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1 **Fructosylation of phenolic compounds by levansucrase from *Gluconacetobacter***  
2 ***diazotrophicus***

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**22 Abstract**

23 Fructosylation can significantly improve the solubility, stability and bioactivity of phenolic  
24 compounds, increasing their health benefits. Levansucrase from *Gluconacetobacter*  
25 *diazotrophicus* (LsdA, EC 2.4.1.10) was found to transfer the fructosyl unit of sucrose to  
26 different classes of phenolic compounds. Among the various acceptors tested, the  
27 isoflavone puerarin and the phenol coniferyl alcohol were the most efficiently fructosylated  
28 compounds, with conversion rates of 93% and 25.1%, respectively. In both cases, mono-,  
29 di-, and trifructosides were synthesized at a ratio of 37:14:1 and 32:8:1, respectively.  
30 Structural characterization of the puerarin mono-fructoside revealed that the enzyme  
31 transferred the fructosyl moiety of sucrose to the O6-position of the glucosyl unit of  
32 puerarin. The water solubility of fructosyl- $\beta$ -(2 $\rightarrow$ 6)-puerarin was increased 23-fold, up to  
33 16.2 g L<sup>-1</sup>, while its antioxidant capacity was only decreased 1.25-fold compared with that  
34 of puerarin.

35 **Keywords:** Phenolic compounds, transfructosylation, levansucrase, *Gluconacetobacter*  
36 *diazotrophicus*

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## 42 **1. Introduction**

43 Phenolic compounds (also called polyphenols) are secondary metabolites that are  
44 widespread in the plant kingdom. Depending on their structure, they can be classified as  
45 hydroxycinnamic acids, hydroxybenzoic acids, stilbenoids, xanthonoids, dihydrochalcones,  
46 flavonoids, phenols or phenolic aldehydes [1]. They have received considerable attention  
47 due to their biological antioxidant, anti-inflammatory, anticancer and anti-viral properties  
48 [2,3]. In nature, phenolic compounds are found either in aglycon or glycosylated forms.  
49 Aglycons are often poorly soluble in water, causing a decrease in their bioavailability and  
50 beneficial effects due to low assimilation [2,4,5]. To overcome these drawbacks,  
51 glycosylation is seen as an attractive means of modifying the physicochemical properties of  
52 phenolic compounds with a view to change their aqueous solubility, stability and  
53 bioavailability [3,6,7].

54 Chemical approaches can be envisioned. However, they require protection and deprotection  
55 steps for the reactive hydroxyl groups, often rely on the use of toxic heavy metals as  
56 catalysts, and may involve hazardous purification steps [7,8]. In contrast, microbial  
57 biotransformation [9,10] and/or direct enzymatic glycosylation are more environmentally  
58 friendly methods [11], which also present the advantage of being regio- and stereo-selective  
59 [12]. Sucrose-active enzymes, and microbial transglucosylases from the GH 13 and GH 70  
60 families in particular, have been described to glycosylate different phenolic compounds,  
61 using either native or engineered enzymes [3,8,11,13–15] Indeed, sucrose is a low-cost and  
62 widely available agro-resource, and is entirely suitable for use as a donor for glucosyl or  
63 fructosyl transfer reactions leading to added-value glycoconjugates. However, the

64 enzymatic fructosylation of polyphenols using sucrose as the donor has been little explored  
65 so far [7].

66 Transfructosylation reactions from sucrose are catalyzed by  $\beta$ -fructosidases and  
67 fructosyltransferases from microbial or plant origins. These enzymes belong to the  
68 glycoside hydrolase families 32 or 68 [16]. Enzymes from the GH32 family have been used  
69 for the fructosylation of different classes of phenolic compounds [7]. Dudíkova et al.[17]  
70 describe the fructosylation of furfuryl alcohol, 4-hydroxybenzyl alcohol, vanillyl alcohol  
71 and coniferyl alcohol catalyzed by GH32  $\beta$ -fructosidases from cell wall preparations of the  
72 fungus *Cryptococcus laurentii* CCY 17-3-6. The xanthonoid mangiferin has also been  
73 fructosylated with a GH32 enzyme from *Arthrobacter arilaitensis* NJEMO1 CCTCC  
74 M2012 [18], and the flavonoid puerarin with a GH 32  $\beta$ -fructosidase from *Arthrobacter*  
75 *nicotianae* CCTCC M2010164 [19], using permeabilized cells of *Microbacterium oxydans*  
76 CGMCC 1788 [20] and cells of *Lysinibacillus fusiformis* CGMCC 4913 [21].

