experiments were measured in the ^1H -detected mode via single quantum coherence with
171 proton decoupling in the ^{13}C domain, using data sets of 2048×256 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).¹⁸ 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: $\rightarrow 4$)- β -D-Glcp-(1 \rightarrow 4)- α -D-GalpA-(1 \rightarrow 4)- α -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: β -D-Glcp-(1 \rightarrow 6)- α -D-Galp-(1 \rightarrow 4)- β -D-GlcpA-(1 \rightarrow 4)- β -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.²⁸ 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 ($R = \beta\text{-Glc}$)¹⁸ and a disulfated octasaccharide repeating unit ($R = \text{SO}_3^-\text{Na}^+$)²⁸

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_{7,8k} of 7,800 g/mol
196 and EPS DR_{19k} 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

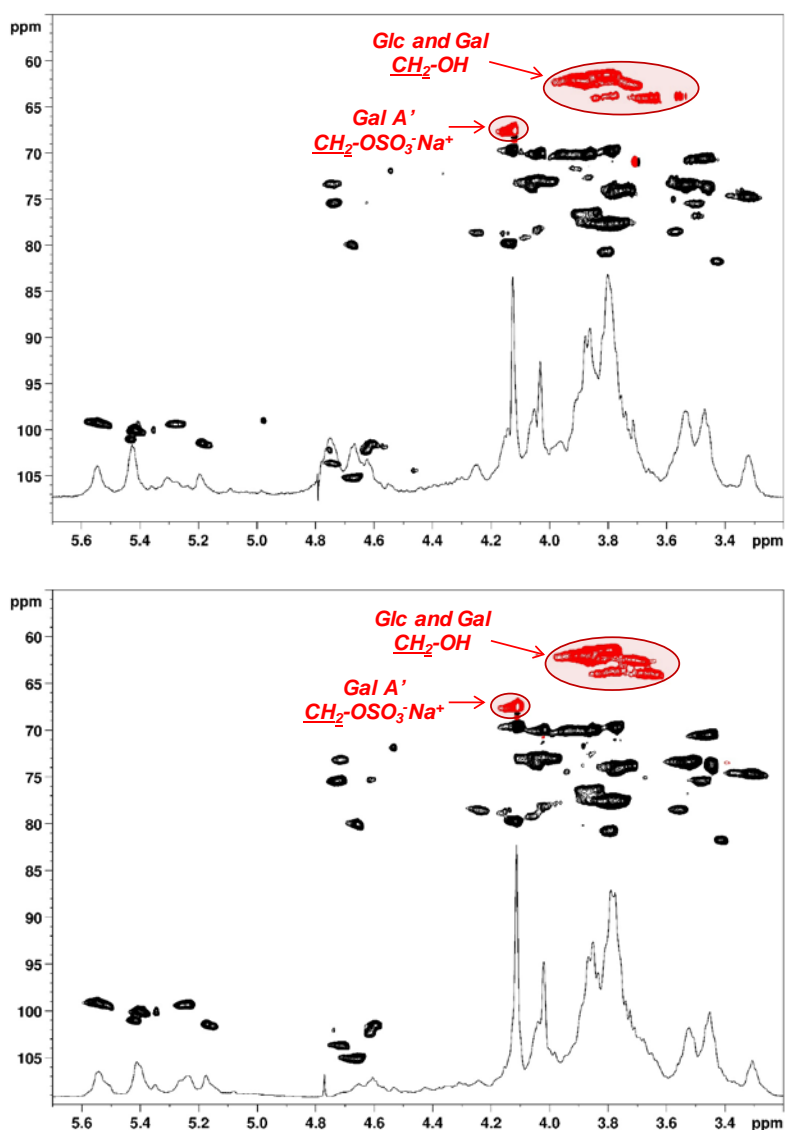
202 **Table 1.** Monosaccharide composition (w/w%), weight average molecular weight, Mw (g/mol),
 203 carbon (%C) and sulfur (%S) contents and degree of sulfation (DS) of the native HMW EPS
 204 and its LMW derivatives, EPS DR_{19k} and EPS DR_{7,8k}.

