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Thioimidoyl furanosides as first inhibitors of the α-L-arabinofuranosidase AbfD3

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Abstract—Two sets of five thioimidoyl α-L-arabino- and β-D-galactofuranosides were designed, synthesized and subjected to docking studies to evaluate their ability to be recognized by the active site of the α-L-arabinofuranosidase AbfD3. Further in vitro assays showed that the targeted furanosides are the first potent inhibitors of this furanosyl hydrolase and that the most efficient one, the thiazolyl α-L-arabinofuranoside **1**, is a competitive inhibitor having a K_I of 1.4 μM.

Furanosides are widely present in nature, notably in nucleic acids. However, significant differences must be noticed for other furanose-containing glycoconjugates: indeed, hexofuranosides, such as D-galactofuranosides (D-Gal*f*), are found in microorganisms but not in mammalian cells.[1] As a result, this observation has increased the number of biological and chemical studies for such potential new pharmacological targets.[2–4] On the other hand, the biodistribution of pentofuranosides is much more important. For example, L-arabinofuranose (L-Ara*f*), structuraly analogous to D-Gal*f*, is found in soil bacteria, fungi and plants.[5] In each case, galactans and arabinans are components of the glycocalix that allows cell survival and that also plays an important morphological role. On this basis, the development of inhibitors of enzymes specifically involved in the biosynthesis and catabolism of glycofuranoconjugates is of great interest to improve knowledge connected with furanosyl transferases and/or hydrolases, and to design new compounds likely to modulate their activity. Moreover, as recently shown, the lack of activity related to the α-L-arabinofuranosidase XYL3 resulted in the reduction of seed size and a delayed germination without affecting the seed viability.[5] In this context, we focused our attention on an arabinofuranosidase, the AbfD3, a thermostable enzyme able to degrade the biomass, and more precisely to cleave α-L-arabinofuranosidic linkages.[6] This enzyme was also used as an efficient biocatalyst to prepare natural and non-natural disaccharides containing either α-L-Ara*f* or β-D-Gal*f* non-reducing residues.[7,8] These studies highlighted the versatility of the AbfD3 for the recognition of substrates other than arabinofuranosyl derivatives.

In this article, we present a short and efficient synthesis of the first inhibitors of the AbfD3. Based on our previous chemo-enzymatic approach, we expected the desired inhibitors to be molecularly built up with (i) a sugar head group recognized by the active site, that is, an α-L-Ara*f* group or a close structure such as β-D-Gal*f* which presents a hydroxymethyl added on C-5, (ii) an aglycon part mimicking a carbohydrate entity, and also able to create hydrogen bonds with suitable amino-acid residues and (iii) a stable connection between these two parts. Based on these assumptions, thioimidoyl α-L-arabinofuranosides **1**–**5** and thioimidoyl β-D-galactofuranosides **6**–**10** could satisfy the given criteria (Table 1), that is, the stability of the thioglycosidic linkage as well as the recognition requirements.

To test the possible potency of these arabinofuranosides as inhibitors, docking studies can nowadays be

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Table 1. Chemical structures of targeted thioimidoyl α-L-arabinofuranosides and β-D-galactofuranosides

	α-L-Araf	β-D-Galf
Thiazolyl	**1**	**6**
Benzothiazolyl	**2**	**7**
Pyrimidinyl	**3**	**8**
Pyridinyl	**4**	**9**
Benzimidazolyl	**5**	**10**

performed and used as a first approach. However, since no crystallographic data for the AbfD3 protein has been published, an in silico model was first constructed by homology modelling to the X-ray structures of the α-L-arabinofuranosidase from *Geobacillus stearothermophilus* T6 and from *Clostridium thermocellum*, sharing 26.1% and 26.0% of identity, respectively.[9,10] Then, molecules **1**–**5** were successfully docked into the active site (Fig. 1), according to the experimental protocol, and compared to the "natural" substrate *p*-nitrophenyl α-L-arabinofuranoside (*p*-NP-α-L-Ara*f*). All of them showed similar conformation inside the active site, with free binding energy in the same order of magnitude as for *p*-NP-α-L-Ara*f*. A difference of 1–10 in their affinity constant was nevertheless obtained when varying the aglycon size, without any incidence due to the heteroatom. Therefore, these compounds were all expected to act as competitive inhibitors of the AbfD3, with increasing effect due to the bigger size of the aromatic substituent. D-Galactofuranosides **6**–**10** were not docked into our computer model since their corresponding natural substrate is known to be weakly recognized.[7] Still they were also expected to be inhibitors.

