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A chemo-enzymatic pathway to expand cellooligosaccharide chemical space through amine bond introduction

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1 **Title:**

2 **A chemo-enzymatic pathway to expand cellooligosaccharide chemical space**
3 **through amine bond introduction**

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31

32 **Abstract:**

33 Enzymatic functionalization of oligosaccharides is a useful and environmentally friendly way to
34 expand their structural chemical space and access to a wider range of applications in the health,
35 food, feed, cosmetics and other sectors. In this work, we first tested the laccase/TEMPO system to
36 generate oxidized forms of cellobiose and methyl β -D-cellobiose, and obtained high yields of novel
37 anionic disaccharides (>60 %) at pH 6.0. Laccase/TEMPO system was then applied to a mix of
38 cellooligosaccharides and to pure D-cellopentaose. The occurrence of carbonyl and carboxyl groups in
39 the oxidation products was shown by LC-HRMS, MALDI-TOF and reductive amination of the carbonyl
40 groups was attempted with *p*-toluidine a low molar mass amine to form the Schiff base, then
41 reduced by 2-picoline borane to generate a more stable amine bond. The new grafted products were
42 characterized by LC-HRMS, LC-UV-MS/MS and covalent grafting was evidenced. Next, the same
43 procedure was adopted to successfully graft a dye, the rhodamine 123, larger in size than toluidine.
44 This two-step chemo-enzymatic approach, never reported before, for functionalization of
45 oligosaccharides, offers attractive opportunities to anionic cellooligosaccharides and derived
46 glucoconjugates of interest for biomedical or nutraceutical applications. It also paves the way for
47 more environmentally-friendly cellulose fabric staining procedures.

48

49

50 **Keywords:**

51 Cellooligosaccharides; Laccase, Oxidation; Reductive amination; Amino-chromophores

52

53

54 **Abbreviations:**

55 LMS, Laccase Mediated System; TvL, *Trametes versicolor* laccase; COS, cellooligosaccharides; MOS,
56 maltooligosaccharides; pic-BH₃, 2-picoline borane; *p*-T, *p*-toluidine; RHO123, rhodamine 123; TEMPO,
57 2,2,6,6-Tetramethyl-1-piperidinyloxy; Glc2, cellobiose; me- β -Glc2, methyl β -D-cellobiose; D-
58 cellopentaose, Glc5;

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62 1. Introduction

63 Functional oligosaccharides are biomolecules that are increasingly in demand in the food, feed,
64 cosmetics, healthcare or agrochemical sectors due to their wide range of applications as food
65 ingredients, non-digestible prebiotic supplements, bulking agents, drug carriers, immunostimulators,
66 antioxidant, anti-inflammatory and more. (Catenza & Donkor, 2021; Ibrahim, 2018; Logtenberg et al.,
67 2021; Mano et al., 2018; Patel & Goyal, 2011). The most common oligosaccharides (fructo-, galacto-,
68 xylo-, gluco-, or manno-oligosaccharides) are homogeneous and neutral. However, oligosaccharide
69 diversity also covers heterogenous and even charged structures such as those found in pectin,
70 alginate or carrageenan (Guo et al., 2022; Liu, Liu, Zhang, Yi, & Everaert, 2021; Vasudevan, Lee, &
71 Lee, 2021). Furthermore, oligosaccharide conjugation can be used to generate structures with unique
72 physico-chemical or biological properties that offer numerous advantages for designing biological
73 probes, carbohydrate-based vaccines, drug delivery agents, etc. (Astronomo & Burton, 2010;
74 Humpierre et al., 2022; Kay, Cuccui, & Wren, 2019).

75 Among oligosaccharides, celooligosaccharides (COS) composed of β -1,4 linked glucosyl units are
76 gaining attention for their functional properties, which are of interest to the food, feed and
77 cosmetics industries (Cangiano, Yohe, Steele, & Renaud, 2020; Jiao et al., 2014; Uyeno, Shigemori, &
78 Shimosato, 2015; Yamasaki, Ibuki, Yaginuma, & Tamura, 2013; Zhong, Ukowitz, Domig, & Nidetzky,
79 2020). They can be produced by controlled chemical or enzymatic hydrolysis of cellulose or using
80 non-thermal technologies such as ultrasound (Billès, Coma, Peruch, & Grelier, 2017; Jérôme, Chatel,
81 & De Oliveira Vigier, 2016). Alternatively, enzyme-based synthetic cascades involving sucrose,
82 cellobiose and cellodextrin phosphorylases have also been proposed. Cell-free or whole cell
83 processes have proven efficient, achieving high production yields with good oligosaccharide size
84 control (Schwaiger, Voit, Wiltschi, & Nidetzky, 2022; Zhong et al., 2020). Surprisingly, the oxidation of
85 COS with oxidases has been little studied to access a wider panel of ionic structures. Using a
86 glucooligosaccharide oxidase, the C1 carbonyl group at the reducing end of COS was efficiently
87 converted to a carboxyl group (Vuong et al., 2013). Recently, COS-based glycoconjugates were also
88 obtained by oxidizing COS with a C4-specific lytic polysaccharide monooxygenase to generate a
89 ketone group at the C-4 position of the non-reducing end of the oxidation product, which then
90 reacted spontaneously with an amino group of 2-(aminoxy)-1-ethanaminium dichloride (Westereng
91 et al., 2020). COS oxidation with laccase (EC 1.10.3.2, benzenediol:oxygen reductases) or using
92 TEMPO chemistry has never been described.

93 Laccases are polyphenol-oxidases belonging to the blue multicopper oxidase family (Morozova,
94 Shumakovich, Shleev, & Yaropolov, 2007; Solomon, Sundaram, & Machonkin, 1996; Yoshida, 1883).

95 They catalyze the reduction of dioxygen to water concomitantly with the oxidation of a substrate,
96 typically a phenolic compound of lignin (Mate & Alcalde, 2017). They are also finding a growing
97 number of applications in organic chemistry, soil and water bioremediation, and biofuel production
98 (Arregui et al., 2019; Mate & Alcalde, 2017; Theerachat, Guieysse, Morel, Remaud-Simeon, &
99 Chulalaksananukul, 2019). Fungal laccases are particularly interesting because of their high redox
100 potential (Baldrian, 2006). When the size of the substrate or its redox potential are too high,
101 mediators can be used in the so called "Laccase Mediator System" (LMS) (d'Acunzo, Galli, Gentili, &
102 Sergi, 2006). Marzorati et al. (2005) first reported the oxidation of methyl glucoside, methyl
103 galactoside, methyl mannoside, trehalose and amygdalin with *Trametes pubescens* laccase and
104 TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl) as mediator and showed the selective oxidation of the
105 C-6 hydroxyl group to the corresponding carboxyl group (Marzorati, Danieli, Haltrich, & Riva, 2005).
106 Similar results were obtained with alkyl glycosides (octyl β -glucoside, dodecyl maltoside and
107 hexadecyl maltoside) using the laccase from *Trametes versicolor* (Ngo, Grey, & Adlercreutz, 2020a,
108 b). In addition, cleavage of the α -1,4-osidic bond of maltoside could be detected, as well as traces of
109 C=C double bond or keto groups at C-3, C-2 or C-4 position of the sugar rings (Ngo et al., 2020a). LMS
110 also served to synthesize glycoconjugates by oxidizing the primary hydroxyl groups of
111 oligosaccharides to carbonyl groups that reacted spontaneously with aminated molecules to form a
112 Schiff base (-CH=N-). This approach was applied to the grafting of tyrosine onto β -cyclodextrin (Yu,
113 Wang, Yuan, Fan, & Wang, 2016). LMS using TEMPO also enabled oxidation of cellulosic pulp and
114 cellulose nanofibers by introducing carbonyl and/or carboxyl groups but was never applied to
115 cellooligosaccharides (Jaušovec, Vogrinčič, & Kokol, 2015; Jiang et al., 2017; Jiang et al., 2021; Patel,
116 Ludwig, Haltrich, Rosenau, & Potthast, 2011; Quintana, Roncero, Vidal, & Valls, 2017).

117 In the present study, we tested for the first time the efficiency of the LMS system to oxidize D-
118 cellobiose (Glc2), methyl β -D-cellobiose (me- β -Glc2), a mixture of COS and D-cellopentaose (Glc5) to
119 generate anionic COS as well as cello-conjugates. The oxidation products were characterized in detail
120 using $^1\text{H}/^{13}\text{C}$ NMR, LC-HRMS and LC-HRMS/MS. We demonstrated the presence of carbonyl groups in
121 the oxidation products of COS and Glc5. Using reductive amination, we successfully grafted Glc5 with
122 the amino chromophores *p*-toluidine (*p*T) and rhodamine 123 (RHO123), showing that this approach
123 effectively expands the COS chemical space, providing access to a wide range of interesting cello-
124 conjugates for diverse applications.

125

126

127

128 **2. Materials and methods**

129 *2.1. Substrates and enzymes*

130 Methyl β -D-cellobiose (me- β -Glc2), and D-cellopentaose (Glc5) were purchased from Carbosynth
131 (United Kingdom). D-cellobiose (Glc2) was from Roth Sochiel (France). 2,2-azino-bis(3-
132 ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,2,6,6-Tetramethyl-1-piperidinyloxy (TEMPO), the
133 laccase from *Trametes versicolor* (TvL) (E.C. 1.10.3.2), the cellulase cocktail (from *Trichoderma reesei*)
134 ≥ 700 units/g, Avicel® PH-101, *p*-toluidine (*p*T), 2-picoline borane (pic-BH₃) and rhodamine 123
135 (RHO123) were purchased from Sigma-Aldrich (St Quentin Fallavier, France). Pellicon® XL50 with
136 Ultracel® 30 kDa membrane was from Merck (Darmstadt, Germany).

137

138 *2.2. Diafiltration of the laccase from Trametes versicolor*

139 Discontinuous diafiltration was performed using a lab-scale TFF system (Merck KGaA, Darmstadt,
140 Germany) with a Pellicon XL filter PXC030C50 (30 kDa) regenerated cellulose membrane (filter area
141 50 m²) at a fixed transmembrane pressure of 2.1 bar at 4°C. The feeding chamber was filled with 50
142 mL of a solution of TvL at 30 g/L. The enzyme solution was diafiltrated twice with 500 mL of water or
143 20 mM acetate buffer pH 6.0, and then concentrated to 15 mL final volume. Before diafiltration, the
144 membrane was previously rinsed with 20 mM acetate buffer pH 6.0 for 15 min. After each trial, the
145 membrane was flushed with distilled water and stored in 50 mM NaOH. Diafiltrate and retentate
146 were analyzed by HPAEC-PAD analysis.

