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Diversion of a thioglycoligase for the synthesis of 1-*O*-acyl arabinofuranoses

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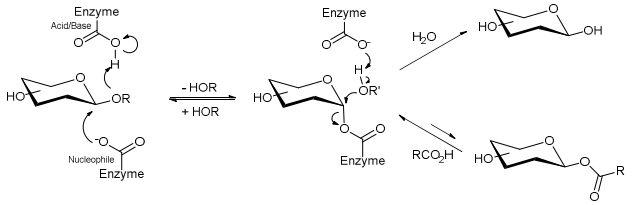
An arabinofuranosylhydrolase from the GH51 family was transformed into an acyl transferase by mutation of the catalytic acid/base amino acid. The resulting enzyme was able to transfer carboxylic acid onto the anomeric position of arabinose with complete chemo- and stereoselectivity. A wide range of acyl α-L-arabinofuranoses was obtained with yields ranging from 25 to 83 %. Using this method, ibuprofen and N-Boc phenylalanine were successfully transformed into their corresponding acyl conjugates, expanding the scope of the reaction to drugs and amino acids.

Glycosyl esters are a family of carbohydrates with increasing potential as bioactive or industrially relevant compounds. Indeed carbohydrates acylated by aryl carboxylic acids,[1] fatty acids[2] or saponins[3] are known high value water soluble preservatives, surfactants or food additives (Stevioside for example).[4] Such conjugates are naturally present in fruits or vegetables, mainly as 1,2-*trans* isomers.[5,6] They constitutes also important metabolites as many xenobiotic carboxylic acids undergo phase II conjugation with glucuronic acid to give the corresponding 1-*O*-acyl β-D-glucuronides that are easily excreted through the bile duct or the kidneys.[7]

The lack of stability of glycosyl esters hampers their extractability and their transfer as manufactured fine chemicals is difficult. Different synthetic strategies were therefore implemented to access such useful intermediates starting from either partially protected or unprotected sugar. Most popular methods relied on S_N2 nucleophilic substitution of glycosyl halides[8,9] or trichloroacetimidate glycosides,[10] remote activation of methoxypyridyl glycoside,[11] Mitsunobu[12] or carbodiimide-promoted coupling[13] to yield either α- or β-glycosyl esters. Such strategies nevertheless lack either efficiency or selectivity. Alternatively, biotechnological tools were developed to access in particular 1-*O*-acyl glucuronides. They relied on microsomial preparation of UDP-glucuronyltransferases[14] or the use of lipases[15,16] as catalyst for the transfer of carboxylic acid to nucleotide- or free sugar respectively. Such seducing approaches are however hampered by either the low availability of the substrate or the lack of versatility of the catalyst.

Very recently, sucrose phosphorylases have been shown to accept carboxylic acids to provide the corresponding 1-*O*-acyl α-D-glucoses.[17,18] The reaction proceeds smoothly with benzoic or acetic acid but is limited to sucrose as substrate. If such phosphorylase could serve as an acyl transferase, there is a possibility that less specific hydrolases could perform the same task. Glycosylhydrolases in the presence of a large excess of alcohol are known to perform transglycosylation.[19] However carboxylic acids are poor nucleophiles and the competition with water would be in favor of the latter (Scheme 1). In addition the resulting glycosyl esters are known to be readily hydrolyzed by native glycosylhydrolases.[20] Interestingly, Withers and co-workers have designed an elegant strategy to favour the attack of various nucleophiles by the mutation of the acid/base residue.[21] Such mutation drastically reduces the kinetic of the attack of water allowing competition with other nucleophiles like mercaptan,[22] fluoride[23] and even carboxylic acid.[24] But for the last two examples, the resulting conjugates were only detected and never isolated. In fact, the thioligases have never been diverted for the synthesis of acyl glycoside derivatives. We thus propose herein a novel strategy for the synthesis of glycosyl esters by the use of a thioligase as innovative and original acyl transfer catalyst.

The drawback associated with the monitoring of glycosyl ester formation is their non-stability due to acyl migration. 1,2-*trans* Acyl arabinofuranoses are known to be more stable and less prone to such rearrangement.[25] Interestingly Davies and collaborators have reported on a versatile, thermostable hydrolase that efficiently degrades polymers of L-arabinose,



Scheme 1 Competition between hydrolysis and acylation of the glycosyl-enzyme intermediate upon action of a glycosylhydrolase.

