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A substrate for the detection of broad specificity α-L-arabinofuranosidases with indirect release of a chromogenic group

Vinciane Borsenberger [a,b,c], Emmie Dornez [d], Marie-Laure Desrousseaux [a,b,c], Christophe M. Courtin [d], Michael J. O'Donohue [a,b,c], Régis Fauré [a,b,c,*]

[a] Université de Toulouse; INSA, UPS, INP; LISBP, 135 Avenue de Rangueil, F-31077 Toulouse, France
[b] INRA, UMR792 Ingénierie des Systèmes Biologiques et des Procédés, F-31400 Toulouse, France
[c] CNRS, UMR5504, F-31400 Toulouse, France
[d] Laboratory of Food Chemistry and Biochemistry, KU Leuven, Kasteelpark Arenberg 20 bus 2463, B-3001 Leuven, Belgium

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ABSTRACT

The synthesis of a compound containing a 4-nitrocatechol bound to two vicinal α-L-arabinofuranosyl moieties through a linker arm was achieved using a sulfate protecting group to facilitate selective alkylation of one aromatic hydroxyl. Several α-L-arabinofuranosidases displaying different selectivities were tested and a simple microtiter plate-based assay was developed. The observed resistance of the compound to α-L-arabinofuranosidase-mediated hydrolysis makes it suitable for the identification of enzymes that are able to accommodate bis-arabinofuranosylated moieties.

Introduction

The increasing penetration of biotechnology into industrial processes is driving high-throughput enzyme discovery.[1–4] In this context, plant cell wall-degrading glycoside hydrolases (GHs) are highly sought after for use in sectors such as the food and animal feed industries, or for the emerging biorefining industry. One example of such GHs is α-L-arabinofuranosidases (Abfs) that are active on a variety of complex L-arabinofuranose-containing polymers, particularly arabinoxylans, which constitute an important and widespread class of plant polymers.[5] When acting on arabinoxylans, Abfs hydrolyze the α-1,2 and/or α-1,3 bonds that link L-arabinofuranosyl (L-Ara*f*) moieties to main-chain D-xylopyranosyl (D-Xyl*p*) units, and thus facilitate the action of main-chain-hydrolyzing GHs, such as endo-β-D-xylanases (Fig. 1). Interestingly, some Abfs are clearly specific for mono-substituted D-Xyl*p*, while others are highly specific for D-Xyl*p* bearing two L-Ara*f* moieties.[6–8]

Chromogenic assays are commonly employed in high-throughput screening, because they provide quick and easy detection of enzyme activity at the microbial colony level, and are compatible with automated protocols.[9] The most commonly used chromogenic substrates for the detection of *exo*-acting GHs are those composed of indolyl[10] or *para*-nitrophenol (PNP) moieties directly attached to a glycoside unit. Although convenient, these types of substrates are almost certainly poor analogues for the natural substrates of most targeted GHs. This is because the aryl moiety can at best only approximately imitate a sugar, while the good leaving group ability of phenolate species,[11] such as PNP, promotes hydrolysis and can thus reveal GH activities that turn out to be essentially minor when considering an enzyme's actual substrate preference.

To facilitate the detection of Abfs that act on disubstituted D-Xyl*p*, we set out to design a substrate that is both simple to use, and whose structure and reactivity might better reflect that of a natural substrate. To achieve this, we decided to move the aryl moiety away from the sugar by introducing an alkyl spacer arm between the two, thus hopefully minimizing the undesirable interactions between the aryl group and the target Abfs. Moreover, to be able to use the substrate in both liquid and solid media-based assays, we designed a compound bearing a 4-nitrocatechol (4NTC) unit. This was attached to two vicinal L-Ara*f* moieties via a cleavable spacer arm, thus affording a compound that is meant to imitate a disubstituted D-Xyl*p* moiety, typical of those found in the main-chain of highly substituted recalcitrant arabinoxylans. Furthermore, in the case of successful bond hydrolysis and release of both L-Ara*f* units, a diol will be generated, which will be vulnerable to oxidative cleavage. Therefore, we expected that, in the presence of $NaIO_4$, an aldehyde could be produced, which would rapidly undergo β-elimination at basic pH,[12–15] thus releasing 4NTC, a chro-

* Corresponding author. Tel.: +33 5 6155 9488; fax: +33 5 6155 9400.
E-mail address: regis.faure@insa-toulouse.fr (R. Fauré).