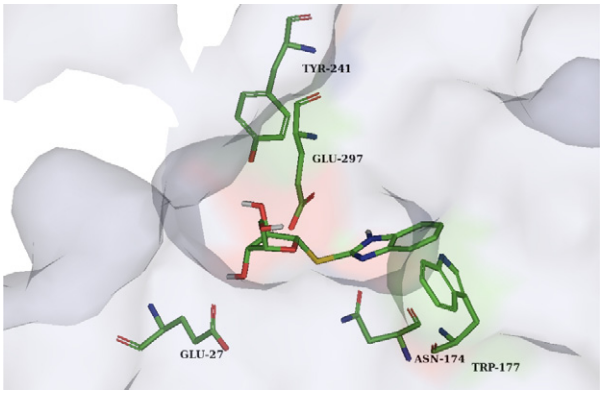
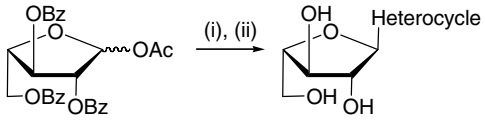


Figure 1. Molecular model of the AbfD3 protein, represented with the docked inhibitor **2**.



Scheme 1. Synthesis of 1-thioimidoyl α-L-arabinofuranosides **2**–**6**. Reagents: (i) Thione, $BF_3{\cdot}OEt_2$, CH_2Cl_2; (ii) MeONa, MeOH (**1**, 49%; **2**, 63%; **3**, 55%; **4**, 61%; **5**, 65%).

As a part of our ongoing research, a general and efficient strategy has recently been developed in our laboratory for the synthesis of 1-thioimidoyl hexofuranosides, readily and exclusively obtained from the corresponding penta-*O*-acetyl hexofuranoses.[11,7] In the present study, we successfully extended this methodology to the pentose analogue of the β-D-Gal*f*, the α-L-Ara*f*, starting from the known 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-α-L- arabinofuranose[12] donor **11** using 2-mercaptothiazole, 2-mercaptobenzothiazole, 2-mercaptopyrimidine, 2-mercaptopyridine or 2-mercaptobenzimidazole, respectively, as an acceptor (Scheme 1). The desired *S*-arabinofuranosides **1**–**5** were thus readily obtained upon activation by boron trifluoride etherate complex and subsequent deacylation under basic Zemplen transesterification (Fig. 1). Careful assignments of the NMR data of these original 1,2-*trans*-thiofuranosides revealed a classical chemical shift around 90 ppm for the anomeric carbon and a coupling constant of $J_{H\text{-}1,H\text{-}2} \approx 4$ Hz.[11–13] In addition to the previously prepared thioimidoyl β-D-galactofuranosides **7**–**10**,[11] thiazolyl 1-thio-β-D-galactofuranoside **6** was prepared from penta-*O*-acetyl galactofuranose[14] and 2-mercaptothiazole, and isolated in 55% yield.

Having thiofuranosides **1**–**10** in hand, their inhibitory activity against the α-L-arabinofuranosidase AbfD3 was studied. The enzymatic assays were performed using an aqueous solution of *p*-NP-α-L-Ara*f* as a substrate, incubated with 0.7 IU AbfD3 at 60 °C.[7] The initial rates of the hydrolytic reactions were determined by measuring the release of *p*-nitrophenol at 400 nm for several concentrations of inhibitor. Preliminary assays using the unglycosylated thiones under similar conditions led to the conclusion that none of these aromatic compounds could act as an inhibitor.

Then, a first set of experiments with both series of sugars, α-L-arabinofuranosides **1**–**5** and β-D-galactofuranosides **6**–**10**, was achieved to evaluate the relative activity of the AbfD3 during the hydrolysis reaction of *p*-NP-α-L-Ara*f* (4.9 mM) at inhibitor concentrations ranging from 0.1 to 6 mM (Fig. 2). First glance at Figure 2 showed an expected global weaker effect of the thiogalactofuranosides **6**–**10** compared to the one caused by the thioarabinofuranosides **1**–**5**. This result is in complete accordance with previous investigations of the AbfD3 specificity towards *p*-nitrophenyl β-D-galactofuranoside (*p*-NP-β-D-Gal*f*) revealing an extremely high K_m

