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► To cite this version:

Syntia Fayad, Benjamin Ayela, Coralie Chat, Philippe Morin, Chrystel Lopin-Bon, et al.. Effect of modified di- and trisaccharides on hyaluronidase activity assessed by capillary electrophoresis-based enzymatic assay. *Carbohydrate Research*, 2019, 475, pp.56 - 64. 10.1016/j.carres.2019.02.006 . hal-03486681

HAL Id: hal-03486681

<https://hal.science/hal-03486681>

Submitted on 20 Dec 2021

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1 **Effect of modified di- and trisaccharides on hyaluronidase activity assessed by capillary**
2 **electrophoresis-based enzymatic assay**

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8

9 **Keywords:** Capillary electrophoresis; enzymatic assay; hyaluronidase; chondroitin sulfate;
10 activation; inhibition constant

11

12 **Abbreviation:** BGE, background electrolyte; CS, chondroitin sulfate; CPA, corrected-peak area;
13 di, disaccharide; HA, hyaluronic acid; IB, incubation buffer; Oligo-HA4, oligohyaluronic acid 4
14 or tetrasaccharide.

15

16

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21 **Abstract**

22 The activity of eukaryote hydrolase-type of hyaluronidases was studied using a miniaturized
23 capillary electrophoresis (CE) assay developed in our laboratory. Few nanoliters of reagents are
24 sufficient and no labeling is required for this assay. The effect of natural and original synthetic
25 effectors of hyaluronidase was evaluated. These di- and trisaccharides from linkage region of
26 proteoglycans were synthesized in 30 to 40 steps from monomeric units using classical
27 protection, deprotection, glycosylation and deoxygenation reactions. The influence of the chain
28 length (di/trisaccharide), the modification type (methoxy/deoxy) and its position (2/4/6) was
29 studied.

30 The inhibition and/or activation percentages were determined at two concentrations of effectors;
31 0.2 mM and 2 mM. The half maximal effective concentration (EC_{50}) values were evaluated
32 ($n=2$) for the most effective inhibitors (~ 1 mM) and activators (~ 0.2 mM). Results showed that
33 hyaluronidase was mostly inhibited in a concentration-dependent fashion by a deoxy
34 modification and activated by a methoxy modification. Trisaccharides were found to be more
35 effective on hyaluronidase activity than disaccharides. Position 4 was found to be more favorable
36 for hyaluronidase activity than position 6 and the activity in position 2 was negligible. For a
37 better understanding of the enzyme function mode, the inhibition constant (K_i) was also
38 evaluated by CE ($K_i \sim 2$ mM). These results are of great interest especially as few activators of
39 hyaluronidase are presented in the literature.

40 **1. Introduction**

41 Hyaluronidases are enzymes that degrade, predominantly, hyaluronan or hyaluronic acid (HA).
42 HA is a simple, high molecular weight glycosaminoglycan (GAG) linear polymer built of large
43 numbers of repeating units consisting of [-D-glucuronic acid- β 1,3-N-acetyl-D-glucosamine- β 1,4-
44]_n. HA, is recognized as a major participant in basic processes such as cell motility, wound
45 healing, embryogenesis, and is implicated in cancer progression [1-3]. The term “hyaluronidase”
46 is a misnomer since they have the limited ability to degrade chondroitin (Ch) and chondroitin
47 sulfates (ChS). Hyaluronidases were classified in 1952 in three classes of enzymes, based on
48 their mechanisms of action (see review [4] and cited references). Two of them are endo- β -N-
49 acetyl-hexosaminidases, cleaving the β -1,4 glycosidic bond.. One group includes the eukaryotic
50 or vertebrate enzymes that utilize substrate hydrolysis for catalysis (EC 3.2.1.35). The vertebrate
51 hyaluronidases degrade HA through a nonprocessive endolytic process, generating mostly
52 tetrasaccharides. They also have transglycosidase activities, with the ability to cross-link chains
53 of HA and the potential ability to cross-link chains of HA with ChS or Ch. This reaction is not
54 well understood, and the precise enzymatic mechanism is not known. The second group, which
55 is predominantly bacterial, includes the eliminases or lyases that function by β -elimination of
56 the glycosidic linkage with introduction of a new double bond (EC 4.2.99.1). The mechanism
57 of action of HA lyases, involving an acid/base-type of proton acceptance and donation reaction is
58 vastly different from the hydrolysis mechanism of vertebrate hyaluronidases. The third group are
59 the endo- β -glucuronidases (EC 3.2.1.36) that cleave the β -1,3 glycosidic bond. Little is known
60 about this class of enzymes. They are characteristic of annelids, such as leeches and certain
61 crustaceans. They utilize the hydrolysis mechanism and thus resemble more to eukaryotic
62 enzymes than to bacterial ones.

63 Vertebrate and bacterial hyaluronidases are involved in tissue degradation, spread of pathogens
64 *e.g* as virulence factor for bacteria, tissue dissection in gas gangrene, treponema spread in
65 syphilis, penetration of skin and gut by nematode parasites and many inflammatory reactions
66 [2,5,6]. In this work, we will be focusing on the study of HA hydrolysis by the vertebrate
67 hyaluronidase.

68 The presence of regulatory or effector molecules (inhibitors or activators) associated with an
69 enzyme or receptor is important to affect the decay of biological activity. Many studies have
70 focused on finding new inhibitors of hyaluronidase to prevent local tissue destruction and also to
71 slow down the diffusion of toxins into the blood, resulting in time delayed death in severe cases
72 of envenomation [7-9]. These inhibitors can be natural or synthetic [10-12]. Compounds such as

73 proteins, glycosaminoglycans, polysaccharides, flavonoids, alkaloids, antioxidants and anti-
74 inflammatory agents have been investigated as hyaluronidase inhibitors [6,13-17]. In addition,
75 aqueous methanolic or ethanolic extracts prepared from different parts of numerous plant species
76 have been investigated for their role in hyaluronidase inhibition [18,19]. The effect of macroalga
77 *Padina pavonica* water extracts was recently studied by our group and the results showed their
78 high capacity in inhibiting hyaluronidase [20].

79 The activation of hyaluronidase is also important despite the fact that only few authors have been
80 interested in finding activators. Hyaluronidase is involved in inflammatory reactions and is
81 usually present in its inactive form, which requires activation. The functions of proteoglycans
82 and HA are altered with aging which requires their elimination by the activation of
83 hyaluronidase. For this, the quantities of elastin, collagen and HA present in the extracellular
84 matrix decrease due to their degradation by different enzymes [21,22]. Moreover, a lack of
85 hyaluronidase activity has been observed in different forms of mucopolysaccharidosis, thus
86 pointing to the need to find activators of this enzyme [23].

87 In 1959, Miyaki *et al.* [5] were the first to study the activation of hyaluronidase by spermine and
88 related diamines. The spermine was shown to be a powerful activator of bovine testicular
89 hyaluronidase. Several other amines, especially polymethylenediamines related to spermine,
90 were also found to activate hyaluronidase with an effect (1 mM) lower than that of spermine (0.1
91 mM). The authors noticed that the spermine is able to increase enzyme's activity due to its anti-
92 heparin action, and heparin is known to be a natural inhibitor of hyaluronidase.

