Biomacromolecules
January 2023, Volume 24 Issue 1 Pages 462-470
https://doi.org/10.1021/acs.biomac.2c01277
https://archimer.ifremer.fr/doc/00813/92487/

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

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Abstract :

Sulfated glycosaminoglycans (GAGs) are fundamental constituents of both the cell surface and extracellular matrix. By playing a key role in cell–cell and cell–matrix interactions, GAGs are involved in many physiological and pathological processes. To design GAG mimetics with similar therapeutic potential as the natural ones, the specific structural features, among them sulfate content, sulfation pattern, and chain length, should be considered. In the present study, we describe a sulfation method based on microwave radiation to obtain highly sulfated derivatives as GAG mimetics. The starting low-molecular-weight (LMW) derivative was prepared from the infernan exopolysaccharide, a highly branched naturally slightly sulfated heteropolysaccharide synthesized by the deep-sea hydrothermal vent bacterium Alteromonas infernus. LMW highly sulfated infernan derivatives obtained by conventional heating sulfation have already been shown to display GAG-mimetic properties. Here, the potential of microwave-assisted sulfation versus that of the conventional method to obtain GAG mimetics was explored. Structural analysis by NMR revealed that highly sulfated derivatives from the two methods shared similar structural features, emphasizing that microwave-assisted sulfation with a 12-fold shorter reaction time is as efficient as the classical one.

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INTRODUCTION

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

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

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

to obtain low-molecular weight (LMW) and highly sulfated derivatives, with molecular weight ranging from 8,000 to 30,000 g/mol and a sulfur content from 12 to 14 %S (w/w).[20] Through their physical interactions with growth factors, e.g. TGF-β1 and BMP-2, these highly sulfated derivatives were able to induce cellular processes essential for tissue regeneration.[21-23] Moreover, their presence allowed to effectively inhibit both migration and invasiveness of osteosarcoma cells *in vitro*, as well as the establishment of lung metastases *in vivo*.[24] LMW highly sulfated derivatives displayed also some anticoagulant properties, remaining however lower compared to LMW heparin and unfractionated heparin (2.5 and 6.5 times, respectively).[25] These bioactive derivatives are classically prepared in the presence of sulfur trioxide pyridine complex (SO_3·py) in dry DMF and the reaction is followed for 2h at 45°C.[20] In this context, the objective of the present study was to prepare highly sulfated infernan derivatives as GAG mimetics using microwave radiation with similar features to those usually prepared by conventional method. By tuning the sulfation conditions, derivatives with different sulfate contents were obtained and fully characterized. The structure of the EPS derivatives with the highest sulfate content obtained by both conventional and microwave radiation sulfation methods was analyzed by NMR and compared with the LMW-infernan precursors. The obtained results clearly emphasized that microwave-assisted sulfation is a fast and efficient method to get suitable GAG-mimetic derivatives.

EXPERIMENTAL SECTION

LMW EPS Derivative Production and Modification. Infernan EPS was produced by fermentation of the marine bacterium *A. infernus* as described previously.[26] LMW EPS derivatives (EPS DR), with an average molecular weight of 7,800 g/mol (EPS $DR_{7,8k}$) and 19,000 g/mol (EPS DR_{19k}) were obtained by the depolymerization of the native EPS using a free-radical process as previously described.[20] After depolymerization, polysaccharide chains

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

Sulfation by Conventional Heating. Oversulfated EPS derivatives (EPS DRS) were obtained by a chemical sulfation of EPS $DR_{7,8k}$, as described previously.[20] Sulfation was performed in duplicate at three different EPS/SO_3·py w/w ratios: 1/5, 1/2.5 or 1/1.25. Briefly, EPS $DR_{7,8k}$ (20 mg) in its pyridinium salt form was firstly solubilized in extra dry DMF over molecular sieve (4 mL) for 2h at 45°C under continuous stirring and then sulfated for the next 2h at 45°C in the presence of SO_3·py (100, 50, or 25 mg). The final aqueous solution (pH 7) was dialyzed against water for three days before freeze-drying.

