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Phosphodiesters serve as potentially tunable aglycones for fluoro sugar inactivators of retaining β-glycosidases†

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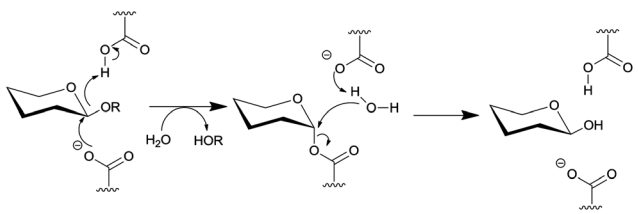
2-Deoxy-2-fluoroglycosides bearing dibenzyl phosphate and phosphonate aglycones were synthesised and tested as covalent inactivators of several retaining α- and β-glycosidases. β-D-Gluco-, -manno- and -galacto-configured benzyl-benzylphosphonate derivatives efficiently inactivated β-gluco-, β-manno- and β-galactosidases, while α-gluco- and α-manno-configured phosphate and phosphonate derivatives served instead as slow substrates.

Glycosidases play key roles in a number of diseases in humans, including metabolic disorders such as Gaucher's disease,[1] cancer,[2] HIV/AIDS,[3] Parkinson's disease,[1] and influenza.[4,5] Specific inhibitors of glycosidases are therefore of interest as potential therapeutics, as well as tools for structural and mechanistic characterisation of the enzymes themselves. Retaining glycosidases are those that hydrolyse the glycosidic bond with net retention of anomeric stereochemistry. Most retaining glycosidases characterized to date employ a double-displacement mechanism. In the first step, nucleophilic attack of an active site carboxylate (Glu or Asp) on the sugar anomeric centre, along with activation of the leaving group through general acid catalysis, generates a covalent glycosyl-enzyme intermediate in what is termed the glycosylation step. During the subsequent deglycosylation step, base-promoted attack of water at the anomeric centre completes the catalytic cycle to regenerate the free enzyme and the sugar residue with the same anomeric configuration as the substrate, as represented in Scheme 1 for a retaining β-glycosidase.[6] Both steps proceed *via* oxocarbenium ion-like transition states. 2-Deoxy-2-fluoroglycosides bearing good leaving groups have been shown to function as highly efficient and selective mechanism-based inactivators for a variety of retaining β-glycosidases.[7] The presence of the highly electronegative fluorine atom on C2 of the pyranosyl ring slows both steps of the reaction by destabilizing both glycosylation and deglycosylation transition states. This occurs through a combination of inductive effects and disruption of critical active site hydrogen-bonding interactions with the hydroxyl normally found on C2 in the natural substrates. The activated leaving group at the anomeric centre selectively accelerates the glycosylation step, leading to an overall accumulation of a highly stabilised covalent glycosyl-enzyme intermediate.[8,9] This strategy is less successful for retaining α-glycosidases: instead the 2-fluorosugars tend to act as slow substrates with a rate-limiting glycosylation step, most likely due to differences in the extent of positive charge development on the anomeric centre *vs.* the ring oxygen atom in each step.[5] Consistent with this, 5-fluoroglycosyl fluorides, wherein the fluorine substituent is close to the endocyclic oxygen, do indeed serve as covalent inactivators of retaining α-glycosidases.[9,10]



Scheme 1 Mechanism for a typical retaining β-glycosidase.

Kinetic studies with a series of aryl 2-deoxy-2-fluoroglycosides revealed that the phenol pK_a should be 5 or lower for the reagents to function as efficient covalent inactivators.[11] Indeed the majority of reported fluorosugar inactivators either use fluoride or 2,-4-dinitrophenol aglycones, although chloride and 2,4,6-trinitrophenol aglycones were used as leaving groups for 2-deoxy-2,2-difluoroglycoside inactivators of retaining α-glycosidases.[12] Further, Overkleeft and co-workers recently employed an *N*-phenyl trifluoroacetimidate as leaving group on a 2-fluorosugar inactivator of human β-glucocerebrosidase.[13] Interestingly, the natural aglycones were also reported

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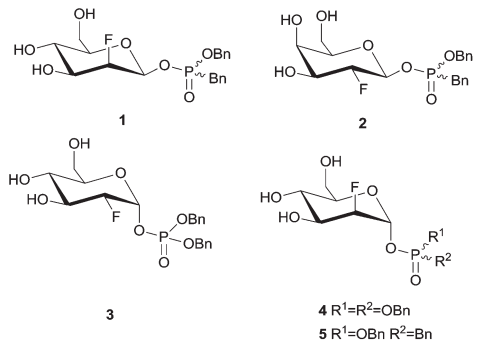
to serve as sufficiently reactive leaving groups for 2-deoxy-2-fluoroglycosides when the rate-limiting step for that natural substrate/enzyme pair was indeed the deglycosylation step.[14,15] In those cases the enzyme recruits transition state-stabilising interactions between the natural leaving group and the aglycone sub-site to accelerate the glycosylation step.