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mogenic moiety that can be monitored in liquid medium and thus used to assay enzyme activity.[16,17]

Results and discussion

The synthesis is described in scheme 1. In order to obtain the target compound **8** as one regioisomer, it was essential to find a method to selectively alkylate the 4NTC at one hydroxyl position only. As direct alkylation of 4NTC under alkaline conditions with an equimolar amount of protected halide **2**[18] resulted mainly in the production of the *O*-1,*O*-2 disubstituted 4NTC (80%) and very little of the *O*-1 mono-substituted species (7%), we adopted a regioselective protection strategy. Esterification of 4NTC using benzoyl chloride resulted in the production of a poorly soluble solid, which appeared to be a mixture of *O*-1 and *O*-2 benzoylated 4NTC. Attempts to separate the two isomers by selective recrystallization failed, because the benzoyl underwent spontaneous intramolecular migration between the aromatic *cis* hydroxyls, resulting in a relatively fast equilibrium between the two regioisomers. Finally, we turned to the commercially available dipotassium salt of 4-nitrocatechol sulfate **1**, and proceeded to a halide coupling under anhydrous conditions. Using a small excess of bromo derivative **2** in the presence of a proton sponge only produced the desired product in low yield (<2%). Therefore, we turned to its more reactive iodo version **3**,[18] which successfully afforded the aryl ether. Next, the sulfate protecting group being more labile than the acetal moiety, was necessary to swap for a benzoyl protecting group. Assuming that in situ deprotection would greatly simplify the coupling reaction work-up, the acid labile sulfate was removed over 1 h by adding a 1.2 equiv of CSA at the end of the coupling reaction, thus affording **4** in 57% yield.[19] Nevertheless, although acetone was employed as the co-solvent in order to prevent the loss of the isopropyledene protecting group, fully deprotected material **9** was obtained in 12% yield.[20] Classic esterification of **4** with benzoyl chloride in pyridine provided the adequately protected acetal, which was quantitatively hydrolyzed in 80% aqueous AcOH, affording **5** in 82% overall yield from **4**.[21] An inverse procedure of Schmidt glycosylation[22] was employed to glycosylate the diol moiety using an excess of the activated sugar donor **6**[23] with a catalytic amount of $BF_3.Et_2O$, which gave the desired compound **7**[24] in 94% yield. Benzoyl protecting groups were removed using sodium methanolate and finally, **8**[25] was obtained in a 40% overall yield in six steps.

The principle of the enzymatic assay is detailed in scheme 2. After enzymatic release of the L-Ara*f* units by Abfs, the free diol can be removed by oxidative cleavage using sodium periodate. After quenching of the excess oxidant with ethylene glycol, the aldehyde **10** undergoes a β-elimination under basic conditions, releasing free 4NTC that displays a characteristic red color above pH 11.[16] Importantly, the pH of the oxidative step must be carefully controlled in order to avoid premature release of 4NTC, which would result in its oxidation and consequent lowered sensitivity of the assay. To determine ideal operating conditions, compound **9** was incubated with 10 equiv of sodium periodate in buffered solutions, in the range pH 3–8.[26] At pH 5, the linker arm remained stable, thus protecting 4NTC from oxidation while allowing complete cleavage of the free diol moiety within 10 min. Longer exposure (1 h) to periodate did not bring about other changes. Further incu-