147

148 *2.3. Laccase activity*

149 Laccase activity was determined using a colorimetric assay and 2,2-azino-bis(3-ethylbenzothiazoline-
150 6-sulphonic acid) (ABTS) as a substrate. A volume of 10 μ L of laccase solution at 0.1 mg/mL, 50 μ L of
151 1 mM ABTS was mixed with 440 μ L of acetate buffer (20 mM, pH 3.5). ABTS oxidation was monitored
152 by measurement of the absorbance at 420 nm ($\epsilon_{420} = 3.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) and 30 °C using a UV-vis
153 spectrophotometer Cary 100 Bio (Agilent Technologies, Santa Clara, CA, United States). One unit (U)
154 of laccase activity is defined as the amount of laccase that oxidizes 1 μ mol of ABTS per minute.

155

156

157

158 *2.4. Preparation of a mix of COS*

159 To produce COS, we used a protocol based on Avicel®PH-101 acid hydrolysis, which was adapted
160 from Zhang & Lynd (2003). Briefly, Avicel® PH-101 (2.5 g) was suspended in cold HCl 37 % (20 mL)
161 and in cold H₂SO₄ 98 % (3.7 mol/L) (5 mL) in an Erlenmeyer flask. The reaction was carried out at
162 room temperature during 2h30 and stirred with a magnetic stir bar. The hydrolyzate was precipitated
163 with acetone at -20 °C (225 mL) and the solution was kept at -20 °C for 2h. The precipitate was
164 recovered by centrifugation at 3500 rpm for 8 min at 4 °C and washed with acetone at -20°C (50 mL)
165 and centrifuged. The pellet was dissolved in water (300 mL) and centrifuged at 5000 rpm for 8 min at
166 4°C to obtain a supernatant containing soluble COS. The COS solution was neutralized to pH 7 with
167 Ba(OH)₂, and the precipitate of BaSO₄ formed was removed by centrifugation (10 000 rpm, 8 min at 4
168 °C). The residual acetone was evaporated and the soluble COS was freeze-dried to form a white
169 powder. To remove the salts, the COS mixture (1 g) was dialyzed overnight against distilled water
170 using 100-500 Da cut-off dialysis tubing from Biotech CE.

171

172 *2.5. Oxidation of COS*

173 Oxidation of D-cellobiose (Glc2), methyl β-D-cellobiose (me-β-Glc2) and COS was performed using 54
174 mM Glc2 or me-β-Glc2 or 17.2 g/L of a mix of cellooligosaccharides, 6 mM TEMPO and 5.4 U/mL of
175 TvL (purified or not) in 20 mM acetate buffer at different pH values (3.0-6.0) and at 30°C (or 25°C).
176 The reaction mixture (5mL) was stirred at 500 rpm (or not stirred) with a magnetic bar in open flask.
177 Samples of 250 μL were collected during 24h for analyses. Either TvL (54 U), TEMPO (9.34 mg) or
178 both were also added to attempt reaction restart after 8h of the reaction, which was initially carried
179 out in 5mL volume with 54 mM Glc2 or me-β-Glc2 and at pH 6 and 30°C. Oxidation of Glc5 was
180 carried out on 5 mL volume with 1.2 mM Glc5, 0.6 mM of TEMPO and 0.27 U/mL of the purified TvL
181 in 20 mM sodium acetate buffer (pH 6.0) at 30 °C under 500 rpm stirring. Samples (250 μL) were
182 taken periodically for 8h. For all oxidation reactions, TvL was inactivated by heating at 95 °C for 5
183 min. Three control experiments were conducted in parallel: control 1 with TvL and TEMPO, control 2
184 with COS and TEMPO, control 3 with COS and TvL.

185

186 *2.6. Purification of oxidized products 2a and 2b*

187 Products 2a and 2b were isolated by chromatography using an Agilent 1260 Infinity chromatographic
188 system equipped with a Refractive Index detector (RI) and a Thermo Scientific™ Dionex UltiMate™
189 3000 automate fraction collector (Thermo Fisher Scientific, San Jose, CA, USA). Analysis was

190 performed with a Shodex™ Asahipak NH2P-50 4E column (5 μm, 19 × 250 mm) maintained at 40 °C at
191 a flow rate of 1 mL/min. Samples (50 μL) were injected and eluted with a solvent composed of
192 water/ammonium acetate (0.3 M), 80:20 (v/v). The fractions containing 2a and 2b were collected
193 and their purity was checked by HPAEC-PAD analysis before lyophilization.

194 *2.7. NMR characterization of products 2a and 2b*

195 For ¹H and ¹³C NMR analyses, 1.5 mg of pure products 2a and 2b were dissolved in 150 μL D₂O
196 containing sodium 2,2,3,3-tetradeuterio-3-trimethylsilylpropanoate (TSP-*d*4) and acetone as internal
197 standards. The chemical shifts were calibrated with respect to TSP-*d*4 (δ¹H 0.00 ppm) and acetone
198 (δ¹³C 30.89 ppm). ¹H and ¹³C NMR spectra were recorded on a Bruker Avance 500-MHz spectrometer
199 operating at 500.13 MHz for ¹H NMR and 125.75 MHz for ¹³C using a 5-mm z-gradient TBI probe. 1D
200 and 2D NMR (COSY, HSQC, HMBC) were recorded in the same conditions in 3 mm tube at 298 K. Data
201 were processed using TopSpin 3.6.2 software.

202

203 *2.8. Grafting of aminated compounds onto oxidized cellopentaose*

204 Glc5 (27 mM) was oxidized using 0.6 mM of TEMPO and 5.4 U/mL of TvL in acetate buffer (20 mM,
205 pH 6.0) at 30°C for 8h. The reaction medium (500 μL) was then incubated with 10.15 mM of *p*-
206 toluidine (*p*T) or rhodamine 123 (RHO123), 2.43 mM of 2-picoline borane (pic-BH₃) and 10 % of acetic
207 acid for 3 h at 40 °C under stirring at 500 rpm. Then, the reaction mixture was centrifugated for 5 min
208 at 10 000 rpm before being diluted for LC-HRMS analysis. After grafting with *p*T or RHO123, the pH of
209 the reaction mixture (500 μL) was adjusted to 4.8 with 2M NaOH before addition of 80 μL of a
210 cellulase cocktail from *T. reesei* (≥700 units/g). Enzymatic hydrolysis was carried out for 24 h at 40 °C
211 and 500 rpm agitation, the reaction was stopped by heating the samples for 10 min at 95 °C. The
212 samples were centrifugated 5 min at 5 000 rpm and filtered on 0.45 μm membrane before HPLC
213 analysis.

214

215 *2.9. Analyses of oxidized oligosaccharides*

216 **HPAEC-PAD analysis.** High performance anion exchange chromatography with pulsed amperometric
217 detection (HPAEC-PAD) analysis was carried out using a Dionex™ ICS-6000 DC (Thermo Fischer
218 Scientific, San Jose, CA, USA) equipped with a Dionex™ CarboPac™ PA100 analytical column (2 x 250
219 mm) at a flow rate of 0.25 mL/min. The mobile phase was A: NaOH 150 mM and B: NaOH 150 mM
220 with 500 mM sodium acetate. The injection volume of the reaction mixture obtained after LMS

221 oxidation was set at 10 μ L. Solvent A was applied for 5 min and the products were eluted using a
222 gradient from 0 to 40% of B over 35 minutes. Before analysis, reaction mixtures obtained from Glc2
223 (54 mM) were diluted 667 times in water and those obtained from me- β -Glc2 (27 mM) or Glc5 (1.2
224 mM) oxidation 40 times. A linear calibration curve was generated for Glc2, me- β -Glc2 and Glc5 using
225 concentrations of commercially available standards in the range of 5 to 30 mg/L. Data acquisition and
226 processing were performed using Chromeleon™ 7.2 data software.

227 **HPLC-CAD analysis.** High performance liquid chromatography with charged aerosol detection (CAD)
228 analysis of the reaction mixture obtained with Glc2, me- β -Glc2 or Glc5 were performed using a
229 Thermo Scientific™ UltiMate™ 3000 system, (Thermo Fisher Scientific, San Jose, CA, USA) with a
230 Shodex™ Asahipak NH2P-50 4E (5 μ m, 4.6 mm x 250 mm) column at 40 °C and at a flow rate of 1
231 mL/min. Samples were not diluted. The mobile phase was composed of solvent A: acetonitrile, B:
232 water and C: ammonium acetate (0.3 M). A gradient starting with solvent A-B 75/25 and decreasing
233 to A-B 40/60 over 30 min was first applied to elute the neutral sugars. Then, solvent B 100% was
234 applied in isocratic mode for 10 min. The oxidized compounds were finally eluted with a step-
235 gradient comprising four steps of 10 min each: step 1 B-C 90/10; step 2 B-C 80/20; step 3 B-C 70/30
236 and step 4 B-C = 0/100. Finally, the column was re-equilibrated during 10 min with A/B =75:25.

237 **HPLC-MS analysis of Glc2 and me- β -Glc2** was performed using an Ultimate 3000 series
238 chromatograph equipped with a Dionex 340 UV/VIS detector coupled with a simple quadrupole mass
239 spectrometer (MSQ Plus, Thermo Fisher Scientific) with a Shodex™ Asahipak NH2P-50 4E column (5
240 μ m, 4.6 x 250 mm) maintained at 40°C. Samples (50 μ L) were analyzed by isocratic elution with
241 water-ammonium acetate (0.3 M), 80:20 at a flow rate of 1 mL/min for 20 min. Mass detection was
242 carried out in a positive and negative heated electrospray ionization (ESI) mode. Mass spectrometer
243 settings were as follows: the spray voltage was 3.5 kV, the voltage cone at 60 V, the temperature of
244 ESI ion source was 350 °C and the gas carrier was nitrogen. The mass spectrometer scanned was
245 from m/z 100 to 1 900. Data acquisition and processing were performed using Chromeleon™ 7.2 data
246 software.