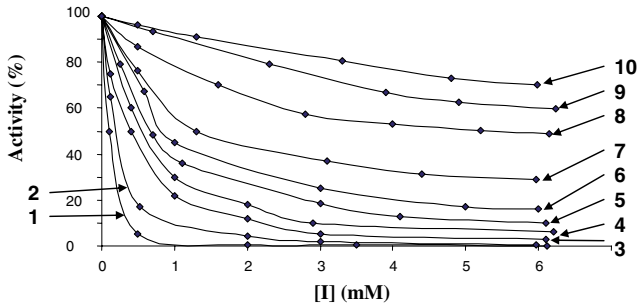


Figure 2. Effects of concentration of 1-thioimidoyl α-L-arabinofuranosides and β-D-galactofuranosides **2**–**6** and **7**–**11**, respectively, on the relative enzymatic activity of AbfD3 (values are means of three experiments).

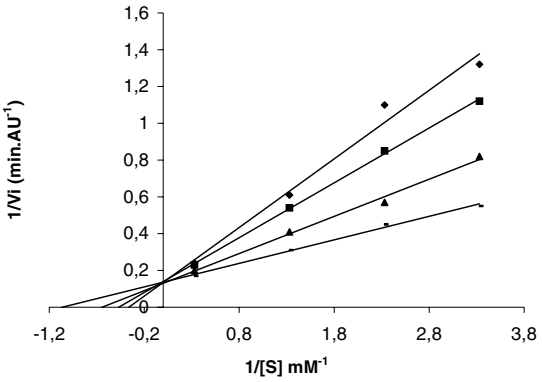


Figure 3. Lineweaver–Burk plots of the AbfD3-catalysed hydrolysis of *p*-NP-α-L-Ara*f* at various concentrations [S] by the arabinofuranoside **1** at concentrations of 2 μM (♦), 1.7 μM (■); 1.3 μM (▲), 1 μM (−). (Values are means of three experiments).

value (>50 mM) compared to the K_m of 0.7 mM for the *p*-NP-α-L-Ara*f*, and suggesting a detrimental effect of the extra hydroxymethyl group at C-5 for substrate binding into the −1 subsite.[15] Moreover, as expected from the described docking study, it is worth mentioning a very coherent influence of the nature of the thioimidoyl aglycons in both series leading. Nevertheless, the in vitro assays showed a decreasing inhibitor effect in the following order: 2-mercaptobenzimidazolyl (**5** and **10**), 2-mercaptopyridinyl (**4** and **9**), 2-mercaptopyrimidinyl (**3** and **8**), 2-mercaptobenzothiazolyl (**2** and **7**) and 2-mercaptothiazolyl (**1** and **6**). We could without ambiguity check, according to the monitoring of the reactions by HPTLC, that none of these thiofuranosides was a substrate of the enzyme under the conditions used since no thione was released. Careful examination of the effects of thioarabinofuranosides **1**–**5** indicated approximate IC_{50} values (molar concentration for 50% inhibition of AbfD3-catalysed hydrolysis of *p*-NP-α-L-Ara*f*) ranging from less than 0.1 mM to around 1 mM.

In both series of furanosides, we could observe a significantly better inhibitor effect when an endocyclic sulfur atom is present within the aglycon (compounds **1**, **2** and **6**, **7**). Therefore, we presumed that the presence of a sulfur atom in the aglycon moiety at an appropriate distance from an electron-deficient residue within the +1 subsite of the enzyme (H^+, NH^+) might be involved in a stabilising hydrogen bond. Moreover, comparison of the relative activity of compounds **1** and **2** suggested a significant effect of the benzene-fused ring attached to the thiazolyl heterocycle leading to a weaker activity for **2**, presumably due to a significant steric hindrance. This observation was corroborated by similar results obtained from β-D-Gal*f* analogues **6** and **7**.

In a second time, considering the greater inhibitor effect of thioimidate **1** among all the compounds tested, we zoomed in the appropriate concentration range (from 0 to 20 μM) the effect of the concentration of **1** on the initial velocity of the hydrolysis reaction and thus evaluate the IC_{50} to be close to 10 μM. The calculated K_I,[16] obtained from this IC_{50}, led to the estimated 1.2 μM.