Sulfation by Microwave Radiation. Oversulfated EPS derivatives (EPS DRS-MW) were obtained in duplicate by a microwave-assisted sulfation of EPS $DR_{7,8k}$ using three different EPS/SO_3·py w/w ratios: 1/5, 1/2.5 or 1/1.25 using a Biotage Initiator Microwave Synthesizer (Sweden). The microwave reactor was equipped with infrared (IR) sensor for reaction temperature control, pressure sensor to monitor the reaction pressure in the closed vessel and a magnetic stirrer to enable proper agitation. EPS $DR_{7,8k}$ (25 mg) in its pyridinium salt form was firstly solubilized in extra dry DMF over molecular sieve (12.5 mL) under stirring for 2 h at 45 °C. Then, SO_3·py (125, 62.5, or 31.2 mg) was added and the mixture was placed in the microwave oven turn table and exposed to 300 Watts maximum power for 10 min at 45°C. No pulsation cycle was used. The obtained solution (pH 7) was then dialyzed against water for three days before freeze-drying. In order to determine the potential effect of microwave

radiation on polysaccharides, EPS $DR_{7,8k}$ was also exposed to microwaves without any SO_3·py addition.

Physico-chemical Analyses. *Monosaccharide Composition***.** Monosaccharide composition was determined in triplicate according to the Kamerling *et al* method as modified by Montreuil *et al*.[27,28] Briefly, infernan and its LMW derivatives (EPS DR) were hydrolyzed for 4h at 100°C by 3 M MeOH/HCl with *myo*-inositol used as internal standard. After re-*N*-acetylation with acetic anhydride overnight at room temperature, the methyl glycosides were converted to their corresponding trimethylsilyl derivatives. Separation and quantification of the per-*O*-trimethylsilyl methyl glycosides were performed by gas chromatography (GC-FID, Agilent Technologies 6890N).

Molecular Weight. The weight-average molecular weight (*Mw*) was determined by high-performance size-exclusion chromatography (HPSEC) coupled with multi-angle light scattering (MALS, Dawn Heleos-II, Wyatt Technology) and differential refractive index (RI) (Optilab Wyatt technology) detectors. HPSEC system was composed of a Prominence Shimadzu HPLC system, a PL aquagel-OH mixed, 8 μm (Varian) guard column (U 7.5mm × L 50 mm), and a PL aquagel-OH mixed (Varian) separation column. Samples (in duplicate) were eluted with 0.1 M ammonium acetate at 1 mL/min flow rate. The mean molecular weight was calculated using a refractive index increment d*n*/d*c* of 0.145 mL/g.

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

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

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

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

NMR Analysis. NMR spectra were recorded on a Bruker (Billerica, MA, USA) Avance-III HD (^{1}H: 400 MHz, ^{13}C: 100 MHz) or on a Bruker Avance-III (^{1}H: 600 MHz, ^{13}C: 150 MHz) instrument – the latter equipped with a cryo-probe – in D_2O (acetone as internal standard, ^{1}H: $(CH_3)_2CO$ at δ 2.22 ppm; ^{13}C:$(CH_3)_2CO$ at δ 31.5 ppm). Data were processed using the data analysis packages integrated with Bruker TopSpin® 4.0.5 software. ^{1}H,^{13}C-HSQC and ^{1}H,^{13}C-HMBC experiments were measured in the ^{1}H-detected mode via single quantum coherence with proton decoupling in the ^{13}C domain, using data sets of 2048 × 256 points and typically 80 increments (160 for HMBC).

RESULTS AND DISCUSSION

Native infernan and its LMW derivatives (EPS DR). Native infernan produced by *A. infernus* is a highly branched anionic heteropolysaccharide with a monosulfated nonasaccharide repeating unit firstly described in 2004 by Roger *et al*. (Figure 1).[18] Infernan main chain is composed of only three residues, glucose (Glc), galacturonic acid (GalA) and galactose (Gal), which are covalently linked in the sequence: →4)-β-D-Glc*p*-(1→4)-α-D-Gal*p*A-(1→4)-α-D-Gal*p*-(1→. GalA residue of the main chain is substituted at *O*-2 by one sulfate group and at *O*-3 by a short side chain composed of two glucuronic acids (GlcA), Gal and Glc units linked through the sequence: β-D-Glc*p*-(1→6)-α-D-Gal*p*-(1→4)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*A-(1→. In addition, each GlcA residue is substituted by a terminal Glc. Recently, a novel disulfated octasaccharide repeating unit was described after mass spectrometry analysis performed on

LMW infernan derivatives obtained after enzymatic depolymerization.[29] In this new repeating unit, the terminal Glc of the side chain initially linked to Gal residue at C-6 was replaced by a sulfate group (Figure 1). It appeared therefore that during EPS biosynthesis two types of repeating unit could be assembled.