247 **HPLC-HRMS of Glc2 and me- β -Glc2** analyses were carried out on a Vanquish™ system coupled to a
248 Thermo Scientific Q Exactive™ Plus hybrid quadrupole-Orbitrap™ mass spectrometer (Thermo Fisher
249 Scientific) with a column Shodex™ Asahipak NH2P-50 4E (5 μ m, 4.6mm x 250 mm) equipped with a
250 Shodex™ Asahipak NH2P-50 4A guard column (4.6 x 10 mm) at a flow rate of 0.5 mL/min. The column
251 and autosampler temperature were set at 40 °C and 4 °C, respectively. Samples were analyzed using
252 the following gradient of A-B 20 mM ammonium acetate-acetonitrile (25/75 at 0 min, 60/40 at 10
253 min, 60/40 at 15 min and 100 % A at 25 min). Injection volume was set at 10 μ L. Conditions for ESI in

254 negative mode were as follows: spray voltage was at 2.75 kV, and capillary and desolvation
255 temperatures were of 400 °C. The maximum injection time was 100 ms. Nitrogen was used as the
256 sheath gas (pressure, 75 units) and auxiliary gas (pressure, 20 units). The automatic gain control
257 (AGC) was set at 10^6 for full-scan mode, with a mass resolution of 70 000 (at 400 m/z). For the full
258 scan MS analysis, the spectra were recorded in the range of m/z 80-1 000. Finally, data acquisition
259 was performed using Thermo Scientific Xcalibur software 4.1.

260 **IC-HRMS analysis of oxidized Glc5.** The mass of Glc5 oxidation products were determined using a
261 liquid anion exchange chromatography on Dionex™ ICS-5000+ Reagent-Free™ HPIC™ system
262 (Thermo Fisher Scientific™, Sunnyvale, CA, USA), coupled to a Thermo Scientific™ LTQ Orbitrap
263 Velos™ mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a heated
264 electrospray ionization probe and equipped with an eluent generator system (ICS-5000+EG, Dionex)
265 for automatic base generation (KOH). Analytes were separated within 50 min, using a linear KOH
266 gradient elution applied to an IonPac AS11-HC column (250 x 2 mm, Dionex) equipped with an AG11-
267 HC guard column (50 x 2 mm, Dionex) at a flow rate of 0.38 ml/min. The gradient program was
268 following: equilibration with 7 mM KOH during 1 min; then KOH ramp from 7 to 15 mM, from 1 to 9.5
269 min; constant concentration 10.5 min; ramp to 45 mM in 10 min; ramp to 70 mM in 3 min; ramp to
270 100 mM in 0.1 min; constant concentration 8.9 min; drop to 7 mM in 0.5 min; and equilibration at 7
271 mM KOH for 7.5 min. The column and autosampler temperatures were thermostated at 25 °C and 4
272 °C, respectively. The injected sample volume was 15 µl. Conditions for electrospray ionization (ESI) in
273 negative mode were as follows: spray voltage was at 2.7 kV, capillary and desolvation temperatures
274 were fixed at 350 °C and the maximum injection time was 50 ms. Nitrogen was used as the sheath
275 gas (pressure, 50 units) and auxiliary gas (pressure, 5 units). AGC was set at 10^6 for full-scan mode,
276 with a mass resolution of 60 000 (at 400 m/z). For the full scan MS analysis, the spectra were
277 recorded in the range of m/z 80.0-1 000.0. Finally, data acquisition was performed using Thermo
278 Scientific Xcalibur software 2.2 SP1.

279 **LC-HRMS/UV analysis of cellopentaose grafted with *p*-toluidine or rhodamine 123.** Analyses were
280 carried out on a Vanquish™ system coupled to a Thermo Scientific Q Exactive™ Plus hybrid
281 quadrupole-Orbitrap™ mass spectrometer (Thermo Fisher Scientific). Samples were separated within
282 95 min using an isocratic elution with a mixture containing 10 % acetonitrile-40 % water-50 % 20 mM
283 ammonium acetate applied to a Shodex™ Asahipak NH2P-50 4E (5 µm, 4.6 mm x 250 mm) column
284 equipped with a Shodex™ Asahipak NH2P-50 4A guard column (4.6 x 10 mm) at a flow rate of 0.7
285 mL/min. The column and autosampler temperatures were set at 40 °C and 4 °C, respectively.
286 Injection volume was of 10 µL. UV/Vis detection was performed at 286 nm for the samples grafted
287 with *p*-toluidine and 500 nm for those grafted with rhodamine 123. ESI in negative mode was

288 performed with spray voltage at 2.75 kV, and capillary and desolvation temperatures of 400 °C.
289 Maximum injection time was 100 ms. Nitrogen was used as the sheath gas (pressure, 75 units) and
290 auxiliary gas (pressure, 20 units). AGC was set at 10^6 for full-scan mode, with a mass resolution of
291 70 000 (at 400 m/z). For the full scan MS analysis, the spectra were recorded in the range of m/z 80-
292 1 000. Finally, data acquisition was performed using Thermo Scientific Xcalibur software V.

293 **LC-MS/MS analysis.** MS/MS analyses of Glc5 grafted with *p*-toluidine were performed on MetaToul-
294 Axiom platform facility at INRAE Toulouse with a Thermo Scientific™ LTQ Orbitrap XL™ mass
295 spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) coupled with an UV detector. Reaction
296 media were diluted 250 times in water-acetonitrile (95.5:4.5, v/v). Separation was performed with a
297 Shodex™ Asahipak NH2P-50 4E (5 μm, 4.6 x 250 mm) column equipped with a Shodex™ Asahipak
298 NH2P-50 4A guard column (4.6 x 10 mm) placed in an oven at 40 °C. Injection was set at 10 μL.
299 Elution was performed isocratically with a water-ammonium acetate 20 mM-acetonitrile mixture
300 (40:50:10, v/v/v) at a flow rate of 700 μL/min. Mass detection was carried out in negative mode over
301 mass ranges of m/z 80 – 1200 in MS mode and m/z 245-950 in MS/MS mode. Spray voltage was set
302 at 2.75 kV. MS/MS analyses in collision-induced dissociation were performed by selected the mass of
303 the precursors of interest as $[M-H]^-$ at m/z 932.32 in the quadrupole prior to their fragmentation in
304 the transfer cell of the instrument (collision energy adjusted at 30 V). Argon was used as the collision
305 gas. UV-detection was set at 286 nm. Data acquisition was carried out using the Xcalibur software.
306 Annotations of spectra and structures were performed according to the nomenclature of Domon and
307 Costello (1988).

308

309 **MALDI-TOF-MS analysis of mix of COS.** Mass spectra were recorded on a Waters Micromass MALDI
310 micro MX mass spectrometer. The measurements were performed with the mass spectrometer in
311 positive reflection mode using an accelerating voltage of 12 kV. Mass spectra were acquired from
312 550 (m/z) to 3000 (m/z). COS were dissolved in water (1 mg/mL) and mixed with the DHB matrix
313 solution (2.5-dihydroxybenzoic acid, 10 mg/mL in H₂O:EtOH, 0.5:0.5; v/v) and NaI solution (sodium
314 iodide 10 mg/mL in H₂O:EtOH, 0.5:0.5, v/v). Samples were prepared by mixing COS solutions, matrix
315 solution, and the cationization agent solution in the ratio 1:3:1 (v/v/v). A total of 1 μL was applied to
316 a stainless steel sample slide and dried at room temperature.

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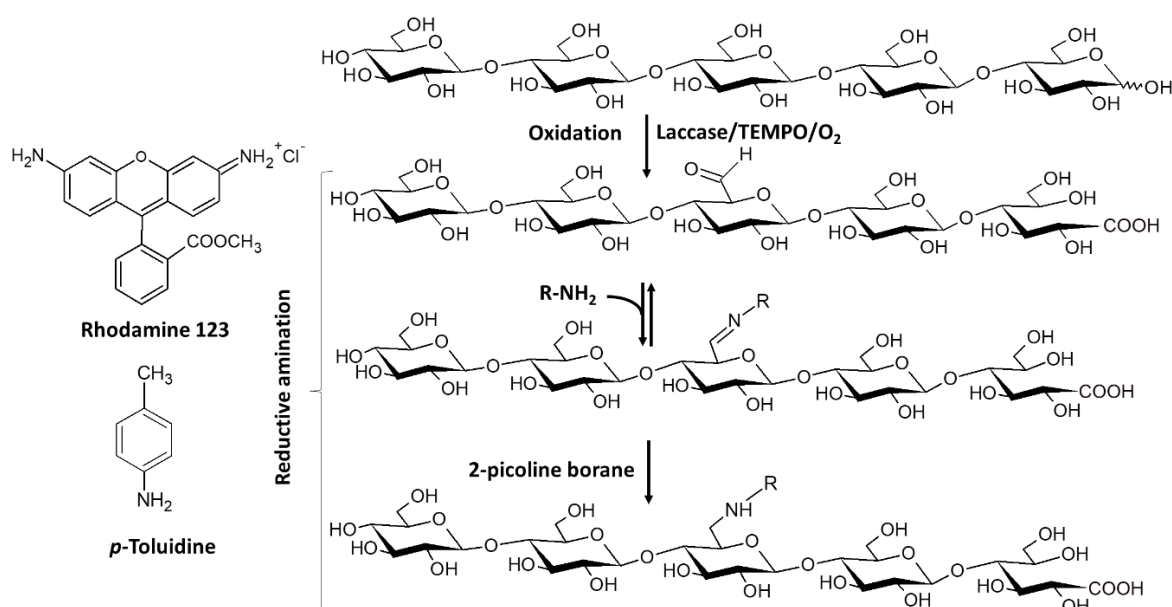
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320 3. Results and discussion

321 To develop an eco-friendly process to graft aminated substances onto COS, we applied the protocol
322 described in (Scheme 1), starting with the oxidation of COS using LMS followed by a direct reaction
323 with an amino-chromophore to form a Schiff base with an imine bond. The imine bond was
324 subsequently reduced using 2-picoline borane to generate a more stable C-N bond. The *Trametes*
325 *versicolor* laccase and TEMPO couple, already used for cellulosic pulp oxidation (Patel et al., 2011),
326 was first tested to oxidize D-cellobiose (Glc2), methyl β-D-cellobiose (me-β-Glc2), a mixture of COS
327 and D-cellopentaose (Glc5). With LMS, the laccase-oxidized redox mediator, in our case TEMPO⁺, is
328 responsible for sugar oxidation.

329



330

331 **Scheme 1.** Two-step chemo-enzymatic procedure for the preparation of celooligosaccharide-
332 conjugates using the laccase/TEMPO/O₂ system for carbohydrate oxidation followed by the reductive
333 amination with amino-chromophores.