93 In 1985, Kakegawa *et al.* [24] studied the effect of some metallic salts on hyaluronidase
94 activation. The authors then investigated the role of metal ions including calcium ions. It was
95 found that hyaluronidase is activated by CaCl_2 and another compound called 48/80. This latter is
96 a polycondensate of N-methyl-p-methoxyphenethylamine and formaldehyde, known to release
97 histamine from mast cells. More recently, Kaessler *et al.* [25] found new heterocyclic activators
98 of bovine hyaluronidase. Searching for new inhibitors for the enzyme, they showed that indole
99 carboxamides inhibited bovine hyaluronidase while indole acetamides activated it. Moreover,
100 bovine serum albumin (BSA), a protein widely used in biology, is known to be an activator of
101 hyaluronidase [26].

102 For the study of hyaluronidase activity, our group has developed an offline assay by capillary
103 electrophoresis (CE) [27-28]. This analytical tool has proved to be very advantageous. It requires
104 only few tenths of nanoliters of reagents, do not necessitate immobilization or derivatization

105 steps, can be coupled to various detectors (UV, fluorescence or mass spectrometry) and is fully
106 automatized [29].

107 The pre-capillary assay developed in CE, aimed to determine the activity of hyaluronidase by
108 following the formation of HA tetrasaccharide fractions [27-29]. The Michaelis-Menten constant
109 (K_m) as well as the maximal velocity (V_{max}) of HA degradation by hyaluronidase were
110 determined by UV detection. Reaction products were identified using high-resolution mass
111 spectrometry (HRMS). The activity of chemically made tetrasaccharides [30] of biotinylated
112 chondroitin sulfate CS-A or CS-C (4- or 6- sulfated in a homogeneous or heterogeneous way) on
113 the hydrolysis reaction of hyaluronidase was thus evaluated. CE-based assays showed that
114 hyaluronidase was mostly inhibited by CS-A tetrasaccharides sulfated in an homogeneous way.
115 The effect of two trisaccharides (non-modified and 6-deoxy) from truncated linkage region of
116 proteoglycans were also tested as inhibitors or activators. A small modification of one hydroxyl
117 group was shown to have an influence on hyaluronidase activity. Based on these promising
118 results, our researches were pursued here by studying the effect of original chemically-made di-
119 and trisaccharides (**Fig. 1**), differing by their substituent (deoxy or methoxy) on different
120 positions (2, 4 or 6), on the activity of hyaluronidase using the same CE-based enzymatic test
121 developed previously. Hydrolysis activity of bovine hyaluronidase was determined by following
122 the formation of tetrasaccharide fractions (oligo-HA4) by CE-UV. In the conditions of this study,
123 HA-tetrasaccharide was the final product of the hyaluronidase hydrolysis of HA [31]; its
124 concentration increases in the reaction mixture with the incubation time. For this reason, we only
125 analyzed the formation of HA-tetrasaccharide. This enzyme was selected since it is
126 commercially available and presents the same activity as the human hyaluronidase [4, 27, 28].
127 The values of IC_{50} and AC_{50} parameters of the most efficient inhibitors and activators were
128 evaluated. Finally, the inhibition constant (K_i) of trisaccharide 6-deoxy was determined in order
129 to better evaluate the type of inhibition of this potent inhibitor towards hyaluronidase.

130

131

132 **2. Materials and methods**

133 **2.1. Chemicals**

134 All reagents were of analytical grade and used as received without further purification.
135 Ammonium acetate (CH_3COONH_4 , purity $\geq 98\%$), apigenin-7-glucoside ($C_{21}H_{20}O_{10}$, purity \geq
136 97% , epigallocatechin gallate (EGCG, purity $\geq 95\%$), hyaluronidase (EC 3.2.1.35) type I-S from
137 bovine testes (BTH, 400-1000 units.mg⁻¹ solid), sodium acetate (CH_3COONa , purity $\geq 99\%$),

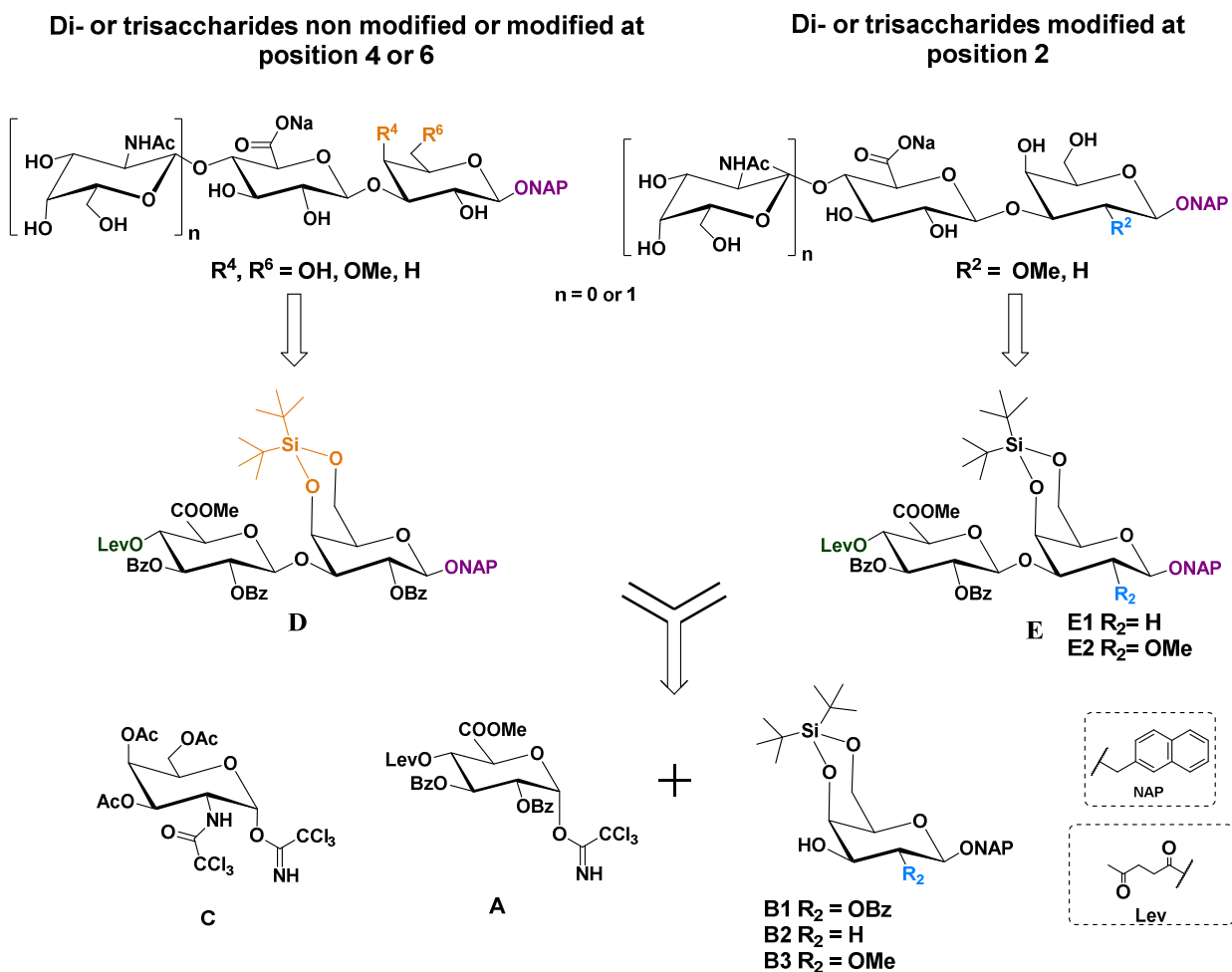
138 sodium hydroxide (NaOH, purity \geq 98%) and oligohyaluronic acid 4 (oligo-HA4 or
139 tetrasaccharide, C₂₈H₄₄N₂O₂₃) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier,
140 France). Hyaluronic acid, sodium salt, *Streptococcus pyogenes* (HA, CAS 9067-32-7 –
141 Calbiochem) was purchased from Merck Millipore (Molsheim, France). Dimethyl sulfoxide was
142 purchased from Fisher scientific ((CH₃)₂SO, purity \geq 99%). Ethanol absolute (EtOH,
143 H₃CCH₂OH, purity \geq 99,7%), glacial acetic acid (CH₃COOH), ammonia (NH₄OH, purity = 28
144 %), were purchased from VWR International (Fontenay-sous-Bois, France). Ultra-pure water (18
145 M Ω -cm) was obtained with an Elga apparatus (Elga, Villeurbanne, France). Syringes and
146 hydrophilic polyvinylidenedifluoride (PVDF) Millex-HV Syringe Filters, pore size 0.45 μ m,
147 were purchased from Merck Millipore.

