



HAL
open science

Synthesis of α -l-Araf and β -d-Galf series furanobiosides using mutants of a GH51 α -l-arabinofuranosidase

Jiao Zhao, Jérémy Esque, Isabelle André, Michael O'Donohue, Régis Fauré

► To cite this version:

Jiao Zhao, Jérémy Esque, Isabelle André, Michael O'Donohue, Régis Fauré. Synthesis of α -l-Araf and β -d-Galf series furanobiosides using mutants of a GH51 α -l-arabinofuranosidase. *Bioorganic Chemistry*, 2021, 116, pp.105245. <10.1016/j.bioorg.2021.105245>. <hal-03371339>

HAL Id: hal-03371339

<https://hal.science/hal-03371339v1>

Submitted on 8 Oct 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

Synthesis of α -L-Araf and β -D-Galf series furanobiosides using mutants of a GH51 α -L-arabinofuranosidase

Jiao Zhao, Jérémy Esque, Isabelle André, Michael J. O'Donohue*, Régis Fauré*

TBI, Université de Toulouse, CNRS, INRAE, INSA, Toulouse, France

* Corresponding authors.

E-mail addresses: michael.odonohue@inrae.fr (M.J. O'Donohue), regis.faire@insa-toulouse.fr (R. Fauré).

ABSTRACT

The GH-51 α -L-arabinofuranosidase from *Thermobacillus xylanilyticus* (TxAbf) possesses versatile catalytic properties, displaying not only the ability to hydrolyze glycosidic linkages but also to synthesize furanobiosides of α -L-Araf and β -D-Galf series. Herein, mutants are investigated to evaluate their ability to perform self-condensation, assessing both yield improvements and changes in regioselectivity. Overall yields of oligo- α -L-arabino- and oligo- β -D-galactofuranosides were increased up to 4.8-fold compared to the wild-type enzyme. In depth characterization revealed that the mutants exhibit increased transfer rates and thus a hydrolysis/self-condensation ratio in favor of synthesis. The consequence of the substitution N216W is the creation of an additional binding subsite that provides the basis for an alternative acceptor substrate binding mode. As a result, mutants bearing N216W synthesize not only (1,2)-linked furanobiosides, but also (1,3)- and even (1,5)-linked furanobiosides. Since the self-condensation is under kinetic control, the yield of homo-disaccharides was maximized using high substrate concentrations. In this way, the mutant R69H-N216W produced oligo- β -D-galactofuranosides in >70% yield. Overall, this study further demonstrates the potential usefulness of TxAbf mutants for glycosynthesis and shows how these might be used to synthesize biologically-relevant glycoconjugates.

Keywords: biocatalysis, self-condensation, retaining glycoside hydrolase, furanobiosides, D-galactofuranosides, regioselectivity, acceptor binding subsites

31

32 *Abbreviations:* L-Araf, α -L-arabinofuranosyl unit; α -L-ArafOpNP, 4-nitrophenyl α -L-
33 arabinofuranoside; D-Galf, β -D-galactofuranosyl unit; β -D-GalfOpNP, 4-nitrophenyl β -D-
34 galactofuranoside; rGH, retaining glycoside hydrolase; pNP, 4-nitrophenol; SA, specific
35 activity; TxAbf, α -L-arabinofuranosidase from *Thermobacillus xylanilyticus*; T/H,
36 transglycosylation/hydrolysis ratio.

37

38

Journal Pre-proofs

39 1. Introduction

40 Furanoses are carbohydrates displaying a five-membered ring structure and are ubiquitous in
41 Nature. An emblematic furanose is (2-deoxy-)D-ribofuranose, which forms an integral part of
42 nucleic acids. Other furanoses are also widespread, with both L-arabinofuranose (L-Araf) and
43 its 5-hydroxymethyl analog D-galactofuranose (D-Galf) frequently found in naturally occurring
44 oligo- and polysaccharides, but absent in mammalian glyco-motifs [1–4]. L-Araf moieties are
45 mostly associated with cell wall, intercellular-matrix and extracellular structures (e.g.
46 arabinoxylans [5,6], arabinogalactans [7] and arabinans [8,9]) in higher plant polysaccharides
47 [6,8,9], while D-Galf is widespread in glycoconjugates produced by pathogenic microorganisms
48 [1,3,10]. Notably, the galactan portion of mycobacterial arabinogalactan is formed by a linear
49 chain of alternating β -(1,5)- and β -(1,6)-linked D-Galf residues [11]. Other glycomotifs, such
50 as β -D-Galf-(1,2)- β -D-Galf are found in the mucins of the protozoan *Trypanosoma cruzi* [12]
51 and β -(1,3)-linked D-Galf moieties are present in oligosaccharidic structures of fungi [13–15]
52 and bacteria [16].

53 Unsurprisingly, the widespread nature of L-Araf and D-Galf makes these two furanose forms
54 interesting targets for synthetic chemistry. In the case of L-Araf-containing compounds, these
55 are of considerable interest for research purposes, particularly as substrates for plant cell wall
56 acting enzymes [17]. Similarly, since L-Araf forms part of antigenic epitopes in glycoconjugates
57 of several pathogenic organisms, it constitutes a target for synthesis aimed at the development
58 of diagnostic tools [18], or chemotherapeutic strategies [1].

59 In biological systems, furanose-containing carbohydrates are synthesized by arabino- and
60 galactofuranosyltransferases, enzymes that use nucleotide sugar donors [18–21]. However, for
61 *in vitro* synthesis, the use of these enzymes is challenging for a variety of reasons, despite recent
62 progress in the field [22–25]. Similarly, the use of well-established synthetic chemistry
63 strategies is fraught with difficulties, particularly because sugars in furanose configuration
64 display less thermodynamic stability than their pyranose counterparts [26–28]. An alternative
65 strategy to access furanose-bearing glycoconjugates is the use of transfuranosylases. Like
66 glycosyltransferases, transfuranosylases are enzymes that possess the ability to synthesize
67 furanose-containing compounds, using reactive furanosyl donor. However, unlike
68 glycosyltransferases, transfuranosylases belong to the glycoside hydrolase (GH) class of
69 enzymes and do not require the use of costly nucleotide sugars.

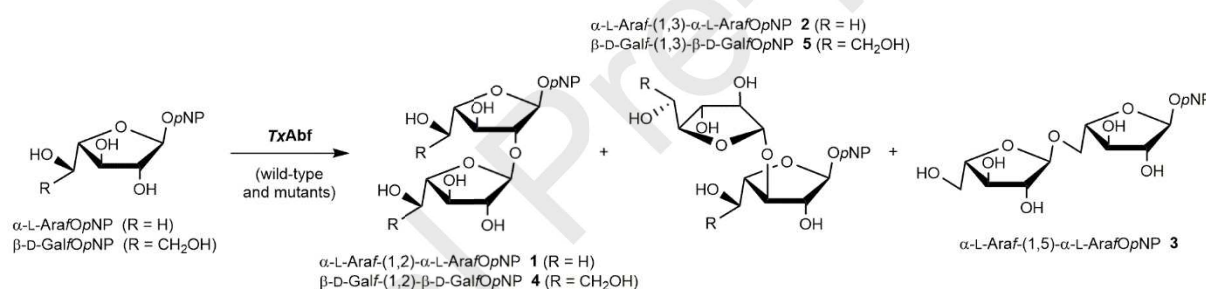
70 Transglycosylases are so-called retaining glycoside hydrolases (rGHs), meaning that these
71 enzymes employ a double displacement mechanism to break down and synthesize glycosidic
72 bonds [29]. In fact, transglycosylases are a specific subtype of rGHs that, while operating the
73 same catalytic mechanism as hydrolytic rGHs, display strong propensity for transglycosylation,
74 a reaction that involves the transfer of glycosyl donors onto glycoside acceptors. Regarding
75 transfuranosylases, to our knowledge naturally-occurring examples are very rare, with
76 transfructosylases being the most well-studied examples [30,31]. Nevertheless, hydrolytic α -L-
77 arabinofuranosidases belonging to the GH51 family and possessing some ability to catalyze
78 transarabinofuranosylation and transgalactofuranosylation have been described [2,32–36].
79 However, while α -L-arabinofuranosidases are relatively widespread and rarer in the GH
80 classification (GH43 and 51 are archetypal arabinofuranosidase families) [37], β -D-
81 galactofuranosidases are rarer, with just a few examples being known to date [38,39]. In
82 particular, using a large scale cloning, expression and screening approach, β -D-
83 galactofuranosidases were recently revealed in GH5 and 43 families [40], but so far little
84 biochemical characterization has been performed [41,42], thus it is too early to say whether
85 these enzymes display significant ability to perform transglycosylation reactions.

86 The GH51 α -L-arabinofuranosidase from *Thermotoga litoralis* (*TxAbf*) is a hydrolytic
87 rGH that is active on substrates bearing α -L-Araf-moieties (*e.g.* wheat, larchwood and oat spelt
88 heteroxylans) [43]. Like other rGHs, *TxAbf* hydrolyses glycosidic bonds using a two-step
89 displacement mechanism that involves the formation of a transient covalent glycosyl-enzyme
90 intermediate with the catalytic nucleophile residue [44]. Formation of this catalytic species is
91 achieved through the glycosylation step. This involves binding of the donor substrate to the
92 enzyme's active site and departure of donor leaving group, which is concomitantly protonated
93 by a catalytic base residue acting as a general acid. In the second so-called deglycosylation,
94 step, the glycosyl-enzyme is usually broken down by a water molecule that displays
95 nucleophilic character under the influence of a catalytic acid/base residue acting as a general
96 base [29,32]. However, deglycosylation can also occur when other suitable incoming species
97 adopt nucleophilic character. When deglycosylation is mediated by an incoming glycoside
98 moiety, a new glycosidic bond is formed and the reaction is described as transglycosylation.
99 Moreover, in the special case where the incoming acceptor species is the same as the donor
100 species, the reaction is described as self-condensation.

101 Regarding *TxAbf*, extensive studies have revealed that this enzyme is able to perform self-
102 condensation using L-Araf, D-Xylp and D-Galf as substrates, the latter reaching particularly

103 elevated yields [33,34]. Furthermore, *TxAbf* has been the subject of protein engineering work
 104 aimed at improving its ability to perform transglycosylation [45–47]. However, so far no
 105 particular focus has been put on improving the self-condensation reactions performed by *TxAbf*
 106 [33,34]. Previous work on self-condensation demonstrated that it was possible to synthesize α -
 107 L-arabino- and β -D-galacto-oligo-furanosides, but reported yields were modest and the
 108 regioselectivity of the reaction was mainly oriented towards the formation of (1,2)-linked
 109 regioisomers. While this regioselectivity might be useful for the synthesis of certain
 110 biologically-relevant α -L-arabino- and β -D-galactofurano-oligosaccharides, it is certainly not
 111 representative of the majority of medically-important glycoconjugates.