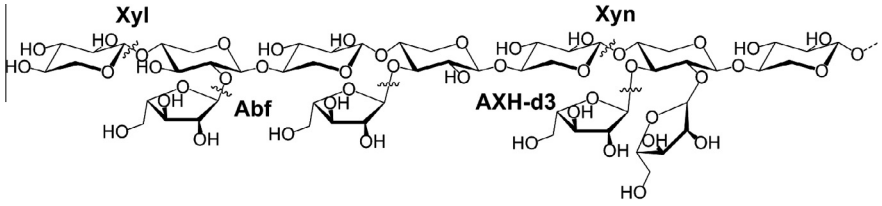
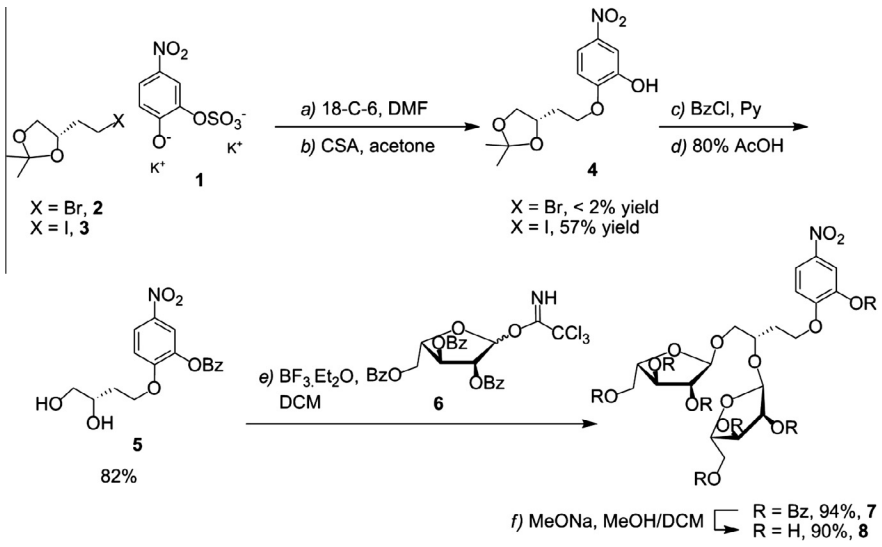
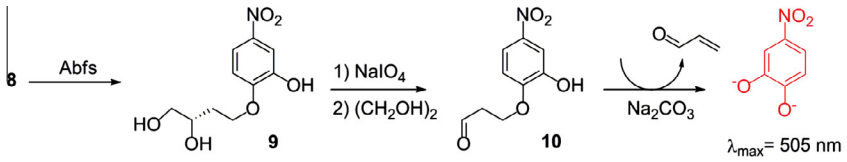


Figure 1. A generic scheme of arabinoxylan structures. The main-chain is composed of β-1,4-linked D-Xyl*p* subunits, decorated with α-1,2 and/or α-1,3-bounded L-Ara*f* residues. Depending on the exact botanic origin, other side-chain decorations are also possible, but these are not shown. Potential enzymatic cleavage sites are indicated by wavy lines (Abf, α-L-arabinofuranosidase; AXH-d_3, double substituted xylan α-1,3-L-arabinofuranosidase; Xyn, endo-β-D-xylanase; Xyl, β-D-xylosidase).[5]



Scheme 1. Reagents and conditions: (a) 1.5 equiv of **3**, 18-*C*-6, DMF, 50 °C, 23 h; (b) CSA 1.2 equiv, acetone, 1 h, 57% over two steps; (c) BzCl 3.7 equiv, pyridine, DCM, 7 h; (d) AcOH, 82% over two steps; (e) 4.4 equiv of **6**, $BF_3.Et_2O$, DCM, 3 h, 94%; (f) MeONa 2.3 equiv, MeOH, DCM, 39 h, 90%.



Scheme 2. Reactions involved in the Abf activity assay.