77 The bacterial levansucrases (EC 2.4.1.10) from the GH68 family are also interesting  
78 fructosylation tools for polyphenol modifications. These  $\beta$ -retaining enzymes catalyze the  
79 synthesis of levan, a fructan composed of  $\beta$ -(2 $\rightarrow$ 6) linkages, from sucrose. Other side-  
80 products are naturally forming, such as: i) glucose and fructose resulting from the transfer  
81 of fructosyl onto water molecules, ii) 1-kestotriose or 6-kestotriose produced by the  
82 acceptor reaction onto sucrose, iii) blastose resulting from the transfer onto glucose, and iv)  
83 inulobiose or levanbiose obtained from the transfer onto fructose [22]. The ratio of each  
84 product is highly dependent on the enzyme's origin and selectivity towards the various  
85 natural acceptors produced during the reaction. For instance, levansucrase from the Gram-

86 positive bacterium *Bacillus subtilis* mainly converts sucrose into levan, whereas  
87 levansucrase from the Gram-negative species *Gluconacetobacter diazotrophicus* catalyses  
88 the formation of relatively high levels of 1-kestotriose and 1-1-kestotetraose, and in  
89 consequence, lower levels of levan, under the same reaction conditions [23]. Levansucrases  
90 can also transfer the fructosyl unit of sucrose to exogenous acceptors, including  
91 monosaccharides such as mannose, galactose, fucose and xylose, or disaccharides such as  
92 maltose, lactose and melibiose, as well as aromatic and aliphatic alcohols [7,22]. The  
93 fructosylation of hydroquinones has previously been performed using levansucrases from  
94 *Leuconostoc mesenteroides* [24] and *Bacillus subtilis* Marburg 168 [25], obtaining a low  
95 yield of 1.14% and 9.51%, respectively. Both reactions were carried out without co-  
96 solvents, and only a mono-fructosylated product was obtained.

97 In this study, the catalytic capacity of the levansucrase secreted by the endophytic  
98 bacterium *G. diazotrophicus* SRT4 (LsdA) was tested for its fructosylation of polyphenolic  
99 compounds. This enzyme is a good candidate for performing such reactions, as it is a robust  
100 enzyme that operates under a wide range of pH conditions (4-7) maintaining 80% of  
101 activity, and in a temperature range between 30°C and 45°C [23]. Eighteen phenolic  
102 compounds of different classes (hydroxycinnamic acids, hydroxybenzoic acids,  
103 hydroxybenzoic methyl esters, stilbenoids, xanthonoids, flavonoids, phenols and phenolic  
104 aldehydes) were tested as acceptors for LsdA, using sucrose as a donor substrate.  
105 Conversion rates were determined in the presence (20%) and absence of DMSO, used to  
106 enhance polyphenol solubility. Puerarin (an isoflavone) and coniferyl alcohol (a phenol)  
107 were identified as the best acceptors, yielding mono-, di- and tri-fructosylated products.

108 The structure, solubility and antioxidant properties of puerarin mono-fructoside were  
109 investigated. This is the first report describing the catalytic versatility of LsdA to  
110 fructosylate phenolic or non-phenolic glycosides compounds that have never been tested  
111 before as levansucrase acceptors.

112

## 113 **2. Materials and methods**

### 114 **2.1 Chemical materials**

115 Vanillin, gallic acid, methyl gallate, caffeic acid, rosmarinic acid, hesperedin, quercetin,  
116 neohesperedin and catechin were supplied by Sigma Aldrich Inc. (MO, USA); puerarin,  
117 resveratrol, ferulic acid, arbutin, coniferyl alcohol, luteolin, myricetin and mangiferin were  
118 supplied by Carbosynth Limited (Compton, UK); gossypin was supplied by Interchim  
119 (Montluçon, France); dimethyl sulfoxide (DMSO) was supplied by Acros Organics; formic  
120 acid (99% purity) by CARLO ERBA Reagents S.A; and acetonitrile (ACN) by Sharlab. S.  
121 L. The sucrose was supplied by Merck KGaA (Darmstadt, Germany).-

### 122 **2.2 Activity measurements**

123 Levansucrase (LsdA) was purified from the culture supernatant of *Gluconacetobacter*  
124 *diazotrophicus* SRT4 using anion exchange chromatography as previously described  
125 Hernandez et al [23]. One unit of LsdA is defined as the amount of enzyme releasing 1  
126  $\mu\text{mol}$  of glucose per minute based on initial velocity measurements of the reaction with 100  
127  $\text{g. L}^{-1}$  sucrose in a 50 mM phosphate buffer at pH 5.8 and 42°C.

128

### 129 **2.3 Acceptor reaction**

130 Fructosylation of the phenolic compounds was performed using fixed concentrations (0.1, 1  
131 or 5 U mL<sup>-1</sup>) of enzyme in the presence or absence of 20% of DMSO (v/v), with sucrose as  
132 the donor (50 g L<sup>-1</sup>, 146 mM) and an amount of phenolic compound corresponding to a  
133 final theoretical concentration of 25 mM, in a 50 mM phosphate buffer at pH 5.8 and 42°C.  
134 As most of the tested acceptors formed a precipitate at the beginning of the reaction, their  
135 real solubility at 42°C was determined using the following procedure. First, a solution  
136 saturated with each acceptor was prepared in 1 ml of ultrapure water (Microtubes) and  
137 incubated at 42°C and 1000 rpm for two hours in a Thermomixer (Eppendorf<sup>TM</sup>, Germany).  
138 Then, the solution was centrifuged at 13,000 g for one minute. The supernatant was diluted  
139 in DMSO to a final concentration comprised between 0.05 and 1 mM and analysed using  
140 HPLC to determine the concentration of the phenolic compound.