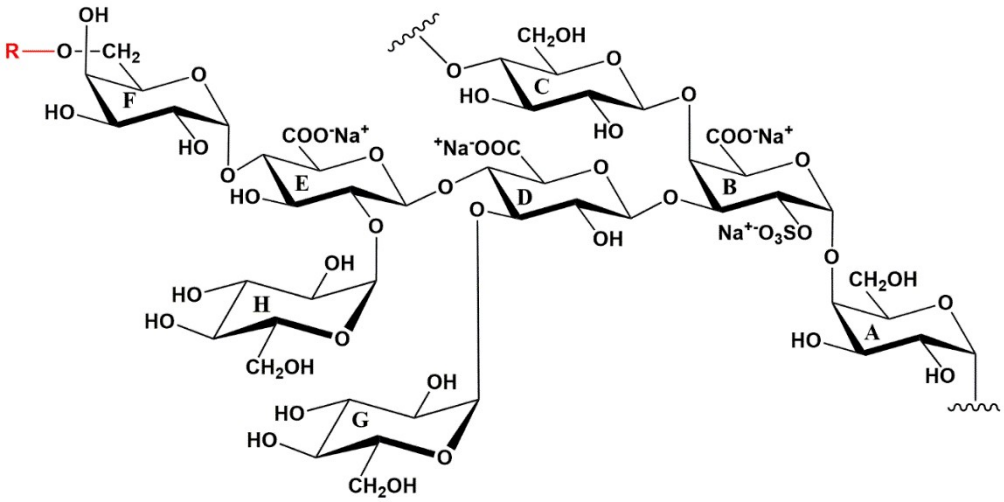


Figure 1. The two types of infernan repeating unit: a monosulfated nonasaccharide repeating unit (R = β-Glc)[18] and a disulfated octasaccharide repeating unit (R = $SO_3^-Na^+$)[29]

In order to prepare GAG-mimetics, native infernan of high-molecular weight (2,000,000 g/mol) was firstly depolymerized using a free-radical process to prepare LMW derivatives (EPS DR). Two derivatives of two different molecular weights were obtained, EPS $DR_{7,8k}$ of 7,800 g/mol and EPS DR_{19k} of 19,000 g/mol. As shown in Table 1, both derivatives presented similar monosaccharide composition as starting EPS, suggesting that the depolymerization process had no major impact on the polysaccharide structure. Elemental analysis performed on the native EPS and its LMW derivatives revealed the DS close to 2 assuming both a nonasaccharide and an octasaccharide repeating units (Table 1).

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

	Monosaccharide composition (w/w%)				**%C**	**%S**	**DS_{nona}**	**DS_{octa}**	**Mw $_{experimental}$** (g/mol)
	Gal	**Glc**	**GalA**	**GlcA**					
Infernan EPS	10.4	18.5	7.6	12.7	29.6	2.9	2.0	1.8	2,000,000 ± 100,000
EPS DR_{19k}	9.7	18.9	10.4	14.7	30.3	3.0	2.0	1.8	19,000 ± 0,400
EPS $DR_{7,8k}$	9.8	18.7	10.3	14.8	30.4	2.7	1.8	1.6	7,800 ± 1,200

Gal, galactose; Glc, glucose; GalA, galacturonic acid; GlcA, glucuronic acid; %C, mean carbon content and %S, mean sulfur content measured by elemental analysis; DS_{nona}, degree of sulfation assuming a nonasaccharide repeating unit (from equation 1); DS_{octa}, degree of sulfation assuming an octasaccharide repeating unit (from equation 1). Mw $_{experimental}$, weight average molecular weight determined by HPSEC-MALS.

These two derivatives were then analyzed by NMR to reveal their fine structures, and in particular to establish the presence of the monosulfated nonasaccharide and/or the disulfated octasaccharide repeating units. ^{1}H-NMR and a set of 2D-NMR ^{1}H,^{1}H-homonuclear (COSY, TOCSY, NOESY) and ^{1}H,^{13}C-heteronuclear spectra (DEPT-HSQC, HSQC-TOCSY, HMBC) were measured for the EPS DR samples in D_2O. Their comparison revealed no significant differences between the two samples (Figure 2 and Figure S1). Chemical shift data were in agreement with those previously reported in 2004 by Roger *et al.*,[18] apart for the Gal F unit (Figure 1). Indeed, in the ^{1}H,^{13}C-DEPT-HSQC spectra the presence of CH_2 signals in two different regions could be detected. The most crowded one at $\delta_{H/C}$ 3.50-4.00/60-65 ppm hosts the methylene signals of Glc and Gal residues carrying a free hydroxyl at C-6, in agreement with Roger *et al.* (2004).[18] The signal at $\delta_{H/C}$ 4.13/67.5 ppm could be assigned to the CH_2O-moiety of 6-*O*-sulfated Gal F unit, in agreement with NMR data previously reported for the synthetic α-D-Gal6S*p*-(1→4)-β-D-Glc*p*A disaccharide (F-E fragment).[30] This assignment was in disagreement with Roger *et al.* (2004), that attributed such signal to a *O*-glycosylated (with