With that idea in mind we recently reported the synthesis and testing of dialkyl phosphate and phosphonate-containing 2-fluorosugar inactivators of human β-glucocerebrosidase that exploit the preference of that enzyme for binding hydrophobic aglycones. The best three inactivators, which inactivate GCase 1000–4000 times faster than does 2-fluoroglucosyl fluoride, were those with di-*O*-octyl phosphate, di-*O*-benzyl phosphate, or benzyl benzylphosphonate leaving groups, with the latter displaying the best balance between hydrolytic stability and enzymatic reactivity.[16] Indeed the di-*O*-alkyl phosphate derivatives underwent spontaneous hydrolysis with a half life of around 15 minutes, while the dibenzylphosphonate derivative was considerably more stable with a half-life around 10 hours.

Since the alkylated phosphate leaving groups are highly activated we were interested in exploring how broadly this new class of activated fluorosugar can be used as covalent inactivators of retaining glycosidases, even in the absence of specific aglycone site interactions. These compounds are of particular interest since their physical, thus pharmacokinetic, properties can be modulated by modifying the alkyl substituent without substantial effect on inherent reactivity of the sugar moiety. To this end, probes **1–5** (Scheme 2) were synthesized and tested as substrates/inactivators for a variety of retaining α- and β-glycosidases. We hypothesized that the α-configured probes would function as slow substrates for the appropriate retaining α-glycosidases without any accumulation of a covalent glycosyl-enzyme intermediate, since it is known that their covalent 2-deoxy-2-fluoroglycosyl-enzyme intermediates are rapidly turned over.[9,10] For the β-configured probes, the dibenzyl phosphonate aglycone was chosen as it had been shown to be relatively stable towards spontaneous hydrolysis while maintaining reactivity with the enzyme.[16] Dibenzyl phosphate and phosphonate derivatives were chosen for the α-configured probes based on their relative synthetic accessibility.

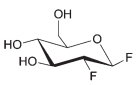
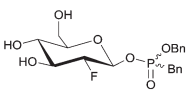
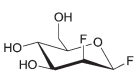
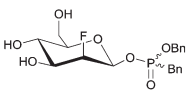
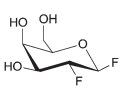
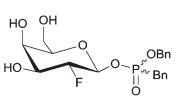


Scheme 2 Compounds tested as covalent inactivators of retaining β-glycosidases (**1** and **2**) and as slow substrates for retaining α-glycosidases (**3–5**).

Probes **1–5** were synthesized from their precursor 2-deoxy-2-fluoroglycopyranosides, as outlined in the ESI (see Schemes S1–S4†). Note that compounds **1**, **2** and **4** were prepared and evaluated as diastereomeric mixtures at phosphorus. While the yields were low for the production of the per-*O*-acetylated precursors to **3–5**, modest amounts could be purified after selective decomposition by exploiting the greater stability of the α-anomer relative to the readily available β-anomer.

Compounds **1** and **2** were tested as covalent inactivators of retaining β-glycosidases by measuring residual enzyme activity as a function of time upon incubation with a range of concentrations of **1** or **2**. Data yielded pseudo-first-order inactivation rate constants at each inhibitor concentration. Fitting of these values to a Michaelis–Menten equation yielded values of k_i, K_i, and k_i/K_i. Kinetic parameters for these compounds and the gluco version synthesised previously[13] as inactivators of the relatively broad specificity retaining β-glucosidase from *Agrobacterium* sp. (Abg)[17] are shown in Table 1, along with previously determined values for other fluorosugars. First order fits are presented as Fig. S1–S2 in the ESI.† The time-dependent loss of enzyme activity measured from treating Abg with either **1** or **2** was too rapid to permit sampling at concentrations approaching saturation, so only k_i/K_i values could be measured. Both **1** (k_i/K_i = 23.9 min^{-1} mM^{-1}) and **2** (k_i/K_i = 3.0 min^{-1} mM^{-1}) functioned as rapid, time-dependent inactivators of Abg, with values bracketing those for the gluco- and manno-analogues.[11,18] This makes the manno-configured compound **1** the second most efficient fluorosugar inactivator of Abg reported to date, with only 2,4-dinitrophenyl 2-deoxy-2-fluoro-

Table 1 Kinetic parameters for selected fluorosugars as inactivators of Abg

Compound	k_i (min^{-1})	K_i (mM)	k_i/K_i (min^{-1} mM^{-1})
	5.9	0.40	14.8
	—	—	11
	5.6	1.2	4.7
	—	—	23.9
	2.6	3.2	0.81
	—	—	3.0

β-D-glucopyranoside (k_i/K_i = 500 min^{-1} mM^{-1})[19] being a better inactivator. The phosphonate derivative of the galacto-sugar, **2**, was also a better inactivator than the corresponding fluoride.[11,18]