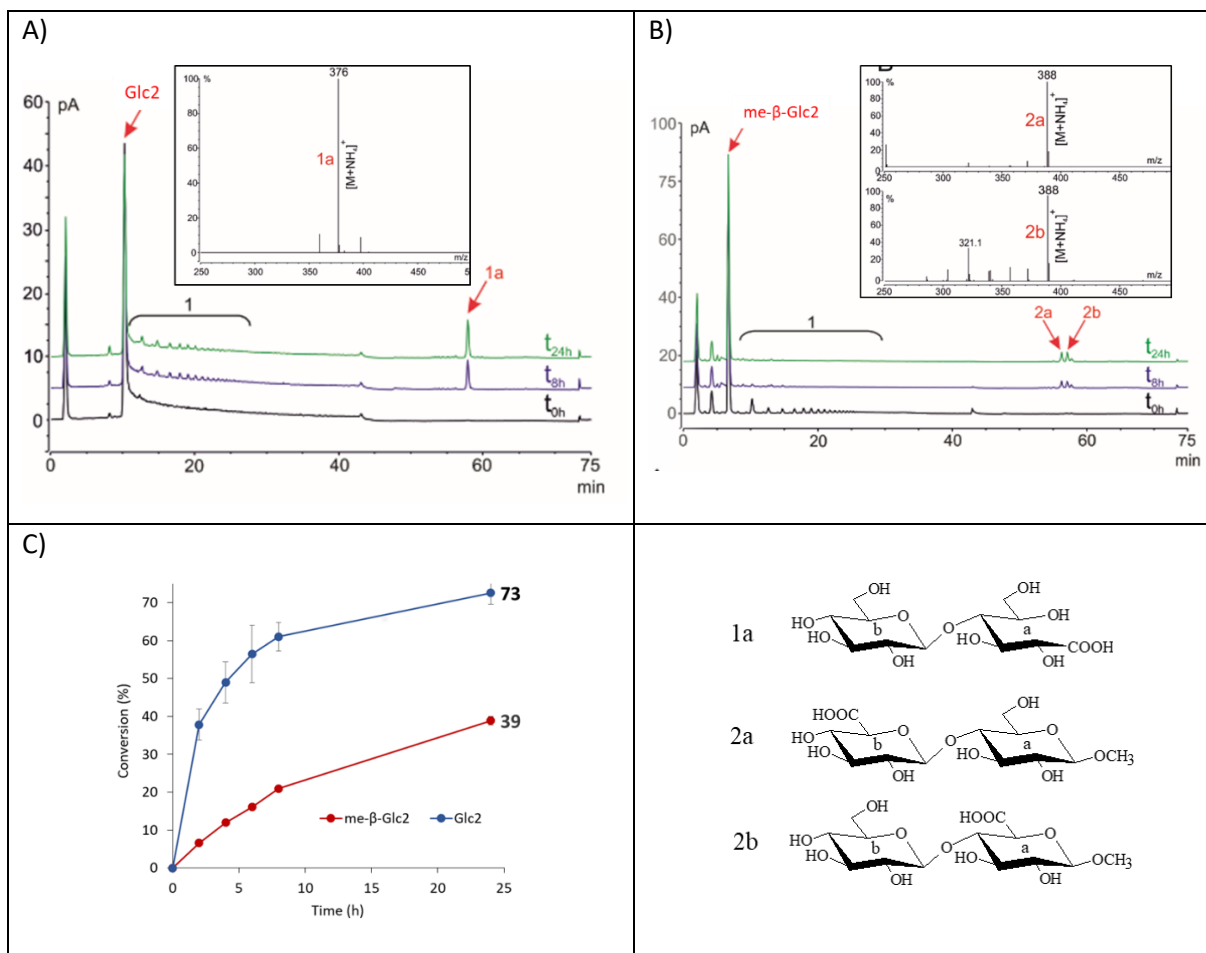
334

335 3.1. Oxidation of cellobiose and methyl cellobiose using the laccase/TEMPO/O₂ system

336 We first performed the oxidation of Glc2 and me-β-Glc2. The product profiles obtained after 8 and 24
337 h oxidation enabled the identification of product 1a (at t_r=57.9 min with m/z 376 [M+NH₄]⁺)
338 corresponding to the aldonic acid form (C₁OOH) of Glc2 (Fig. 1A). With me-β-Glc2, two oxidation
339 products 2a and 2b were obtained (at t_r=56.3 and 57.0 min) both with m/z 388 [M+NH₄]⁺ (Fig. 1B).

340

341



342 **Fig. 1.** Oxidation of Glc2 or me-β-Glc2 with LMS system. (A) HPLC-CAD chromatograms of reaction
 343 mixture with Glc2; 1= maltooligosaccharides (t_r between 5-26 min present in commercial preparation
 344 of TvL); 1a monoacid formed during the reaction $t_r=57.9$ min and ESI-mass spectrum, m/z 376
 345 $[M+NH_4]^+$. (B) HPLC-CAD chromatograms of reaction mixture with me-β-Glc2; 1=
 346 maltooligosaccharides ($t_r= 5-26$ min); 2a and 2b monoacid formed during the reaction, 2a $t_r= 56.3$
 347 min, 2b $t_r= 57.1$ min and ESI-mass spectra of 2a and 2b, m/z 388 $[M+NH_4]^+$. (C) Glc2 and me-β-Glc2
 348 conversion versus reaction time, conversions were determined by HPAEC-PAD analysis. Reaction
 349 conditions: 54 mM Glc2 or me-β-Glc2 TEMPO 6 mM, TvL 5.4 U/mL, 10 mL sodium acetate buffer (20
 350 mM; pH 4.5), 30°C, 24 h, 500 rpm. The experiments have been carried out in triplicates and some
 351 standard deviation are too small to be visualized. In the control experiments without laccase or
 352 without TEMPO, no oxidation products were detected (data not shown).

353

354 The mass increase ($m/z +14$) indicates the presence of one carboxyl group on C-6 of the a or b
 355 glucosyl ring of me-β-Glc2. The structures of 2a and 2b, isolated with a purity higher than 98% were
 356 determined using 1D-, 2D-NMR analyses (Fig. S1, S2, Table S1). In the ^{13}C -NMR spectrum of 2a and
 357 2b, the signals of the primary C-6 OH of me-β-Glc2 at 61.2 ppm and 60.6 ppm disappeared to the
 358 profit of the characteristic signals of C-6 carboxylic acid group at 176.1 ppm and 175.7 ppm for 2a
 359 and 2b, respectively (Fig. S2B). 2D-NMR confirms that 2a and 2b are oxidized on the C6 of glucosyl
 360 ring b and a of me-β-Glc2, respectively. Conversion of Glc2 was fivefold faster than that of me-β-Glc2
 361 and a maximum conversion of 73% and 39% was reached after 24 h reaction for Glc2 and me-β-Glc2,

362 respectively (Fig. 1C). This can be explained by the higher reactivity of the C1 aldehyde of Glc2
363 compared to the C6-hydroxyl of me- β -Glc2. HPLC-CAD analysis also revealed traces of
364 oligosaccharides in the control reaction (with only TvL and TEMPO, Fig. 1A, 1B), corresponding to
365 maltooligosaccharides (MOS) contained in the laccase preparation (Fig. S3 A). To avoid any
366 interferences between COS and MOS oxidation, we prepared a MOS-free preparation of the
367 commercial laccase by diafiltration and used it in the following experiments (Fig. S3B).

368 To date, oligosaccharide or polysaccharide oxidation with Laccase/TEMPO system has mainly been
369 performed under acidic conditions at pH 4.5 to 5.0 (Jaušovec et al., 2015; Marzorati et al., 2005;
370 Quintana et al., 2017). As chemical oxidation with TEMPO is more efficient under basic conditions,
371 we increased the reaction pH to 6 after verifying that TEMPO (alone) was sufficiently oxidized to
372 oxoammonium (TEMPO⁺) at pH 6 (figure S4). Remarkably, me- β -Glc2 conversion was four times
373 faster at pH 6 than at any other pHs increasing from 40 % to 62 % (Fig. 2A). Regardless of reaction pH,
374 a plateau is reached - after 8 h at pH 6 and 24 h at pH 3.5 and 4.5 - which may be due to either
375 enzyme inactivation or TEMPO (TEMPO⁺) oxidation as described by Jiang et al. (2021). Stirring at 500
376 rpm enabled us to further increase me- β -Glc2 conversion value from 58% (without stirring) to 69%,
377 probably owing to better oxygen supply (Fig. 2B). The me- β -Glc2/TEMPO molar ratio is also an
378 important parameter to consider. Oxidation is faster at me- β -Glc2/TEMPO molar ratio 9 (54 mM/6
379 mM) in comparison to a ratio of 4.5 (27 mM/6 mM) but the best conversion (77 %) is obtained with a
380 ratio of 4.5. (Fig. 2C). Over 8h, conversion varied very little. As shown in Fig. 2D, the addition of
381 laccase (54 U), TEMPO (9.34 mg) or both resulted in an inflexion in the reaction rate decrease, and
382 conversion resumed upon complete consumption of me- β -Glc2, 16 h after addition, with a
383 concomitant decrease in pH (Fig. S5). This indicates that the enzyme initially used may have been
384 partially inactivated, limiting TEMPO⁺ formation and carbohydrate oxidation. The addition of TEMPO⁺
385 also restarted the reaction, showing that at least some of the initially introduced enzyme was still
386 active in oxidizing TEMPO[•] to TEMPO⁺. Altogether, these results suggest that TEMPO⁺ was the
387 limiting reagent, due to ion instability or to the slow regeneration of TEMPO[•] from TEMPOH under
388 acidic pH as reported by Arends et al. (2006).