a β-Glc residue) rather than 6-*O*-sulfated Gal F unit, in spite of the absence of any n.O.e. between β-Glc and Gal F residues[18] as well as of any correlation between the signal at $\delta_{H/C}$ 4.13/67.5 ppm and anomeric signals in the $^{1}H,^{13}C$-HMBC spectrum (Figure S2). NMR data together with the DS values determined for both derivatives, indicating the presence of 2 sulfate groups per repeating unit (Table 1), suggested that a revision of the structure of the repeating unit of infernan should be done. Indeed, a disulfated octasaccharide, in agreement with our recent mass spectrometry data on LMW infernan derivatives,[29] rather than a monosulfated nonasaccharide[18] should only be considered.

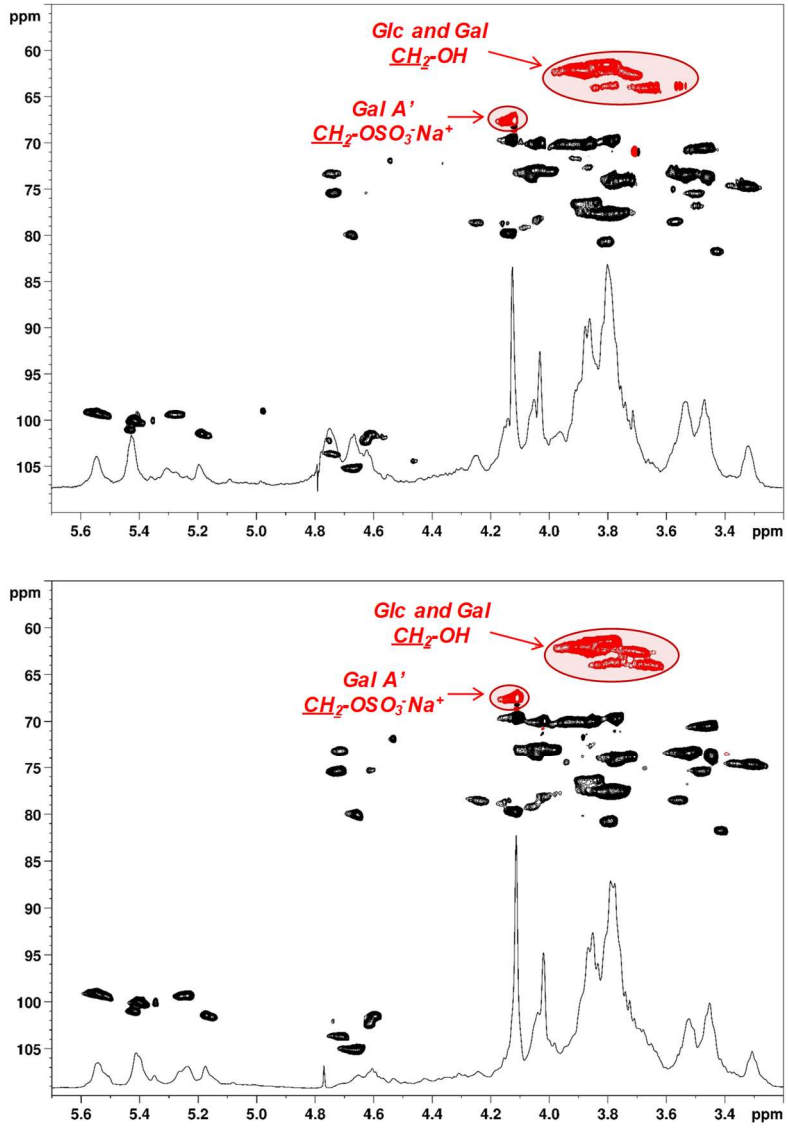


Figure 2. ^{1}H- and $^{1}H,^{13}C$-DEPT-HSQC NMR spectra (400 MHz, D_2O, 298K, zoom) of EPS DR_{19k} (top) and EPS $DR_{7,8k}$ (bottom).