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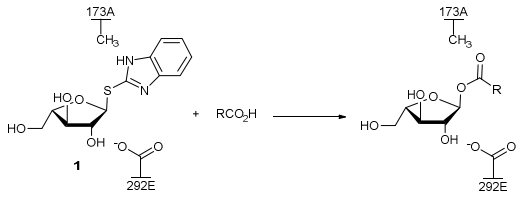

Scheme 2 Strategy of acylation of L-arabinose by the thioligase *Ct*Ara*f*51 E173A

the arabinofuranosidase 51 from *Rhuminiclostridium thermocellum* (*Ct*Ara*f*51).[26] Latter on Ferrières and co-workers used this enzyme as efficient biocatalyst in both self-condensation and transglycosylation of L-arabinofuranose donor.[27,28] Furthermore, *Ct*Ara*f*51 was successfully transformed into the corresponding thioligase by the mutation of the glutamic acid 173 into the alanine. The resulting mutant *Ct*Ara*f*51 E173A was able to recognize a thioimidoyl arabinofuranoside **1** as a donor and transfer the furanose to different thiophenol via a remote activation mechanism.[29] From there, we decided to perform the 1-*O*-acylation of arabinose from **1** with *Ct*Ara*f*51 E173A as catalyst to provide a wide range of 1-*O*-acyl arabinofuranoses (Scheme 2).

We tested first our strategy starting from **1**[30] and 4-methoxybenzoic acid as the acylating agent (Figure 1). The mutant Ara*f*51 E173A was expressed according to known procedure.[29] The reaction was performed in 50 mM phosphate buffer pH 7 at 25 °C in the presence of an excess of 4-methoxybenzoic acid. In the absence of the catalyst, no reaction occurred. Gratifyingly, when Ara*f*51 E173A was added, TLC analysis showed the formation of a new spot identified after purification as the 1-*O*-methoxybenzoyl α-L-arabinofuranose **2**. The isolated yield reached 52 %. The structure of **2** was unambiguously confirmed by NMR spectroscopy. ^{1}H and ^{13}C NMR showed in particular signals at 6.05 ppm and 102.3 ppm respectively, characteristic of an acylated anomeric position. The associated coupling constant reached 1.1 Hz, typical of a 1,2-*trans* configuration. Such complete diastereoselectivity of the reaction was expected as the selected furanosidase works with retention of configuration. The resulting 1-*O*-methoxybenzoyl α-L-arabinofuranose **2** was surprisingly very stable and did not undergo acyl migration or hydrolysis in the course of the reaction unlike others acyl glycosides[31] (monitored by ^{1}H NMR. See ESI). Interestingly, using the native Ara*f*51 as catalyst, sole the arabinose was formed. These preliminary results validated our model and confirm the possibility to implement a new reactivity, the acylation, to a glycosidase after mutation of the acid/base residue. It also confirmed the crucial role of the mutation to prevent the competition of the carboxylic acid acceptor with water.

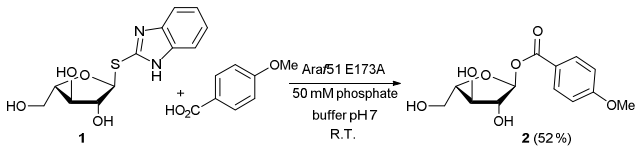

Figure 1 Synthesis of 4-methoxybenzoyl α-L-arabinofuranose **2** catalysed by *Ct*Ara*f*51 E173A

Table 1 Screening of pH and acceptor amount for the acylation of thioimidoyl arabinofuranoside **1** by 4-methoxybenzoic acid. Conditions: *Ct*Ara*f*51 E173A, 50 mM phosphate buffer, R.T.

Entry	Equivalent of acceptor	pH	Time (h)	Yield of **2** (%)	Ratio Ara/**2**[a]
1	4	4.5	16	-	1/0
2	4	8	16	-	1/0[b]
3	4	7	16	52	0.3/1
4	4	6	16	67	0.1/1
5	4	6	24	78	0.15/1
6	1	6	24	60	0.4/1

[a] determined by NMR. [b] in this case large amount of **1** remained.