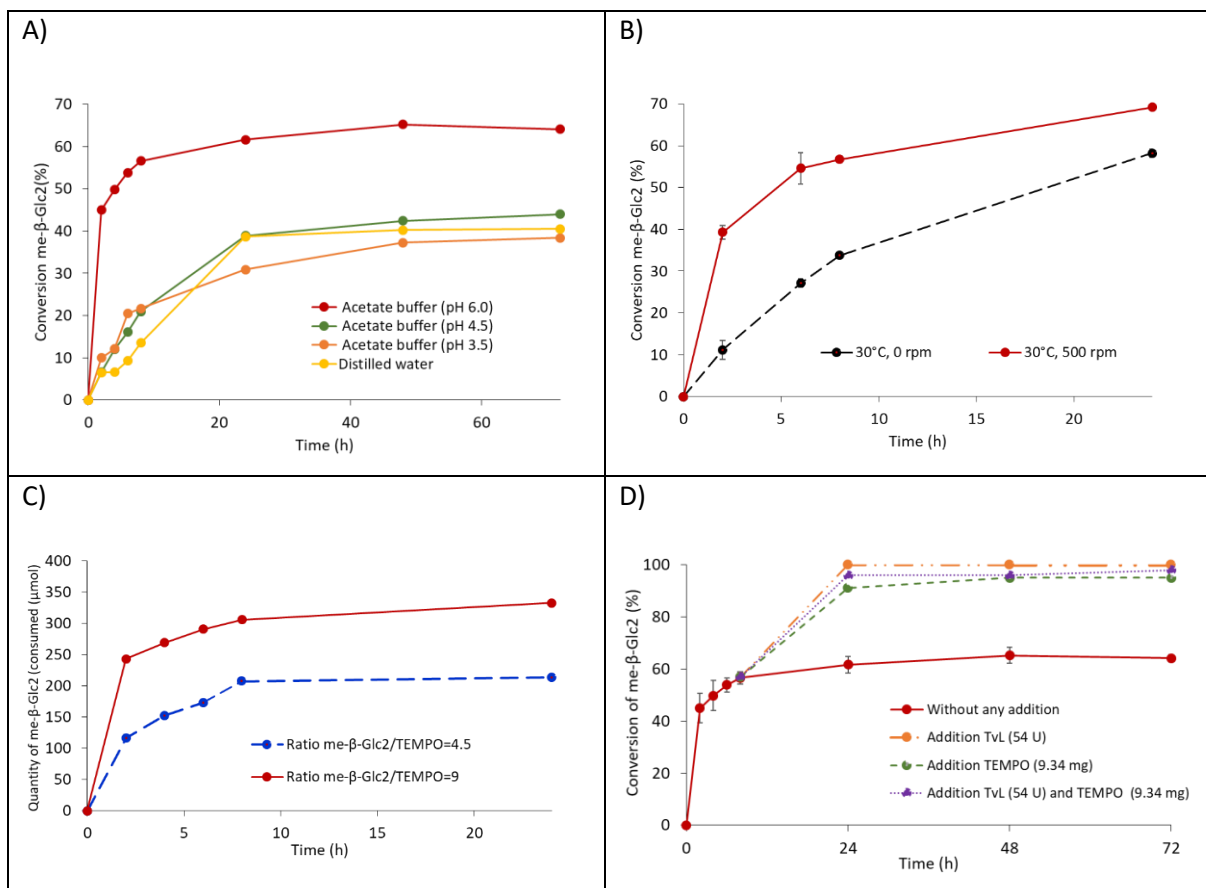
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394 Fig. 2. Effect of pH (A), agitation (B) and sugar/TEMPO molar ratio (C) on conversion of me-β-Glc2.
 395 The me-β-Glc2 conversion was determined by HPAEC-PAD analysis. Reaction conditions: (A)
 396 oxidation of me-β-Glc2 was performed in 20 mM sodium acetate buffer at pH 3.5, 4.5 and 6.0 or in
 397 distilled water (pH 6.5) with 54 mM me-β-Glc2 (540 μmol), 6 mM TEMPO (60 μmol), 5.4 U.mL⁻¹ TvL
 398 under 500 rpm or (B) without agitation, sodium acetate buffer at pH 6.0) at 30°C in open flask ;(C, D)
 399 me-β-Glc2 54 mM or 27 mM (540 or 270 μmol), TEMPO 6 mM, TvL 5.4 U/mL (54 U), volume 10 mL
 400 (sodium acetate buffer 20 mM, pH 6), 500 rpm, 30 °C in open flask. (D) conversion progress after
 401 addition of TvL, TEMPO, or both after 8 h reaction. The experiments have been carried out in
 402 triplicates and some standard deviation are too small to be visualized.

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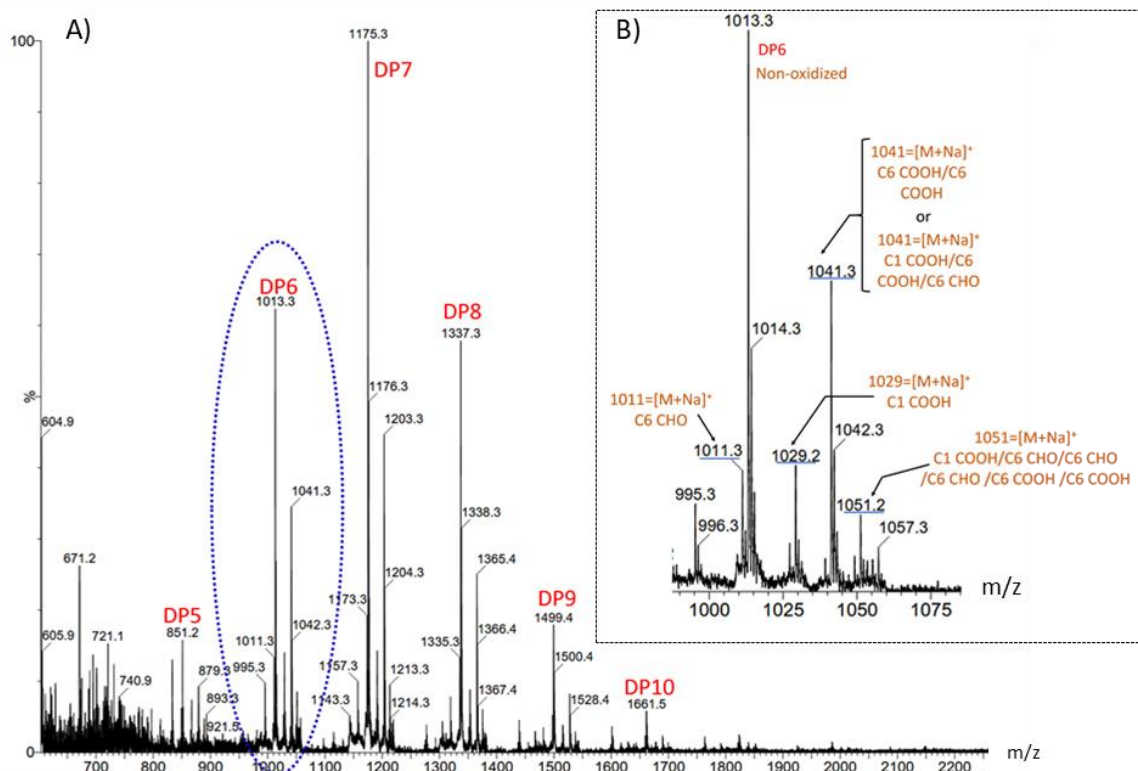
404 3.2. Oxidation of COS

405 3.2.1. Oxidation of a mixture of COS

406 We first produced a mixture of COS with degrees of polymerization ranging from 1 to 13 by acid
 407 hydrolysis of Avicel®PH-101 (Fig. S6) and then applied to COS the protocol used for me-β-Glc2
 408 oxidation. HPLC profiles (Fig. S7A, S7B) and MALDI-TOF analyses of the reaction products revealed
 409 the presence of oxidized compounds (Fig. 3A). MALDI-TOF spectrum of the DP6 at m/z 1 013
 410 ([M+Na]⁺) (Fig. 3B, Table 1) reveals several different ions with m/z-values varying slightly compared
 411 to the unoxidized DP6 (Fig. S6B). These ions could be attributed to different structures and only mass
 412 spectrometry fragmentation could help to discriminate more precisely the structures obtained.
 413 However, the peak at m/z 1 011 indicates that carbonyl functions are formed, which augurs well for

414 subsequent functionalization with compounds bearing an amine group. In addition, given the
 415 specificity of TEMPO⁺ for oxidation of aldehyde and primary hydroxyl groups, we can assume that
 416 oxidation occurred mainly on the C-1 at the reducing end or on the C-6 of the different rings of DP6,
 417 which is interesting for functionalization with an amine group-bearing substituent.

418



419 Fig. 3. MALDI-TOF spectrum (DHB matrix with NaI salt in positive mode) of oxidized COS (DP 1-13).
 420 (A) After oxidation with laccase/TEMPO System. (B) Zoom on the DP6. In brown, are given some
 421 examples of the structural modifications due to oxidation. The reaction was carried out with 17
 422 mg/mL of substrate, 54 U of laccase from Tvl and 0.6 mM of TEMPO in 5 mL acetate buffer (20 mM,
 423 pH 4.5) at 30 °C for 24 h.

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434 Table 1. Mass of the oxidized products formed from DP6 COS oxidation determined by MALDI-TOF
 435 analysis.

m/z detected [M+Na] ⁺	Assignment of possibly introduced oxidized functions*
m/z = 1013	DP6 (non-oxidized)
m/z = 1011	m/z DP6 - 2: presence of one carbonyl group on the C-6, C-3 or C-2 of one of the ring <i>a</i> , <i>b</i> , <i>c</i> , <i>d</i> , <i>e</i> or <i>f</i> of DP6
m/z = 1029	m/z DP6 + 16: presence of C ₁ OOH, in ring <i>a</i> or geminal diols at C-2, C-3, or C-6 of rings <i>a</i> , <i>b</i> , <i>c</i> , <i>d</i> , <i>e</i> or <i>f</i>
m/z = 1041	m/z DP6 + 14 + 14: presence of two carboxylic acid groups in the DP6 or presence of one C ₆ HO, one C ₆ OOH and C ₁ OOH (m/z DP6 - 2 + 14 + 16). Many other combinations could match with a mass increase of 28
m/z = 1051	m/z DP6 (- 2) x3 + (14) x2 + 16: possible presence of three carbonyl groups, two carboxyl groups (C ₆ OOH) and carboxyl group on C-1 in the DP6. Many other profiles of oxidation could match with this mass increase

* The m/z values were compared to m/z 1013 ([M+Na]⁺) of DP6 (M=990 Da)