148

149 All the di- and trisaccharides from linkage region of proteoglycans were synthesized in 30-40
150 steps from three different monomeric units (compounds **A**, **B (1, 2, 3)** and **C**) [30-32] using
151 classical protection, deprotection, glycosylation and deoxygenation reactions (data not shown
152 and will be the topic of a future research paper). A convergent strategy allowed us to obtain the
153 different di- and trisaccharides, modified or not with good yields (see retrosynthesis **Scheme 1**).
154 Coupling reaction between **A** and **B1** gave disaccharide **D** bearing a silylene acetal for the
155 modification at position 4 or 6. Glycosylation between **A** and modified saccharides **B2** or **B3**
156 afforded the disaccharides **E** modified at position 2. Access to the trisaccharides was managed
157 after deprotection of the levulinoyl ester (Lev) of the modified disaccharides followed by
158 coupling with **C**. Overall, we synthesized fourteen compounds, selectively altered on the position
159 2, 4, and 6 by methoxy or deoxy modifications (**Fig. 1**). NMR, HRMS and polarimetry of the
160 compounds **8**, **10** and **12** (the most potent compounds) are presented in the **supporting**
161 **information**.

162

163



164

165 **Scheme 1:** Retrosynthesis of the tested di- and trisaccharides

166

167 2.2. Solutions

168 All solutions for CE assays were prepared with ultra pure water, filtered through PVDF Millex-

169 HV Syringe Filter before use and stored at 4°C (except for the enzyme that was stored at 0°C).

170 The pH of the buffers was measured with a MeterLab PHM201 Portable pH-Meter (Radiometer
 171 Analytical, Villeurbanne, France). Buffers were daily prepared and their parameters given by

172 Phoebus software (Analis, Namur, Belgium). Sodium acetate at 2 mM was used as the

173 incubation buffer (IB). Its pH was fixed at 4.0 by adding glacial acetic acid. The background

174 electrolyte (BGE) was ammonium acetate 50 mM with a pH of 9.0 adjusted with ammonia.

175 Stock solutions of hyaluronidase, HA, EGCG and Tet were prepared in the IB at a concentration

176 of 2 mg.mL⁻¹ and diluted to the appropriate concentrations in the same buffer. Three stock

177 solutions of apigenin-7-glucoside were prepared in different solvents: ultra-pure water, ethanol

178 absolute and DMSO, at a concentration of 2 mg.mL⁻¹ and diluted to the appropriate

179 concentrations in IB. The enzyme stock solution was prepared daily and stored at -20°C.

180 Stock solutions of modified saccharides were prepared at 4 mM in pure water and then diluted in
181 the IB to the appropriate concentration.

182

183 ***2.3. Instrumentation and operating conditions***

184 Throughout this study, uncoated fused-silica capillaries purchased from Polymicro Technologies
185 (Phoenix, AZ, USA) were used. New capillaries were conditioned by performing the following
186 rinse cycles: 1 M NaOH (20 min), water (5 min) and BGE (10 min). At the end of each working-
187 day, the capillary was rinsed with NaOH 1 M (10 min) and water (10 min) to ensure good
188 cleaning of the inner capillary surface. All rinse cycles were carried out at 30 psi. Capillaries
189 were stored overnight in water. The capillary temperature was 25°C. Hydrodynamic injections at
190 0.5 psi for 5 s were performed.

191 The corrected-peak area (CPA) of an analyte represents the peak area corrected of the migration
192 time. The relative standard deviation (RSD) of the corrected peak-areas and of the migration
193 times was used to assess repeatability. The limit of quantification (LOQ) was calculated as the
194 concentration corresponding to a signal-to-noise ratio of 10.

195 The capillary electrophoresis system used was a PA800+ automated CE instrument from AB
196 Sciex (Brea, CA, USA) equipped with a photodiode array detection system. The control of CE
197 was performed using 32 Karat software (Beckman Coulter). Between two runs, the capillary was
198 flushed with NaOH 1 M (1 min), water (0.5 min) and BGE (3 min). The UV detection of the
199 substrate (HA) and the products was performed at 200 nm (bandwidth 10 nm) using a capillary
200 of 60 cm total length (49.8 cm effective detection length) and 50 µm internal diameter (i.d.).
201 Injections (5 nL) were performed at the anodic side of the capillary and normal polarity mode
202 was used with a separation voltage of + 20 kV.

203 High-resolution mass spectrometry (HRMS) was used for peak identification. For CE-HRMS
204 on-line coupling [28], the sheath liquid was MeOH/water (80/20; v/v) with 0.02 % formic acid at
205 10 µL min⁻¹. N₂ was used as dry gas at 6 L min⁻¹. Nebulizer pressure was 1.1 bar and
206 electrospray ionization voltage was - 4 kV.

207

208 ***2.4. Effector assays - Evaluation of chemically made oligosaccharide effect on hyaluronidase*** 209 ***activity***

210 Hydrolysis activity of bovine hyaluronidase was determined by following the formation of
211 tetrasaccharide fractions (oligo-HA4) by CE-UV. This enzyme was chosen since it is
212 commercially available and presents comparable activity as the one of human hyaluronidase

213 [4,27,28]. The previously developed pre-capillary CE-assay [20, 27] was validated for this study.
214 For this, two natural standards, EGCG and apigenin-7-glucoside, were tested. The enzymatic
215 reaction was then conducted at 37 °C in a microvial in a total volume of 50 µL of IB. 5 µL of
216 hyaluronidase (0.2 mg mL⁻¹) were pre-incubated with each natural compound or saccharide for
217 10 min. Then, a small volume (10 µL) of HA (0.4 mg mL⁻¹, corresponding to the *K_m* value in the
218 literature of 0.46 mg mL⁻¹ [28,33]) was added to the mixture and incubated for 1 h. The reaction
219 was stopped by heating for 10 min at 90 °C. After cooling down the reaction vial, 5 nL (0.5 psi
220 for 5 s) of the reaction mixture was introduced into the capillary. Blank assays, without the
221 addition of the enzyme, the substrate and/or the natural compound/saccharide were conducted
222 before each set of CE analysis.

223

224 **2.5. Determination of I % and EC₅₀**

225 Each di- and trisaccharide was tested at two concentrations, 0.2 and 2 mM (Table 1). Non-
226 modified di- and trisaccharides as well as di- and trisaccharides modified in position 2 or 4 or 6
227 with a deoxy or methoxy group were used (compounds **1-14**; **Fig. 1**).