	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 _{19k}	9.7	18.9	10.4	14.7	30.3	3.0	2.0	1.8	19,000 ± 0,400	1.9
EPS DR _{7,8k}	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. ¹H-NMR and a set of 2D-NMR ¹H,¹H-homonuclear (COSY,
 212 TOCSY, NOESY) and ¹H,¹³C-heteronuclear spectra (DEPT-HSQC, HSQC-TOCSY, HMBC)
 213 were measured for the EPS DR samples in D₂O. 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.*,¹⁸ apart for the Gal F unit
 216 (Figure 1). Indeed, in the ¹H,¹³C-DEPT-HSQC spectra the presence of CH₂ signals in two
 217 different regions could be detected. The most crowded one at δ_{H/C} 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).¹⁸ The signal at δ_{H/C} 4.13/67.5 ppm could be assigned to the CH₂O-
 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).²⁹ This assignment was
 222 in disagreement with Roger *et al.* (2004), that attributed such signal to a *O*-glycosylated (with

223 a β -Glc residue) rather than 6-*O*-sulfated Gal F unit, in spite of the absence of any n.O.e.
224 between β -Glc and Gal F residues¹⁸ 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,²⁸ rather than a monosulfated
230 nonasaccharide¹⁸ should only be considered.



231 **Figure 2.** ^1H - and $^1\text{H},^{13}\text{C}$ -DEPT-HSQC NMR spectra (400 MHz, D_2O , 298K, zoom) of EPS
232 DR_{19k} (top) and EPS DR_{7,8k} (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_{7,8k}, 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.²⁰ 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_{HS}) and microwave radiation (EPS DRS-MW_{HS}). 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.¹² 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/SO₃·py w/w ratios.

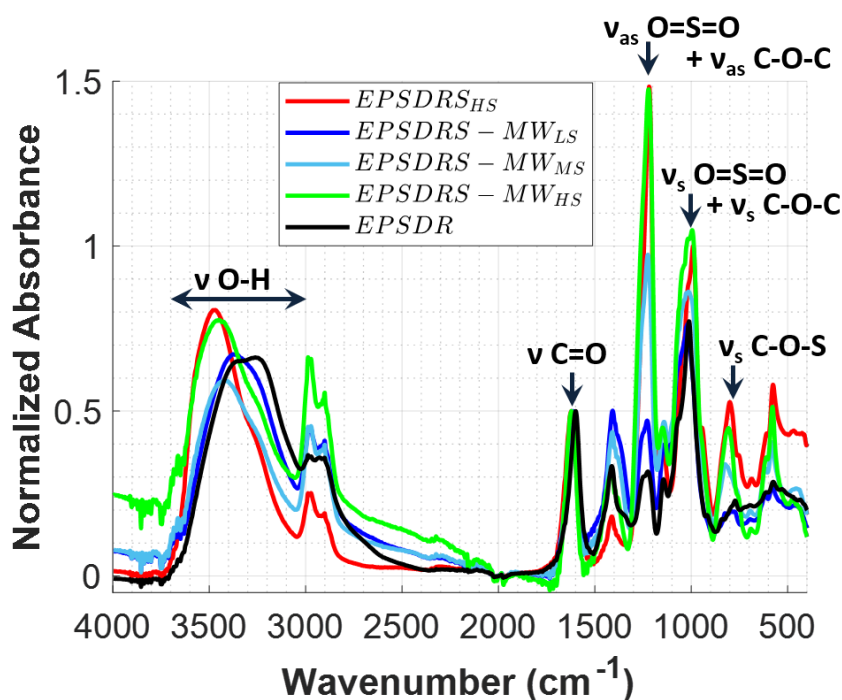
EPS derivative	T (°C)	t (min)	EPS/SO ₃ ·py	%S	Mw (g/mol)	<i>I_p</i> Mw/Mn
EPS DR-MW	45	120	-	2.5	6,900 ± 0,600	1.3
EPS DRS _{HS}	45	120	1/5	12.2	17,200 ± 3,400	1.6
EPS DRS _{MS}	45	120	1/2.5	6.1	8,000 ± 0,200	1.7
EPS DRS _{LS}	45	120	1/1.25	2.6	6,800 ± 0,200	1.5
EPS DRS-MW _{HS}	45	10	1/5	13.7	12,600 ± 0,800	1.5
EPS DRS-MW _{MS}	45	10	1/2.5	7.1	11,700 ± 0,500	1.4
EPS DRS-MW _{LS}	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; *I_p* (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_{7,8k} 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⁻¹ 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⁻¹ was considerably narrowed for both EPS DRS_{HS} and
 268 EPS DRS-MW_{HS} samples having the highest sulfur content, due to the esterification of the
 269 hydroxyl groups, when compared to EPS DR_{7,8k} or EPS DRS_{LS, MS} with low and medium sulfur
 270 contents. The strong O=S=O asymmetric stretching band at 1262 cm⁻¹ clearly increased with
 271 increasing sulfur content. For both highly sulfated derivatives, EPS DRS_{HS} and EPS DRS-
 272 MW_{HS}, 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⁻¹. An important