### 141 **2.4 Determination of phenolic compound conversion value**

142 At the beginning of the reaction and at a theoretical concentration of 25 mM, the tested  
143 phenolic compounds (excepting gallic acid, methyl gallate, catechin, coniferyl alcohol  
144 arbutin, and vanillin) were not entirely soluble, and a proportion of acceptor remained in a  
145 precipitate. During the course of the reaction with puerarin, the precipitate disappeared  
146 indicating a slow phase transfer of phenolic compounds from the solid to the liquid phase.  
147 For others, some precipitate remains. Consequently and to determine accurate conversion  
148 value, 20 µl of each reaction mixture were taken at the final reaction time and dissolved in  
149 980 µL of DMSO in order to solubilize all undissolved phenolic acceptors. The samples  
150 were then analysed by HPLC/MS using UV detection at 254 nm. The concentration of each

151 phenolic compound was determined from its UV response at 254 nm using a standard curve  
152 established with concentrations comprised between 0 to 1 mM. Finally, the phenolic  
153 compound conversion was determined as the ratio of consumed phenolic compounds versus  
154 theoretical concentration at initial time (25mM).

## 155 **2.5 HPLC-MS analysis of the fructosylation reaction mixture**

156 HPLC-MS analysis of phenolic compounds was performed using an Ultimate 3000 series  
157 chromatograph equipped with a Dionex 340 UV/VIS detector and coupled with a simple  
158 quadruple mass spectrometer (MSQ Plus, Thermo Scientific). The column was a  
159 Phenomenex PFP C18 (Luna 5 $\mu$ m, 100 A, 250 x 4.6 mm, USA) maintained at 40°C.  
160 Samples were analysed using the following gradient of water/formic acid 0.05% (v/v) and  
161 acetonitrile/formic acid 0.05% (v/v) (90/10 at 0 min, 50/50 at 20 min, 5/95 at 20.1 min and  
162 90/10 at 35 min) at a flow rate of 1 ml min<sup>-1</sup>. The different phenolic compounds were  
163 quantified using UV detection at 254 nm. The mass spectrometer was in negative and  
164 positive mode with a voltage cone at 50, 80 and 110 V, the temperature of the electrospray  
165 ionization (ESI) ion source was 450°C and the gas carrier was nitrogen. The mass  
166 spectrometer scanned from m/z 100 to 1,500. The data acquisition and processing were  
167 performed using Chromeleon™ 7.2 data systems.

## 168 **2.6 Large-scale preparation of the fructosylated product**

169 Mono-fructosyl puerarin was produced on a preparative scale using LsdA. The reaction  
170 conditions were the following: 146 mM of sucrose (50 g L<sup>-1</sup>) and 25 mM of puerarin (10.4  
171 g L<sup>-1</sup>) in 400 mL of 50 mM phosphate buffer at pH 5.8 with 0.1 U mL<sup>-1</sup> of enzyme, agitated  
172 at 250 rpm for a period of four hours at a temperature of 42°C. The kinetic of puerarin

173 consumption was monitored using HPLC-MS. When the puerarin conversion rate reached  
174 90%, the reaction was stopped by heating the mixture at 95°C for five minutes. The  
175 reaction mixture was then subjected to column chromatography using a Purosorb PAD 910  
176 resin column (30 x 2 cm). The column was washed with pure water to remove sugars.  
177 Puerarin fructosides were eluted using an ethanol/H<sub>2</sub>O solution (20:80, v/v). The fractions  
178 containing the highest amounts of mono-fructoside were collected, mixed and analysed  
179 using HPLC-MS. The puerarin, mono- and di-fructosides were then separated using flash  
180 chromatography on a silica column (Reveleris® Flash Chromatography System, Grace,  
181 USA), with the following solvents used as eluents: pure acetonitrile, acetonitrile/H<sub>2</sub>O  
182 (90/10) and acetonitrile/H<sub>2</sub>O (80/20).

### 183 **2.7 Structural analysis of puerarin mono-fructoside**

184 <sup>1</sup>H, <sup>13</sup>C and 2D-NMR spectra were acquired on an Advance 500 MHz spectrometer  
185 (Bruker) operating at 500 MHz for <sup>1</sup>H, and 125 MHz for <sup>13</sup>C. The data were processed  
186 using Topspin 3 software. All measurements were recorded at 298 K, and chemical shifts  
187 were given in ppm relative to the residual signal of dimethyl sulfoxide.