Conventional *versus* microwave-assisted sulfation of EPS DR. In the next step, the LMW derivative of the lowest molecular weight, namely EPS $DR_{7,8k}$, was selected to explore the potential of microwave-assisted sulfation to obtain a highly sulfated compound similar to GAGs, such as LMW heparin. Firstly, the effect of microwave radiation on the polysaccharide structural integrity was assessed by exposing the derivative to the radiation without the SO_3·py addition. Weight average molecular weight was similar before and after microwave exposure suggesting that radiation had no destructive effect on the polysaccharide structure (EPS DR-MW, Table 2).

Sulfation was then performed in the presence of SO_3·py complex using microwave radiation, in parallel to the conventional heating method.[20] By tuning EPS/SO_3·py ratio, the sulfur content, corresponding to the amount of added sulfate groups, could be modulated (Table 2). Highly sulfated derivatives were obtained with the highest EPS/SO_3·py ratio (1/5) using both conventional heating (EPS DRS_{HS}) and microwave radiation (EPS DRS-MW_{HS}). The amount of sulfur was above 10%, which corresponds to a ~32% of sulfate groups, similarly to the amount typically found in sulfated GAGs. A two-fold decrease in the sulfur content was measured for the derivatives sulfated using the EPS/SO_3·py ratio of 1/2.5. The lowest EPS/SO_3·py ratio (1/1.25) applied was not sufficient to sulfate the derivative, as the sulfur content was comparable to the derivative before sulfation. In addition, a molecular weight increase was observed with the increase of added sulfate groups, suggesting that the backbone of the EPS derivatives was not degraded during the reaction (Table 2), in contrast to other widely used sulfation reagents such as chlorosulfonic acid or sulfuric acid.[12] In particular, weight-average molecular weight determined by HPSEC-MALS ($Mw_{experimental}$) was compared

to the theoretical one ($Mw_{theoretical}$) calculated following the equation (2). It appeared that for highly sulfated derivative, EPS DRS_{HS} with DS 14 (12.2% S), the experimental *Mw* was slightly higher compared to the theoretical value (Table 2). Conversely, *Mw* determined by HPSEC-MALS was slightly lower for EPS DRS-MW_{HS} with DS 18 (13.7% S) with respect to the theoretical value. However, by considering the measurement uncertainty, this difference seems relatively low, thus confirming the integrity of the EPS backbone after sulfation reactions.

Table 2. Characterization of LMW EPS derivatives sulfated by either conventional heating (EPS DRS) or microwave radiation (EPS DRS-MW) at three different EPS/SO_3·py w/w ratios.

EPS derivative	T (°C)	t (min)	EPS/SO_3·py	%C	%S	DS_{octa}	Mw $_{experimental}$ (g/mol)	Mw $_{theoretical}$ (g/mol)
EPS DR-MW	45	120	-	30.1	2.5	1.5	6,900 ± 0,600	-
EPS DRS_{HS}	45	120	1/5	15.3	12.2	14.4	17,200 ± 3,400	14,400 ± 2,200
EPS DRS_{MS}	45	120	1/2.5	20.4	6.1	5.4	8,000 ± 0,200	9,700 ± 1,500
EPS DRS_{LS}	45	120	1/1.25	29.6	2.6	1.6	6,800 ± 0,200	7,800 ± 1,200
EPS DRS-MW_{HS}	45	10	1/5	13.6	13.7	18.1	12,600 ± 0,800	16,400 ± 2,500
EPS DRS-MW_{MS}	45	10	1/2.5	19.3	7.1	6.6	11,700 ± 0,500	10,400 ± 1,600
EPS DRS-MW_{LS}	45	10	1/1.25	27.5	3.1	2.0	7,700 ± 1,200	8,000 ± 1,200

HS, high sulfate; MS, medium sulfate; LS, low sulfate; %S, mean sulfur content measured by elemental analysis; Mw $_{experimental}$, weight average molecular weight determined by HPSEC-MALS; Mw $_{theoretical}$, weight average molecular weight calculated following the equation (2):

$$Mw_{theoretical} = MwEPS\ DR_{7,8k} \frac{(DS_{octa} * MwS) + MwRUnit}{\left(DS_{octaEPS\ DR_{7,8k}} * MwS\right) + MwRUnit} \quad (2)$$

where, $Mw\ EPS\ DR_{7,8k}$ of 7,800 g/mol is the molecular weight of initial derivative EPS $DR_{7,8k}$, DS_{octa} is the degree of sulfation of octasaccharide repeating unit of sulfated derivative EPS DRS (as calculated from %C and %S by applying equation 1), $DS_{octa\ EPS\ DR\ 7,8k}$ is the degree of sulfation of octasaccharide repeating unit of initial derivative EPS $DR_{7,8k}$ (from equation 1), *MwS* of 103 g/mol is the molecular weight increase by a single sulfate group (SO_3^- Na^+), *MwRUnit* of 1383 g/mol is the molecular weight of octasaccharide repeating unit with DS = 0.