274 absorption band was observed for all derivatives at 1100-1010 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 cm^{-1} of slightly
278 sulfated EPS DR_{7,8k} before sulfation, highly sulfated EPS DRS_{HS} obtained by conventional
279 sulfation method and EPS DRS-MW_{LS, MS, HS} prepared using microwave radiation. HS = high
280 sulfate, MS = medium sulfate, LS = low sulfate, MW = Microwave.

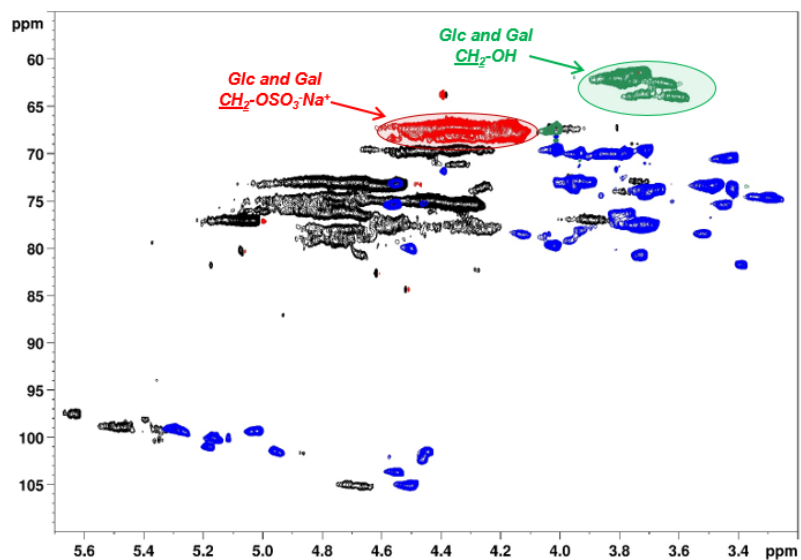
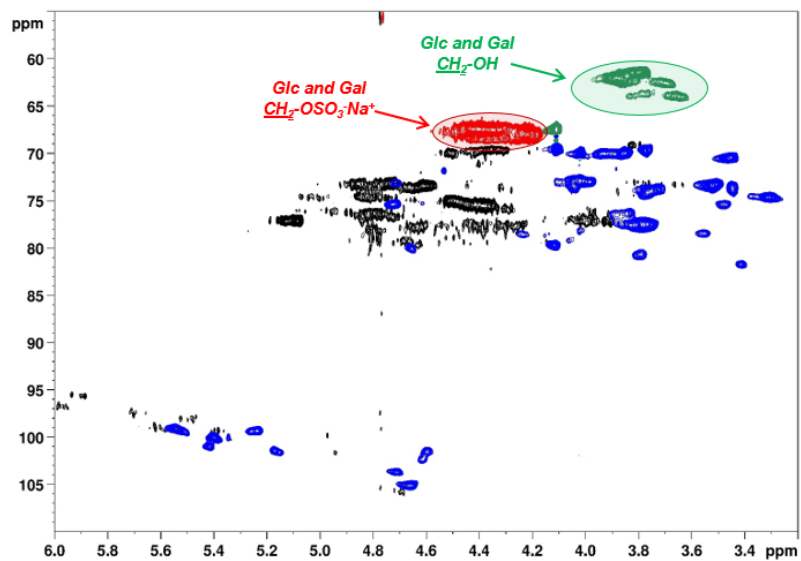
281
282 By taking into account the physico-chemical characterizations, it appeared clearly that similarly
283 sulfated derivatives were obtained using both conventional and microwave-assisted sulfation
284 methods. However, the main advantage of the microwave-assisted sulfation is the reaction time
285 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_{HS} and EPS DRS-MW_{HS}, were further analyzed by NMR to get some
288 insights into their sulfation pattern. A comparison of their ^1H , ^{13}C -DEPT-HSQC with respect to
289 EPS DR_{7,8k} clearly confirmed an extensive sulfation for both samples, as indicated by a marked