112 Herein, we focus on previously generated *TxAbf* mutants [47,48], using these to evaluate their
 113 ability to perform self-condensation. In the course of this study we have carefully characterized
 114 regioselectivity in order to reveal the potentiality of the different *TxAbf* mutants for use as
 115 synthetic tools to access a variety of α -L-arabinofurano- and β -D-galactofurano-containing
 116 oligosaccharides (Fig. 1).



117
 118 **Fig. 1.** Structures of the 4-nitrophenyl furanobiosides synthesized through self-condensation
 119 reactions catalyzed by *TxAbf* and its mutants. Compounds **1-3** and **4-5** were products of self-
 120 condensation using α -L-ArafOpNP and β -D-GalfOpNP as substrate respectively.
 121 Stereochemistry at C-5 position is depicted for D-Galf series (β -D-GalfOpNP and compounds **4**
 122 and **5**, with R = CH₂OH).

123

124 2. Materials and methods

125 2.1. Substrates and chemicals

126 The substrates 4-nitrophenyl α -L-arabinofuranoside (α -L-ArafOpNP) and 4-nitrophenyl β -D-
 127 galactofuranoside (β -D-GalfOpNP) were obtained from CarboSynth. Molecular biology
 128 reagents were purchased from New England BioLabs.

129

130 2.2. Mutagenesis, protein expression and purification

131 *In vitro* site-directed mutagenesis was achieved using the QuikChange II Site-Directed
132 Mutagenesis Kit (Agilent). The plasmid pET24-*TxAbf* (original pET vector was from Novagen)
133 containing the complete *TxAbf* coding sequence (GenBank accession no [CAA76421.2](https://www.ncbi.nlm.nih.gov/nuccore/CAA76421.2)) was
134 used as the DNA template for mutagenesis. Mutations were introduced using a series of
135 oligonucleotide primers [48]. Mutagenesis, expression and purification of *TxAbf* and mutants
136 thereof were performed as previously described [43,47,48]. Protein concentrations were
137 determined by measuring absorbance at 280 nm using a NanoDrop ND-2000 spectrophotometer.
138 Theoretical molecular weight and extinction coefficients were calculated using the ExPASy
139 ProtParam tool (<https://web.expasy.org/protparam/>).

140

141 2.3. Enzymatic assay

142 Enzyme activities were measured using a discontinuous assay [48]. Reactions were performed
143 in triplicate at 45 °C, using 5 mM β -D-Gal fOp NP as donor in 50 mM sodium phosphate buffer
144 at pH 7.0 with 1 mg·mL BSA, in a final reaction volume of 350 μ L. Prior to enzyme addition,
145 reaction mixtures were pre-incubated at 45 °C. Once launched, reactions were conducted for
146 10-20 min, removing 40 μ L samples at regular intervals. These samples were immediately
147 mixed with 200 μ L of 1 M Na₂CO₃ and placed on ice. The release of 4-nitrophenol (*p*NP) was
148 monitored at 401 nm using a spectrophotometer (Infinite M200 Microplate reader, Tecan) and
149 quantified using an appropriate standard curve, prepared using pure *p*NP. Negative controls
150 containing all of the reactants except the enzyme were used to correct for spontaneous
151 hydrolysis of the donor substrate. Initial reaction rates were determined from the linear regions
152 of time-dependent plots, which correspond to less than 15% consumption of the donor substrate.
153 One unit (IU) of enzyme specific activity (SA) corresponds to the amount of enzyme releasing
154 one μ mol of *p*NP per minute. The kinetic parameters K_M , k_{cat} and k_{cat}/K_M were determined by
155 measuring enzyme SA at various substrate concentrations. The β -D-Gal fOp NP was varied from
156 0.2-30 mM. A modified version of Michaelis-Menten equation (Table 2) was applied to fit the
157 nonlinear regression plot of SA versus [S] (substrate concentration) using SigmaPlot 11.0
158 software.

159

160

161

162 2.4. NMR analysis

163 To monitor reactions, ^1H NMR spectra were collected using a Bruker Avance II spectrometer
164 equipped with a TCI probe and operating at 500 MHz. Reactions were performed at 45 °C with
165 α -L-ArafOpNP or β -D-GalfOpNP containing appropriately diluted enzymes in a total volume of
166 600 μL (containing 10% D_2O in 10 mM sodium phosphate buffer at pH 7.0, v/v; with 1 $\text{mg}\cdot\text{mL}^{-1}$
167 BSA). To initiate reactions, a fixed (10% of total reaction volume) aliquot of enzyme solution
168 was added, with the enzyme concentration being chosen to reach maximal transglycosylation
169 yield within a timeframe that was similar for all reactions. In the α -L-ArafOpNP self-
170 condensation reaction, 0.003 μM wild-type TxAbf, 0.27 μM R69H-N216W, 0.45 μM R69H-
171 N216W-L352M and 1.01 μM F26L-R69H-N216W were used while, in the β -D-GalfOpNP self-
172 condensation reaction, 0.27 μM wild-type TxAbf, 4.00 μM R69H-N216W and 41.80 μM
173 R69H-N216W-L352M were used. Time course NMR monitoring was achieved by performing
174 pseudo-2D kinetics experiments based on a phase sensitive NOESY sequence with pre-
175 saturation, with spectra being accumulated every 8.7 min (2×32 scans). Using previously
176 determined SAs, it was possible to adjust enzyme quantities to suit a 18-h reaction time frame,
177 although the actual duration of the reaction was dependent on the enzyme used. The pH in 10%
178 D_2O was measured using a glass pH electrode, applying the modified equation $\text{pH}_{10\% \text{D}_2\text{O}} =$
179 $\text{pH}_{\text{electrode}} + 0.04$ [49]. The chemical shift reference was based on the HOD signal calibrated at
180 4.55 ppm at 45 °C [50].

181 Donor (*i.e.* pNP-glycofuranoside) consumption and the apparition of self-condensation
182 furanobiosides **1-5** were quantified by integrating the anomeric proton signals from the pNP-
183 linked furanosyl moiety (H-1a). For self-condensation products, the blinded anomeric protons
184 associated with the non-reducing end of the furanosyl unit (H-1b) were not taken into account,
185 because they overlap with the anomeric protons of the monosaccharide hydrolysis product. The
186 selected anomeric protons and their chemical shifts are shown in Table 1. Donor substrate was
187 quantified by integrating its relevant anomeric proton signals (Fig. 1 and Supplementary Figs.
188 S1-S5). The molarity of all compounds was normalized relative to the initial proton integral
189 of the donor compound. Yields of self-condensation products were maximal yields expressed
190 as a percentage of initial donor concentration. The mean value of the *ortho* and *meta* protons of
191 the linked pNP were regarded as the sum of the donor and self-condensation products. Thereby,
192 the overall yield of self-condensation products was calculated by subtracting the value of the
193 integral of the anomeric donor signal from half the value of the average integral of *ortho* and

194 *meta* aromatic protons of the linked *p*NP signals (Supplementary Fig. S3). In doing so, we were
 195 able to account even for minor products, which taken alone were barely detectable.

196

197 **Table 1**

198 Chemical shift of the substrates and self-condensation products **1-5** (Fig. 1) used for quantifying
 199 the evolution of enzymatic reaction.^a

Compound	δ (ppm)	
	H-1a	Aromatic H _m and H _o of <i>p</i> NP moiety
α -L-ArafOpNP	5.78	
1 α -L-(1,2)	5.89	8.20-8.18 and 7.18-7.16
2 α -L-(1,3)	5.81	
3 α -L-(1,5)	5.79	
β -D-GalfOpNP	5.74	
4 β -D-(1,2)	5.82	8.20-8.18 and 7.18-7.16
5 β -D-(1,3)	5.78	

200 ^a The chemical shifts were analyzed at 45 °C and pH 7 in buffered 10% D₂O. Chemical shifts
 201 of self-condensation furanobiosides **1-5** were referred to previous work [2,33,34,48].

202

203 *2.4. Modelling TxAbf and R69H-N216W-L352M in complex with self-condensation products*

204 Crystallographic data of the inactive TxAbf E176Q (PDB: 2VRQ) and the mutant R69H-
 205 N216W-L352M (PDB: 6ZT8) [48], were used to construct a complete 3D model of the mutant
 206 R69H-N216W-L352M. Next, α -L-Araf disaccharides (without *p*NP) were built using Glycam
 207 Force Field and tleap program from AMBER16 software suite [51]. For each α -L-Araf
 208 disaccharide, a short minimization in implicit solvent (Generalized Born) with 250 steps of
 209 steepest descent and 250 steps of conjugated gradient was performed, using a cut-off of 99 Å.
 210 *p*NP aglycon was added at the reducing end and geometrically optimized using Avogadro
 211 software [52]. β -D-Galf disaccharide structures were derived from the corresponding α -L-Araf
 212 disaccharides by adding hydroxymethyl on each α -L-Araf unit using Avogadro software. The
 213 complexes formed by the enzyme and the self-condensation products (**1-5**) were initially
 214 assembled in PyMol [53] by pair fitting onto bound α -L-Araf (occupying subsite -1) in X-ray
 215 structure of the inactive TxAbf E176Q (PDB: 2VRQ). Complexes were further subjected to
 216 energy minimization macro supplied in the YASARA package (version 12.8.1) [54], using

217 AMBER and GAFF Force Fields for the protein and the ligand, respectively. The figure was
218 then prepared using PyMol Molecular Graphics System, v0.99 (Schrödinger).

219

220 3. Results

221 3.1. Kinetic analysis of *TxAbf* mutants

222 Measurement of steady state kinetic parameters of α -L-ArafOpNP conversion (monitoring *p*NP
223 release) catalyzed by *TxAbf* and its mutants revealed that all mutations severely affected
224 catalytic efficiency (Table 2). This was also the case for *TxAbf* acting on β -D-GalfOpNP. The
225 values were reduced by more than two and three orders of magnitude in reactions containing α -
226 L-ArafOpNP and β -D-GalfOpNP respectively. Comparing the k_{cat}/K_M values of reactions
227 catalyzed by the wild-type enzyme acting on either of the two substrates (α -L-ArafOpNP and
228 β -D-GalfOpNP) revealed a more than 6 000-fold difference. Regarding the reaction involving
229 α -L-ArafOpNP and R69H-N216W-L352M the K_M value was relatively similar to that of the
230 reaction catalyzed by the wild-type enzyme, whereas the same reaction catalyzed by F26L-
231 R69H-N216W is characterized by significant decreases in both k_{cat} and K_M . Assuming that
232 deglycosylation is rate-limiting, the lowered k_{cat} value leads us to postulate that R69H-N216W-
233 L352M is mostly impaired with respect to deglycosylation, whereas the impact of F26L-R69H-
234 N216W appears to be more complex, affecting both steps [47]. Regarding the reaction
235 involving R69H-N216W and β -D-GalfOpNP, a two-phase reaction profile was observed that
236 did not reach saturation (Fig. 2) [47,48]. Remarkably, unlike wild-type *TxAbf*, the mutant
237 R69H-N216W yielded a measurable K_M value (0.45 mM) for the reaction containing β -D-
238 GalfOpNP, which is in the same order of magnitude to that of wild-type *TxAbf* acting on α -L-
239 ArafOpNP (0.25 mM). Nevertheless, because the k_{cat} value of the reaction is also extremely
240 low, the overall catalytic efficiency of R69H-N216W acting on β -D-GalfOpNP is unchanged
241 compared to the reaction involving wild-type *TxAbf*.