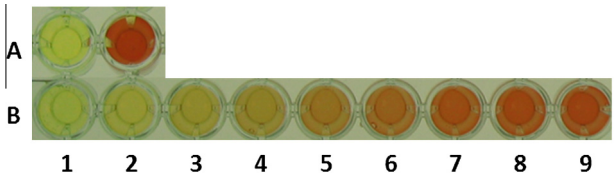


Figure 2. Hydrolysis of **8** in 96-well microtiter plates. A1: compound **8** (negative control), A2: compound **9** (positive control), B1-9: discontinuous monitoring of enzyme-mediated hydrolysis of **8** by an Abf. Each well contains a 50 pmol of substrate to which 500 pmol of periodic acid, 50 μL ethylene glycol and 150 μL of 1 M Na_2CO_3 are added successively.[30]

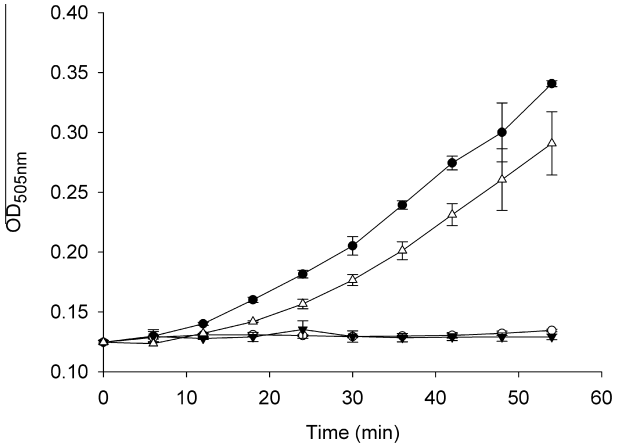


Figure 3. Time-dependent hydrolysis of **8** by various Abfs, revealing selectivity differences (△, *Tx*Abf; ●, AbfB; ○, AbfA; and ■, AXH-d_3).[30] Reactions were discontinuously monitored at 505 nm.

bation (10 min) in the presence of ethylene glycol was sufficient to ensure complete quenching of unreacted sodium periodate. Finally, the addition of a 1 M Na_2CO_3 solution prompted β-elimination of the aldehyde. The reaction was complete within 5 min at 0 °C, thus the presence of free 4NTC, which is red at highly basic pH could be monitored at OD_{505nm}. In our tests, the final concentration of free 4NTC corresponded to a quantitative conversion of the starting material. Importantly, when submitted to this assay compound **8** remained perfectly stable, with no detectable increase of absorbance at 505 nm being observed (Fig. 2).

To further validate the assay, compound **8** was submitted to the hydrolytic action of several Abfs that display different specificities. Two of the Abfs used belong to the family GH43 of the CAZy classification[27] and are known to selectively release α-L-Ara*f* from D-Xyl*p* units, with AbfA (GenBank accession no. BAF39204.1),[28] only being able to act on mono-substituted units, and AXH-d_3 (AAO67499.1),[6] specifically, removing α-1,3-bonded L-Ara*f* from disubstituted D-Xyl*p* moieties. The other Abfs employed, AbfB (BAF40305.1)[28] and *Tx*Abf (CAA76421.2),[29] are family GH51 members. These present broader specificities and have the potential to hydrolyze doubly-substituted D-Xyl*p* units to a small extent.[16,28] Duplicate or triplicate experiments with discontinuous monitoring were performed over 1 h in order to observe enzymatic hydrolysis of **8**, operating at the optimum pH and temperature of the Abf being used.[30] At regular intervals, 50 μL aliquots were removed and added to cold (0 °C) periodate solution (pH 2) in 96-wells microtiter plates and kept at 0 °C throughout, followed by treatment using the previously established procedure. Hence, samples were exposed to the oxidative cleavage for 10 min, before another 10 min quenching with ethylene glycol, after which absorbance was measured at 505 nm. As shown in Fig. 3, AbfB and *Tx*Abf successfully removed both sugars from compound **8**, while AbfA and AXH-d_3 failed to produce any free 4NTC. Interestingly, ^{1}H NMR analysis of the AXH-d_3-mediated reaction indicated that the enzyme had removed one L-Ara*f*, hence proving that the substrate successfully mimics the recalcitrant bis-arabinofuranosylated motif (unpublished data). Moreover, regarding the GH51 enzymes, the enzyme concentration needed to hydrolyze 50% of **8** was at least 10-fold higher than that required to hydrolyze PNP α-L-arabinofuranoside under identical conditions, indicating that catalytic efficiency on **8** was greatly diminished, and thus confirming that this compound is well-suited to the identification of enzymes displaying high catalytic efficiency on both mono-substituted and disubstituted D-Xyl*p* moieties. Interestingly, using ^{1}H NMR we observed that *Tx*Abf does indeed release L-Ara*f* from disubstituted D-Xyl*p* moieties in arabinoxylo-oligosaccharides provided that these are located at a terminal position (unpublished data).