Sulfated EPS derivatives obtained by conventional and microwave-assisted sulfation methods were analyzed by ATR-FTIR and compared to EPS $DR_{7,8k}$ before sulfation (Figure 3 and S3).

The raw spectra (Figure S3) were normalized with respect to the C=O stretching band at 1650 cm^{-1} to better visualize the differences in the sulfur content between the samples (Figure 3). Indeed, carboxylic acid groups are not involved in sulfation reaction and their band intensity could be considered constant. On normalized ATR-FTIR spectra (Figure 3), the broad band corresponding to O-H at 3600-3000 cm^{-1} was considerably narrowed for both EPS DRS_{HS} and EPS DRS-MW_{HS} samples having the highest sulfur content, due to the esterification of the hydroxyl groups, when compared to EPS $DR_{7,8k}$ or EPS $DRS_{LS, MS}$ with low and medium sulfur contents. The strong O=S=O asymmetric stretching band at 1262 cm^{-1} clearly increased with increasing sulfur content. For both highly sulfated derivatives, EPS DRS_{HS} and EPS DRS-MW_{HS}, prepared by the two sulfation methods, the intensity of O=S=O band was comparable. Similar results were observed for C-O-S symmetric vibration band at 816 cm^{-1}. An important absorption band was observed for all derivatives at 1100-1010 cm^{-1} attributed to the C-O stretching vibration of pyranosyl rings.

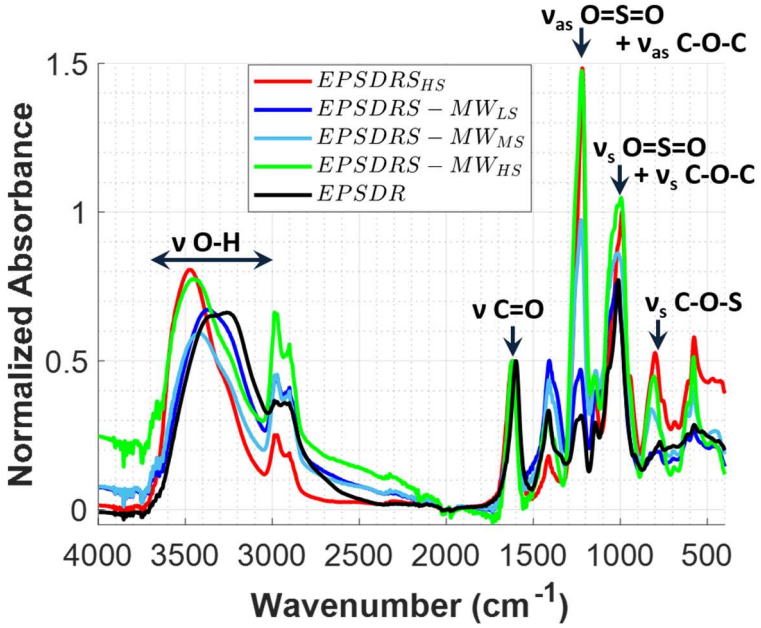


Figure 3. Normalized ATR-FTIR spectra with respect to the C=O band at 1650 cm^{-1} of slightly sulfated EPS $DR_{7,8k}$ before sulfation, highly sulfated EPS DRS_{HS} obtained by conventional sulfation method and EPS DRS-$MW_{LS, MS, HS}$ prepared using microwave radiation. HS = high sulfate, MS = medium sulfate, LS = low sulfate, MW = Microwave.