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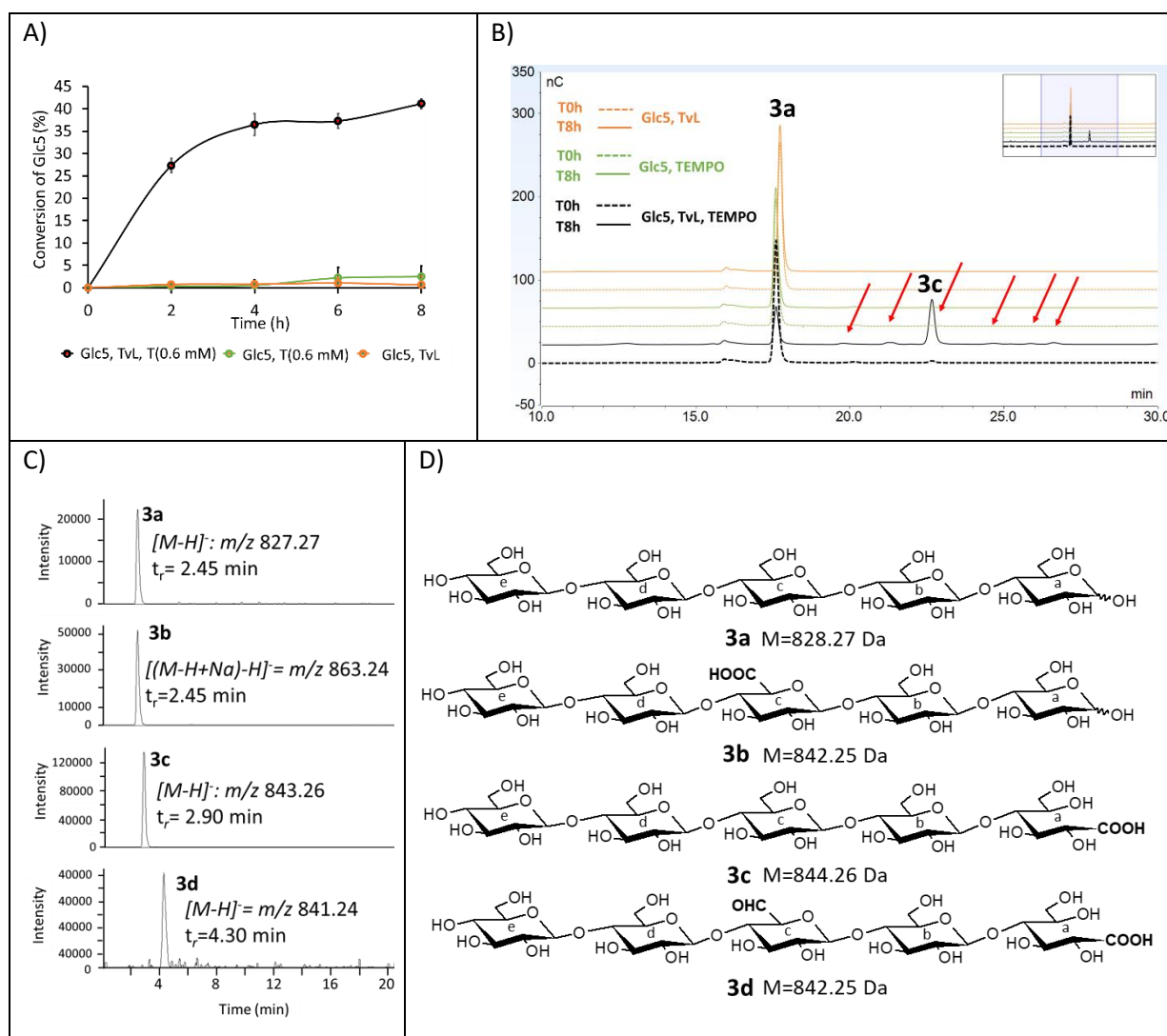
437 3.2.2 Oxidation of cellopentaose

438 In order to facilitate structural analysis of the oxidation products and their derivatives resulting from
 439 subsequent functionalization, we chose to oxidize pure Glc5 and monitored the reaction by HPAEC-
 440 PAD analysis (Fig. 4A-D). We obtained 41 % conversion and reached a plateau after 4 h of reaction,
 441 indicating laccase inactivation and/or a problem of TEMPO* regeneration at acidic pH as previously
 442 seen for me-β-Glc2 oxidation (Fig. 4A). HPAEC-PAD chromatogram revealed the presence of 5
 443 products that can be attributed to product 3c (t_r= 22.92 min) and of at least four minor products of
 444 oxidation (Fig. 4B). Their structure was further investigated by ion exchange chromatography-high
 445 resolution (Orbitrap) mass spectrometry (IC-HRMS). Based on the extracted ion chromatogram (Fig.
 446 4C), the product 3c m/z 843.26 ([M-H]⁻) with a m/z +16 mass increase relative to that of Glc5 m/z
 447 827.27 ([M-H]⁻) could be a C-1-oxidized Glc5 (aldonic acid form) or a Glc5 with a geminal diol
 448 (hydrated aldehyde or ketone) at C-2, C-3 or C-6 position of its glucosyl ring. The products 3d m/z
 449 841.24 ([M-H]⁻) and 3b m/z 863.24 ([M-H+Na]-H⁻), both with a m/z +14 mass increase are consistent
 450 with the presence of a carboxyl group at the C-6 position in one of the Glc5 glucosyl rings, with five
 451 positional isomers possible. It can also reflect, for example, the occurrence of an aldonic acid at C-1
 452 plus a carbonyl group at C-2, C-3 or C-6 position in one of the Glc5 rings. The presence of a geminal
 453 diol (m/z +16 mass increase) and a carbonyl (m/z -2 mass decrease) in the Glc5 could also explain the
 454 m/z +14 mass increase and cannot be excluded. No species with only one carbonyl group (m/z -2

455 mass decrease) were detected. In summary, several structures of oxidized products account for the
 456 variations of mass observed. It is not possible to distinguish these isomers without additional
 457 analyses. However, in products 3b or 3d, we clearly demonstrate that a carbonyl group is present in
 458 addition to a carboxyl or geminal diol group, a new result never before reported for oligosaccharide
 459 oxidation opening up good prospects for future reductive amination. Oxidation of insoluble cellulose
 460 nanofibers, cellulosic fibers or fabrics by LMS with TEMPO also resulted in the formation of carboxyl
 461 and carbonyl groups due to C6-OH oxidation. The ratio of -COOH to -CHO was shown to be
 462 influenced by the type and amount of laccase, mediator and substrate, as well as by the reaction
 463 conditions (Jaušovec et al., 2015; Jiang et al., 2017, 2021; Patel et al., 2011; Quintana et al., 2017). In
 464 soluble COS, COOH groups were mainly introduced. It is likely that by adjusting the reaction
 465 conditions, the carbonyl content can be increased, as is the case with insoluble material.

466

467



468 Fig. 4. Oxidation of cellopentaose (Glc5) by laccase/TEMPO system. (A) Glc5 conversion versus
469 reaction time, conversions were determined by HPAEC-PAD analysis. (B) HPAEC-PAD profiles of Glc5
470 oxidation products. Control without TEMPO and control without laccase are shown in orange and
471 green, respectively. The main product 3c ($t_r = 22.92$ min) and other minor products are showed by red
472 arrows. (C) Extracted ion chromatograms of oxidized products after ionic chromatography high mass
473 spectrometry in negative mode. (D) Glc5 and potential structures of oxidized Glc5 products and their
474 calculated molecular mass. The carbonyl (3d^f) and carboxylic acid (3b^f) groups are arbitrarily placed
475 on glucose unit 'c'. Reactions performed with Glc5 (1.2 mM, 6 μ mol), TEMPO (0.3 mM, 1.5 μ mol),
476 laccase (0.27 U/mL) in 5 mL of acetate buffer (20 mM pH 6.0), open flask at 500 rpm, 30 °C, 8 h.

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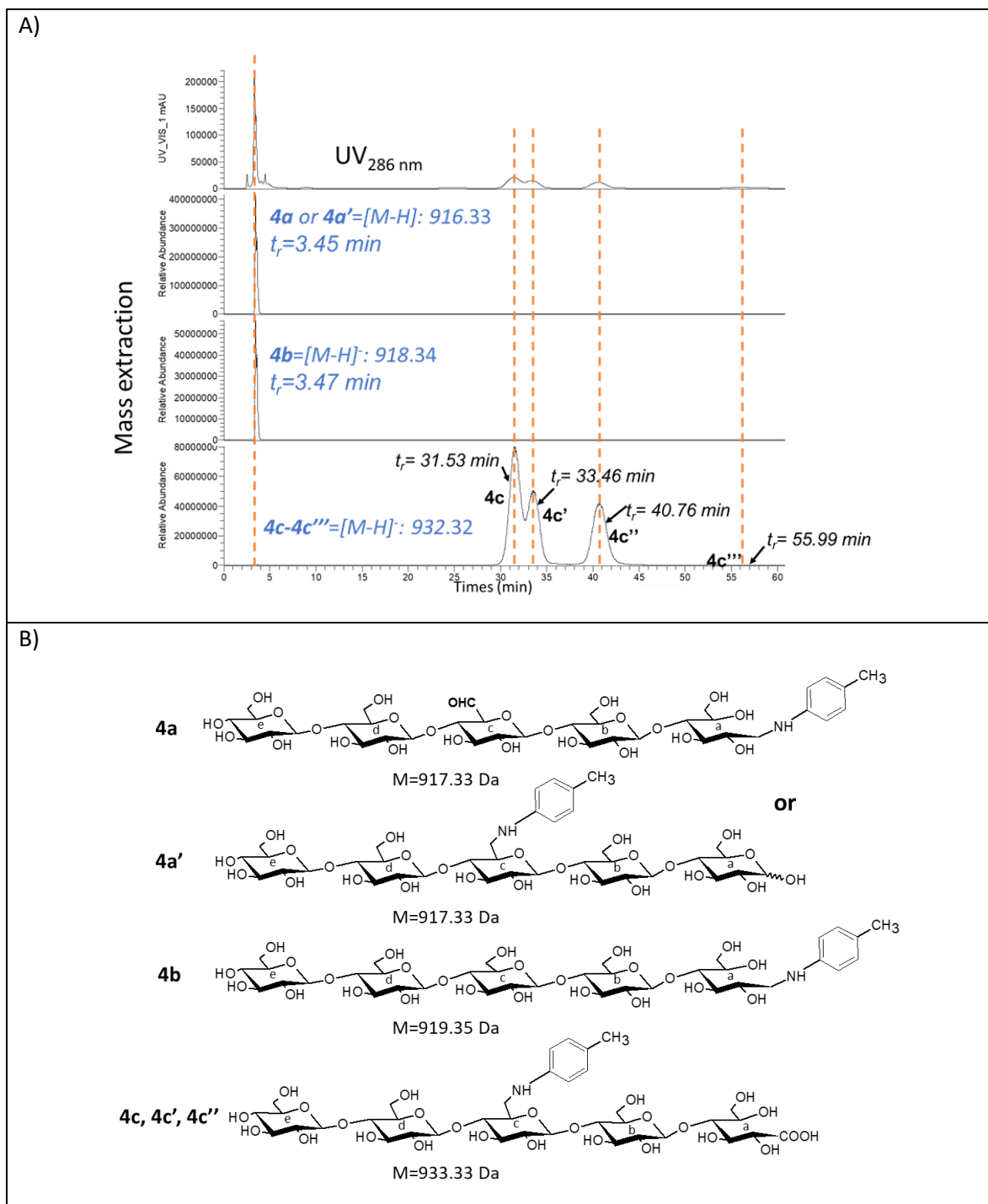
478 **3.3. Grafting of amino compounds onto oxidized Glc5**

479 The plan was to exploit the presence of a carbonyl group in the oxidized products to attempt
480 reactions with the amine group of amino-chromophores, first synthesizing an imine (-C=N-) and then
481 reducing it to give a secondary amine with a strong covalent bond between the substituent and the
482 oligosaccharide (Scheme 1).