Conclusions

In summary, we have shown that sulfate is a good protecting group to selectively alkylate one hydroxyl of a catechol moiety, because it does not migrate and is easily removed, although the solubility issue that arises from having an ionic component in organic mixture might limit its scope. Nevertheless, this procedure has afforded a new chromogenic substrate that can be used to detect a specific class of Abfs that selectively release L-Ara*f* from disubstituted D-Xyl*p* moieties. This substrate, which we believe better imitates natural osidic linkages than conventional chromogenic substrates, might be used in combination with an Abf that displays tight selectivity for monosubstituted D-Xyl*p* moieties, thus providing the means to detect 4NTC, the ultimate product of the two-enzyme cascade reaction, or alone to detect Abfs that have broader substrate specificity, being able to release L-Ara*f* from both mono- and disubstituted D-Xyl*p* moieties. This molecule would enable the selection of 'GH51-like' enzymes of high catalytic efficiency.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2013.03.136.

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19. Procedure for the synthesis of (*S*)-4-*O*-(2-hydroxy-4-nitrophenyl)-1,2-*O*-isopropylidene-1,2,4-butanetriol **4**: to a solution of 4-nitrocatechol sulfate dipotassium salt **1** (312 mg, 1.0 mmol) and proton sponge (135 mg, 0.5 mmol) in anhydrous DMF (10 mL) under an N_2 atmosphere, was added (*S*)-1-iodo-3,4-*O*-isopropylidene-3,4-butanediol **3** (250 μL or 388 mg, 1.5 mmol) by a syringe. The reaction mixture was stirred for 23 h at 50 °C, then allowed to reach room temperature, and poured into a solution of camphor sulfonic acid (274 mg, 1.2 mmol) in acetone (70 mL). Solvents were removed under reduced pressure and the residue was taken in a saturated $NaHCO_3$ solution, extracted with ethyl acetate. The combined organic extracts were washed in turn with water and brine, dried ($MgSO_4$), filtered, and evaporated. The product was isolated by flash column chromatography using a gradient of acetone in toluene (10–20%), which provided 161 mg (0.57 mmol, 57%) of **4** as white crystals: ^{1}H NMR (500 MHz, $(CD_3)_2CO$) δ 8.56 (1H, s), 7.79 (ddd, 1H, 8.9, 2.8, 0.6), 7.67 (dd, 1H, *J* = 2.8, 1.1), 7.18 (d, 1H, *J* = 8.9), 4.39–4.28 (m, 3H), 4.11 (dd, 1H, *J* = 8.1, 6.0), 3.65 (dd, 1H, *J* = 8.1, 6.9), 2.15–2.3 (m, 2H), 1.35 (s, 3H), 1.29 (s, 3H); ^{13}C NMR (126 MHz, $(CD_3)_2CO$) δ 153.5, 147.7, 142.6, 117.1, 112.4, 110.9, 109.3, 73.9, 69.9, 67.4, 34.0, 27.3, 26.0; HRMS (ESI) calcd for $C_{13}H_{17}NO_6Na$ $[M+Na]^+$: 306.0954 found: 306.0952 (0 ppm).