242

243

244

245

246

247

248 **Table 2**

249 Kinetic parameters of reactions catalyzed by *TxAbf* and mutants thereof, using α -L-Araf/OpNP
 250 or β -D-Galf/OpNP as substrate.

Enzyme	SA _{th} (IU·mg ⁻¹)	K _M (mM)	k _{cat} (s ⁻¹)	k _{cat} /K _M (s ⁻¹ ·mM ⁻¹)	N _S ^a (s ⁻¹ ·mM ⁻¹)
<u>α-L-Araf/OpNP</u>					
<i>TxAbf</i> ^b	615	0.72	575	795	-
<i>TxAbf</i> ^c	145	0.25	139	556	-
R69H-N216W-L352M ^c	0.60	0.48	0.58	1.21	0.034
F26L-R69H-N216W ^c	0.16	0.01	0.15	10.81	0.034
<u>β-D-Galf/OpNP</u>					
<i>TxAbf</i> ^b	15.6	>50 ^d	15.02	0.13	-
R69H-N216W ^e	0.065	0.45	0.06	0.14	0.008

251 ^a N_s is a nonspecific constant that is included in the modified Michaelis-Menten equation to
 252 account for activation of the enzyme by the self-condensation product: $SA_{app.} = SA_{th.} \cdot [S] / (K_M$
 253 $+ [S]) + N_S \cdot [S]$ where SA_{th.} is the theoretical maximum activity achieved if the enzyme operates
 254 according to the Michaelis-Menten model.

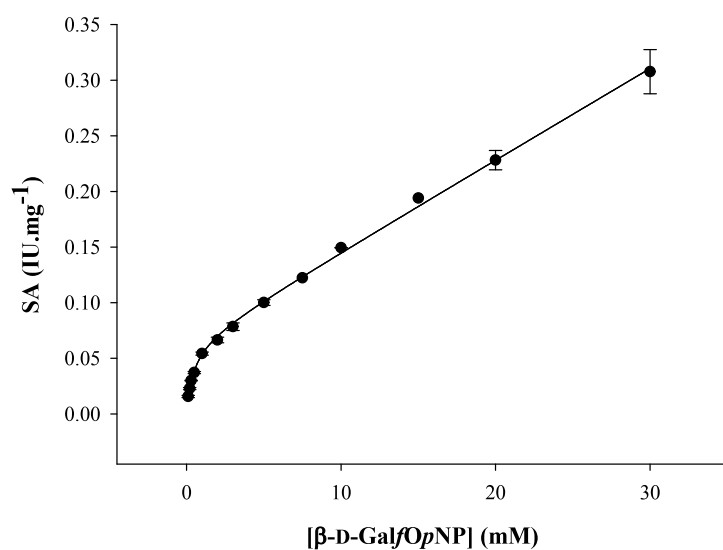
255 ^b The kinetic parameters were referred to previous work at 60 °C in 50 mM sodium acetate
 256 buffer pH 5.8 [55,56].

257 ^c The kinetic parameters were referred to previous work at 45 °C in 50 mM sodium phosphate
 258 buffer pH 7. ⁴⁸¹

259 ^d The K_M v estimate, because its measurement was unfeasible due to the limited
 260 solubility of the substrate.

261 ^e Kinetic assays were carried out in triplicate at 45 °C in 50 mM sodium phosphate buffer pH
 262 7.0. Relative errors were inferior to 10%.

263



264

265 **Fig. 2.** Specific activity (SA) as a function of β -D-GalOpNP concentration for R69H-N216W.

266

267 3.2. Self-condensation profile using α -L-ArafOpNP as donor

268 When using TxAbf, the yield of the major self-condensation product **1**, α -L-Araf-(1,2)- α -L-
 269 ArafOpNP (Fig. 1 and Table 3), is consistent with that previously reported [34]. This compound
 270 was accompanied by trace amounts of compounds **2** and **3**, corresponding to (1,3) and (1,5)
 271 linkages respectively. Compared to TxAbf, the three R69H-N216W-containing mutants
 272 exhibited enhanced ability to promote self-condensation, with overall yields being 3.5- to 3.8-
 273 fold higher. Moreover, increased yield was coupled to significant changes in enzyme
 274 regioselectivity, with the proportion (% of total yield) of (1,3)-linked product **2** increasing from
 275 30% (TxAbf) to >70% in reactions catalyzed by the mutants, and even reaching 82% in the case
 276 of R69H-N216W. Overall, the three mutants were similar in terms of both regioselectivity and
 277 yields.

278 Using real time NMR spectroscopy (Supplementary Fig. S1), it was possible to monitor the
 279 evolution of each self-condensation product, and thus observe secondary hydrolysis during the
 280 latter stages of the reaction. For wild-type TxAbf (Fig. 3A and Supplementary Fig. S4A), the
 281 maximum yield (7%) of the major product **1** was reached at 68% conversion of α -L-ArafOpNP,
 282 meaning that hydrolysis was the predominant reaction. Concerning the R69H-N216W-
 283 containing mutants, maximal yields were achieved at later stages in the reaction, when the
 284 conversion of α -L-ArafOpNP was higher, *i.e.* 88, 90 and 92% for F26L-R69H-N216W, R69H-
 285 N216W and R69H-N216W-L352M respectively (Fig. 3B-D and Supplementary Fig. S4B-
 286 D). This means that compared to wild-type TxAbf, secondary hydrolysis was delayed in

287 reactions catalyzed by the mutants. When comparing the decomposition rate of major
 288 compound **2**, it is clear that R69H-N216W performs secondary hydrolysis (v_{HII}) at a higher rate
 289 than R69H-N216W-L352M and F26L-R69H-N216W (Supplementary Table S1). Additionally,
 290 the overall self-condensation yield of this double mutant is slightly lower than that of the other
 291 two mutants. However, as a synthetic tool, R69H-N216W can be considered the best all-round
 292 performer. This conclusion is based on its combined performance: yield of the major product
 293 **2**, synthesis rate v_{S} and SA (Table 3 and Supplementary Table S1). Finally, R69H-N216W-
 294 L352M displayed the highest self-condensation/secondary hydrolysis ($v_{\text{S}}/v_{\text{HII}}$) ratio with a value
 295 of 3.0 (Supplementary Table S1).

296

297 **Table 3**298 Maximal self-condensation yields (in %) using α -L-ArafOpNP as donor.

Enzyme	SA (IU·mg ⁻¹) ^a	1 α -L-(1,2)	2 α -L-(1,3)	3 ^b α -L-(1,5)	Overall yield ^c
<i>TxAbf</i>	261.79 ± 10.72	7	3	2	10
R69H-N216W	1.60 ± 0.02	6	37	8 (3)	45
R69H-N216W-L352M	0.97 ± 0.11	10	34	6 (3)	47
F26L-R69H-N216W	0.32 ± 0.01	6	38	8 (4)	48

299 ^a Specific activities (SA) referred to previous work at 45 °C in 50 mM sodium phosphate buffer
 300 pH 7.0 containing 5 mM α -L-ArafOpNP and were measured by monitoring *p*NP release using
 301 a discontinuous assay.

302 ^b Since maximal yield of product **3** was reached later than those of products **1** and **2**, the yield
 303 of **3** when **1** and **2** are maximum is shown in brackets.

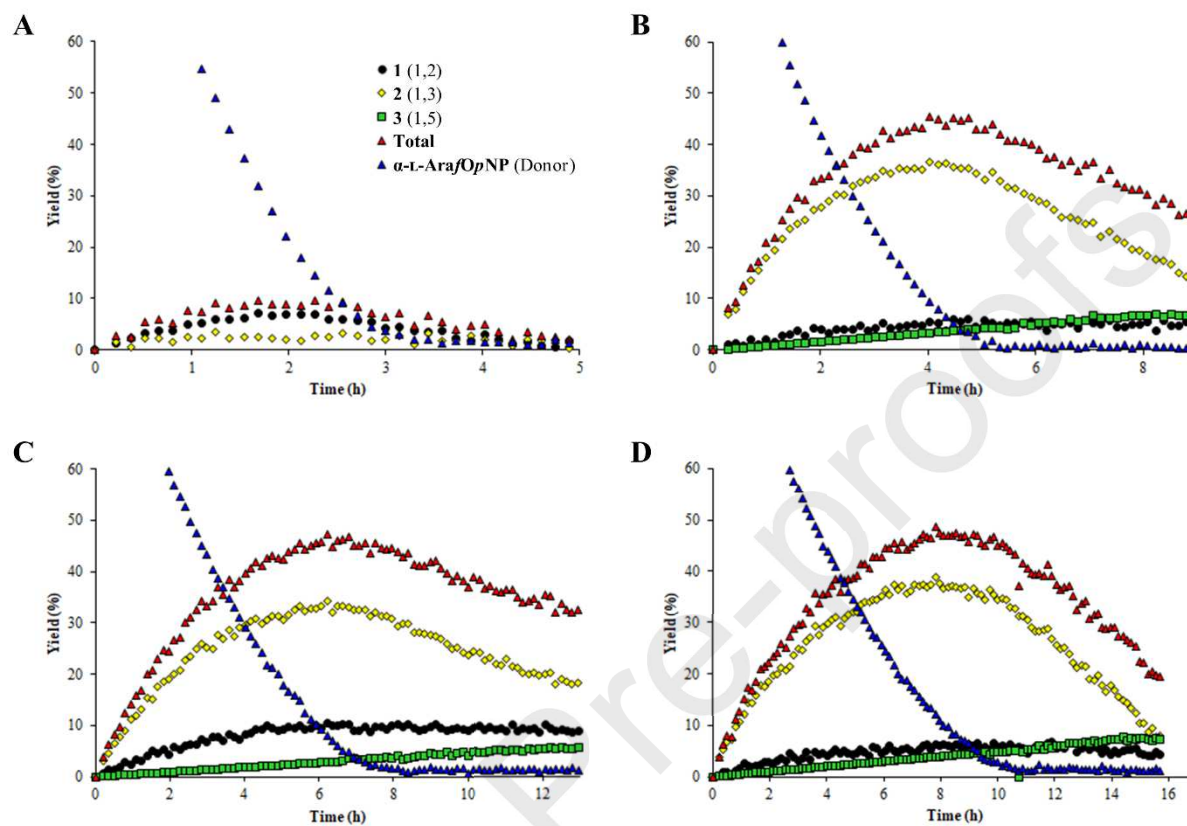
304 ^c Maximal yields of each disaccharide are reached after different incubation times. This explains
 305 why overall yields are not equal to the sum of the maximum yield of each disaccharide.