In order to optimize this reaction, different conditions (pH, time, donor/acceptor ratio) were then screened (Table 1). On one hand, at pH below 4.5 (entry 1), the thioimidoyl arabinofuranose **1** was quickly chemically hydrolysed due to proton activation of the benzimidazole. On the other hand, in alkaline conditions, typically the one used for thioligation, no product of acylation could be detected and only the starting thioimidoyl arabinofuranoside **1** and arabinose were found (entry 2). Interestingly, improved isolated yield compared to neutral pH was obtained at acidic pH (entry 3 *vs.* entry 4). Complete disappearance of **1** was achieved after 24h and the isolated yield reached 78 % (entry 5). It nevertheless dropped to 60 % when using an equimolar equivalent of 4-methoxybenzoic acid (entry 6). In these conditions the kinetic slowed down and a fair amount of hydrolysis occurred. In addition, using ^{1}H NMR, we confirmed that in neutral and alkaline media, the resulting product **2** was more sensitive to chemical hydrolysis (See ESI). It correlated the larger amount of arabinose found when the reaction occurred at pH above 6 (entry 4 *vs.* entries 2 and 3).

In term of mechanism, the reaction should proceed according to the canonical mechanism described by Withers.[21] The first half-reaction followed a two-steps process i) attack of the nucleophilic residue glutamate 292 and ii) subsequent departure of thiobenzimidazole *via* a remote activation mechanism[29] (Scheme 3). The second-half of the reaction, that is to say the attack by the carboxylic acid, can proceed according to two different scenarios with either the carboxylic acid or the carboxylate as the nucleophile. Sugimoto *et al.* demonstrated that the acylation of glucose catalysed by sucrose phosphorylase proceeded best at pH below the pK_a of acetic or benzoic acid meaning that the protonated form of the acid probably act as the nucleophile.[17] In our case, the difference in efficiency between pH 6 and pH 7 was not striking and it is probably the carboxylate form that act as the nucleophile. Such form does not need to be activated unlike water. As the mutation of the glutamate residue 173 by an alanine one reduced drastically the activation of water, the carboxylate can compete efficiently to give the corresponding acyl 1,2-*trans* arabinofuranose as the only product of the reaction (Scheme 3).

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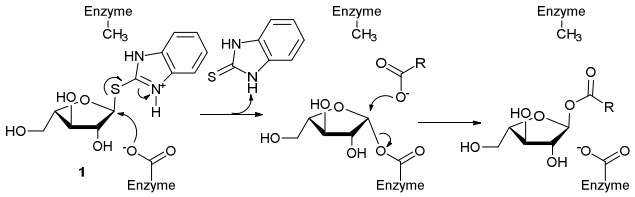
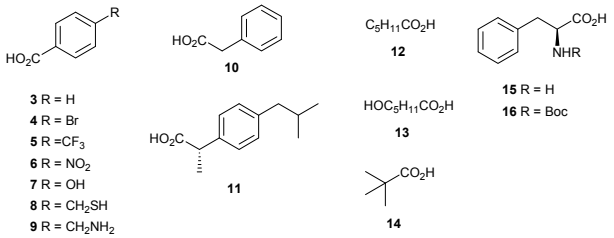


Scheme 3 Hypothesized mechanism of 1-*O*-acylation of thioimidoyl arabinofuranoside **1** catalysed by Ara*f*51 E173A.

According to our data, the pH nevertheless influenced greatly the kinetic of the reaction. The Ara*f*51 is known to be stable and active in a large range of pH.[26] It is therefore not the potential degradation of the enzyme that can explain such difference. We suspect that the difference in reactivity is linked to the first half-reaction rather than the second-half one. Acidic media could indeed contribute to accelerate the departure of the thiobenzimidazole aglycon thus increasing the turn-over of the reaction.