20. (*S*)-4-*O*-(2-hydroxy-4-nitrophenyl)-1,2,4-butanetriol **9** was obtained after the extraction step and before the chromatography purification of **4**. It can be isolated from the crude mixture by triturating the yellow oil in toluene (4 mL) and then, cooling it to −20 °C for 1 h, which results in the formation of a white precipitate. Filtration and drying, provided 29 mg (0.12 mmol, 12%) of white crystals. ^{1}H NMR (500 MHz, CD_3OD) δ 7.77 (dd, 1H, *J* = 8.9, 2.8), 7.64 (d, 1H, *J* = 2.8), 7.08 (d, 1H, *J* = 8.9), 4.34 (ddd, 1H, *J* = 9.7, 8.1, 5.4), 4.30 (dt, 1H, *J* = 9.7, 5.6), 3.90 (dtd, 1H, *J* = 9.0, 5.4, 3.6), 3.57 (dd, 1H, *J* = 11.1, 6.0), 3.55 (dd, 1H, *J* = 11.1, 5.8), 2.14 (dddd, 1H, *J* = 14.4, 8.1, 6.2, 3.6), 1.89 (ddt, 1H, *J* = 14.4, 9.0, 5.3); ^{13}C NMR (126 MHz, CD_3OD) δ 154.2, 148.2, 142.8, 117.2, 112.5, 111.2, 70.2, 67.5, 67.3, 33.8; HRMS (ESI) calcd $C_{10}H_{13}NO_6Na$ $[M+Na]^+$: 266.0641 found: 266.0641 (0 ppm).
21. (*S*)-4-*O*-(2-*O*-benzoyl-4-nitrophenyl)-1,2,4-butanetriol **5**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.22–8.18 (m, 3H), 8.11 (d, 1H, *J* = 2.7), 7.68 (t, 1H, *J* = 7.5),), 7.54 (t, 2H, *J* = 7.5), 7.11 (d, 1H, *J* = 9.1), 4.28 (dd, 2H, *J* = 6.5, 5.3), 3.80–3.75 (m, 1H), 3.51 (dd, 1H, *J* = 11.1, 3.3), 3.37 (dd, 1H, *J* = 11.1, 7.0), 2.17 (s, 2H), 1.89–1.79 (m, 2H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 164.6, 156.1, 139.9, 134.4, 130.5, 129.0, 128.5, 123.5, 119.4, 112.5, 68.9, 66.6, 66.4, 32.2; HRMS (ESI) calcd for $C_{17}H_{17}NO_7Na$ $[M+Na]^+$: 370.0903 found: 370.0905 (1 ppm).
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24. (*S*)-1,2-*O*-bis-(2,3,5-tri-*O*-benzoyl-α-L-arabinofuranosyl)-4-*O*-(2-*O*-benzoyl-4-nitrophenyl)-1,2,4-butanetriol **7**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.16-8.12 (m, 2H), 8.04–7.98 (m, 9H), 7.96–7.93 (m, 2H), 7.91–7.87 (m, 2H), 7.85 (dd, 1H, *J* = 9.1, 2.7), 7.61 (tt, 1H, *J* = 7.5, 1.3), 7.58–7.41 (m, 10H), 7.39–7.31 (m, 4H), 7.22–7.31 (m, 6H), 6.71 (d, 1H, *J* = 9.1), 5.61 (s, 1H), 5.56 (d, 1H, *J* = 5.0), 5.50 (d, 1H, *J* = 4.4), 5.48 (d, 1H, *J* = 1.0), 5.47 (d, 1H, *J* = 1.2), 5.24 (s, 1H), 4.76 (dd, 1H, *J* = 11.9, 3.6), 4.74 (dd, 1H, *J* = 12.2, 3.9), 4.63 (dd, 1H, *J* = 11.9, 4.9), 4.60–4.54 (m, 2H), 4.41–4.37 (m, 1H), 4.30–4.24 (m, 1H), 4.21–4.15 (m, 2H), 3.89 (dd, 