228 The activity of hyaluronidase in the absence of the tested saccharides was defined as 100%. The
229 half maximal effective concentration (EC₅₀) was determined as the saccharide concentration at
230 which half of the difference between the maximum and the minimum enzymatic activity was
231 obtained. IC₅₀ was defined for inhibition studies whereas AC₅₀ was defined for activation assays.
232 The following equation was used and EC₅₀ values were obtained by fitting data using the
233 nonlinear curve fitting program PRISM®5.04 (GraphPad, San Diego, CA, USA):

$$234 \quad EC_{50}(\%) = \left(1 - \frac{A_x}{A_0}\right) \times 100 \quad (1)$$

235 where *A_x* is the CPA of the tetrasaccharide product formed in the presence of the saccharide and
236 *A₀* is the CPA of the product formed in the absence of the saccharide.

237 Inhibition assays with two natural inhibitors, EGCG and apigenin-7-glucoside, were performed
238 to validate the developed CE-based enzymatic assay. Concentration-response curves for
239 hyaluronidase inhibition by EGCG and apigenin-7-glucoside were constructed by plotting the
240 percentage of inhibition (I %) versus log [inhibitor]. Twelve inhibitor concentration values,
241 ranging from 0.01 to 1 µg.mL⁻¹ were tested. Moreover, IC₅₀ and AC₅₀ were evaluated for the
242 most potent inhibitors (**8** and **12**) and activator (**10**), respectively. Thus, inhibition assays were
243 performed with 6 concentrations of **8** (trisaccharide 4-deoxy) and **12** (trisaccharide 6-deoxy),
244 ranging from 0.1 to 3 mM. The concentration-response curves for hyaluronidase inhibition by **8**

245 and **12** were constructed to obtain IC₅₀. In the same manner, activation assays were performed
246 with six concentrations of **10** (trisaccharide 4-methoxy), ranging from 0.5 to 1.5 mM. The
247 concentration-response curve for hyaluronidase activation by **10** was constructed by plotting the
248 percentage of activation (A %) *versus* log [trisaccharide 4-methoxy] to obtain AC₅₀. Each assay
249 was repeated twice (n=2).

250

251 **2.6. Determination of K_i**

252 To identify the inhibition mode of hyaluronidase by the compound **12** using CE, the reaction rate
253 was determined for a range of HA concentrations (0.1-0.7 mM) in the absence and presence of
254 the inhibitor. The corresponding relative Lineweaver-Burk plots were assessed at different
255 inhibitor concentrations of trisaccharide 6-deoxy (0.1, 0.5, 1, 1.5, and 3.0 mM). The
256 hyaluronidase activity (%) was defined as the ratio of the hyaluronidase reaction rate in the
257 presence and absence of the inhibitor.

258

259

260 **3. Results and discussion**

261 **3.1. Natural compound inhibition evaluated by CE/UV**

262 To validate the CE assay that will be used throughout this study, two referenced natural
263 inhibitors were tested: the epigallocatechin gallate (EGCG) and the apigenin-7-glucoside. This
264 latter was expected to present lower affinity towards hyaluronidase (higher value of IC₅₀) since a
265 similar compound, the apigenin-7-O-β-glucuronide, showed in literature an IC₅₀ of 0.25 mg.mL⁻¹
266 or 0.55 mM [34]. Water was not adequate to ensure enough inhibitor solubility. The presence
267 of DMSO in the reaction mixtures at quantities higher than 2 % caused a loss in the catalytic
268 activity of hyaluronidase, probably because the DMSO alters the tridimensional integrity of the
269 enzyme (**Fig. 2.a**). On the other hand, better results were obtained using the CE assay when
270 ethanol was used as solvent (**Fig. 2b**).

271 For EGCG, the IC₅₀ value found was 0.02 mg.mL⁻¹, which is in the same range of the value
272 reported in literature of 0.09 mg.mL⁻¹ [35]. The coefficients of determination (r²) value was
273 0.9941 demonstrating the quality of the data fitting.

274 For apigenin-7-glucoside, the IC₅₀ value was found to be 0.07 mg.mL⁻¹ or 0.16 mM (r² =
275 0.9815). Apigenin-7-glucoside has thus affinity in the same range towards hyaluronidase as
276 EGCG. The good results obtained confirm the reliability of the CE-based hyaluronidase assay.

277

278 **3.2. Di- and trisaccharide activity evaluated by CE/UV**

279 The effect of different di- and trisaccharides (structure in **Fig. 1**) on the hydrolysis of HA by
280 hyaluronidase was monitored. The inhibition percentage (I %) of each saccharide is presented in
281 **Table 1**. The peak area of **Tet** product was not modified in the presence of the non-modified
282 saccharides **1** and **2**, showing the absence of any activation or inhibition of the enzyme by these
283 saccharides. This result is not surprising since these compounds are the ones with the most
284 similarities to HA, thus not modifying the activity of hyaluronidase.

285 The modified compounds 2-deoxy or 2-methoxy (**3**, **4**, **5** and **6**) were then tested. No variation
286 was noticed for the product peak with these oligosaccharides regardless of the concentration,
287 modification type or the length of the saccharidic chain. In fact, position 2 is known to have no
288 effect on the enzyme but the test was performed to see if the methoxy or deoxy modifications
289 may have some influence in this case.

290 The effect of 4- and 6-deoxy and methoxy (**7** to **14**) was also studied. Despite the chain length
291 (di- or trisaccharides) or the position (4 or 6), the deoxy modification inhibited the enzyme while
292 the methoxy modification activated it. It is very important to specify here that exactly the same
293 CE- based assay allowed to conduct inhibition as well as activation analyses. On other terms, the
294 herein presented assay is generic and needs no further optimization to screen various inhibitors
295 or activators for their influence on hyaluronidase activity. Moreover, the obtained results indicate
296 that a minor modification in the oligosaccharide structure can affect the activity of the enzyme.
297 This effect can be explained by the fact that with the deoxy modification, the number of
298 interactions between the enzyme and the substrate decreases with the loss of hydrogen bonds and
299 electronic interactions. On the other hand, the methoxy modification increases the number of
300 interactions by expanding the structure. The electropherograms reported in **Fig. 3.a)** and **Fig.**
301 **3.b)** using CE-UV/HRMS illustrate the inhibition of hyaluronidase in the presence of 6-
302 deoxytrisaccharide (**12**) and its activation with 4-methoxytrisaccharide (**10**).

303 For the same position and same modification but different chain length, for example for 4-deoxy
304 disaccharide (**7**) and 4-deoxy trisaccharide (**8**), the trisaccharide has a greater effect than the
305 disaccharide. The corresponding I % was 35 % versus 61 % at 2 mM inhibitor concentration
306 (**Table 1**). Indeed, in previous studies, it was found that when the chain length is longer, the
307 effect is stronger [32]. This could be due to a better recognition of the substrates by the enzyme,
308 since the HA length recognized by the enzyme needs usually to be greater than four saccharidic
309 units.

310 By comparing the different positions of each modification, it appears that position 4 is more
311 favorable for enzyme interaction than position 6. Results obtained show that 6-deoxy
312 trisaccharide (**12**) inhibits hyaluronidase with an inhibition up to 65 % at 2 mM, as mentioned in
313 a previous study (I % = 55 %) [28]. This difference could be due to the use of two different
314 batches of the same oligosaccharide.