290 ^1H downfield shift for several CH and CH₂ signals (Figure 4).³⁰ 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 CH₂ 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_{HS} and
297 EPS DRS-MW_{HS} samples revealed no significant differences (Figures S4-S6). This confirmed
298 that conventional and microwave-assisted sulfation reactions proceeded similarly on EPS
299 DR_{7,8k}. From elemental analysis, the DS of 14 and 18 per octasaccharide repeating unit were
300 determined, respectively, for EPS-DRS_{HS} (12.2 %S, 15.3 %C) and EPS DRS-MW_{HS} (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_{HS} and EPS DRS-MW_{HS}. 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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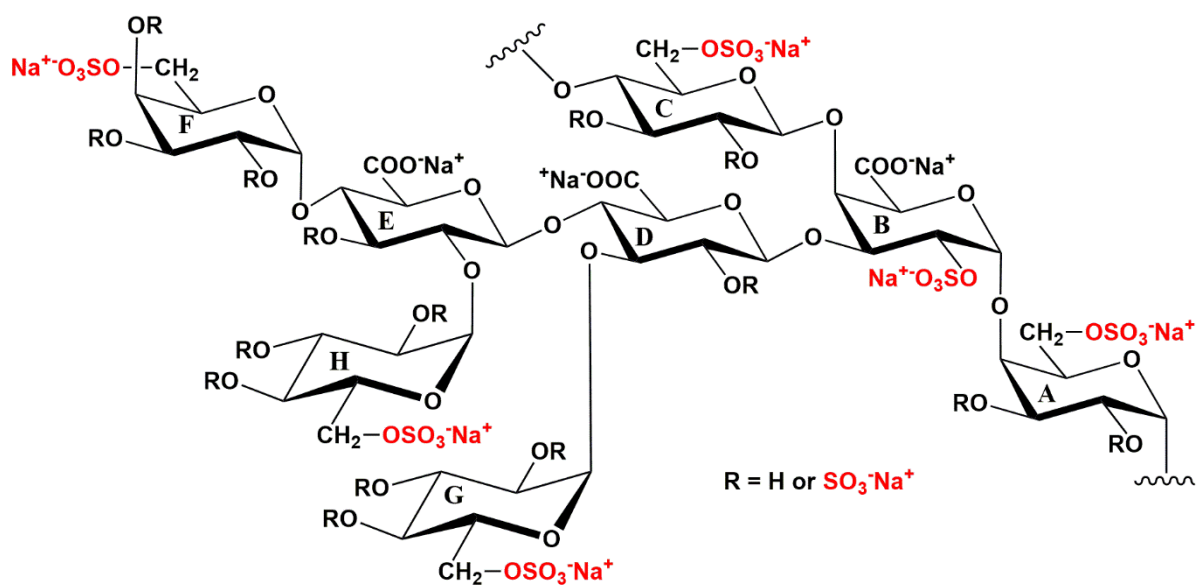