Finally, by the analysis of the Lineweaver–Burk plots, we checked the competitive inhibitor character of this type of substrate analogue for AbfD3 (Fig. 3). Moreover, the intersections of the different lines with the 1/[S] axis provided a mean K_I value of 1.4 μM,[17] in complete agreement with the previous value estimated using the IC_{50}. This K_I value for **1** is about 700-fold lower than the K_m (0.7 mM) of the *p*-NP-α-L-Ara*f* under these conditions, suggesting a much favourable binding of the thiofuranoside **1** in the active site than the preferred synthetic substrate of the AbfD3.[11,7,15] We assumed that in this compound, the thiazolyl heterocycle may accommodate within the +1 subsite and that the endocyclic sulfur atom may act as a stabilising element for the inhibitor–enzyme complex.

In conclusion, we have prepared the first thioimidoyl furanosides able to inhibit the hydrolytic activity of the arabinofuranosyl hydrolase AbfD3. These compounds were readily obtained in only two steps from peracyl pentose or hexose and a commercially available thione. As it could not be predicted by preliminary in silico study, the best inhibitor was the thiazolyl arabinoside **1**. To explain this difference and in the absence of 3D structure of the AbfD3, we assume that hydrogen bonding may compensate the hydrophobic interactions between a tryptophan residue present in the active site of the enzyme and the aromatic aglycon. The present study supplements well previous structure–activity relationship studies, but further X-ray data of the crystallized or co-crystallized AbfD3 will probably give more details about its active site and would help us in designing better inhibitors.

Homology modelling and docking studies. The homology model was based on the two described structures of L-arabinofuranosidase from the family 51.[9,10] It was constructed using the first approach mode of the Swiss Model website—an automated comparative protein modelling server.[18] Rotatable bonds in the ligands were assigned with Autodock Tools—an accessory program that allows the user to interact with Autodock. Docking simulations were performed with AutoDock 3.0.5 using a Lamarckian genetic algorithm.[19] The standard docking procedure was used for a rigid protein and a flexible ligand whose torsion

angles were identified (for 20 independent runs per ligand). A grid of 60, 60 and 60 points in *x*, *y* and *z* directions was built, centred on the acid/base catalyst residue Glu-176. A grid spacing of 0.375 Å and a distance-dependent function of the dielectric constant were used for the calculation of the energetic map. The default settings were used for all parameters. At the end of docking, ligands with the most favourable free energy of binding and a similar orientation as the 'natural' substrate were selected as the resultant complex structures. All calculations were carried out on PC based machines. The resultant structure files were analyzed using Pymol visualization programs.[20]

Synthesis. Optical rotations were measured on a Perkin-Elmer 341 polarimeter. Melting points were determined on a Reichert microscope and are uncorrected. Thin-layer chromatography (TLC) analyses were conducted on E. Merck 60 F_{254} Silica Gel non-activated plates and compounds were revealed using a 5% solution of H_2SO_4 in EtOH followed by heating. For column chromatography, Geduran Si 60 (40–63 μm) Silica Gel was used. ^1H, ^{13}C, HMQC and COSY NMR spectra were recorded on a Bruker ARX 400 spectrometer at 400 MHz for ^1H and 100 MHz for ^{13}C, respectively. Chemical shifts are given in δ-units (ppm) measured downfield from Me_4Si. The HRMS were measured at the CRMPO with a MS/MS ZabSpec TOF Micromass using *m*-nitrobenzylic alcohol as a matrix and accelerated caesium ions for ionisation.

Thiazol-2-yl 1-thio-α-L-arabinofuranoside (***1***). CH_2Cl_2/MeOH (9:1), R_f 0.5; mp: 157 °C; $[\alpha]_D^{20}$ +25 (*c* 1, MeOH); ^1H NMR (CD_3OD) δ (ppm): 7.70 (d, 1H, CHS, J = 3.3 Hz), 7.50 (d, 1H, CHN), 5.60 (d, 1H, H-1, $J_{1,2}$ 3.3 Hz), 4.07 (dd, 1H, H-2, $J_{2,3}$ 4.2 Hz), 3.99–4.03 (m, 1H, H-4), 3.94 (dd, 1H, H-3, $J_{3,4}$ = 6.4 Hz), 3.72 (dd, 1H, H-5a, $J_{4,5a}$ = 3.3 Hz, $J_{5a,5b}$ = 12.4 Hz), 3.63 (dd, 1H, H-5b, $J_{4,5b}$ = 4.9 Hz); ^{13}C NMR (CD_3OD) δ(ppm): 160.1 (SCN), 144.1 (CHS), 123.5 (CHN), 94.3 (C-1), 88.4 (C-4), 86.5 (C-2), 79.8 (C-3), 62.7 (C-5); HRMS (ESI^+) for $C_8H_{11}O_4NS_2Na$: *m*/*z* $[M]^+$: calcd 272.0027; found 272.0031.