188 **P1:** <sup>1</sup>H-NMR (500MHz, DMSO-d<sub>6</sub>, δ<sub>H</sub> ppm): 9,57 (1H, s, 4'-OH), 8,29 (1H, s, H<sub>2</sub>), 7,94  
189 (1H, d, J=8.8 Hz, H<sub>5</sub>), 7,40 (2H, d, J=8,5 Hz, H<sub>2</sub>', H<sub>6</sub>'), 6,80 (2H, d, J=8.5 Hz, H<sub>3</sub>',H<sub>5</sub>'),  
190 4,81 (1H, d, J=9,1 Hz, H<sub>1</sub>''), 3,90-4,00 (2H, m, H<sub>2</sub>'', H<sub>3</sub>'''), 3,90 (1H, d, H<sub>6</sub>''), 3,80 (1H,  
191 t, H<sub>4</sub>'''), 3,51-3,65 (2H, m, H<sub>6</sub>'', H<sub>5</sub>'''), 3,38-3,51 (2H, m, H<sub>6</sub>'''), 3,38-3,28 (6H, m,  
192 H<sub>3</sub>'', H<sub>4</sub>'', H<sub>5</sub>'', 2H<sub>1</sub>''')

193 **P1:**  $^{13}\text{C}$ -NMR (125 MHz, DMSO- $d_6$ ,  $\delta_{\text{C}}$  ppm) : 175.08 (C-4), 161.15 (C-6), 157.22 (C-9,  
 194 C-4'), 152.72 (C-2), 130.17 (C-2', C-6'), 126.36 (C-5), 123.17 (C-3), 122.67 (C-1'), 116.90  
 195 (C-10), 115.08 (C-6, C-3', C-5'), 112.55 (C-8), 104.24 (C-2'''), 82.21 (C-5'''), 79.83 (C-  
 196 5''), 78.56 (C-3''), 76.77 (C-3'''), 75.23 (C-4''), 73.57 (C-1''), 70.88 (C-2''), 70.46 (C-  
 197 4''), 62.52 (C-6'''), 61.58 (C-6''), 61.21 (C-1''')

## 198 **2.8 Solubility determination of puerarin mono-fructoside**

199 Puerarin mono-fructoside and puerarin were solubilized until saturation in 1 ml of ultrapure  
 200 water (Microtubes). The solutions were then incubated at 25°C and 250 rpm for two hours,  
 201 before undergoing centrifugation at 13,000 g for five minutes. The supernatant was diluted  
 202 in water and analysed using HPLC to determine the concentration of the mono-fructoside.

## 203 **2.9 Antioxidant activity of puerarin mono-fructoside**

204 The antioxidant activity of puerarin and puerarin mono-fructoside was estimated using a  
 205 DPPH assay as described in [26]). 150  $\mu\text{l}$  of DPPH at 0.1 mM in ethanol (95%) were mixed  
 206 with 150  $\mu\text{l}$  of puerarin or puerarin mono-fructoside at 1  $\text{mg L}^{-1}$ , also in ethanol (95%). The  
 207 reaction mixtures were then incubated in the dark at 37°C for 30 minutes, and the  
 208 absorbance measured at 517 nm. The assay was carried out in triplicate. The percentage of  
 209 DPPH inhibition was calculated using the following equation:

$$210 \quad \% \text{ DPPH inhibition} = ((1 - (A_{\text{sample}} / A_{\text{control}})) \times 100$$

211 where  $A_{\text{sample}}$  is the absorbance of the puerarin–DPPH or puerarin monofructoside–DPPH  
 212 mixtures, and  $A_{\text{control}}$  is the absorbance of the DPPH solution. Trolox<sup>TM</sup> (6-hydroxy-2,5,7,8-

213 tetramethylchroman-2-carboxylic acid) was used as a positive control to determine  
214 antioxidant activity.

215

### 216 **3. Results and discussion**

#### 217 **3.1 LsdA fructosylates a broad spectrum of phenolic compounds in an aqueous** 218 **medium**

219 A preliminary screening was performed to evaluate the capacity of LsdA to fructosylate 18  
220 different phenolic compounds, and examine the acceptor specificity of this enzyme. A ratio  
221 sucrose/acceptor was arbitrarily fixed at 5.8. This is a value commonly reported in the  
222 literature [7], ensuring an excess of sucrose to maximize fructosylation yield while  
223 avoiding possible enzyme inhibition by the phenolic compounds.

224 The reactions were first performed in an aqueous medium without using DMSO as a co-  
225 solvent (Table 1). Out of the 18 assayed compounds, only puerarin [19–21], coniferyl  
226 alcohol [17] and mangiferin [18] had previously been fructosylated with other  
227 fructosyltransferases. No data on the fructosylation of the other compounds are available.

228 The highest conversion rate (93.8%) was obtained for the isoflavone puerarin after six  
229 hours of LsdA reaction. Such a conversion rate significantly outperforms those previously  
230 reported for the conversion of 0.48 and 9.6 mM of this flavonoid using either permeabilized  
231 cells of *Microbacterium oxydans* without a co-solvent, or whole cells of *Lysinibacillus*  
232 *fusiformis* with 10% of ethanol as the co-solvent, which were found to be 54.5% and  
233 92.6%, respectively, after a reaction time of 48 hours [20,21]. The synthesis of puerarin

234 fructosides using a  $\beta$ -fructosidase ( $0.24 \text{ U mL}^{-1}$ ) from *Arthrobacter nicotianae* has also  
235 been described in Wu et al. [19]. Mono- and di-fructosides with a conversion rate of 75.6%  
236 were produced from 265.5 mM of acceptor after a reaction time of 72 hours in the presence  
237 of 25% of a DMSO co-solvent.