312

313 **Figure 4.** Superimposition of $^1\text{H},^{13}\text{C}$ -DEPT-HSQC NMR spectra (400 MHz, D_2O , 298 K,
 314 zoom) of EPS DR_{7,8k} (blue/green) and EPS DRS-MW_{HS} (black/red; top) or EPS DRS_{HS}
 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_{HS} and EPS DRS-MW_{HS}.

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/SO₃·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/SO₃·py ratio, the sulfur content was increased to reach
 332 the amount known for sulfated GAGs (EPS/SO₃·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.

363 **REFERENCES**

- 364 (1) Karamanos, N. K.; Piperigkou, Z.; Theocharis, A. D.; Watanabe, H.; Franchi, M.; Baud, S.; Brezillon, S.; Gotte,
365 M.; Passi, A.; Vigetti, D.; Ricard-Blum, S.; Sanderson, R. D.; Neill, T.; Iozzo, R. V. Proteoglycan Chemical Diversity
366 Drives Multifunctional Cell Regulation and Therapeutics. *Chemical Reviews* **2018**, *118*, 9152-9232.
- 367 (2) Gandhi, N. S.; Mancera, R. L. The Structure of Glycosaminoglycans and their Interactions with Proteins.
368 *Chemical Biology & Drug Design* **2008**, *72*, 455-482.
- 369 (3) Schnabelrauch, M.; Scharnweber, D.; Schiller, J. Sulfated Glycosaminoglycans As Promising Artificial
370 Extracellular Matrix Components to Improve the Regeneration of Tissues. *Curr. Med. Chem.* **2013**, *20*, 2501-
371 2523.