483 **3.3.1 Grafting of *p*-toluidine**

484 Reductive amination was first tested with *p*-toluidine (*p*T), directly on the oxidized Glc5. The amine
485 (10 % (v/v)) was added along with 2-picoline borane (pic-BH₃) as a reductant, and contact was
486 maintained for 3 h at 40 °C under stirring. Pic-BH₃ was chosen over the more usual NaBH₃CN, as it is a
487 less hazardous reductant that prevents the production of toxic by-products such as cyanide residues
488 (Sato, Sakamoto, Miyazawa, & Kikugawa, 2004). LC-HRMS analysis of the products in negative mode
489 revealed several signals that can be attributed to functionalized derivatives (Fig. 5). Products, 4a'
490 ($t_r = 3.45$ min, m/z 916.33, [M-H]⁻) and 4b ($t_r = 3.47$ min, m/z 918.34, [M-H]⁻) correspond to grafted Glc5
491 without carboxyl group. Product 4a' (m/z 916.33) with a $m/z + 89$ mass increase relative to Glc5 mass
492 corresponds to a toluidine grafted to the C-2, C-3, or C-6 of one of the 5 glucose rings of Glc5, the
493 grafting on the C-6 position being the most probable. Another possible structure for 4a would be a *p*T
494 grafted to the C-1 plus an aldehyde group on the C-2, C-3 or C-6 of units a, b, c, d or e. Product 4b
495 (m/z 918.34) with a $m/z + 91$ mass increase is a Glc5 with *p*T grafted at C-1 of unit a (reducing end),
496 which supports the hypothesis that enzymatic oxidation of the reducing end was not complete,
497 leaving the anomeric aldehyde free for subsequent reductive amination. The other products eluting
498 after 30 min (4c, 4c', 4c'' and 4c''') have the same m/z value of 932.32 ([M-H]⁻), with $m/z + 105$ mass
499 increase compared to Glc5. The $m/z + 105$ increase can be broken down in $m/z + 89$ and $m/z + 16$
500 increase, accounting for a C-6-grafted toluidine (or C-2, C-3) on one of the Glc5 units, and a carboxyl
501 group at the C-1 of Glc5 reducing end.

502



503 Fig. 5. LC-UV-HRMS analysis of the glucoconjugate obtained by reductive amination of oxidized Glc5
 504 with toluidine. (A) UV_{286nm} chromatogram and extracted ion chromatograms of products 4a, 4a', 4b,
 505 4c, 4c' and 4c''. (B) Potential structures of compounds 4 and their calculated molecular mass. In the
 506 structures 4a, note that the carbonyl function could also be on the C-2, or C-3 of the glucosyl units,
 507 similarly we cannot rule out the pT being grafted onto the C-2 or C-3, although these grafting
 508 positions are less likely. Reaction conditions: after 8 h of enzymatic oxidation of Glc5 with
 509 TvL/TEMPO, 500 μL of the oxidized reaction medium was mixed for 3 h with 10.15 mM pT, 2.43 mM
 510 pic-BH₃ and 10 % acetic acid at 40 °C.

511

512 Given the complexity of the products obtained and the multiple structures that could match with the
513 mass analysis, we turned to LC-HRMS/MS using collision induced dissociation fragmentation (CID-
514 MS/MS) of the molecular ions at m/z 932.32, $[M-H]^-$ in negative mode to analyze the three most
515 abundant isomers 4c ($t_r= 31.53$ min), 4c' ($t_r= 33.46$ min) and 4c'' ($t_r= 40.76$ min).

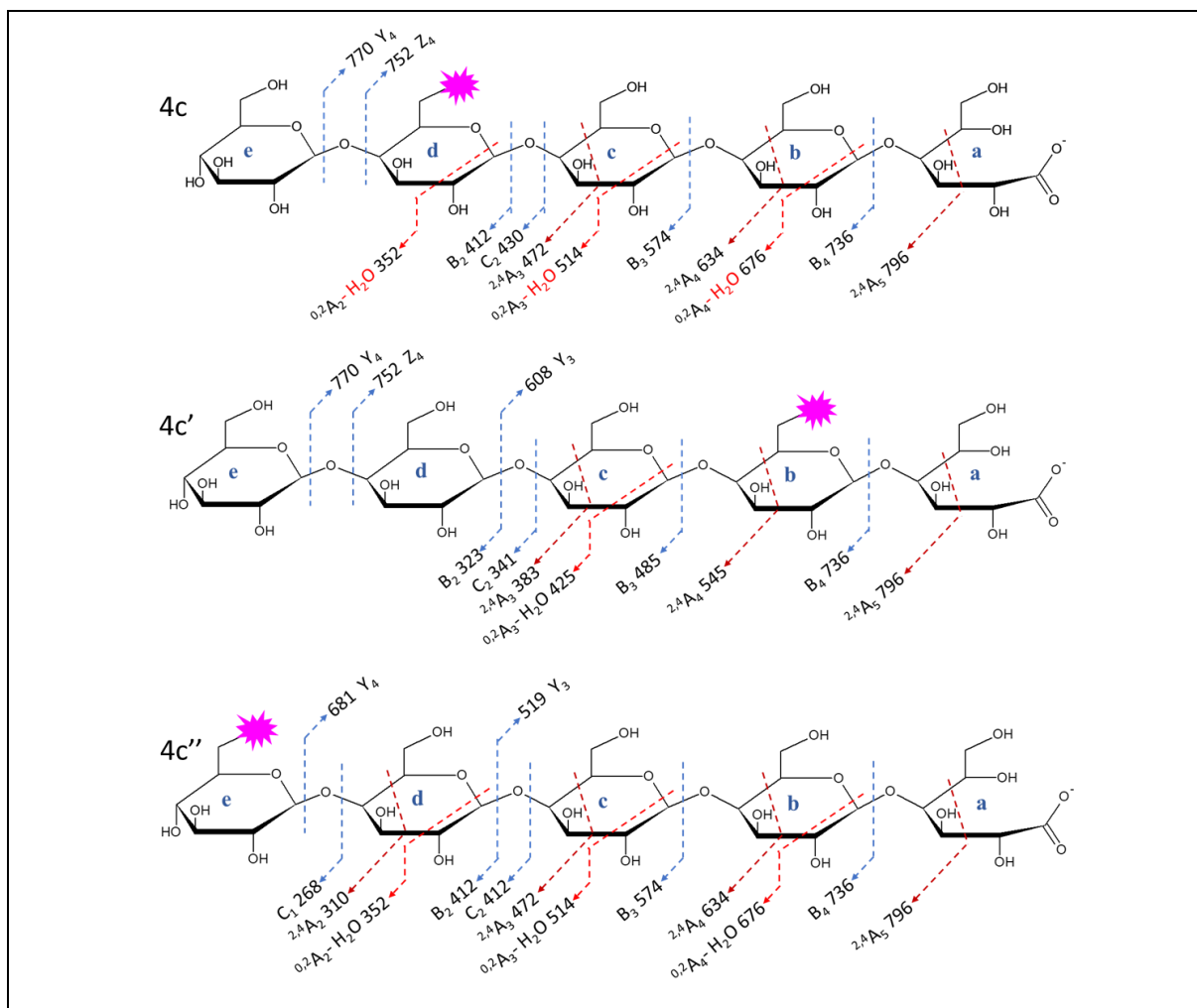
516 Annotation was performed according to the nomenclature developed by Domon & Costello (1988)
517 (Fig. 6 A-C, Fig. S8). MS/MS fragmentation of the three molecular ions displayed mostly the same
518 fragmentation patterns as those described by Sun et al. (2020) for C-1-oxidized cellooligosaccharides
519 (Fig. 6 A-C, Fig. S8). Overall, B-/Y- and C-/Z-fragments arising from glycosidic bond cleavage are less
520 abundant than those resulting from ring cleavage, such as $^{0,2}A_n$ and $^{2,4}A_n$ fragments, with the $^{2,4}A_n$
521 fragments overall dominating. The MS/MS spectra of the three isomers 4c, 4c' and 4c'' showed,
522 among the most abundant fragments, a characteristic intracyclic fragment $^{2,4}A_5$ at m/z 796,
523 confirming the grafting of *pT* on the C-6 or C-2 carbon atom of the glucose units *b*, *c*, *d*, or *e* or on the
524 C-3 of glucose units *a*, *b*, *c*, *d*, or *e* or on C-4 of the glucose unit *e* with an aldonic acid residue on
525 glucosyl unit *a*.

526 In addition to the fragment $^{2,4}A_5$, product 4c fragmentation yielded fragments B_3 (m/z 574), Y_4 (m/z
527 770) and the abundant fragment $^{2,4}A_4$ at m/z 634 (Fig. 6A). These fragments are matching with *pT* on
528 units *c* or *d*. Due to i) the presence of a low signal intensity of the fragment at m/z 323, which could
529 correspond to B_2 fragment without *pT*, ii) the B_2 fragment at m/z 412 and iii) the very low intensity of
530 Y_3 (m/z 519), we cannot determine whether *pT* is linked to unit *c* or *d*, the two isomers could be
531 present and coelute together.