238 A fairly good conversion rate of 25% was also achieved in the presence of a coniferyl  
239 alcohol acceptor at 25 mM, yielding 6 mM of fructosylated products. Using *Cryptococcus*  
240 *laurentii* cell walls in the presence of a co-solvent of 30% of acetone (v/v), Dudíkova et al.  
241 [17] describe a 2% conversion rate for 200 mM coniferyl alcohol, resulting in 4mM mono-  
242 fructoside. Fructoside yields obtained using LsdA and in the absence of a co-solvent are  
243 thus slightly higher than those obtained using *C. laurentii* cells.

244 LC/MS analyses of the puerarin acceptor reaction products further revealed that LsdA  
245 catalyses the formation of mono-, di-, tri and tetra-fructosylated forms, with mono-  
246 fructoside always being preponderant in the mixture (Fig. 1). The proportions of  
247 fructosylated compounds is reported in Table 2. Under the conditions of this experiment,  
248 mono-fructoside accounted for more than 70% of the reaction products (based on peak area  
249 integration on HPLC chromatograms). This was also observed with other phenolic  
250 compounds, such as coniferyl alcohol and rosmarinic acid (data not shown).

251 The fructosylation of mangiferin was also tested under the same conditions. Only  
252 mangiferin mono-fructoside was produced, with a conversion rate of 3.7%, which is much  
253 lower than the rate reported by Wu et al. [18] they achieved a conversion rate of 67.5%  
254 from 112 mM of acceptor using the  $\beta$ -fructofuranosidase of *Arthrobacter arilaitensis* in  
255 DMSO (20% v/v). Aiming to enhance the fructosylation of mangiferin, the LsdA reaction

256 was performed in 20% (v/v) DMSO. This increased the conversion rate up to 13.5 % but  
257 remained very low compared with the values reported by Wu et al. [18], indicating that the  
258  $\beta$ -fructofuranosidase of *Arthrobacter arilaitensis* recognizes mangiferin more efficiently  
259 than LsdA.

260 It is difficult to directly compare the results for the fructosylation of puerarin, coniferyl  
261 alcohol and mangiferin available in the literature with those reported here, as the acceptor  
262 specificity of each given enzyme, and the operational conditions, are important factors that  
263 can significantly affect fructosylation yield. LsdA was found to be a particularly attractive  
264 prospect for the fructosylation of puerarin and coniferyl alcohol, especially in reactions  
265 conducted using water without any co-solvent.

266 The catalytic versatility of LsdA was further investigated by evaluating the acceptor  
267 efficiency of phenolic compounds never tested before in transfructosylation reactions  
268 (Table 1). Hydroxycinnamic acids such as ferulic acid, caffeic and rosmarinic acid were  
269 fructosylated with conversion rates of between 6.4% and 15.2%. Other phenolic  
270 compounds such as methyl gallate (a hydroxybenzoic methyl ester) and resveratrol (a  
271 stilbenoid) were also converted up to 5.9% and 7.2%, respectively. With regard to  
272 flavonoids, fructosylation was observed for catechin and neohesperidin only, with  
273 conversion rates of 10.9% and 2.5%, respectively.

274 These findings confirm that LsdA is reasonably versatile and useful when it comes to  
275 fructosylating polyphenolic compounds. Fructosylation is clearly dependent on acceptor  
276 structure, and appears to be influenced to some degree by the presence of DMSO, which  
277 should enhance polyphenol solubility. The number of phenolic rings, presence of sugar

278 substituents and reactivity of the hydroxyl groups are thus key determinants of the acceptor  
279 recognition and correct positioning that allow productive fructosylation.

### 280 **3.2 Characterization of the puerarin acceptor reaction**

#### 281 **3.2.1 Effect of LsdA concentration on puerarin fructosylation**

282 The reactions were performed using 0.1, 1.0 and 5.0 U mL<sup>-1</sup> of enzyme, with puerarin at 25  
283 mM as the acceptor. Acceptor conversion was monitored over time. As shown in Figure 2,  
284 the highest conversion rate (93%) was obtained using 0.1 U mL<sup>-1</sup> of enzyme and a reaction  
285 time of four hours. In the presence of 1.0 U mL<sup>-1</sup> of enzyme, a 66 % conversion rate was  
286 obtained after four hours, and when the reaction was performed using 5.0 U mL<sup>-1</sup>, a  
287 maximum conversion rate of 82 % was obtained after a reaction time of two hours.  
288 However, at 5 U mL<sup>-1</sup> of enzyme, a significant decrease in conversion occurs, suggesting  
289 that fructosides are used as the fructosyl donor once sucrose is depleted. This highlights the  
290 importance of using a small amount of enzyme (0.1 U mL<sup>-1</sup>) in order to limit puerarin  
291 fructoside degradation.

#### 292 **3.2.2 Structural characterization of puerarin mono-fructoside**

293 A larger volume puerarin acceptor reaction was performed, and the mono-fructoside  
294 subsequently purified for NMR characterization.