315 The corresponding 6-deoxy disaccharide (**11**) appeared to follow the same tendency, also being
316 an inhibitor of the hyaluronidase, but less effectively than (**12**) at 2 mM, and showing no activity
317 at lower concentrations. The compounds with a 4-deoxy modification were shown to be
318 inhibitors of the hyaluronidase activity, with a higher effect of the tri- than disaccharide. The
319 trisaccharide 4-deoxy (**8**) was way more effective than the 6-deoxy (**12**) at lower concentration
320 (0.2 mM), with an inhibition up to 48% versus 26% for (**12**). As for the 6-deoxy compounds, the
321 4-deoxy disaccharide (**7**) had the same effect on the activity than the trisaccharide while being
322 less effective.

323 Position 4-methoxy or deoxy is thus more efficient for interactions with hyaluronidase than
324 positions 6 and 2, which are not. These results correspond well to the ones obtained in our
325 previous study and by other authors [28,33]. Indeed, in a previous study on another enzyme
326 responsible for the chondroitin sulfate chain elongation, Guilberti *et al.* [36] obtained similar
327 results, showing the importance of chain length and that position 4 seems to affect most the
328 enzyme's activity.

329 In order to have a better idea of how these different compounds affect the enzyme, IC₅₀ and AC₅₀
330 for the most efficient inhibitors (trisaccharides 4 and 6-deoxy) and activators (trisaccharide 4-
331 methoxy) were evaluated by assessing the corresponding concentration-response curves.

332

333 **3.3. Evaluation of IC₅₀ and AC₅₀**

334 Concentration-response curves of 4-deoxy and 6-deoxy trisaccharides (**8** and **12**) were plotted
335 (**Fig. 4**) with good coefficients of determination ($r^2 > 0.98$, $n=2$). IC₅₀ for **8** and **12** were found to
336 be 1.48 ± 0.09 mM (1.04 mg.mL⁻¹) and 1.03 ± 0.08 mM (0.76 mg.mL⁻¹), respectively. These
337 results show that acceptable affinity is noticed for the trisaccharides compared, for example to
338 the referenced natural inhibitor apigenin-7-O-β-glucuronide (IC₅₀ = 0.55 mM) [34].

339 Moreover, the most potent inhibitor presents the same I % as the homogeneously 4-sulfated
340 chondroitin tetrasaccharide mentioned in our previous study [28], thus assuring its efficiency
341 since a smaller chain was implicated. This facilitates synthesis and modulation of the saccharide.

342 The activity of hyaluronidase in the presence of the most potent activator, the 4-methoxy
343 trisaccharide (**10**), was also monitored. AC_{50} was found to be equal to 0.24 ± 0.04 mM or 0.17
344 $mg \cdot mL^{-1}$ ($n=2$). This result is very interesting since the affinity of this activator is relatively
345 higher than the affinity of the inhibitors previously tested. Furthermore, only few activators of
346 hyaluronidase are presented in the literature.

347

348 **3.4. Evaluation of K_i**

349 To investigate the mode of trisaccharide 6-deoxy (**12**) action against BTH, the Lineweaver-Burk
350 plots were obtained with and without the inhibitor (**Fig. 5**). Therefore, several concentrations of
351 **12** were tested with varying concentrations of HA.

352 In the presence of 0.1, 0.5 and 1 mM concentrations of the compound **12**, V_{max} was reduced. If
353 interactions are competitive then V_{max} should not change [37], which is not the case here.
354 However, apparent K_m values in the presence of different concentrations of compound **12**
355 seemed to be in the same range. This observation is only possible in the non-competitive
356 inhibition. This indicates that the trisaccharide 6-deoxy probably interacts with hyaluronidase in
357 a non-competitive inhibition mode.

358 K_i was determined from the equation corresponding to non-competitive inhibition [37] and was
359 equal to 2.05 ± 0.12 mM (**Fig. 4**). From the **Fig. 5** and the Cheng-Prusoff equation [38], K_i value
360 was also obtained as 1.75 ± 0.23 mM. Both K_i values are in the range confirming the hypothesis
361 of non-competitive inhibition mode of hyaluronidase by **12**. Supplementary data needs to be
362 acquired using a complementary technique (spectrophotometry- or HPLC- based assays) to
363 confirm the obtained inhibition mode proposed in this study.

364

365 **4. Conclusions**

366 An efficient and cost-effective CE-based kinetic assay was used to screen a small library of
367 natural and original compounds, synthesized from monosaccharide units in our laboratory, for
368 hyaluronidase inhibition and activation. This CE test is generic and needs no optimization to
369 screen various inhibitors as well as activators for their influence on hyaluronidase activity., The
370 influence of the chain length of saccharides from linkage region of proteoglycans
371 (di/trisaccharide), the modification type (methoxy/deoxy) and its position (2/4/6) on the activity
372 of hyaluronidase was evaluated.

373 Results showed that hyaluronidase is mainly inhibited by a deoxy-modification and activated by
374 a methoxy-modification. In addition, the length of the saccharides chain of the tested

375 compounds was shown to be very important; short disaccharides were shown to affect less the
376 hyaluronidase activity than trisaccharides. Moreover, the effect of the position 2 was found to be
377 negligible compared to the position 6 and mostly to the position 4 that was found to be more
378 favorable for hyaluronidase activity. IC_{50} obtained for the most efficient trisaccharides (4-deoxy
379 and 6-deoxy trisaccharides or compounds **8** and **12**) showed acceptable affinity towards
380 hyaluronidase ($IC_{50} = 1.48$ mM and 1.03 mM, respectively) compared to the referenced natural
381 inhibitor apigenin-7-O- β -glucuronide ($IC_{50} = 0.55$ mM) [34]. K_i was evaluated for the compound
382 **12** suggesting a non-competitive inhibition mode. AC_{50} of the most potent activator, the 4-
383 methoxy trisaccharide (compound **10**) was found to be 0.24 ± 0.04 mM. This shows rather a
384 good affinity of this activator towards hyaluronidase.
385 This approach of combining original synthetic di- and trisaccharides to a CE-based enzymatic
386 assay is very interesting for understanding the hyaluronidase activity in a fast, simple, economic
387 and green manner. These data are thus of great interest since they may serve as a benchmark for
388 other researchers to unravel mechanisms of hyaluronidase activity and to develop reliable
389 effectors toward this interesting enzyme for cosmetic or therapeutic applications.

390

391

392 **Acknowledgements**

393 Authors gratefully acknowledge Labex SynOrg Grant ANR-11-LABEX-0029 for financial
394 support and the Région Centre-Val de Loire (research projects PODOUCE and BPCO-Lyse).

395

396 *The authors declare that there are no conflicts of interest.*

397

398 **List of Figures**

399

400 **Figure 1:** Names and structures of the different oligosaccharides modified and non-modified in
401 positions 2, 4 or 6.

402 **Figure 2:** Concentration-response curves obtained for hyaluronidase inhibition with **a)**
403 Apigenin-7-glucoside prepared in DMSO 2 % and **b)** Apigenin-7-glucoside and EGCG prepared
404 in EtOH.

405 Pre-capillary assay conditions: reaction mixture in IB: 0.2 mg.mL⁻¹ hyaluronidase and HA at 0.4
406 mg.mL⁻¹; incubation: at 37°C during 1 h; IB: 2 mM sodium acetate (pH 4.0). Blank assay
407 conducted in the absence of inhibitor in the reaction mixture. Electrophoretic separation
408 conditions: BGE: 50 mM ammonium acetate (pH 9.0); injection at the anodic side: 0.5 psi for 5 s
409 (5 nL); separation: + 20 kV at 25°C; detection: $\lambda = 200$ nm; rinse between analyses at 30 psi: 1
410 min NaOH (1 M), 0.5 min water and 3 min BGE; bare-silica capillary: 60 cm total length, 49.8
411 cm detection length, 50 μ m i.d.