By taking into account the physico-chemical characterizations, it appeared clearly that similarly sulfated derivatives were obtained using both conventional and microwave-assisted sulfation methods. However, the main advantage of the microwave-assisted sulfation is the reaction time that can be considerably decreased (10 min instead of 120 min). To confirm that both methods lead to similar derivatives, the structures of the EPS derivatives with the highest sulfate contents, EPS-DRS_{HS} and EPS DRS-MW_{HS}, were further analyzed by NMR to get some insights into their sulfation pattern. A comparison of their ^{1}H,^{13}C-DEPT-HSQC with respect to EPS $DR_{7,8k}$ clearly confirmed an extensive sulfation for both samples, as indicated by a marked ^{1}H downfield shift for several CH and CH_2 signals (Figure 4).[31] Their quite broad shape suggested that the obtained polysaccharides have a heterogeneous structure. Indeed, the only insight into their sulfation pattern could be inferred from the absence of any signal assignable to unsulfated CH_2 moieties. This indicated that a quantitative degree of sulfation at the primary hydroxyl of Gal and Glc residues of both polysaccharide samples was achieved. This is not unexpected, due to the generally higher reactivity of primary *vs.* secondary alcohols. Furthermore, a comparison between the ^{1}H,^{13}C-DEPT-HSQC NMR spectra of EPS-DRS_{HS} and EPS DRS-MW_{HS} samples revealed no significant differences (Figures S4-S6). This confirmed that conventional and microwave-assisted sulfation reactions proceeded similarly on EPS $DR_{7,8k}$. From elemental analysis, the DS of 14 and 18 per octasaccharide repeating unit were determined, respectively, for EPS-DRS_{HS} (12.2 %S, 15.3 %C) and EPS DRS-MW_{HS} (13.7 %S, 13.6 %C) (Table 2). By considering that (i) the highest DS theoretically possible is 21, (ii) two sulfated positions are already present in native infernan and (iii) all the four primary hydroxyl groups (2 on the backbone and 2 on the side chain) result sulfated as evidenced by NMR (Figure 5), 8 and 12 sulfate groups remain, respectively, in EPS-DRS_{HS} and EPS DRS-MW_{HS}. Although, the accessibility of secondary hydroxyl groups on the side chains (11 groups available) during sulfation seems higher in contrast to the backbone (4 groups available), the

$^{1}H,^{13}C$-DEPT-HSQC spectra suggesting a heterogeneous structure for both derivatives do not indicate a high regioselectivity for sulfate group insertion on side chain *vs.* backbone secondary positions.

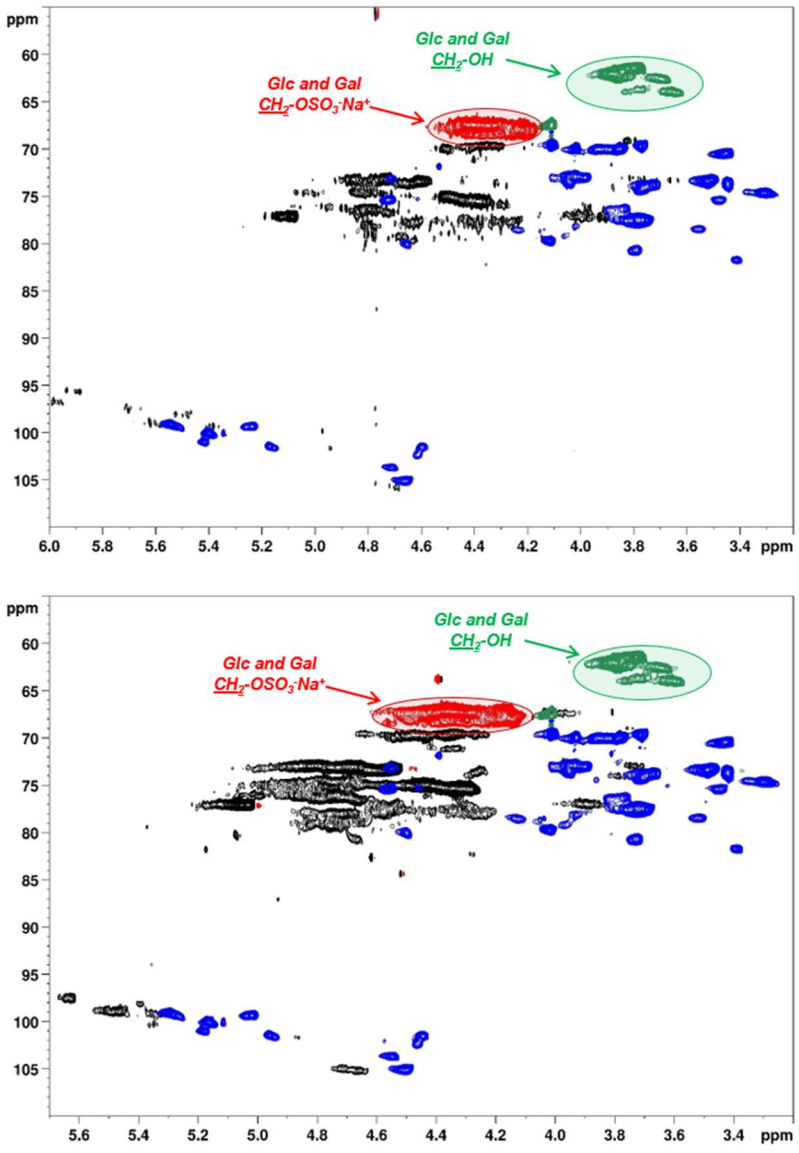
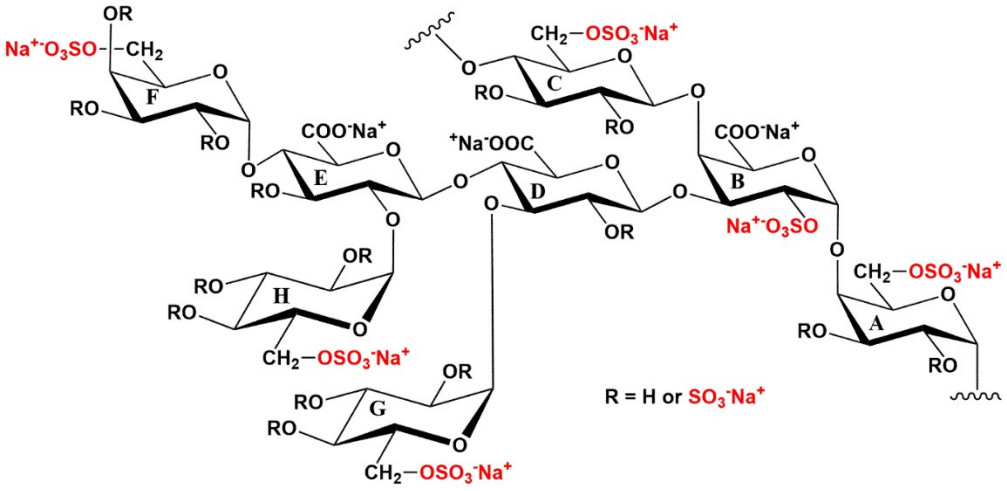