306

307 In absolute terms, the yield of α -L-Araf-(1,5)- α -L-ArafOpNP **3** was also slightly (3- or 4-fold)
 308 increased in reactions catalyzed by the mutants. However, in relative terms the proportion of
 309 compound **3** was slightly reduced from 20% of total yield (*TxAbf*) to as little as 6% (R69H-
 310 N216W-L352M) when maximal total transglycosylation yield was reached. Significantly,
 311 although the yield of compound **3** is low for the mutants, it continues to be produced, even when
 312 α -L-ArafOpNP is consumed (Fig. 3B-D). This observation suggests that at later stages in the

313 reaction, compound **3** is formed via hydrolysis of compounds **1** and **2** and the rate of hydrolysis
 314 of compound **3** is inferior to that of its synthesis.

315



316
 317 **Fig. 3.** Monitoring of α -L-ArafOpNP consumption and the apparition of the different self-
 318 condensation products **1-3** as a function of time in reactions catalyzed by (A) TxAbf, (B) R69H-
 319 N216W, (C) R69H-N216W-L352M, and (D) F26L-R69H-N216W.

320

321 3.3. Self-condensation profile using β -D-GalfOpNP as donor

322 Despite being a poor substrate for TxAbf-mediated hydrolysis, β -D-GalfOpNP readily
 323 participated in the self-condensation reaction (Fig. 1), procuring a 24% overall yield of
 324 oligosaccharide products (Table 4). The major product **4**, β -D-Galf-(1,2)- β -D-GalfOpNP (18%
 325 yield), represents 75% of the regioisomeric mixture, revealing the good regioselectivity of wild-
 326 type TxAbf in this reaction. This observation is highly consistent with previous work performed
 327 both on TxAbf [33] and other GH51 Abf [2] and further confirms the natural propensity of the
 328 wild-type enzyme to hydrolyze and synthesize (1,2)-linkages. Characterization of mutants
 329 containing single substitutions (R69H and N216W) revealed that these display
 330 regioselectivities and yields similar to those of TxAbf (Table 4, Fig. 4A and Supplementary Fig.

331 S5). However, combination of these point mutations (*i.e.* R69H-N216W and R69H-N216W-
 332 L352M) yielded enzymes that double the total product yield and procure regioisomeric mixtures
 333 containing a (1,3)-linked disaccharide **5** at a yield of 18 (R69H-N216W-L352M) and 22%
 334 (R69H-N216W) respectively. In this respect, the yield of product **4** was relatively unaltered,
 335 meaning that the gains in overall yield can be completely attributed to the vast improvement in
 336 the enzyme's ability to form regioisomer **5** (Table 4 and Supplementary Fig. S2).

337

338 **Table 4**339 Maximal self-condensation yields (in %) using β -D-Gal/OpNP as donor.

Enzyme	SA (IU·mg ⁻¹) ^a	4		5		Overall yield ^b
		β -D-(1,2)	β -D-(1,3)	β -D-(1,2)	β -D-(1,3)	
TxAbf	1.23 ± 0.09	18	2	24		24
R69H	0.05 ± 0.0003	18	3	24		24
N216W	1.33 ± 0.035	21	3	24		24
R69H-N216W	0.09 ± 0.001	15	22	47		47
R69H-N216W-L352M	0.025 ± 0.001	16	18	45		45

340 ^a SA was measured by monitoring *p*NP release using a discontinuous assay. Assays were carried
 341 out at 45 °C in 50 mM sodium phosphate buffer pH 7.0 using 5 mM β -D-Gal/OpNP.

342 ^b Overall yield is higher than the sum of compound **4** and **5** due to the presence of small amounts
 343 of β -D-(1,5) and β -D-(1,6)-linked disaccharides and traces of *p*NP oligo- β -D-galactofuranosides
 344 displaying degrees of polymerization >2.

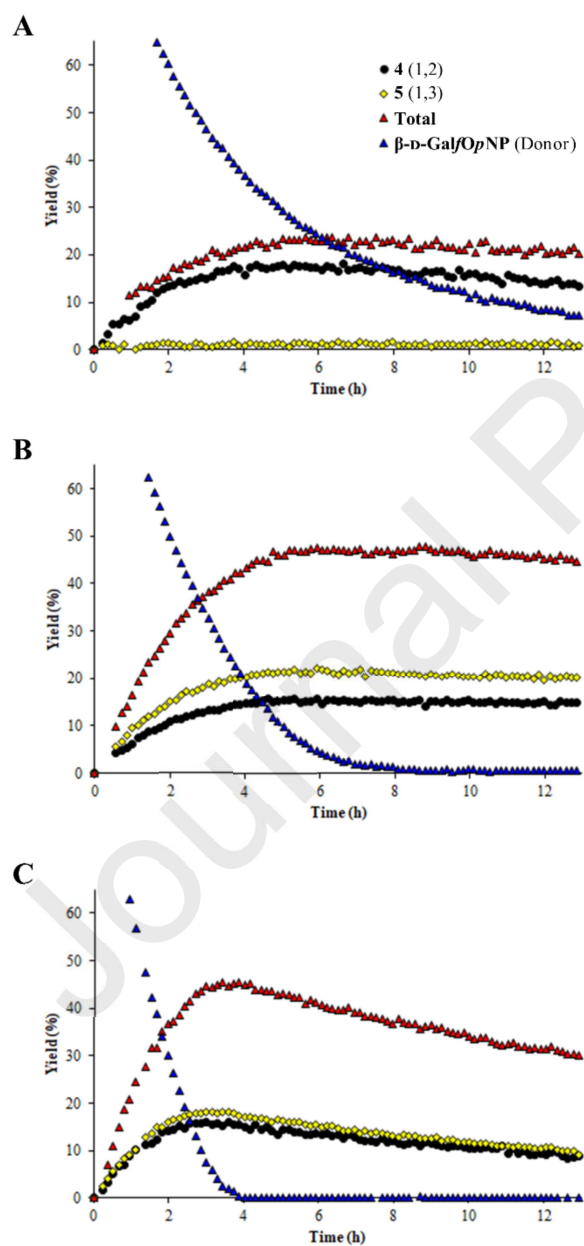
345

346 In reactions catalyzed by wild-type TxAbf, the maximum yield of galactofuranobiosides **4** and
 347 **5** was reached when 70% of the substrate had been consumed. However, in the case of the
 348 mutants, this point was shifted to 92 (R69H-N216W-L352M) and 96% (R69H-N216W)
 349 respectively. Despite this difference between reactions catalyzed by wild-type and mutant
 350 enzymes, it is noteworthy that in all three cases the self-condensation products displayed
 351 remarkable stability, meaning that secondary hydrolysis was strongly diminished, even at very
 352 low donor substrate concentrations (Fig. 4B-C). This was particularly noticeable for the reaction
 353 involving the mutant R69H-N216W (Fig. 4B and Supplementary Fig. S6), since product
 354 decomposition was barely perceptible. Therefore, accounting for the additional fact that R69H-

355 N216W displayed 3.6-fold higher catalytic activity than the triple mutant (Table 4), this enzyme
356 can be regarded as the best performing enzyme in terms of β -D-Gal $fOpNP$ self-condensation.

357 Although the major products of α -L-Araf $fOpNP$ and β -D-Gal $fOpNP$ self-condensation were
358 disaccharides, in reactions catalyzed by *TxA*b enzymes (more for the mutants), trisaccharides
359 were also observed. However, due to the small quantity produced and the fact that NMR signals
360 overlapped with those of the disaccharides, these trisaccharides were neither quantified, nor
361 fully characterized. Nevertheless, their detection further demonstrates the aptitude of the mutant
362 enzymes to synthesize L-Araf and D-Gal f -oligosaccharides.

363



364

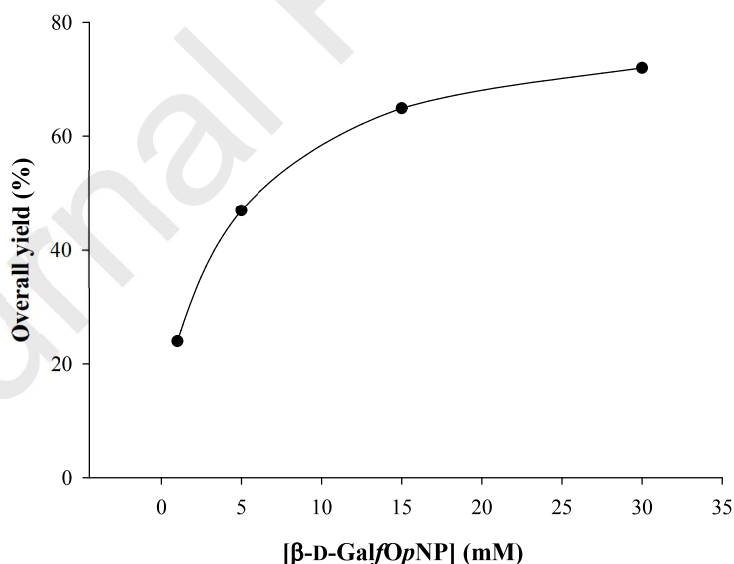
365 **Fig. 4.** Monitoring of β -D-Gal β NP consumption and the apparition of the different self-
366 condensation products **4-5** as a function of time in reactions catalyzed by (A) *TxA*bf, (B) R69H-
367 N216W, and (C) R69H-N216W-L352M.

368

369 3.4. Probing the relationship between substrate concentration and global synthesis yield

370 In view of the catalytic superiority of R69H-N216W for the self-condensation of β -D-Gal β NP,
371 this enzyme was employed to explore how substrate concentration influences the global yield
372 of products (Fig. 5). Increasing substrate concentration clearly elevated both total self-
373 condensation yield and the rate of donor conversion into self-condensation products (Fig. S6).
374 Moreover, increasing β -D-Gal β NP concentration delayed the (tipping) point at which
375 secondary hydrolysis became dominant, outweighing self-condensation. Consequently, the
376 linear phase of self-condensation of the R69H-N216W-catalyzed reactions was extended at
377 higher substrate concentration, meaning that the reaction is mainly under kinetic control. An
378 overall yield of 72% was achieved when using 30 mM of β -D-Gal β NP. Considering substrate
379 solubility, this is close to the maximum achievable yield in an aqueous reaction system.

380



381 **Fig. 5.** Relationship between β -D-Gal β NP substrate concentration and overall self-
382 condensation yield in the R69H-N216W-catalyzed reaction.