532 In the case of product 4c' (Fig. 6B), the abundant fragment $^{2,4}A_4$ at m/z 545 and the fragment $^{2,4}A_5$ at
533 m/z 796 with a mass increase of 162 and $m/z + 89$ ($89 =$ mass of toluidine) plus the fragment B_4 at
534 m/z 736 are consistent with *pT* bound at the C-6 of the glucosyl unit *b*. Although less probable, we
535 cannot exclude *pT* binding at C-2 of unit *b*.

536 Regarding product 4c'' (Fig. 6C), a fragment Y_4 at m/z 681 is seen with a mass decrease of m/z 89
537 compared to the mass of fragment Y_4 at m/z 770 observed from the fragmentation of products 4c
538 and 4c'. This fragment plus the C_1 fragment at m/z 268 clearly indicates that *pT* is carried at the
539 position C-6, C-2, C-3 or C-4 of glucosyl unit *e*. Other fragments such as $^{2,4}A_4$ (m/z 634), B_3 (m/z 574),
540 $^{2,4}A_3$ (m/z 472) or the m/z 383 result from successive fragmentation $Y_4/^{2,4}A_4$ are consistent with the
541 proposed structure.

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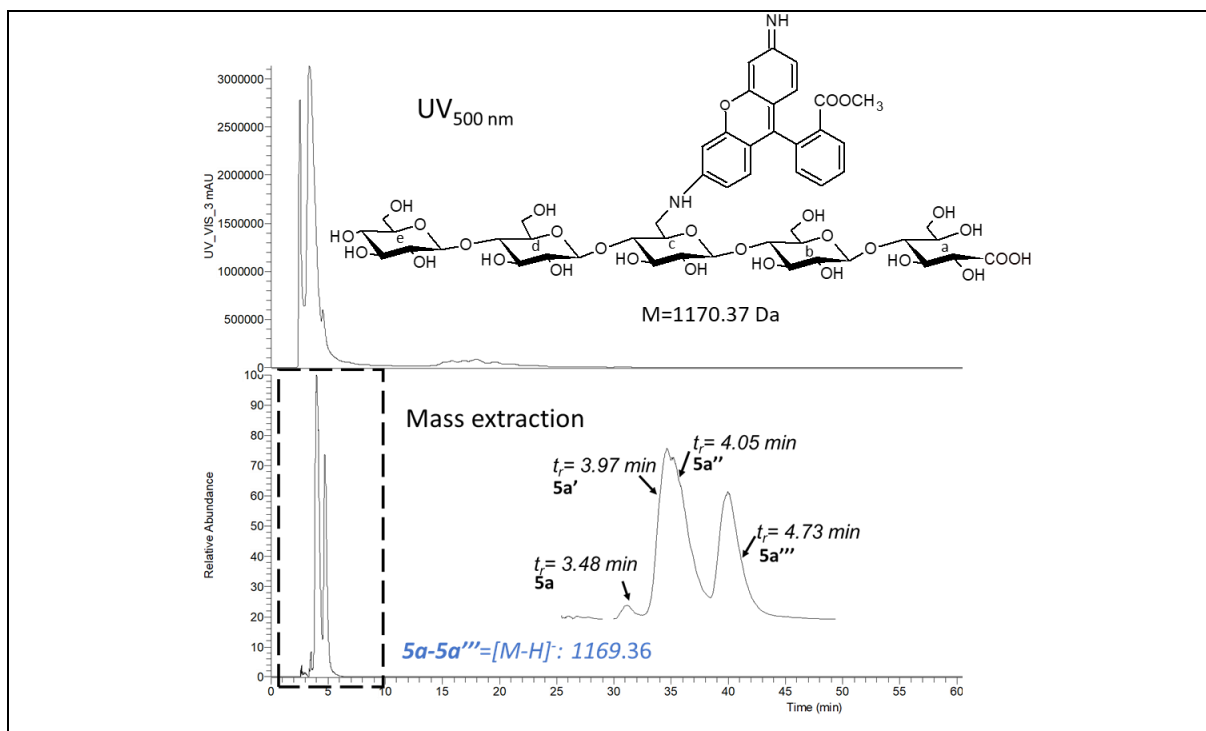
543 Fig. 6. Summary of fragmentation observed by LC-ESI-CID-MS/MS analysis in negative ion mode of
 544 products 4c, 4c' and 4c'', m/z 932.32 $[M-H]^-$. The fragments are annotated according to Domon &
 545 Costello (1988).

546

547 3.3.2 Grafting of rhodamine

548 Encouraged by the results obtained with *pT*, we conducted the same reductive amination but with a
 549 bulkier aminated molecule: the "rhodamine 123 (RHO123)". HPLC-UV-HRMS (Fig. 7) showed the
 550 presence of at least 4 different products 5a-5a''', at m/z 1 169.36 ($[M-H]^-$). This mass is consistent
 551 with a Glc5 molecule, on which a RHO123 molecule is grafted and that displays a carboxyl group
 552 (most probably at the reducing end). Further MS/MS analysis was not performed to determine the
 553 exact position of grafting.

554



555 Fig. 7. LC-ESI-HRMS analysis of the glycoconjugate obtained by reductive amination of oxidized Glc5
 556 with RHO123. UV_{500 nm} chromatogram and extracted ion chromatogram of product 5a, m/z 1169.3.
 557 We cannot exclude that RHO123 is grafted onto the C-2 or C-3, even if these positions of grafting are
 558 less probable. The structure gives an example of a possible isomer. Reaction conditions: After 8h of
 559 enzymatic oxidation of Glc5 with TvL/TEMPO, 500 μ L of the oxidized reaction medium was mixed for
 560 3 h with 10.15 mM RHO123, 2.43 mM pic-BH₃ and 10 % acetic acid at 40°C.

561

562 3.3.3 Hydrolysis of the grafted products obtained by reductive amination

563 Glc5 grafted with toluidine or rhodamine was subjected to hydrolysis with a cocktail of cellulases.
 564 After reductive amination, the pH of the reaction medium was set at 4.8 and incubated with a
 565 commercial preparation of cellulases from *Trichoderma reesei* for 24 hours. HPLC-UV-HRMS analyses
 566 of the reaction mixture confirmed the presence of covalent bond between pT or RHO123 and Glc5
 567 (Table S2). It is worth mentioning that we extracted a mass corresponding to a glucose molecule
 568 substituted with two toluidine molecules (m/z 359.19), which reflects the great diversity of products
 569 that can be generated (Table S2 entry 3). In the case of RHO123, the hydrolysis of the functionalized
 570 Glc5 was more difficult, resulting in the detection of non-hydrolyzed substituted DP4. It should be
 571 noted that four molecules with m/z 505.16 were identified. This mass is representative of a glucose
 572 unit substituted by one rhodamine molecule. It also proves that the substitution took place not only
 573 at the C-6 position but also at C-2, C-3 or even C-4 (when the latter is not involved in an osidic bond).

574

575

576 4. Conclusions

577 The laccase/TEMPO/O₂ system was successfully applied to the oxidation of cellobiose, methyl β-
578 cellobiose, COS and Glc5. Oxidation of these type of oligosaccharides with LMS had never been
579 described before. The conditions for oxidation reaction were optimized in terms of pH, temperature,
580 time and oxygen supply for me-β-Glc2. Among the different pHs tested, the highest conversion
581 (>70%) was obtained at pH 6, a pH value that could favour TEMPO regeneration. The introduction of
582 both carboxyl and carbonyl groups into the COS mixture and into Glc5 was demonstrated by
583 extensive structural analyses combining LC-MS, LC-HRMS/MS and NMR. The carbonyl groups
584 incorporated in these oligosaccharides allowed the advantageous grafting of chromophores (pT and
585 RHO123) by reductive amination. It should be noted that the combination of LMS-oxidation to
586 reductive amination for the derivatization of oligosaccharides is effective and had never been
587 proposed before. We showed that reductive amination can occur on different sugar units of the COS
588 but could not define with high precision on which carbon of Glc5 glucosyl units the substituent is
589 attached to. Only NMR or further MS studies could help clarify this point. However, the C-6 position
590 is probably the most reactive one.

591 LMS-based oxidation coupled with reductive amination represents an efficient "green" route to
592 generate negatively charged or functionalized cellooligosaccharides. The molecular diversity
593 obtained provides access to new structures with potential prebiotic, immunostimulant, antioxidant
594 or antitumor activities, highly sought after in the food, cosmetics and healthcare sectors. In addition,
595 its versatility renders it applicable to different types of oligosaccharides. Although it still needs to be
596 optimized to demonstrate its economic relevance, the proposed process is particularly attractive
597 compared with chemical methods that require multiple protection and deprotection steps for sugar
598 functionalization. In addition, oxidation of sugars by the laccase/Tempo system is also
599 environmentally beneficial compared with oxidation based on TEMPO chemistry, which is generally
600 carried out at a pH above 8 in the presence of NaOCl/NaBr and can induce cleavage between C2 and
601 C3 of the sugar rings ([Hillscher et al., 2024](#); [Thaburet, Merbouh, Ibert, Marsais, & Queguiner, 2001](#)).
602 Finally, our results clearly highlight the potential of reductive amination to modify cellulose-based
603 materials via a simple process with reduced use of hazardous or waste-generating procedures. We
604 believe this could be of great interest to the textile industry for dyeing or modifying cotton fabrics in
605 an eco-friendlier way.

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609 **CRedit authorship contribution statement**

610 **Awilda Maccow:** Investigation, Formal analysis, visualization, Writing - Original Draft, Writing -
611 Review & Editing. **Hanna Kulyk:** Formal analysis. **Etienne Severac:** Conceptualization, Methodology,
612 Formal analysis, manuscript Review. **Sandrine Morel:** Conceptualization, Methodology. **Claire**
613 **Moulis:** Conceptualization, Methodology, Formal analysis, manuscript Review. **Guillaume**
614 **Boissonnat:** Conceptualization, Validation, Supervision, Project administration, Funding acquisition,
615 manuscript Review. **Magali Remaud-Simeon:** Conceptualization, Methodology, Validation, Writing -
616 Review & Editing, Visualization, Supervision, Project administration, Funding acquisition. **David**
617 **Guieysse:** Conceptualization, Methodology, Validation, Investigation, Formal analysis, Writing -
618 Review & Editing, Visualization, Supervision, Project administration, Funding acquisition.

619

620 **Declaration of competing interest**

621 The authors declare that they have no conflict of interest.

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633

634 **Appendix A. Supplementary data**

635 Figures S1 to S7 and Tables S1 and S2.

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