412 **Figure 3:** Example of electropherograms obtained by CE/UV for hyaluronidase inhibition by **a)**
413 trisaccharide 6-deoxy at 2 mM and activation by **b)** trisaccharide 4-methoxy at 1 mM. Blank
414 assay conducted in the absence of effector in the reaction mixture. Peak identification was
415 conducted by CE-HRMS. Other conditions: Fig. 2.

416 **Figure 4:** Concentration-response curves obtained for hyaluronidase inhibition with **a)**
417 trisaccharide 6-deoxy **b)** trisaccharide 4-deoxy and **c)** for activation by trisaccharide 4-methoxy.
418 Conditions: Fig 2.

419 **Figure 5:** Lineweaver-Burk plot obtained by offline BTH assay of reciprocal rates of **Tet**
420 formation ($1/V_i$) as a function of $1/[HA]$. Each line represents linear regression of average rates
421 ($n=2$) for different trisaccharide 6-deoxy (**12**) concentrations: 0.0 mM (**■**), 0.1 mM (**▲**), 0.5 mM
422 (**◆**) and 1 mM (**x**). Other conditions as in **Fig. 2**.

423

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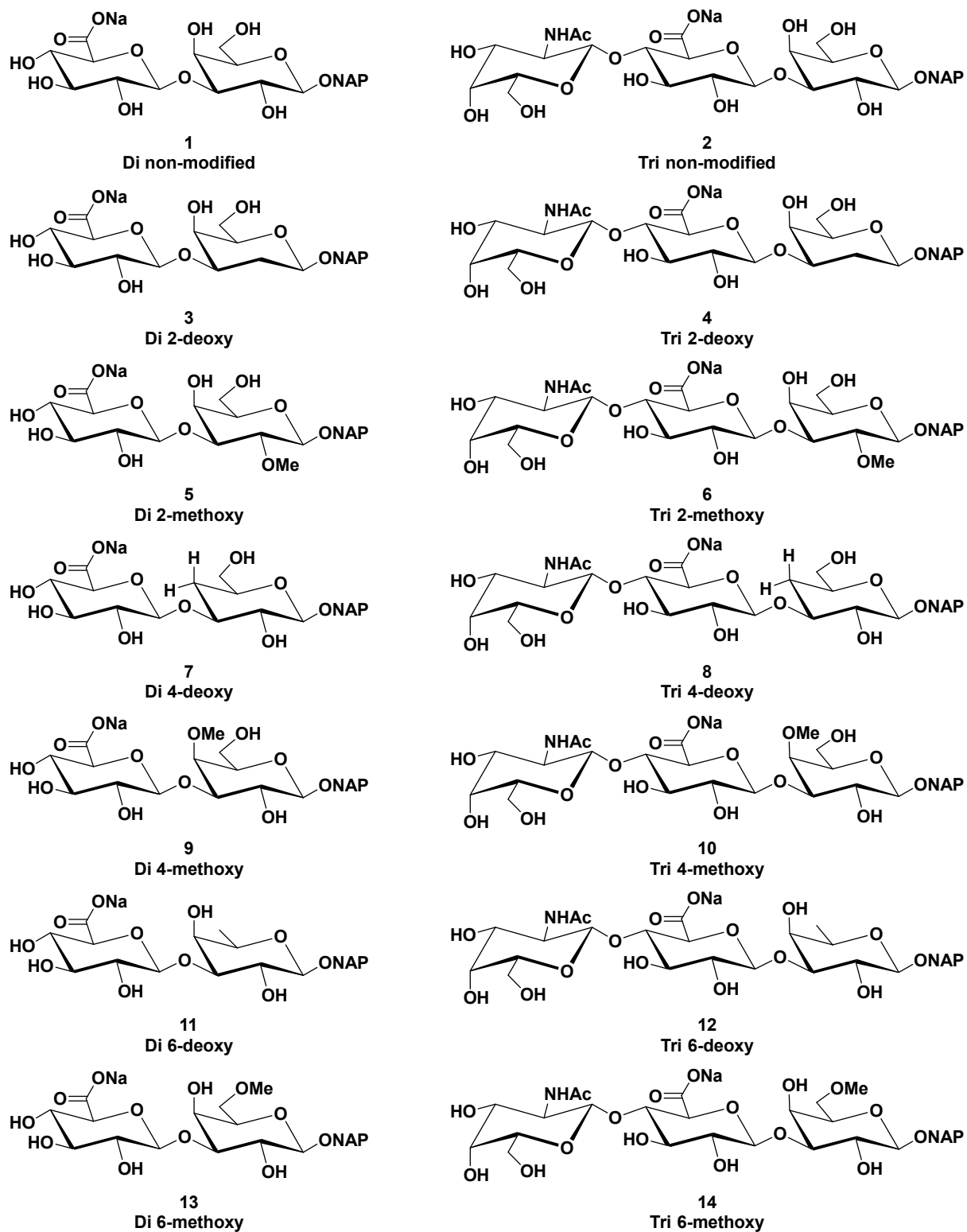


Figure 1: Names and structures of the different oligosaccharides modified and non-modified in positions 2, 4 or 6.

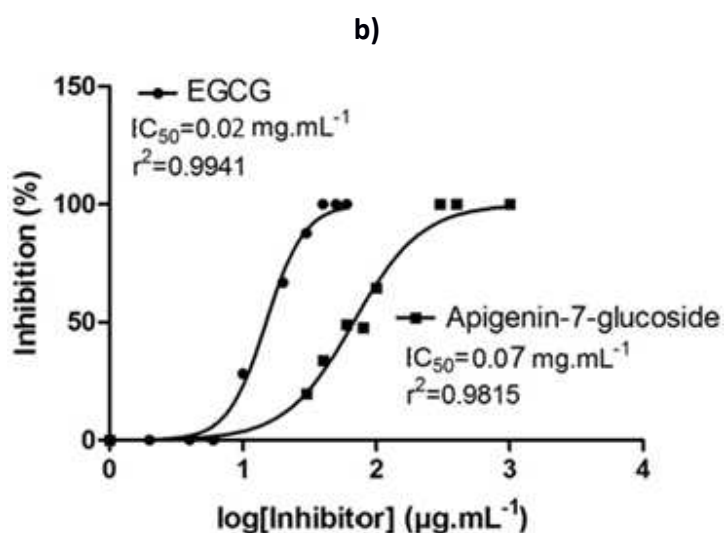
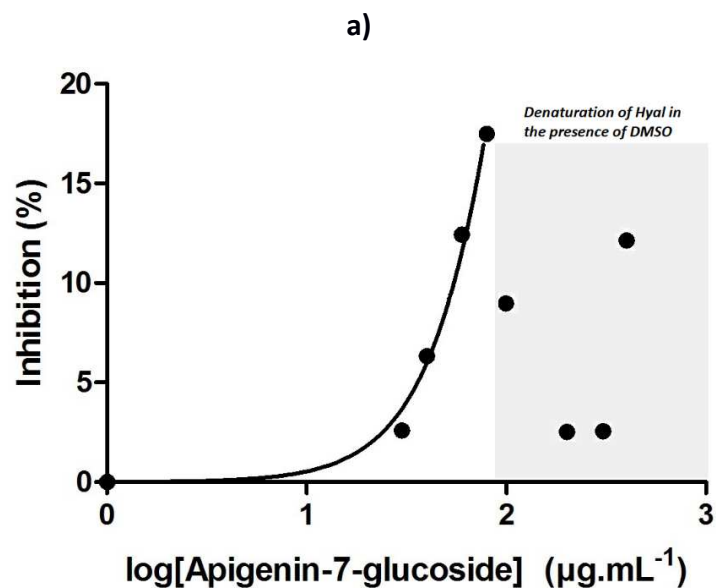