Compound **1** was also tested as a covalent inactivator of the retaining β-mannosidase from *Cellulomonas fimi* (Man2A)[20] yielding inactivation parameters of k_i = 0.52 min^{-1}, K_i = 3.7 mM, and k_i/K_i = 0.14 min^{-1} mM^{-1}. Interestingly this is almost a 10-fold worse inactivator of Man2A than is 2-deoxy-2-fluoro-β-D-mannopyranosyl fluoride (k_i = 0.57 min^{-1}, K_i = 0.41 mM, and k_i/K_i = 1.4 min^{-1} mM^{-1}),[20] due almost entirely to an increase in K_i. This suggests that the benzyl benzylphosphonate aglycone is less well accommodated in the active site than is the much smaller fluoride aglycone. However, there does not appear to be a significant effect on the energies of the two transition states relative to their respective ground states, as the k_i values are extremely close in magnitude.

Compound **2** proved to be a useful inactivator of the retaining β-galactosidase from *Escherichia coli* (Lac-Z), with kinetic parameters of k_i = 0.14 min^{-1}, K_i = 0.058 mM, and k_i/K_i = 2.5 min^{-1} mM^{-1}. It is therefore a tighter binding inactivator than is 2-deoxy-2-fluoro-β-D-galactopyranosyl fluoride (k_i = 13.2 min^{-1}, K_i = 1.3 mM, and k_i/K_i = 10.2 min^{-1} mM^{-1}),[21] but undergoes reaction almost 10 fold slower. Fluoride thus appears to be the aglycone that is best accommodated in the glycosylation transition state, as can be seen from a comparison of the relative k_i values, while the benzyl benzylphosphonate aglycone appears to be the one that binds tightest to the enzyme in the ground state. This finding is consistent with the fact that Lac-Z is known to have a relatively open active site that can accommodate both of the natural substrates, allolactose and lactose.[22]

Probe **3** was incubated with yeast α-glucosidase (Yag) to test whether it functioned as an inactivator, or as a slow substrate. When residual enzyme activity was measured as a function of time, no time-dependent decrease in activity was observed either at 37 °C or 4 °C, demonstrating that no substantial accumulation of the covalent intermediate was occurring. A TLC analysis of the reaction products from incubation of Yag and **3** showed the enzyme-dependent formation of 2-deoxy-2-fluoro-D-glucose, confirming that **3** was indeed acting as a substrate. By testing **3** and 4-nitrophenyl α-D-glucopyranoside[23] as competitive substrates for Yag under steady state conditions, an apparent K_i' value for **3** (corresponding to the K_m value for **3** as a substrate) of 0.7 mM was obtained (see Fig. S6†).

Probes **4** and **5** were separately evaluated as substrates for Jack bean α-mannosidase (JBAM). Neither behaved as a covalent inactivator at either 37 °C or 4 °C when enzyme activity was monitored as a function of incubation time. Further, TLC analysis indicated that only **4** was hydrolysed by the enzyme. Evaluation of **4** as a competitive substrate for the known substrate 2,4-dinitrophenyl α-D-mannopyranoside[24] for JBAM under steady state conditions gave an apparent K_i' value of ~5 mM, indicating that **4** was a relatively poor substrate for JBAM (see Fig. S8†).

In conclusion, 2-deoxy-2-fluoro-β-D-gluco-, -manno- and -galactosides bearing benzyl-benzylphosphonate aglycones were each shown to function as covalent inactivators for their cognate β-glucosidase, β-mannosidase, and β-galactosidase. In addition, all three compounds functioned as inactivators of a single broad-spectrum β-glycosidase (Abg). These results demonstrate the broad applicability of this new class of compounds as covalent inactivators for retaining β-glycosidases. In general the inactivators were of comparable reactivity to analogous inactivators bearing fluoride or dinitrophenolate aglycones, consistent with their roughly comparable chemical reactivities. As anticipated, none of the alpha-linked versions, 2-deoxy-2-fluoro-α-D-glucopyranosyl dibenzyl phosphate **3**, 2-deoxy-2-fluoro-α-D-mannopyranosyl dibenzyl phosphate **4**, or 2-deoxy-2-fluoro-α-D-mannopyranosyl benzyl-benzylphosphonate **5** functioned as covalent inactivators of their cognate α-glycosidases. While both the dibenzyl phosphate derivatives were hydrolysed as slow substrates, 2-deoxy-2-fluoro-α-D-mannopyranosyl benzyl-benzylphosphonate **5** did not bind to the jack bean α-mannosidase at any concentration tested. In general, the use of substituted phosphonate aglycones as leaving groups in fluorosugar-based inactivators of β-glycosidases yields reagents of generally comparable reactivity to the commonly employed fluoride aglycone. However the alkyl substituents employed on the phosphonate may be chosen to either modulate pharmacokinetic behaviour of the inhibitors or to harness specific interactions with the enzyme's aglycone subsite to improve both efficiency and selectivity.

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