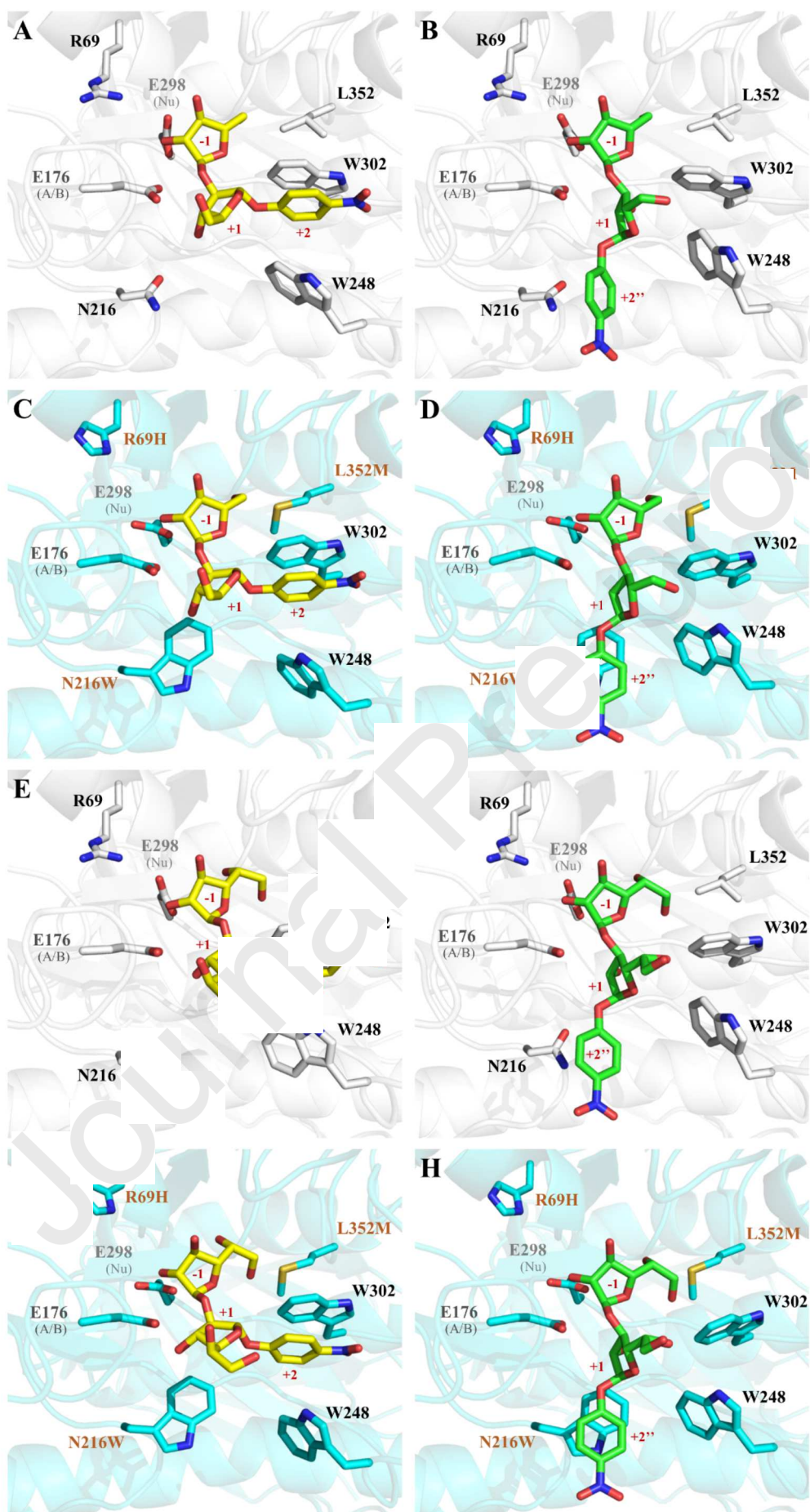
384

385

386 3.5. Getting molecular insight on the regioselectivity of the mutant enzymes

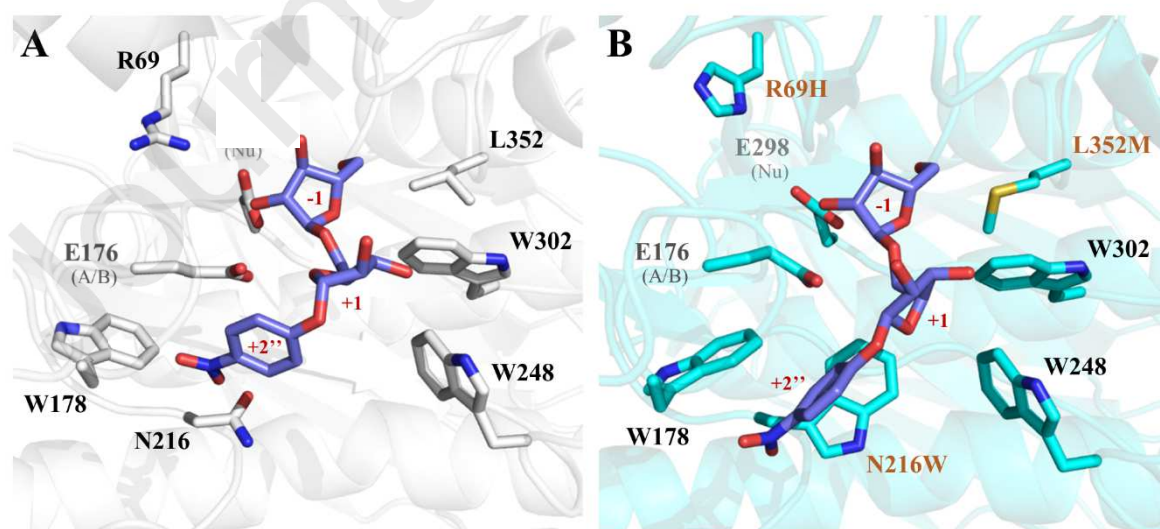
387 To investigate the regioselectivity of the mutant enzymes, 3D models of *TxA*b_f and R69H-
388 N216W-L352M complexed to self-condensation products **1-5** were built (Fig. 6). Strikingly,
389 analysis of the models reveals that mutation of Asn216 by a tryptophan residue (N216W)
390 engenders cleft topology better-adapted for binding to the more linear (1,3)-linked
391 furanobiosides. The presence of tryptophan provides a putative stacking interaction with *p*NP
392 group. This observation is fully consistent with the experimental results associated with both
393 the double and triple mutants. Moreover, 3D models suggest that the nature of the furanosyl
394 moieties (*L*-Araf vs *D*-Gal_f) does not significantly alter product positioning and binding
395 interactions in the active site, consistent with the fact that the impact of N216W on
396 regioselectivity is equivalent when either α -*L*-Araf_{Op}NP or β -*D*-Gal_{Op}NP are used as
397 substrates. Comparing binding of the (1,2)- and (1,3)-linked compounds, occupation of subsite
398 -1 is globally very similar, probably because the spatial confinement of this pocket-like subsite,
399 combined with a significant number of specific enzyme-substrate interactions therein does not
400 provide room for alternative binding modes [57]. However, some differences are observed in
401 subsite +1 depending on the nature of the furanosyl moiety occupying the site and on how the
402 *p*NP moiety is accommodated (Fig. 6). In the case of (1,2)-linked compounds, 3D models
403 suggest that both the wild-type and mutant enzymes accommodate the *p*NP moiety in subsite
404 +2 [57], which contains residue Trp302, likely establishing a π - π stacking interaction (distances
405 < 3.8 Å) with the *p*NP moiety (Fig. 6A, C, E and G). Regarding (1,3)-linked products, the model
406 reveals that the *p*NP moiety lies in a different spatial position and thus occupies an alternative
407 subsite, designated here as +2''. In the wild-type enzyme, subsite +2'' contains Asn216 (Fig.
408 6B and F), where this residue is substituted by a tryptophan in the mutant enzymes. This
409 forms a novel hydrophobic platform, favorable for a π - π interaction (distances < 4.1 Å) with
410 the *p*NP moiety (Fig. 6D and H). Consequently, this modification putatively creates a new
411 binding mode for (1,3)-linked furanobiosides that is absent in wild-type *TxA*b_f. It is noteworthy
412 that the emergence of an alternative subsite +2'' in the mutant enzymes does not alter subsite
413 +2, which explains why the mutants do not lose their ability to form (1,2)-linked self-
414 condensation products. Indeed, the initial interactions of *p*NP from compound **1** and **4** with Trp
415 248, Trp302 and putatively Lys251 are retained for R69H-N216W-L352M (Supplementary Fig.
416 S7).

417



419 **Fig. 6.** Wild-type *TxAbf* (grey) and R69H-N216W-L352M (cyan) bound to products **1** or **4**
 420 (yellow) and **2** or **5** (green): (A) *TxAbf* bound to its major product **1**, α -L-Araf-(1,2)- α -L-
 421 ArafOpNP; (B) *TxAbf* bound to its minor product **2**; (C) R69H-N216W-L352M bound to its
 422 minor product **1**; (D) R69H-N216W-L352M bound to its major product **2**, α -L-Araf-(1,3)- α -L-
 423 ArafOpNP; (E) *TxAbf* bound to its major product **4**, β -D-Galf-(1,2)- β -D-GalfOpNP; (F) *TxAbf*
 424 bound to its minor product **5**; (G) R69H-N216W-L352M bound to its minor product **4**; and (H)
 425 R69H-N216W-L352M bound to its major product **5**, β -D-Galf-(1,3)- β -D-GalfOpNP. The
 426 protein is shown in cartoon, whereas the key labelled residues and the ligands are shown in
 427 stick representation. Mutations and subsites are labelled (brown and red characters respectively).
 428

429 Finally, experimental data revealed that, in the presence of α -L-ArafOpNP, mutant enzymes
 430 displayed greater ability to produce (1,5)-linked products. 3D modelling of α -L-Araf-(1,5)- α -L-
 431 ArafOpNP **3** shows that this disaccharide adopts a conformation that is similar (albeit more
 432 extended) to that of α -L-Araf-(1,3)- α -L-ArafOpNP **2**. Accordingly, it is plausible that these
 433 compounds occupy the same subsites and that the production of compound **3** is increased by
 434 the mutation N216W and the unveiling of subsite +2'' (Fig. 7). However, considering the
 435 experimental and *in silico* data, it is likely that the linkage of pNP and its orientational
 436 constraints in compound **3** leads to less favorable interactions in subsite +2'', in particular with
 437 respect to residue Trp216 compared to compound **2** (parallel π - π stacking in Fig. 6D and H).
 438



439
 440 **Fig. 7.** Enzymes bound to the (1,5)-linked product **3**: (A) wild-type *TxAbf* and (B) R69H-
 441 N216W-L352M. The protein is shown in cartoon, whereas the key labelled residues and the

442 ligands are shown in stick representation. Mutation and subsites are labelled (brown and red
443 characters respectively).

444

445 **4. Discussion**

446 The α -L-arabinofuranosidase, *TxAbf* is probably the best studied member of GH51, especially
447 regarding its ability to perform glycosynthesis [33,34,45–48,58,59]. So far, we have shown that
448 this enzyme possesses a wide range of capabilities, including the ability to synthesize D-
449 galactofuranosides and is relatively amenable to enzyme engineering [33]. In the present study,
450 we have revisited some of the more fundamental features of *TxAbf*, in particular its ability to
451 catalyze self-condensation reactions using either pento- or hexo-furanosides, focusing on the
452 way in which mutagenesis of key residues modifies this property.