Finally, to demonstrate the versatility of the reaction, a large panel of carboxylic acid was screened as acceptor of the acylation reaction (Table 2).[‡] It included aryl and alkyl carboxylic acid as well as amino acids or drugs like ibuprofen. The yields for the furanosylation of aryl carboxylic acid ranged from 18 to 83 % (entries 1 to 5). The lowest yields were found with electron-withdrawing groups on the aromatic ring like bromide, trifluoromethyl or nitro (entries 2 to 4). A follow-up of the reaction between **1** and 4-nitrobenzoic acid was performed and showed the concomitant formation of the corresponding acyl arabinofuranose **20** and arabinose. We then compared the stability of the resulting conjugates at pH 6 in the presence or the absence of Ara*f*51 E173 (See ESI). A strong increase of the kinetic of hydrolysis was found when the enzyme was added. The presence of the electron-withdrawing group must reduce the nucleophilicity of the carboxylic acid and increase the nucleofuge property of the acyl group of the product. Both effects contributed then to increase the competition with water. The same trend was observed using salicylic acid as substrate (See ESI). The strong hydrolysis observed was probably linked to the activation of the ester function by the phenol in ortho position. Interestingly, the alkyl carboxylic acids 2-phenylacetic acid **10** and hexanoic acid **12** were readily converted to the corresponding acyl arabinofuranoses **22** and **23** with yields up to 66 % (entries 6 and 7). For the bulkier pivaloic acid **14**, the conversion remained low probably because of the steric hindrance (entry 8). Gratifyingly ibuprofen **11** was transformed into the acyl conjugate **25** with an excellent 83 % yield (entry 9) opening the possibility to transfer the methodology to the synthesis of acyl glucuronides that result from phase II metabolism of drugs. It was worth reporting that no stereoselection occurred when using racemic ibuprofen and a 1:1 ratio of diastereoisomers was obtained (See ESI). Also with 6-hydroxyhexanoic acid **13** no product of *O*-glycosylation was formed and the corresponding acyl arabinofuranose **26** was obtained in 53 % yield (entry 10). The same chemoselectivity was found using hydroxybenzoic acid **7** (entry 5). Even the 4

Table 2 Extension to various carboxylic acid acceptors[‡]



Entry	Acceptor RCO_2H	Isolated Yield (%)	1-*O*-Acyl Ara*f*
1	**3**	57	**17**
2	**4**	47	**18**
3	**5**	57	**19**
4	**6**	18[a]	**20**
5	**7**	78	**21**
6	**10**	66	**22**
7	**12**	77	**23**
8	**14**	25	**24**
9	**11**	83[a]	**25**
10	**13**	53	**26**
11	**8**	100[b]	**27**
12	**9**	-	**28**
13	**15**	-	**29**
14	**16**	38	**30**

[a] 1:1 ratio of diatereoisomer with (±) ibuprofen [b] Conversion yield determined by NMR.

-(mercaptomethyl)benzoic acid **8** reacted with **1** without formation of the thiobenzyl arabinoside isomer (entry 11). Thus it showed in our conditions that carboxylic acid was a better nucleophile than the phenol, the alcohol or the aliphatic thiol. Finally, no reaction was detected in the presence of 4-(aminomethyl)benzoic acid **9** (entry 12) or phenylalanine (entry 13). The presence of the ammonium group seemed to be detrimental to the approach of the acceptor in the +1 sub-site. Indeed, when the amine of phenylalanine was protected as a carbamate, the resulting acyl conjugate **30** was obtained with 38 % yield (entry 14). Such result paves the way to the extension of the scope of reaction in the domain of protein glycosylation.

To summarize, we report here a novel catalytic pathway for the synthesis of glycosyl esters using a mutated glycosylhydrolase. This methodology has the advantage to use stable reactant and readily available enzyme. It is performed in mild reaction conditions and with a large range of carboxylic acids with good yields and excellent selectivity. This study in particular shows the plasticity of glycosylhydrolases and in particular the arabinofuranosidase towards numerous catalytic activities. In addition to transglycosylation and thioligation, this enzyme is also able to perform acylation of arabinose. Interestingly the reaction could be extended to mimics of L-arabinose, like D-galactofuranose with potential biological

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properties. Also the extension of the methodology to thioligases with greater interest like glucuronidase would be a definite advantage for the synthesis of the corresponding acyl glucuronides.

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Conflicts of interest

There are no conflicts to declare

Notes and references

‡ General procedure for the enzymatic synthesis of glycosyl esters: A mixture of 4 equivalent of carboxylic acid acceptor in phosphate buffer (20 mL, 50 mM, pH = 6) was prepared and pH was adjusted to 6 with NaOH 2N. The mutant enzyme Araf51 E173A (≈ 5 mg, 0.00025 eq) and 2'-benzimidazolyl-1-thio-α-*L*-arabinofuranoside **1** (≈ 100 mg, 0.35 mmol, 1 eq) were added. The mixture was stirred at RT for 16 hours. After freeze-drying, the mixture was purified by column chromatography on silica gel to yield the corresponding 1-*O*-acyl arabinofuranose.

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