295 The chemical structure of the compound corresponding to peak P1 was determined using  
296 mass spectrometry, <sup>1</sup>H-NMR, <sup>13</sup>C-NMR and 2D NMR. LC-MS analysis of the compound  
297 under peak P1 corresponds to puerarin derivatives with a molecular mass of 577 g.mol<sup>-1</sup>  
298 (m/z = 577 [M-H]<sup>-</sup>), confirming the transfer of one fructosyl unit onto puerarin (Figs. 3A

299 and 3B). The  $^{13}\text{C}$  spectrum revealed 27 signals (three overlapped), of which 12 carbon  
300 signals were in the range 80–110 ppm and assigned to puerarin, and 11 signals were in the  
301 range 60–90 ppm and assigned to the two monosaccharides (fructose and glucose).  
302 Compared with the  $^{13}\text{C}$  for puerarin, six additional signals were identified in the spectrum  
303 for fructosyl puerarin, with the chemical shift at 104.1 ppm being characteristic of the C2''''  
304 of the fructosyl moiety. The HMBC spectrum of P1 was then used to deduce the position  
305 of fructosylation (Fig. 3C). It showed a long-range correlation between the C2'''' of  
306 fructose ( $\delta_{\text{C}}$  104.24 ppm) and H6''' ( $\delta_{\text{H}}$  3.57-3.88) of the glucose unit, demonstrating that  
307 the fructoside produced by LsdA corresponds to  $\beta$ -D-mono-fructofuranosyl-(2-6)-puerarin  
308 (Fig. 3A), the same mono-fructoside as previously described in Wu et al. [19], where it was  
309 obtained using *A. nicotianae*  $\beta$ -fructosidase. In contrast, bioconversion of puerarin using  
310 permeabilized cells of *Microbacterium oxylans* or whole cells of *Lysinibacillus fusiformis*  
311 [20,21] resulted in a different structure – puerarin-7-O-fructoside – indicating that the  
312 position of fructosylation is dependent on enzyme specificity.

### 313 3.2.3 Water solubility and antioxidant activity of the fructosyl- $\beta$ -(2 $\rightarrow$ 6)-puerarin

314 We further investigated whether, and to what extent, fructosylation had altered the  
315 solubility and antioxidant activity of puerarin. As shown in Table 3, the  $\beta$ -D-  
316 fructofuranosyl-(2 $\rightarrow$ 6)-puerarin was 23 times more soluble in water at room temperature  
317 (25°C) than puerarin. Wang et al. [21] reported a 5.6-fold increase in puerarin-7-O-  
318 fructoside solubility compared with that of puerarin (0.7 to 16.2 gL $^{-1}$ ). A comparison of  
319 these results indicates that the solubility of puerarin mono-fructoside in water is dependent  
320 on the position of the fructosyl substituent.

321 The antioxidant activity of  $\beta$ -D-fructofuranosyl-(2 $\rightarrow$ 6)-puerarin decreased compared with  
322 that of puerarin, as evidenced by its free radical scavenging capacity, which dropped from  
323 33% to 26%. Notably, an inverse effect has been described with the same method (DPPH)  
324 for puerarin-7-O-fructoside, which displayed a 5% increase in its anti-oxidant activity  
325 compared with puerarin [27]. As with solubility, antioxidant activity is influenced by the  
326 position of the fructosyl  
327 moiety on puerarin.

328

#### 329 **4. Conclusion**

330 This paper describes the first assays of polyphenolic compound fructosylation using the  
331 FOS-producing levansucrase from *Gluconacetobacter diazotrophicus* (LsdA). Reactions  
332 were deliberately performed in water to avoid any addition of co-solvents. Out of the 18  
333 phenolic compounds tested, 11 were fructosylated, with conversion rates ranging from  
334 2.5% to 93%. The main product obtained when using puerarin as the fructosyl acceptor was  
335 characterized as  $\beta$ -D-fructofuranosyl-(2 $\rightarrow$ 6)-puerarin. This reveals that LsdA is a  
336 promising biocatalyst with a high biotechnological potential to be used in the fructosylation  
337 of different types of phenolic compound for the purposes of generating new glycosides of  
338 interest in terms of their solubility and antioxidant properties.

#### 339 **Acknowledgements**

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343 discovery, part of the Integrated Screening Platform of Toulouse (PICT, IBiSA), for  
344 providing the HPLC equipment and protein purification system.

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346 M14A01, CONACYT project CB-2012-01/000000000181766 and CONACYT [grant  
347 number 454708].

#### 348 **Conflict of interest**

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

#### 350 **Compliance with ethical standards**

351 This article does not contain any studies conducted by any of the authors involving human  
352 participants or animals.