453

454 *4.1. Key factors favoring self-condensation*

455 Both mutations F26L and L352M have been reported to enhance the ability of *TxAbf* to perform
456 transglycosylation [46–48,60]. Moreover, in the case of L352M, we have shown that
457 modification of *TxAbf* properties is due to quite complex long-range effects that affect both the
458 negative and positive subsites, whereas F26L only affects subsite -1 [48]. Here, we reveal that
459 neither of these mutations produce major effects on self-condensation when compared to the
460 more determining effect of the double mutation R69H-N216W. Clearly, these latter mutations
461 are responsible for the most overall improvements in self-condensation reported in this work
462 and, in the case of N216W mutation, for the acquisition of enhanced aptitude to form (1,3)-
463 linked furanobiosides and α -L-(1,5)-linked arabinofuranobiosides. It is noteworthy that
464 mutation R69H alone does not enhance the self-condensation ability of β -D-Gal/*OpNP*
465 compared to the wild-type *TxAbf*. Compared to α -L-Ara/*OpNP*, the global repositioning of β -
466 D-Gal/*OpNP* within subsites -1 of wild-type *TxAbf* and the R69H-mutated enzyme is caused
467 by the presence of the extra hydroxymethyl moiety at C-5. However, in R69H disruption is
468 further compounded by the presence of H69, which impacts the positioning of both catalytic
469 residues [47,48]. However, regarding self-condensation these two factors clearly do not produce
470 a positive additive effect.

471

472

473 4.2. Improving self-condensation of β -D-Gal/OpNP

474 One of the aims of this work was to improve the ability of TxAbf to synthesize D-galactofurano-
475 oligosaccharides. In previous work, we have shown that it is extremely difficult to determine
476 the K_M value for reactions containing β -D-Gal/OpNP, catalyzed by the wild-type enzyme
477 [33,55,56]. Moreover, performing both STD-NMR and ITC failed to reveal any measurable
478 interactions between TxAbf and β -D-Gal/OpNP [55]. Therefore, it was extremely gratifying to
479 observe that the double mutant R69H-N216W not only improves self-condensation of β -D-
480 Gal/OpNP, but also provides the basis to measure a K_M value that is similar to that measured
481 for the wild-type enzyme in the presence of α -L-Ara/OpNP. Since the constant $K_M (= [k_3 \cdot (k_{-1} +$
482 $k_2)]/[k_1 \cdot (k_2 + k_3)])$ is composed of both an affinity component ($1/K_d = k_1/k_{-1}$) and constants
483 related to both glycosylation (k_2) and deglycosylation (k_3) steps, it is difficult to further analyze
484 this improvement [32,61]. However, considering that β -D-Gal/OpNP possesses a good leaving
485 group, we postulate that improvements in self-condensation of this compound are in part due
486 to improved substrate binding.

487

488 4.3. Insights related to secondary hydrolysis

489 Data related to the mutants investigated herein supply insight on how secondary hydrolysis of
490 glycosynthetic products might be modulated. In the case of the synthesis of D-galactofuranosyl
491 disaccharides, secondary hydrolysis is clearly reduced or even imperceptible. One reason for
492 this might be suboptimal positioning of the scissile inter-glycosidic bond with respect to the
493 catalytic residues. In this regard, comparison of the 3D models of TxAbf enzymes bound to
494 compounds **1**, **2**, **4** and **5** reveals that the oxygen atom of the scissile bond of the self-
495 condensation products are similarly distant (1.7-2.0 Å) from protonated Glu176 (acid/base).
496 Likewise, the anomeric carbon is similarly located at 3.0-3.4 Å from Glu298 (nucleophile)
497 (Supplementary Fig. S8). Therefore, in the absence of visible differences between the self-
498 condensation product/enzyme pairs, it is plausible that the scissile inter-glycosidic bond in the
499 D-galactofuranosyl disaccharides is dynamically mis-orientated (*i.e.* during conformational
500 preactivation [62,63]) for hydrolysis. This might be the result of the additional
501 hydroxymethyl group at the C-5 position on the furanose ring bound in subsite -1 and/or enzyme
502 mutations.

503 It is worth recalling that compared to R69H-N216W, the introduction of L352M or F26L
504 reveals decreased secondary hydrolysis (v_{HII}) of product **2** (Supplementary Table S1). Similarly,

505 the synthesis rates of **2** are decreased, especially for F26L-containing triple-mutant, although
506 exactly how this affects both self-condensation and secondary hydrolysis is unclear. However,
507 modification of the internal volume of the pocket-like subsite -1 and/or the interactions with
508 the donor substrate might modulate the behavior of the triple-mutants.

509

510 *4.4. Introduction of a tryptophan at position 216 forms a new binding subsite*

511 A key finding in this study is the emergence of new regioselectivity, yielding (1,3)-linked
512 pentofurano- and hexofurano-disaccharides. The introduction of tryptophan at position 216
513 contributes to this, although as a standalone mutation N216W is insufficient to procure altered
514 regioselectivity. Nevertheless, regarding N216W, introduction of a tryptophan provides the
515 basis for a new π - π interaction between the planar indolyl ring and the *p*NP aglycon moiety. It
516 is relevant that the introduction of tryptophan into other GHs has also produced changes in
517 regioselectivity, either by forcing the repositioning of the glycoside acceptor [64], or by creating
518 a favorable interaction with the acceptor or its linked aromatic moiety [65,66]. It is also
519 noteworthy that in *TxA*bF, unveiling of the new subsite +2'' does not interfere with the original
520 subsite +2. This infers that it could be possible through further enzyme engineering to block the
521 subsite +2 and thus obtain an enzyme that procures mainly (1,3)-linked pentofurano- and
522 hexofurano-disaccharides, perhaps with small traces of (1,5)-linked compounds; or inversely
523 for the synthesis of (1,2)-linked oligosaccharides.

524

525 **5. Conclusion**

526 This study contributes to our understanding of how *TxA*bF catalyzes self-condensation of pento-
527 and hexofurano- and provides new mutants that can be added to the growing toolbox of
528 biocatalysts suitable for glycosynthesis. Self-condensation reactions remain under kinetic
529 control, meaning that high yields can be obtained using higher substrate concentrations, and
530 that regioselectivity can be quite efficiently modulated. Bearing this in mind, it should be
531 possible in the very near future to define reactions catalyzed by mutated enzymes that will
532 procure high yields of relatively pure (1,3)-linked regioisomers. Likewise, with the knowledge
533 of how to enhance the formation of (1,3)-linked regioisomers, it should also be possible to
534 repress their formation, while identifying ways to enhance the regioselective formation of (1,2)
535 linkages.

536

537 Acknowledgments

538 NMR analyses were performed using facilities at MetaToul (Metabolomics & Fluxomics
539 Facilities, Toulouse, France, www.metatoul.fr), which is part of the national infrastructure
540 MetaboHUB (The French National infrastructure for metabolomics and fluxomics,
541 www.metabohub.fr) and is supported by grants from the Région Midi-Pyrénées, the European
542 Regional Development Fund, SICOVAL, IBiSa-France, CNRS, and INRAE.

543

544 Funding

545 The PhD fellowship of J. Zhao was supported by CSC (China Scholarship Council).

546

547 Declaration of Competing Interest

548 The authors declare no conflict of interest.

549

550 Author contributions

551 R.F. and M.J.O designed and supervised the study. J.Z. performed the biochemical experiment.
552 J.Z., J.E. and I.A. performed the docking experiment. All authors analyzed data and contributed
553 to write the manuscript.

554

555 Appendix A. Supplementary material

556 Supplementary data for this article can be found online at .

557

558 References

- 559 [1] P. Peltier, R. Euzen, R. Daniellou, C. Nugier-Chauvin, V. Ferrières, Recent knowledge
560 and innovations related to hexofuranosides: structure, synthesis and applications,
561 *Carbohydr. Res.* 343 (2008) 1897–1923. <https://doi.org/10.1016/j.carres.2008.02.010>.
- 562 [2] I. Chlubnová, D. Filipp, V. Spiwok, H. Dvoráková, R. Daniellou, C. Nugier-Chauvin, B.
563 Králová, V. Ferrières, Enzymatic synthesis of oligo-D-galactofuranosides and L-
564 arabinofuranosides: from molecular dynamics to immunological assays., *Org. Biomol.*
565 *Chem.* 8 (2010) 2092–2102. <https://doi.org/10.1039/b926988f>.
- 566 [3] L.L. Pedersen, S.J. Turco, Galactofuranose metabolism: a potential target for
567 antimicrobial chemotherapy, *Cell. Mol. Life Sci.* 60 (2003) 259–266.