Figure 2: Concentration-response curves obtained for hyaluronidase inhibition with a) Apigenin-7-glucoside prepared in DMSO 2 % and b) Apigenin-7-glucoside and EGCG prepared in EtOH. Pre-capillary assay conditions: reaction mixture in IB: 0.2 mg.mL^{-1} hyaluronidase and HA at 0.4 mg.mL^{-1} ; incubation: at 37°C during 1 h; IB: 2 mM sodium acetate (pH 4.0). Blank assay conducted in the absence of inhibitor in the reaction mixture. Electrophoretic separation conditions: BGE: 50 mM ammonium acetate (pH 9.0); injection at the anodic side: 0.5 psi for 5 s (5 nL); separation: +20 kV at 25°C ; detection: $\lambda = 200 \text{ nm}$; rinse between analyses at 30 psi: 1 min NaOH (1 M), 0.5 min water and 3 min BGE; bare-silica capillary: 60 cm total length, 49.8 cm detection length, 50 μm i.d..

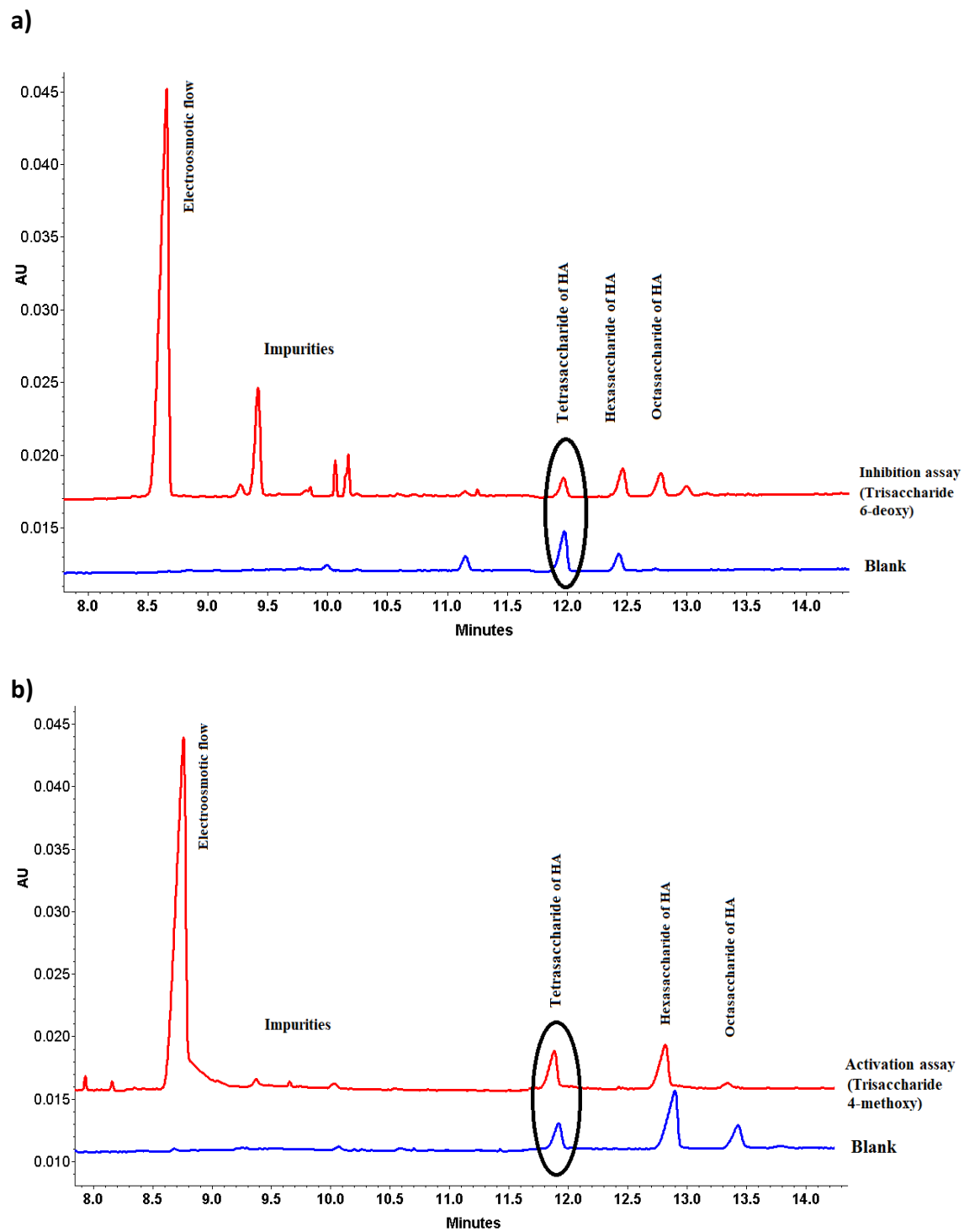


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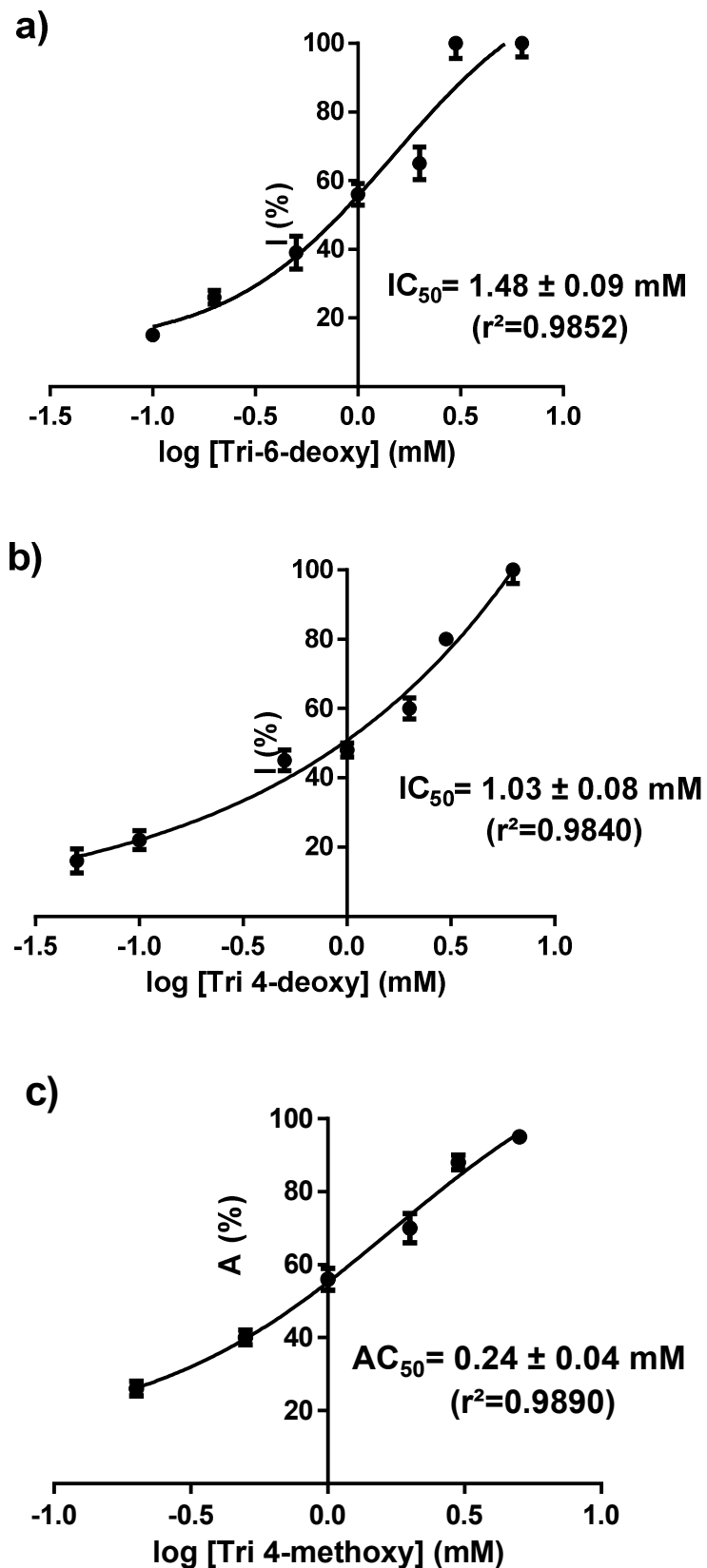