353

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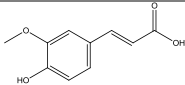
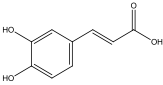
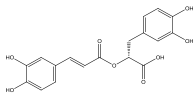
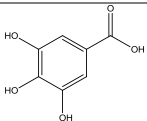
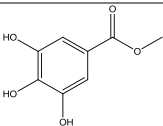
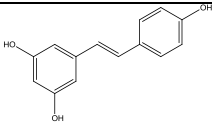
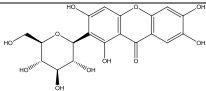
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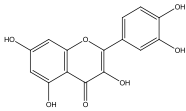
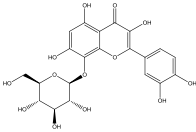
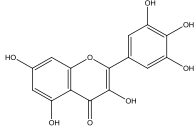
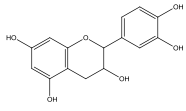
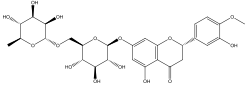
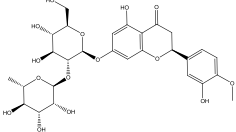
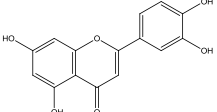
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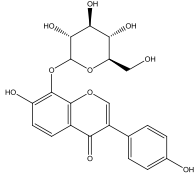
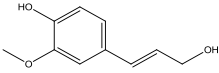
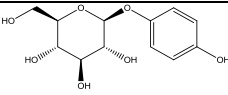
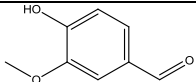
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446 **Tables and Figures**

447 **Table 1.** Screening of the fructosylation of different phenolic compounds by levansucrase  
 448 from *G. diazotrophicus*

Class of compound	Compound	Structure	Solubility (mM) at 42 °C in water	% conversion without DMSO <sup>b</sup>
Hydroxycinnamic acids	Ferulic acid <sup>a</sup>		7.4 ± 1.3	6.4 ± 2.3
	Caffeic acid <sup>a</sup>		4.7 ± 0.2	11.6 ± 1.7
	Rosmarinic acid <sup>a</sup>		4.0 ± 0.01	15.2 ± 0.3
Hydroxybenzoic acid	Gallic acid <sup>a</sup>		78.2 ± 0.1	0
Hydroxybenzoic methyl ester	Methyl gallate <sup>a</sup>		103.7 ± 5.2	5.9 ± 0.6
Stilbenoid	Resveratrol <sup>a</sup>		0.5 ± 0.02	7.2 ± 0.2
Xanthonoid	Mangiferin		0.66 ± 0.02	3.7 ± 0.1

Flavonoids	Quercetin <sup>a</sup> (Flavonol)		0.07 ± 0.01	0
	Gossypin <sup>a</sup> (Flavonol)		1.3 ± 0.3	0
	Myricetin <sup>a</sup> (Flavonol)		0.18 ± 0.01	0
	Catechin <sup>a</sup> (Flavanol)		69.3 ± 0.01	10.9 ± 1.4
	Hesperidin <sup>a</sup> (Flavanone)		0.07 ± 0.004	0
	Neohesperidin <sup>a</sup> (Flavanone)		2.8 ± 0.3	2.5 ± 1.4
	Luteolin <sup>a</sup> (Flavone)		0.09 ± 0.01	0

	Puerarin (Isoflavone)		$9.9 \pm 1.0$	$93.0 \pm 2.3$
Phenol	Coniferyl alcohol		$34.2 \pm 0.2$	$25.1 \pm 6.3$
	Arbutin <sup>a</sup>		$581.5 \pm 18.9$	0
Phenolic aldehyde	Vanillin <sup>a</sup>		$133.3 \pm 9.0$	$4.7 \pm 0.8$

449 Each phenolic compound was tested at a concentration of 25 mM. The enzyme  
450 concentration in all reactions was 0.1 U mL<sup>-1</sup>.

451 <sup>a</sup> Never tested before for enzymatic fructosylation.

452 <sup>b</sup> The conversion rate was calculated from the amount of acceptor remaining. Phenolic  
453 compound conversion = 100 \* ([phenolic compound]<sub>initial</sub> - [phenolic compound]<sub>final</sub>) /  
454 [phenolic compound]<sub>initial</sub>.

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460 **Table 2.** Conversion rates and proportions of fructosylated puerarin compounds

Compound	Conversion (%) <sup>a</sup>	Fructosylated compound (%) <sup>b</sup>			
		Mono	Di	Tri	Tetra
<b>Puerarin</b>	93.8 ± 2.4	70.9	26.4	1.9	Traces

461 <sup>a</sup>The conversion rate was calculated from the amount of acceptor remaining. Phenolic  
 462 compound conversion=100\*([phenolic compound]<sub>initial</sub> - [phenolic compound]<sub>final</sub>)/  
 463 [phenolic compound]<sub>initial</sub>.

464 <sup>b</sup>The % of fructosylated compounds was calculated using the formula (P<sub>n</sub> peak area)/Σ (P<sub>1</sub>,  
 465 P<sub>2</sub>...P<sub>n</sub> peak areas), where 1≤n≤4.

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475 **Table 3.** Solubility and free radical scavenging of puerarin and puerarin mono-fructoside

Compound	Solubility (g L <sup>-1</sup> )	Free radical scavenging activity (%) <sup>a</sup>
Puerarin	0.7 ± 0.09	33.3 ± 0.9
Puerarin mono- fructoside	16.2 ± 1.7	26.2 ± 1.3

476 <sup>a</sup>The free radical scavenging activity of DPPH was considered to be 100%.

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488 **Figure captions**

489 **Figure 1.** HPLC-UV/MS chromatograms after a reaction time of 24 hours with LsdA (S:  
490 puerarin, P1: puerarin mono-fructoside, P2: puerarin di-fructoside, P3: puerarin tri-  
491 fructoside) under the following conditions: 25 mM of puerarin and 146 mM of sucrose at  
492 pH 5.8 (50 mM phosphate buffer) and 42°C.