- 568 <https://doi.org/10.1007/s000180300021>.
- 569 [4] J.B. Houseknecht, T.L. Lowary, Chemistry and biology of arabinofuranosyl- and
570 galactofuranosyl-containing polysaccharides, *Curr. Opin. Chem. Biol.* 5 (2001) 677–682.
571 [https://doi.org/10.1016/S1367-5931\(01\)00265-4](https://doi.org/10.1016/S1367-5931(01)00265-4).
- 572 [5] H. Gruppen, R.A. Hoffmann, F.J.M. Kormelink, A.G.J. Voragen, J.P. Kamerling, J.F.G.
573 Vliegthart, Characterisation by ¹H NMR spectroscopy of enzymically derived
574 oligosaccharides from alkali-extractable wheat-flour arabinoxylan, *Carbohydr. Res.* 233
575 (1992) 45–64. [https://doi.org/10.1016/S0008-6215\(00\)90919-4](https://doi.org/10.1016/S0008-6215(00)90919-4).
- 576 [6] B. Seiboth, B. Metz, Fungal arabinan and L-arabinose metabolism, *Appl. Microbiol.*
577 *Biotechnol.* 89 (2011) 1665–1673. <https://doi.org/10.1007/s00253-010-3071-8>.
- 578 [7] B. Classen, A. Baumann, J. Utermoehlen, Arabinogalactan-proteins in spore-producing
579 land plants, *Carbohydr. Polym.* 210 (2019) 215–224.
580 <https://doi.org/10.1016/j.carbpol.2019.01.077>.
- 581 [8] Y. Kawabata, S. Kaneko, I. Kusakabe, Y. Gama, Synthesis of regioisomeric methyl α -L-
582 arabinofuranobiosides, *Carbohydr. Res.* 267 (1995) 39–47.
583 [https://doi.org/10.1016/0008-6215\(94\)00290-V](https://doi.org/10.1016/0008-6215(94)00290-V).
- 584 [9] J.P. Joseleau, G. Chambat, M. Vignon, F. Barnoud, Chemical and ¹³C N.M.R. studies on
585 two arabinans from the inner bark of young stems of *Rosa glauca*, *Carbohydr. Res.* 58
586 (1977) 165–175. [https://doi.org/10.1016/S0008-6215\(00\)83412-6](https://doi.org/10.1016/S0008-6215(00)83412-6).
- 587 [10] C. Marino, L. Baldoni, Synthesis of D-galactofuranose-containing molecules: design of
588 galactofuranosyl acceptors, *ChemBioChem.* 15 (2014) 188–204.
589 <https://doi.org/10.1002/cbic.201300638>.
- 590 [11] T.L. Lowary, Synthesis and conformational analysis of arabinofuranosides,
591 galactofuranosides and fructofuranosides, *Curr. Opin. Chem. Biol.* 7 (2003) 749–756.
592 <https://doi.org/10.1016/j.cob.2003.10.005>.
- 593 [12] C. Jones, A.R. Todeschini, A. Agrellos, J.O. Previato, L. Mendonça-Previato,
594 Heterogeneity in the biosynthesis of mucin O-glycans from *Trypanosoma cruzi*
595 Tulahuen strain with the expression of novel galactofuranosyl-containing
596 oligosaccharides, *Biochemistry.* 43 (2004) 11889–11897.
597 <https://doi.org/10.1021/bi048942u>.
- 598 [13] J.A. Leal, J. Gomez-Miranda, A. Prieto, J. Domenech, O. Ahrazem, M. Bernabé,
599 Possible chemotypes from cell wall polysaccharides, as an aid in the systematics of
600 *Leptothyrium* and its teleomorphic states *Eupenicillium* and *Talaromyces*, *Mycol. Res.* 101
601 (2001) 1259–1264. <https://doi.org/10.1017/S0953756297004012>.
- 602 [14] A. Frenkel, M. Bernabé, J.A. Leal, Isolation, purification and chemical characterization of
603 alkali-extractable polysaccharides from the cell walls of *Talaromyces* species, *Mycol.*
604 *Res.* 99 (1995) 69–75. [https://doi.org/10.1016/S0953-7562\(09\)80318-3](https://doi.org/10.1016/S0953-7562(09)80318-3).
- 605 [15] E.A. Leitão, V.C.B. Bittencourt, R.M.T. Haido, A.P. Valente, J. Peter-Katalinic, M.
606 Letzel, L.M. de Souza, E. Barreto-Bergter, β -Galactofuranose-containing O-linked
607 oligosaccharides present in the cell wall peptidogalactomannan of *Aspergillus fumigatus*
608 contain immunodominant epitopes, *Glycobiology.* 13 (2003) 681–692.
609 <https://doi.org/10.1093/glycob/cwg089>.
- 610 [16] U. Sørum, B. Robertsen, L. Kenne, Structural studies of the major polysaccharide in the
611 cell wall of *Renibacterium salmoninarum*, *Carbohydr. Res.* 306 (1998).
612 [https://doi.org/10.1016/S0008-6215\(97\)10071-4](https://doi.org/10.1016/S0008-6215(97)10071-4).

- 613 [17] A. Trincone, Uncommon glycosidases for the enzymatic preparation of glycosides,
614 *Biomolecules*. 5 (2015) 2160–2183. <https://doi.org/10.3390/biom5042160>.
- 615 [18] C. Marino, A. Rinflerch, R.M. De Lederkremer, Galactofuranose antigens, a target for
616 diagnosis of fungal infections in humans, *Futur. Sci. OA*. 3 (2017) FSO199.
617 <https://doi.org/10.4155/fsoa-2017-0030>.
- 618 [19] T. Konishi, H. Ono, M. Ohnishi-Kameyama, S. Kaneko, T. Ishii, Identification of a mung
619 bean arabinofuranosyltransferase that transfers arabinofuranosyl residues onto (1,5)-
620 linked α -L-arabino-oligosaccharides, *Plant Physiol.* 141 (2006) 1098–1105.
621 <https://doi.org/10.1104/pp.106.080309>.
- 622 [20] N.L. Rose, G.C. Completo, S.J. Lin, M. McNeil, M.M. Palcic, T.L. Lowary, Expression,
623 purification, and characterization of a galactofuranosyltransferase involved in
624 *Mycobacterium tuberculosis* arabinogalactan biosynthesis, *J. Am. Chem. Soc.* 128 (2006)
625 6721–6729. <https://doi.org/10.1021/ja058254d>.
- 626 [21] M. Belanova, P. Dianiskova, P.J. Brennan, G.C. Completo, N.L. Rose, T.L. Lowary, K.
627 Mikusova, Galactosyl transferases in mycobacterial cell wall synthesis, *J. Bacteriol.* 190
628 (2008) 1141–1145. <https://doi.org/10.1128/JB.01326-07>.
- 629 [22] A. Castilho, G. Beihammer, C. Pfeiffer, K. Göritzer, L. Montero-Morales, U. Vavra, D.
630 Maresch, C. Grünwald-Gruber, F. Altmann, H. Steinkellner, R. Strasser, An
631 oligosaccharyltransferase from *Leishmania major* increases the N-glycan occupancy on
632 recombinant glycoproteins produced in *Nicotiana benthamiana*, *Plant Biotechnol. J.* 16
633 (2018) 1700–1709. <https://doi.org/10.1111/pbi.12906>.
- 634 [23] S. Kallolimath, C. Gruber, H. Steinkellner, A. Castilho, Promoter choice impacts the
635 efficiency of plant glyco-engineering, *Biotechnol. J.* 13 (2018) 1700380.
636 <https://doi.org/10.1002/biot.201700380>.
- 637 [24] L. Montero-Morales, H. Steinkellner, Advanced plant-based glycan engineering, *Front.*
638 *Bioeng. Biotechnol.* 9 (2018) 81. <https://doi.org/10.3389/fbioe.2018.00081>.
- 639 [25] B. Nidetzky, A. Gutmann, C. Zhong, Leloir glycosyltransferases as biocatalysts for
640 chemical production, *ACS Catal.* 8 (2018) 6283–6300.
641 <https://doi.org/10.1021/acscatal.8b00710>.
- 642 [26] M.R. Richards, T.L. Lowary, Chemistry and biology of galactofuranose-containing
643 polysaccharides, *ChemBioChem.* 10 (2009) 1920–1938.
644 <https://doi.org/10.1002/cbic.200900208>.
- 645 [27] J. Houseknecht, T.L. Lowary, Oligofuranosides containing conformationally
646 restricted furanose residues: synthesis and conformational analysis, *J. Org. Chem.* 67 (2002)
647 64–64. <https://doi.org/10.1021/jo011127p>.
- 648 [28] H.A. Taha, M.R. Richards, T.L. Lowary, Conformational analysis of furanoside-
649 containing mono- and oligosaccharides, *Chem. Rev.* 113 (2013) 1851–1876.
650 <https://doi.org/10.1021/cr300249c>.
- 651 [29] D.E. Koshland, Stereochemistry and the mechanism of enzymatic reactions, *Biol. Rev.*
652 28 (1953) 416–436. <https://doi.org/10.1111/j.1469-185X.1953.tb01386.x>.
- 653 [30] S.A. Barker, E.J. Bourne, M. Stacey, R.B. Ward, Some paper-chromatographic studies
654 with *Aspergillus niger* ‘152’ transfructosylase, *Biochem. J.* 69 (1958) 60–62.
655 <https://doi.org/10.1042/bj0690060>.
- 656 [31] Y.K. Park, M.M. Almeida, Production of fructooligosaccharides from sucrose by a

- 657 transfructosylase from *Aspergillus niger*, World J. Microbiol. Biotechnol. 7 (1991) 331–
658 334. <https://doi.org/10.1007/BF00329399>.
- 659 [32] B. Bissaro, P. Monsan, R. Fauré, M.J. O’Donohue, Glycosynthesis in a waterworld: new
660 insight into the molecular basis of transglycosylation in retaining glycoside hydrolases,
661 Biochem. J. 467 (2015) 17–35. <https://doi.org/10.1042/BJ20141412>.
- 662 [33] C. Rémond, R. Plantier-Royon, N. Aubry, M.J. O’Donohue, An original
663 chemoenzymatic route for the synthesis of β -D-galactofuranosides using an α -L-
664 arabinofuranosidase, Carbohydr. Res. 340 (2005) 637–644.
665 <https://doi.org/10.1016/j.carres.2005.01.016>.
- 666 [34] C. Rémond, R. Plantier-Royon, N. Aubry, E. Maes, C. Bliard, M.J. O’Donohue,
667 Synthesis of pentose-containing disaccharides using a thermostable α -L-
668 arabinofuranosidase, Carbohydr. Res. 339 (2004) 2019–2025.
669 <https://doi.org/10.1016/j.carres.2004.04.017>.
- 670 [35] I. Chlubnová, B. Králová, H. Dvořáková, P. Hošek, V. Spiwok, D. Štěpánek, C. Nugier-
671 Chauvin, R. Daniellou, V. Ferrières, The versatile enzyme Ara51 allowed efficient
672 synthesis of rare pathogen-related β -D-galactofuranosyl-pyranoside disaccharides, Org.
673 Biomol. Chem. 12 (2014) 3080–3089. <https://doi.org/10.1039/c3ob42519c>.
- 674 [36] I. Chlubnová, B. Králová, H. Dvořáková, V. Spiwok, D. Štěpánek, C. Nugier-Chauvin, R.
675 Daniellou, V. Ferrières, Biocatalyzed synthesis of β -D-galactofuranosyl-pyranosides and their ability to
676 trigger production of TNF- α , Bioorg. Med. Chem. Lett. 26 (2016) 1550–1553.
677 <https://doi.org/10.1016/j.bmcl.2016.02.018>.
- 678 [37] V. Lombard, H. Golaconda Ramulu, E. Drula, P.M. Coutinho, B. Henrissat, The
679 carbohydrate-active enzymes database (CAZy) in 2013, Nucleic Acids Res. 42 (2014)
680 D490–D495. <https://doi.org/10.1093/nar/gkt1178>.
- 681 [38] M. Seničar, L. Legentil, V. Ferrières, S. V. Eliseeva, S. Petoud, K. Takegawa, P. Lafite,
682 R. Daniellou, Galactofuranosidase from JHA 19 *Streptomyces* sp.: subcloning and
683 biochemical characterization, Carbohydr. Res. 480 (2019) 35–41.
684 <https://doi.org/10.1016/j.carres.2019.05.011>.
- 685 [39] M. Seničar, P. Lafite, S. V. Eliseeva, S. Petoud, L. Landemarre, R. Daniellou,
686 Galactofuranose-related enzymes: challenges and hopes, Int. J. Mol. Sci. 21 (2020) 3465.
687 <https://doi.org/10.3390/ijms21103465>.
- 688 [40] W. Helbert, L. Poulet, S. Drouillard, S. Mathieu, M. Loiodice, M. Couturier, V. Lombard,
689 N. Terrapon, J. Turchetto, R. Vincentelli, B. Henrissat, Discovery of novel carbohydrate-
690 active enzymes through the rational exploration of the protein sequences space, Proc.
691 Natl. Acad. Sci. U.S.A. 116 (2019) 6063–6068.
692 <https://doi.org/10.1073/pnas.1815791116>.
- 693 [41] E. Matsunaga, Y. Higuchi, K. Mori, N. Yairo, T. Oka, S. Shinozuka, K. Tashiro, M.
694 Izumi, S. Kuhara, K. Takegawa, Identification and characterization of a novel
695 galactofuranose-specific β -D-galactofuranosidase from *Streptomyces* species, PLoS
696 ONE. 10 (2015) e0137230. <https://doi.org/10.1371/journal.pone.0137230>.
- 697 [42] E. Matsunaga, Y. Higuchi, K. Mori, N. Yairo, S. Toyota, T. Oka, K. Tashiro, K.
698 Takegawa, Characterization of a PA14 domain-containing galactofuranose-specific β -D-
699 galactofuranosidase from *Streptomyces* sp., Biosci. Biotechnol. Biochem. 81 (2017)
700 1314–1319. <https://doi.org/10.1080/09168451.2017.1300518>.
- 701 [43] T. Debeche, N. Cummings, I. Connerton, P. Debeire, M.J. O’Donohue, Genetic and