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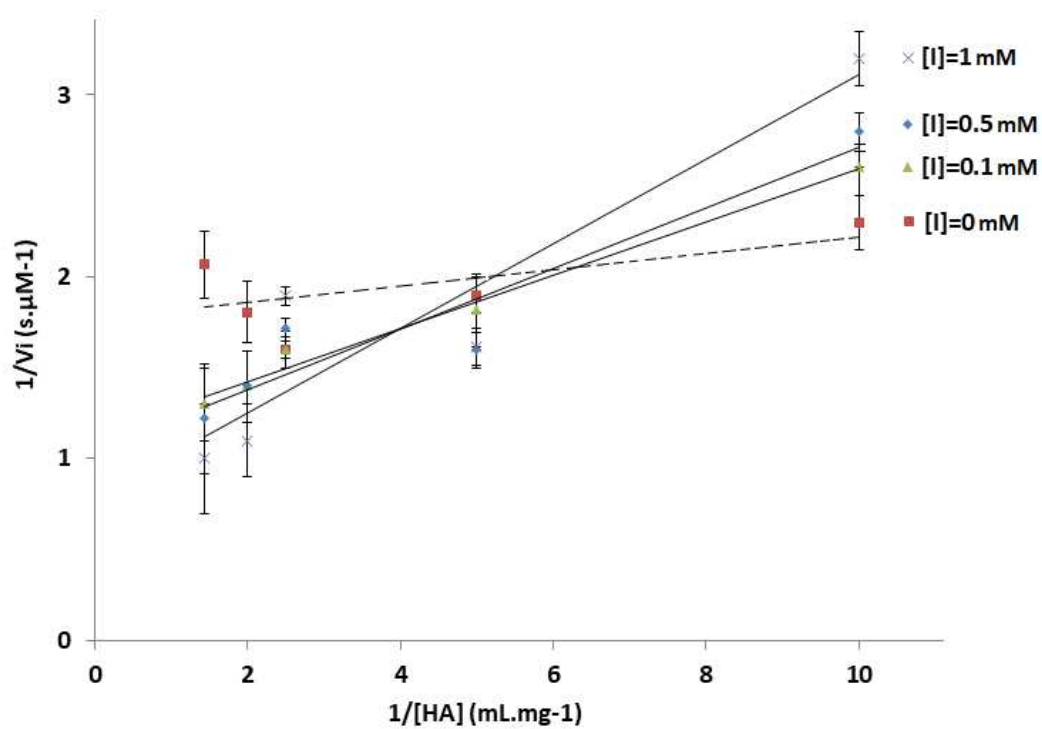
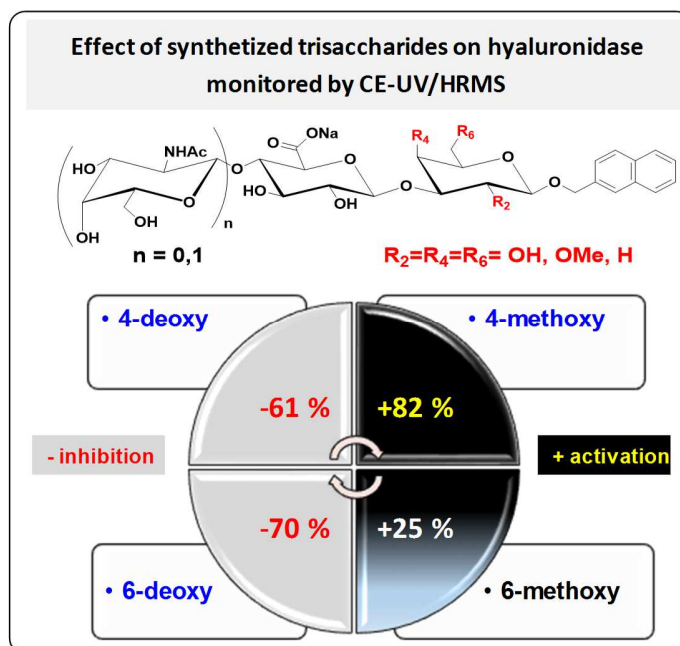
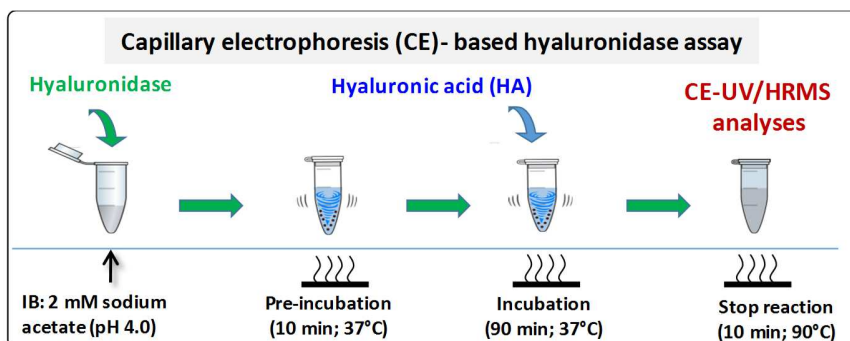


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Table 1: Effect (%) of tested oligosaccharides on hyaluronidase obtained by CE-based assay.*(- : inhibition ; +: activation)*

Oligosaccharide type		N°	Effect (%) at 0.2 mM	Effect (%) at 2.0 mM	Effect on the activity
Not modified	Di	1	0	0	no
	Tri	2	0	0	no
2-deoxy	Di	3	0	0	no
	Tri	4	0	0	no
2-methoxy	Di	5	0	0	no
	Tri	6	0	0	no
4-deoxy	Di	7	0	-35	Inhibitor
	Tri	8	-48	-61	Inhibitor
4-methoxy	Di	9	0	+40	Activator
	Tri	10	+48	+82	Activator
6-deoxy	Di	11	0	-30	Inhibitor
	Tri	12	-26	-65	Inhibitor
6-methoxy	Di	13	0	+12	Activator
	Tri	14	+15	+25	Activator



Graphical abstract