Benzothiazol-2-yl 1-thio-α-L -arabinofuranoside (***2***). CH_2Cl_2/MeOH (9:1), R_f 0.5; mp: 192 °C; $[\alpha]_D^{20}$ +74 (*c* 1, MeOH); ^1H NMR (CD_3OD) δ (ppm): 7.83–7.81 (m, 2H, CHCN, CHCS), 7.49–7.47 (m, 2H, C*H*CHCN, C*H*CHCS), 5.80 (d, 1H, H-1, $J_{1,2}$ = 4.2 Hz), 3.88 (dd, 1H, H-2, $J_{2,3}$ 4.4 Hz), 3.85–3.75 (m, 2H, H-4, H-3), 3.60 (dd, 1H, H-5a, $J_{4,5a}$ = 3.4 Hz, $J_{5a,5b}$ = 12.1 Hz), 3.48 (dd, 1H, H-5b, $J_{4,5b}$ = 5.0 Hz); ^{13}C NMR (CD_3OD) δ (ppm): 150.6 (SCS), 134.9, 134.3 (CH*C*N, CH*C*S), 131.1, 129.8 (*C*HCHCS, *C*HCHCN), 124.9, 122.3 (*C*HCS, *C*HCN), 90.7 (C-1), 85.7 (C-4), 83.9 (C-2), 78.2 (C-3), 62.8 (C-5); HRMS (ESI^+) for $C_{12}H_{13}O_4NS_2Na$: *m*/*z* $[M]^+$: calcd 322.0184; found 322.0187.

Pyrimidin-2-yl 1-thio-α-L -arabinofuranoside (***3***). CH_2Cl_2/MeOH (9:1), R_f 0.5; mp: 201 °C; $[\alpha]_D^{20}$: +107 (*c* 1, MeOH); ^1H NMR (CD_3OD) δ (ppm): 8.42 (d, 2H, CHN, J = 4.9 Hz), 7.40–7.35 (m, 1H, C*H*CHN), 6.20 (d, 1H, H-1, $J_{1,2}$ = 2.4 Hz), 4.13 (dd, 1H, H-2, $J_{2,3}$ = 4.2 Hz), 4.09–3.92 (m, 2H, H-4 H-3), 3.65–3.55 (m, 2H, H-5a, H-5b); ^{13}C NMR (CD_3OD) δ(ppm): 160.9 (SCS), 120.7 (2 CHN), 111.5 (*C*HCHCN), 92.8 (C-1), 89.2 (C-4), 85.7 (C-2), 81.2 (C-3), 65.8 (C-5); HRMS (ESI^+) for $C_9H_{12}O_4N_2SNa$: *m*/*z* $[M]^+$: calcd 267.0415; found 267.0415.

Pyridin-2-yl 1-thio-α-L-arabinofuranoside (***4***). CH_2Cl_2/MeOH (9:1), R_f 0.6; mp: 169 °C; $[\alpha]_D^{20}$ +22 (*c* 1, MeOH); ^1H NMR (CD_3OD) δ (ppm): 8.31 (ddd, 1H, CHN, J = 0.9 Hz, J = 1.8 Hz, J = 4.9 Hz), 7.52 (ddd, 1H, C*H*CHCHN, J = 7.5 Hz, J = 7.6 Hz), 7.21 (ddd, 1H, C*H*CHCHCHN, J = 1.1 Hz), 7.00 (ddd, 1H, C*H*CHN), 6.05 (d, 1H, H-1, $J_{1,2}$ = 3.1 Hz), 4.10 (dd, 1H, H-2, $J_{2,3}$ = 4.3 Hz), 4.00 (dd, 1H, H-3, $J_{3,4}$ = 4.0 Hz), 3.96-3.92 (m, 1H, H-4), 3.64 (dd, 1H, H-5a, $J_{5a,5b}$ = 11.9 Hz $J_{4,5a}$ = 3.3 Hz), 3.56 (dd, 1H, H-5b, $J_{4,5b}$ = 4.0 Hz); ^{13}C NMR (CD_3OD) δ (ppm): 159.2 (SCN), 149.7 (CHN), 137.0 (*C*HCHCHN), 123.0 (*C*HCHCHCHN), 120.5 (*C*HCHN), 89.6 (C-1), 85.8 (C-4), 83.3 (C-2), 77.8 (C-3), 62.3 (C-5); HRMS (ESI^+) for $C_{10}H_{13}O_4NSNa$: *m*/*z* $[M]^+$: calcd 243.0565; found 243.0569.