- 372 (4) DeAngelis, P. L. Glycosaminoglycan polysaccharide biosynthesis and production: today and tomorrow.
373 *Applied Microbiology and Biotechnology* **2012**, *94*, 295-305.
- 374 (5) Mende, M.; Bednarek, C.; Wawryszyn, M.; Sauter, P.; Biskup, M. B.; Schepers, U.; Brase, S. Chemical
375 Synthesis of Glycosaminoglycans. *Chemical Reviews* **2016**, *116*, 8193-8255.
- 376 (6) Zhang, X.; Lin, L.; Huang, H.; Linhardt, R. J. Chemoenzymatic Synthesis of Glycosaminoglycans. *Acc. Chem.*
377 *Res.* **2020**, *53*, 335-346.
- 378 (7) Gao, C. Z.; Edgar, K. Efficient synthesis of novel glycosaminoglycan analogs. *Abstracts of Papers of the*
379 *American Chemical Society* **2019**, *257*.
- 380 (8) Oreste, P.; Zoppetti, G. Semi-synthetic heparinoids. *Handb. Exp. Pharmacol.* **2012**, 403-422.
- 381 (9) Badri, A.; Williams, A.; Linhardt, R. J.; Koffas, M. A. G. The road to animal-free glycosaminoglycan
382 production: current efforts and bottlenecks. *Curr. Opin. Biotechnol.* **2018**, *53*, 85-92.
- 383 (10) Bedini, E.; Laezza, A.; Parrilli, M.; Iadonisi, A. A review of chemical methods for the selective sulfation and
384 desulfation of polysaccharides. *Carbohydr. Polym.* **2017**, *174*, 1224-1239.
- 385 (11) Wang, J. L.; Bao, A. J.; Meng, X. H.; Guo, H. Y.; Zhang, Y. D.; Zhao, Y. L.; Kong, W. B.; Liang, J. Y.; Yao, J.;
386 Zhang, J. An efficient approach to prepare sulfated polysaccharide and evaluation of anti-tumor activities in
387 vitro. *Carbohydr. Polym.* **2018**, *184*, 366-375.
- 388 (12) Feng, Y. L.; Li, W. Q.; Wu, X. Q.; He, L. A.; Ma, S. Y. Rapid and efficient microwave-assisted sulfate
389 modification of lentinan and its antioxidant and antiproliferative activities in vitro. *Carbohydr. Polym.* **2010**, *82*,
390 605-612.
- 391 (13) Deming, J. Deep ocean environmental biotechnology. *Curr. Opin. Biotechnol.* **1998**, *9*, 283-287.
- 392 (14) Finore, I.; Di Donato, P.; Mastascusa, V.; Nicolaus, B.; Poli, A. Fermentation Technologies for the
393 Optimization of Marine Microbial Exopolysaccharide Production. *Mar. Drugs* **2014**, *12*, 3005-3024.
- 394 (15) Delbarre-Ladrat, C.; Siquin, C.; Lebellenger, L.; Zykwinska, A.; Collic-Jouault, S. Exopolysaccharides
395 produced by marine bacteria and their applications as glycosaminoglycan-like molecules. *Frontiers in Chemistry*
396 **2014**, *2*.
- 397 (16) Zykwinska, A.; Marchand, L.; Bonnetot, S.; Siquin, C.; Collic-Jouault, S.; Delbarre-Ladrat, C. Deep-sea
398 Hydrothermal Vent Bacteria as a Source of Glycosaminoglycan-Mimetic Exopolysaccharides. *Molecules* **2019**,
399 *24*.
- 400 (17) Raguenes, G. H.; Peres, A.; Ruimy, R.; Pignet, P.; Christen, R.; Loaec, M.; Rougeaux, H.; Barbier, G.;
401 Guezennec, J. G. *Alteromonas infernus* sp. nov., a new polysaccharide-producing bacterium isolated from a
402 deep-sea hydrothermal vent. *J. Appl. Microbiol.* **1997**, *82*, 422-430.
- 403 (18) Roger, O.; Kervarec, N.; Ratiskol, J.; Collic-Jouault, S.; Chevolut, L. Structural studies of the main
404 exopolysaccharide produced by the deep-sea bacterium *Alteromonas infernus*. *Carbohydr. Res.* **2004**, *339*,
405 2371-2380.
- 406 (19) Rederstorff, E.; Rethore, G.; Weiss, P.; Sourice, S.; Beck-Cormier, S.; Mathieu, E.; Maillason, M.; Jacques,
407 Y.; Collic-Jouault, S.; Fella, B. H.; Guicheux, J.; Vinatier, C. Enriching a cellulose hydrogel with a biologically
408 active marine exopolysaccharide for cell-based cartilage engineering. *Journal of Tissue Engineering and*
409 *Regenerative Medicine* **2017**, *11*, 1152-1164.
- 410 (20) Chopin, N.; Siquin, C.; Ratiskol, J.; Zykwinska, A.; Weiss, P.; Cerantola, S.; Le Bideau, J.; Collic-Jouault, S. A
411 Direct Sulfation Process of a Marine Polysaccharide in Ionic Liquid. *BioMed Research International* **2015**, *2015*,
412 508656. doi:.
- 413 (21) Merceron, C.; Portron, S.; Vignes-Colombeix, C.; Rederstorff, E.; Masson, M.; Lesoeur, J.; Sourice, S.;
414 Siquin, C.; Collic-Jouault, S.; Weiss, P.; Vinatier, C.; Guicheux, J. Pharmacological modulation of human
415 mesenchymal stem cell chondrogenesis by a chemically oversulfated polysaccharide of marine origin: potential
416 application to cartilage regenerative medicine. *Stem cells (Dayton, Ohio)* **2012**, *30*, 471-480.
- 417 (22) Zykwinska, A.; Marquis, M.; Godin, M.; Marchand, L.; Siquin, C.; Garnier, C.; Jonchere, C.; Chedeville, C.;
418 Le Visage, C.; Guicheux, J.; Collic-Jouault, S.; Cuenot, S. Microcarriers Based on Glycosaminoglycan-Like Marine
419 Exopolysaccharide for TGF-beta 1 Long-Term Protection. *Mar. Drugs* **2019**, *17*.