493

494 **Figure 2.** Effect of enzyme concentration (●: 0.1 U/ml, ○: 1 U/ml, and ▼: 5 U/ml) on  
495 puerarin conversion at different times and under the following conditions: 25 mM of  
496 puerarin and 146 mM of sucrose at pH 5.8 (50 mM phosphate buffer) at 42°C, over 24  
497 hours.

498

499 **Figure 3.** Enzymatic fructosylation of puerarin by LsdA under the following conditions: 25  
500 mM of acceptor and 146 mM of sucrose at pH 5.8 (50 mM phosphate buffer) and 42°C with  
501 0.1 U/ml of activity. A) Schematic mono-fructosylation of puerarin by LsdA, B) MS  
502 spectra of mono-fructosylated puerarin and C) 2D  $^1\text{H}$   $^{13}\text{C}$  HMBC NMR spectrum of  $\beta$ -D-  
503 fructofuranosyl-(2->6)-puerarin.

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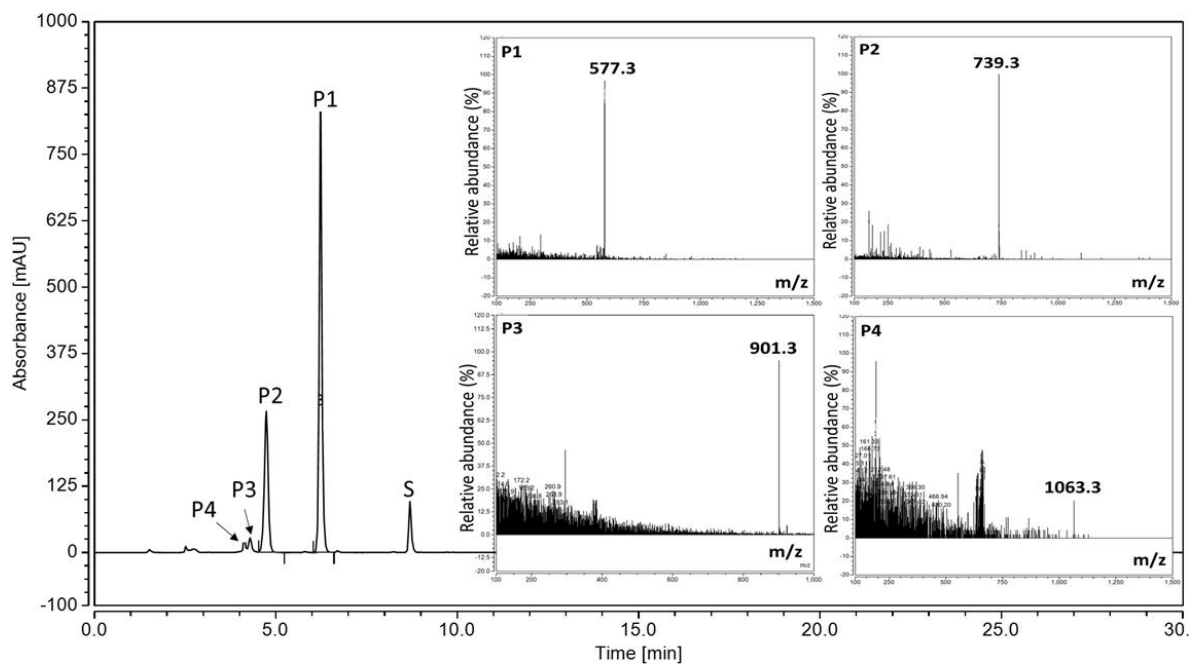
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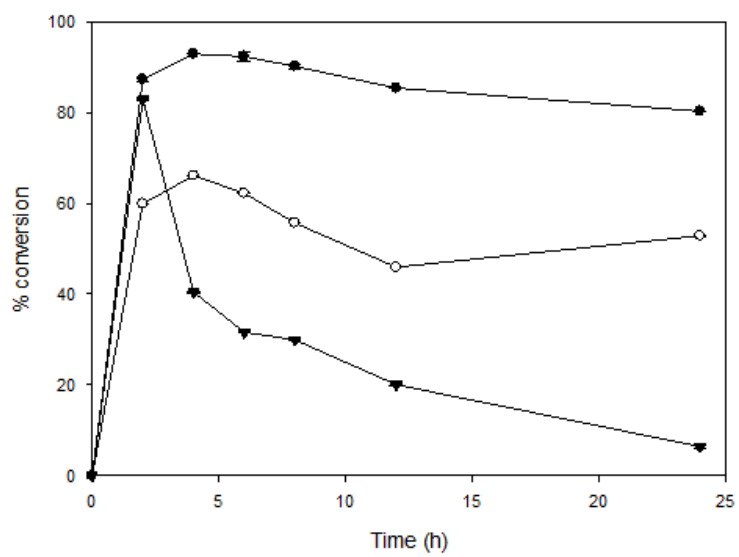
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