Benzimidazol-2-yl 1-thio-α-L-arabinofuranoside (***5***). CH_2Cl_2/MeOH (9:1), R_f 0.61; mp: 158 °C; $[\alpha]_D^{20}$ +69 (*c* 1, MeOH); ^1H NMR (CD_3OD) δ (ppm): 7.51–7.49 (m, 2H, CHCN, C*H*CNH), 7.21–7.19 (m, 2H, C*H*CHCN, C*H*CHCNH), 5.76 (d, 1H, H-1, $J_{1,2}$ = 3.8 Hz), 4.10 (dd, 1H, H-3, $J_{2,3}$ = 4.4 Hz, $J_{3,4}$ = 3.9 Hz), 4.08 (ddd, 1H, H-4, $J_{4,5a}$ = 2.8 Hz, $J_{4,5b}$ = 5.5 Hz), 3.88 (dd, 1H, H-2), 3.78 (dd, 1H, H-5a, $J_{5a,5b}$ = 12.2 Hz), 3.66 (dd, 1H, H-5b); ^{13}C NMR (CD_3OD) δ (ppm): 149.6 (SCN), 134.3, 134.1 (CN, CNH), 130.1, 128.9 (*C*HCHCN, *C*HCHCNH), 123.9, 121.3 (*C*HCN, *C*HCNH), 92.3 (C-1), 85.7 (C-4), 83.7 (C-2), 77.9 (C-3), 62.5 (C-5); HRMS (ESI^+) for $C_{12}H_{14}O_4N_2SNa$: *m*/*z* $[M]^+$: calcd 305.0572; found 305.0576.

Thiazol-2-yl 1-thio-β-D-galactofuranoside (***6***). CH_2Cl_2/MeOH (9:1), R_f 0.4; ^1H NMR (CD_3OD) δ (ppm): 7.72 (d, 1H, CHN, J = 3.3 Hz), 7.56 (d, 1H, CHS), 5.56 (d, 1H, H-1, $J_{1,2}$ = 3.8 Hz), 4.12 (dd, 1H, H-3, $J_{2,3}$ = 4.6 Hz, $J_{3,4}$ = 6.6 Hz), 4.07–4.04 (m, 2H, H-2, H-4), 3.72 (ddd, 1H, H-5, $J_{4,5}$ = 2.8 Hz, $J_{5,6a}$ = 6.1 Hz, $J_{5,6b}$ = 6.1 Hz), 3.60–3.58 (m, 2H, H-6a, H-6b); ^{13}C NMR (CD_3OD) δ (ppm): 160.2 (SCN), 144.3 (CHS), 123.5 (CHN), 93.8 (C-1), 85.0 (C-4), 78.7 (C-2), 78.3 (C-3), 72.0 (C-5), 64.4 (C-6); HRMS (ESI^+) for $C_9H_{13}NO_5S_2Na$: *m*/*z* $[M]^+$: calcd 302.0133; found 302.00136.

Enzymatic inhibition. The hydrolytic activity of AbfD3 was quantified after incubation of the enzyme (0.7 IU) with *p*-NP-α-L-Ara*f* at 60 °C in water in the absence or presence of inhibitor. Continuous release of *p*-nitrophenol was measured at 400 nm. One unit of activity corresponds to the amount of enzyme releasing 1 μmol of *p*-nitrophenol per minute. The inhibitor and the substrate aqueous solutions were mixed and prewarmed before the addition of enzyme. Initial rates of the hydrolytic reaction were used in order to determine the relative activity using various inhibitor concentrations between

0.1 μM and 6 mM (the maximal activity corresponding to the initial rate of the reaction without any inhibitor) and each value is the mean of three experiments. The inhibition constant K_I was determined from Lineweaver–Burk plots using substrate concentrations between 0.3 and 3 mM and inhibitor concentrations between 1 and 2 μM, thanks to the following Michaelis–Menten equation for competitive inhibition:

$$1/V_0 = K_m(1 + [I]/K_I)/V_m[S] + 1/V_m$$

K_I value was corroborated by the following equation:[17]

$$K_I = IC_{50}/(1 + [S]/K_m).$$

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