Figure 4. Superimposition of $^{1}H,^{13}C$-DEPT-HSQC NMR spectra (400 MHz, D_2O, 298 K, zoom) of EPS $DR_{7,8k}$ (blue/green) and EPS DRS-MW_{HS} (black/red; top) or EPS DRS_{HS} (black/red; bottom).



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329 **Figure 5**. The postulated structure for the octasaccharide repeating unit of infernan highly-
330 sulfated derivatives EPS DRS_{HS} and EPS DRS-MW_{HS}.

331

332 **CONCLUSIONS**

333 The present study explored a microwave-assisted sulfation method applied to LMW derivative
334 prepared from infernan EPS to obtain sulfated GAG-mimetics. The new method was compared
335 to the conventional one that we classically use to prepare highly sulfated polysaccharides
336 displaying biological properties similar to GAGs. For both methods, the same sulfation
337 conditions, including EPS/SO_3·py ratios and temperature were applied, except for the reaction
338 time, which was twelve-fold shorter for the microwave-assisted sulfation (10 min) in
339 comparison to the classical one (120 min). Derivatives obtained in both cases displayed very
340 similar physico-chemical characteristics in terms of sulfur content and molecular weight
341 distribution. Moreover, by tuning EPS/SO_3·py ratio, the sulfur content was increased to reach
342 the amount known for sulfated GAGs (EPS/SO_3·py 1/5 w/w ratio). In order to get further insight
343 into their fine structure, and in particular the sulfation pattern, LMW derivatives displaying the
344 highest sulfur content prepared by both methods were analyzed by NMR. An extensive sulfation
345 was evidenced for both samples, with a slightly higher degree for the one obtained through the
346 microwave-assisted reaction. Both derivatives showed a highly heterogeneous structure

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

ASSOCIATED CONTENT

Supporting information

1D- and 2D-NMR spectra, and raw ATR-FTIR spectra of LMW infernan derivatives before and after sulfation reactions.

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Acknowledgment

The authors would like to acknowledge the French GagoSciences Network (GdR GAG 3739). Financial supports were also provided by University of Naples Federico II (FRA-2020-B grant, project GLYCOPLAGENT, to E.B.) and Région Centre Val de Loire (APR-IR FLOWSYN and INFLUX). Authors thank the projects CHemBio (FEDER-FSE 2014-2020-EX003677), Techsab (FEDER-FSE 2014-2020-EX011313), QUALICHIM (APR-IA-PF 2021-00149467), the RTR Motivhealth (2019-00131403), and the Labex programs SYNORG (ANR-11-LABX-0029) and IRON (ANR-11-LABX-0018-01) for their financial support of ICOA, UMR 7311, University of Orléans, CNRS.

Notes

The authors declare no competing financial interest.

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