420 (23) Gelebart, P.; Cuenot, S.; Sinquin, C.; Halgand, B.; Sourice, S.; Le Visage, C.; Guicheux, J.; Collic-Jouault, S.;
421 Zykwinska, A. Microgels based on Infernan, a glycosaminoglycan-mimetic bacterial exopolysaccharide, as BMP-
422 2 delivery systems. *Carbohydr. Polym.* **2022**, *284*.

423 (24) Heymann, D.; Ruiz-Velasco, C.; Chesneau, J.; Ratiskol, J.; Sinquin, C.; Collic-Jouault, S. Anti-Metastatic
424 Properties of a Marine Bacterial Exopolysaccharide-Based Derivative Designed to Mimic Glycosaminoglycans.
425 *Molecules* **2016**, *21*, 309.

426 (25) Zykwinska, A.; Berre, L. T.-L.; Sinquin, C.; Ropartz, D.; Rogniaux, H.; Collic-Jouault, S.; Delbarre-Ladrat, C.
427 Enzymatic depolymerization of the GY785 exopolysaccharide produced by the deep-sea hydrothermal
428 bacterium *Alteromonas infernus*: Structural study and enzyme activity assessment. *Carbohydr. Polym.* **2018**,
429 *188*, 101-107.

430 (26) Kamerling, J. P.; Gerwig, G. J.; Vliegthart, J. F.; Clamp, J. R. Characterization by gas-liquid
431 chromatography-mass spectrometry and proton-magnetic-resonance spectroscopy of pertrimethylsilyl methyl
432 glycosides obtained in the methanolysis of glycoproteins and glycopeptides. *Biochemical Journal* **1975**, *151*,
433 491-495.

434 (27) Montreuil, J.; Bouquelet, S.; Debray, H.; Fournet, B.; Spik, G.; Strecker, G.: *Carbohydrate Analysis: a*
435 *practical approach*; Oxford University Press: New York, 1986.

436 (28) Akoumany, K.; Zykwinska, A.; Sinquin, C.; Marchand, L.; Fanuel, M.; Ropartz, D.; Rogniaux, H.; Pipelier, M.;
437 Delbarre-Ladrat, C.; Collic-Jouault, S. Characterization of New Oligosaccharides Obtained by An Enzymatic
438 Cleavage of the Exopolysaccharide Produced by the Deep-Sea Bacterium *Alteromonas infernus* Using its Cell
439 Extract. *Molecules* **2019**, *24*.

440 (29) Zsiska, M.; Meyer, B. SYNTHESSES OF DISACCHARIDES WITH (1- 4)-BETA GLYCOSIDIC LINKAGES RELATED TO
441 THE 4-SULFATES AND 6-SULFATES AND THE 4,6-DISULFATES OF CHONDROITIN. *Carbohydr. Res.* **1991**, *215*, 261-
442 277.

443 (30) Vessella, G.; Traboni, S.; Pirozzi, A. V. A.; Laezza, A.; Iadonisi, A.; Schiraldi, C.; Bedini, E. A Study for the
444 Access to a Semi-synthetic Regioisomer of Natural Fucosylated Chondroitin Sulfate with Fucosyl Branches on N-
445 acetyl-Galactosamine Units. *Mar. Drugs* **2019**, *17*.

446