1H, *J* = 10.6, 3.7), 3.53 (dd, 1H, *J* = 10.6, 5.9), 2.09–2.03 (2H, m); ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.3, 166.3, 165.8, 165.6, 165.5, 165.4, 164.2, 155.9, 141.1, 139.9, 134.1, 133.8, 133.6, 133.6, 133.2, 133.2, 130.4, 130.0, 130.0, 129.9, 129.9, 129.8, 129.8, 129.8, 129.2, 129.2, 129.2,129.0, 128.9, 128.7, 128.6, 128.6, 128.5, 128.4, 128.4, 123.2, 119.4, 111.8, 106.0, 104.5, 82.3, 82.1, 82.0, 81.4, 78.2, 77.8, 70.9, 68.7, 65.6, 64.0, 63.8, 31.8; HRMS (ESI) calcd $C_{69}H_{57}NO_{21}Na$ [M+Na]: 1258.3321 found: 1258.3318 (0 ppm).
25. (*S*)-1,2-*O*-bis-(α-L-arabinofuranosyl)-4-*O*-(2-*O*-benzoyl-4-nitrophenyl)-1,2,4-butanetriol **8**: ^{1}H NMR (500 MHz, D_2O) δ 7.73 (dd, 1H, *J* = 9.0, 2.8), 7.59 (d, 1H, *J* = 2.8), 6.98 (d, 1H, *J* = 9.0), 5.23 (d, 1H, *J* = 1.6), 5.07 (d, 1H, *J* = 1.6), 4.27–4.16 (m, 3H), 4.14 (dd, 1H, *J* = 3.5, 1.7), 4.10 (dd, 1H, *J* = 3.6, 1.8), 4.09 (td, 1H, *J* = 5.9, 3.3), 3.99–3.92 (m, 3H), 3.89–3.86 (m, 1H), 3.84 (dd, 1H, *J* = 12.3, 3.3), 3.73 (dd, 1H, *J* = 12.3, 5.6), 3.73 (dd, 1H, *J* = 12.3, 5.6), 3.63 (dd, 1H, *J* = 11.1, 6.0), 3.52 (d, 2H, *J* = 4.2), 2.17–2.08 (m, 1H), 2.07–2.0 (m, 1H); ^{13}C NMR (126 MHz, D_2O) δ 152.9, 144.9, 140.6, 117.6, 111.4, 110.3, 107.9, 106.7, 83.7, 83.6, 81.4, 81.0, 76.4, 76.2, 72.8, 69.9, 65.6, 61.1, 60.5, 30.5; HRMS (ESI) calcd $C_{20}H_{29}NO_{14}$ $[M+Na]^+$: 530.1486 found: 530.1482 (1 ppm).
26. All experiments were carried in the presence of 2 equiv of L-Ara in order to account for the consumption of oxidant by the free sugar in the enzymatic hydrolysis of **8**.
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30. In a typical experiment, discontinuous enzyme assays were performed in triplicate in buffered conditions (50 mM sodium acetate, pH 6.0) in the presence of **8** (1 mM) and 1 g L^{-1} of BSA. Reactions were incubated at 30 (AXH-d_3, 22 μg mL^{-1}, and AbfB, 36 μg mL^{-1}), 50 (AbfA, 97 μg mL^{-1}), or 60 °C (*Tx*Abf, 28 μg mL^{-1}) for 60 min. Aliquots (50 μL) were removed every 6 min and mixed with 50 μL of cooled (0 °C) 10 mM $NaIO_4$ solution (pH 2) in 96-well microtiter plates. After keeping 10 min at 0 °C throughout, 50 μL of ethylene glycol were added to each of the wells and incubated, followed by 150 μL of 1 M Na_2CO_3 after 10 min. The optical densities at 505 nm were recorded for all wells on a VersaMax microplate reader (molecular devices). Control reactions containing all reactants except enzyme were prepared and incubated for 1 h at 30, 50, or 60 °C.