- 702 biochemical characterization of a highly thermostable α -L-arabinofuranosidase from
703 *Thermobacillus xylanilyticus*, Appl. Environ. Microbiol. 66 (2000) 1734–1736.
704 <https://doi.org/10.1128/AEM.66.4.1734-1736.2000>.
- 705 [44] T. Debeche, C. Bliard, P. Debeire, M.J. O'Donohue, Probing the catalytically essential
706 residues of the α -L-arabinofuranosidase from *Thermobacillus xylanilyticus*, Protein Eng.
707 Des. Sel. 15 (2002) 21–28. <https://doi.org/10.1093/protein/15.1.21>.
- 708 [45] F. Arab-Jaziri, B. Bissaro, C. Tellier, M. Dion, R. Fauré, M.J. O'Donohue, Enhancing
709 the chemoenzymatic synthesis of arabinosylated xylo-oligosaccharides by GH51 α -L-
710 arabinofuranosidase, Carbohydr. Res. 401 (2015) 64–72.
711 <https://doi.org/10.1016/j.carres.2014.10.029>.
- 712 [46] F. Arab-Jaziri, B. Bissaro, M. Dion, O. Saurel, D. Harrison, F. Ferreira, A. Milon, C.
713 Tellier, R. Fauré, M.J. O'Donohue, Engineering transglycosidase activity into a GH51
714 α -L-arabinofuranosidase, New Biotechnol. 30 (2013) 536–544.
715 <https://doi.org/10.1016/j.nbt.2013.04.002>.
- 716 [47] B. Bissaro, J. Durand, A. Planas, P. Monsan, X. Biarnès, A. Planas, P. Monsan, M.J.
717 O'Donohue, R. Fauré, Molecular design of non-Leloir transglucosylating enzymes
718 from an α -L-arabinofuranosidase: a rationale for the engineering of evolved
719 transglycosylases, ACS Catal. 5 (2015) 4598–4611.
720 <https://doi.org/10.1021/acscatal.5b00949>.
- 721 [48] J. Zhao, T. Tandrup, B. Bissaro, S. Barbe, J.-C.N. Poulsen, I. André, C. Dumon, L. Lo
722 Leggio, M.J. O'Donohue, R. Fauré, Probing the determinants of the
723 transglycosylation/hydrolysis partition of a retaining α -L-arabinofuranosidase, New
724 Biotechnol. 62 (2021) 68–78. <https://doi.org/10.1016/j.nbt.2021.01.008>.
- 725 [49] P.K. Glasoe, F.A. Long, Use of glass electrodes to measure acidities in deuterium oxide,
726 J. Phys. Chem. 64 (1960) 188–190. <https://doi.org/10.1021/j100830a521>.
- 727 [50] H.E. Gottlieb, V. Kotlyar, S. J. Edelmann, NMR chemical shifts of common laboratory
728 solvents as trace impurities, J. Org. Chem. 62 (1997) 7512–7515.
729 <https://doi.org/10.1021/jo9711176v>.
- 730 [51] D.A. Case, R.M. Betz, D.S. Cerutti, T.E. Cheatham III, T.A. Darden, R.E. Duke, T.J.
731 Giese, H. Gohlke, A.W. Goetz, N. Homeyer, S. Izadi, P. Janowski, J. Kaus, A.
732 Kovalenko, T.S. Lee, S. LeGrand, P. Li, C. Lin, T. Luchko, R. Luo, B. Madej, D.
733 Mermelstein, K.M. Merz, G. Monard, H. Nguyen, H.T. Nguyen, I. Omelyan, A. Onufriev,
734 D.R. Roe, A. Roitberg, C. Sagui, C.L. Simmerling, W.M. Botello-Smith, J. Swails, R.C.
735 Walker, J. Wang, R.M. Wolf, X. Wu, L. Xiao, P.A. Kollman, AMBER 2016, University
736 of California, San Francisco (2016). <https://ambermd.org/doc12/Amber16.pdf>
- 737 [52] M.D. Hanwell, D.E. Curtis, D.C. Lonie, T. Vandermeersch, E. Zurek, G.R. Hutchison,
738 Avogadro: an advanced semantic chemical editor, visualization, and analysis platform,
739 J. Cheminform. 4 (2012) 17. <https://doi.org/10.1186/1758-2946-4-17>.
- 740 [53] The PyMOL molecular graphics system, Version 1.8 Schrödinger, LLC.
741 <https://pymol.org/2/support.html>
- 742 [54] E. Krieger, G. Vriend, YASARA view -molecular graphics for all devices- from
743 smartphones to workstations, Bioinformatics 30 (2014) 2981–2982.
744 <https://doi.org/10.1093/bioinformatics/btu426>.
- 745 [55] F. Arab-Jaziri, B. Bissaro, S. Barbe, O. Saurel, H. Débat, C. Dumon, V. Gervais, A.
746 Milon, I. André, R. Fauré, M.J. O'Donohue, Functional roles of H98 and W99 and β 2 α

- 747 loop dynamics in the α -L-arabinofuranosidase from *Thermobacillus xylanilyticus*, FEBS
748 J. 279 (2012) 3598–3611. <https://doi.org/10.1111/j.1742-4658.2012.08720.x>.
- 749 [56] R. Euzen, G. Lopez, C. Nugier-Chauvin, V. Ferrières, D. Plusquellec, C. Rémond, M.
750 O'Donohue, A chemoenzymatic approach for the synthesis of unnatural disaccharides
751 containing D-galacto- or D-fucofuranosides, Eur. J. Org. Chem. (2005) 4860–4869.
752 <https://doi.org/10.1002/ejoc.200500525>.
- 753 [57] G. Paës, L.K. Skov, M.J. O'Donohue, C. Rémond, J.S. Kastrup, M. Gajhede, O. Mirza,
754 The structure of the complex between a branched pentasaccharide and *Thermobacillus*
755 *xylanilyticus* GH-51 arabinofuranosidase reveals xylan-binding determinants and
756 induced fit, Biochemistry. 47 (2008) 7441–51. <https://doi.org/10.1021/bi800424e>.
- 757 [58] C. Rémond, M. Ferchichi, N. Aubry, R. Plantier-Royon, C. Portella, M.J. O'Donohue,
758 Enzymatic synthesis of alkyl arabinofuranosides using a thermostable α -L-
759 arabinofuranosidase, Tetrahedron Lett. 43 (2002) 9653–9655.
760 [https://doi.org/10.1016/S0040-4039\(02\)02381-X](https://doi.org/10.1016/S0040-4039(02)02381-X).
- 761 [59] B. Bissaro, O. Saurel, F. Arab-jaziri, L. Saulnier, A. Milon, M. Tenkanen, P. Monsan,
762 M.J. O'Donohue, R. Fauré, Mutation of a pH-modulating residue in a GH51 α -L-
763 arabinofuranosidase leads to a severe reduction of the secondary hydrolysis of
764 transfuranosylation products, Biochim. Biophys. Acta - Subj. 1840 (2014) 626–636.
765 <https://doi.org/10.1016/j.bbagen.2013.10.013>.
- 766 [60] D. Teze, J. Zhao, M. Wiemann, Z.G.A. Kazi, R. Li, M. Weuner, M. Vuillemin, M.E.
767 Rønne, G. Carlström, J.Ø. Duus, Y.-H. Sanejouand, M.J. O'Donohue, E. Nordberg
768 Karlsson, R. Fauré, H. Stålbrand, B. Svensson, Rational enzyme design without
769 structural knowledge: a sequence-based approach for efficient generation of
770 transglycosylases, Chem. Eur. J. 27 (2021) 10323–10334.
771 <https://doi.org/10.1002/chem.202100110>.
- 772 [61] K.A. Johnson, A century of enzyme kinetic analysis, 1913 to 2013, FEBS Lett. 587 (2013)
773 2753–2766. <https://doi.org/10.1016/j.febslet.2013.07.012>.
- 774 [62] N. G. S. McGregor, M. A. Nin-Hill, D. Linzel, M. Haon, J. Reijngoud, A. Ram,
775 M.-N. Rosso, G. A. S. Farel, J. D. C. Codée, G. P. van Wezel, J.-G. Berrin, C.
776 Rovira, H. S. Overkleeft, G. J. Davies, Rational design of mechanism-based inhibitors
777 and activity-based probes for the identification of retaining α -L-arabinofuranosidases, J.
778 Am. Chem. Soc. 142 (2020) 4648–4662. <https://doi.org/10.1021/jacs.9b11351>.
- 779 [63] K. Hövel, D. Shallom, K. Niefind, V. Belakhov, G. Shoham, T. Baasov, Y. Shoham, D.
780 Schomburg, Crystal structure and snapshots along the reaction pathway of a family 51
781 α -L-arabinofuranosidase, EMBO J. 22 (2003) 4922–4932.
782 <https://doi.org/10.1093/emboj/cdg494>.
- 783 [64] V. Tran, L. Hoffmann, C. Rabiller, C. Tellier, M. Dion, Rational design of a GH1 β -
784 glycosidase to prevent self-condensation during the transglycosylation reaction, Protein
785 Eng. Des. Sel. 23 (2010) 43–49. <https://doi.org/10.1093/protein/gzp068>.
- 786 [65] J. Madhuprakash, A. Singh, S. Kumar, M. Sinha, P. Kaur, S. Sharma, A.R. Podile, T.P.
787 Singh, Structure of chitinase D from *Serratia proteamaculans* reveals the structural basis
788 of its dual action of hydrolysis and transglycosylation, Int. J. Biochem. Mol. Biol. 4
789 (2013) 166–178.
- 790 [66] A. Rosengren, S.K. Reddy, J.S. Sjöberg, O. Aurelius, D.T. Logan, K. Kolenová, H.
791 Stålbrand, An *Aspergillus nidulans* β -mannanase with high transglycosylation capacity

792 revealed through comparative studies within glycosidase family 5, Appl. Microbiol.
793 Biotechnol. 98 (2014) 10091–10104. <https://doi.org/10.1007/s00253-014-5871-8>.

794

795

796

Journal Pre-proofs