Glycosidase-catalyzed hydrolysis of 2-deoxyglucopyranosyl pyridinium salts: effect of the 2-OH group on binding and catalysis

Kelly S. E. Tanaka, Jiang Zhu, Xicai Huang, Francesco Lipari, and Andrew J. Bennet

Abstract: Three 2-deoxy-α-D-glucopyranosyl pyridinium tetrafluoroborates were tested for their binding affinity to a range of α-glucosidases and α-mannosidases. The α-isoquinolinium salt (**11**) binds approximately 275–fold more tightly to yeast α-glucosidase than does the isomeric quinolinium salt (**12**). In addition, compound **11** binds to the yeast enzyme approximately two-fold tighter than the corresponding glucopyranosyl isoquinolinium salt (**9**). The (k_{cat}/k_{hyd}) values for the yeast α-glucosidase-catalyzed reactions of **11** and **9** are 1.6×10^5 and 2.0×10^9, respectively, when compared to the spontaneous uncatalyzed reactions. Thus, the interaction of the 2-OH group in compound **9** with the yeast enzyme's active site generates a relative transition state stabilization of about 23.5 kJ mol^{-1}. For both compounds **11** and **12**, the observed rate accelerations for the yeast α-glucosidase-catalyzed hydrolysis, relative to the spontaneous reaction in solution, (k_{cat}/k_{hyd}) are identical within experimental error.

Key words: glycosidase, inhibitor, 2-deoxyglucose, pyridinium, catalysis.

Résumé : On a évalué les affinités de liaison de trois fluoroborates de 2-désoxy-α-D-glucopyranosyl pyridinium avec un ensemble d'α-glucosidases et d'α-mannosidases. Le sel d'α-isoquinoléinium (**11**) se lie approximativement 275 fois fortement à l'α-glucosidase de la levure que le sel quinoléinium isomère (**12**). De plus, le composé **11** se lie à l'enzyme de la levure approximativement deux fois mieux que le sel du glucopyranosyl isoquinoléinium correspondant (**9**). Les valeurs de (k_{cat}/k_{hyd}) pour les réactions des composés **11** et **9** catalysées par l'α-glucosidase de la levure sont égales respectivement à $1{,}6 \times 10^5$ et $2{,}0 \times 10^9$ lorsqu'on les compare aux réactions spontanées non catalysées. On en déduit que l'interaction du groupe 2-OH du composé **9** avec le site de l'enzyme de la levure génère une stabilisation relative de l'état de transition d'environ 23,5 kJ mol^{-1}. Pour chacun des composés **11** et **12**, les accélérations de vitesse observées pour les hydrolyses catalysées par l'α-glucosidase de la levure, comparées à la réaction spontanée en solution (k_{cat}/k_{hyd}) sont identiques aux erreurs expérimentales près.

Mots clés : glucosidase, inhibiteur, 2-désoxyglucose, pyridinium, catalyse.

Introduction

Recent advances in the rapidly growing field of glycobiology have provided insights into the fundamental importance of glycoproteins, glycolipids, and oligosaccharides in a myriad of biological events (1). Clearly, glycoconjugate-processing enzymes play a crucial role in the scheme of these various biological events (2). As a key subclass of glycoconjugate-processing enzymes, glycosidases are the focus of ongoing research aimed at the discovery of new glycosidase inhibitors (3). One class of naturally occurring glycosidase inhibitors are carbohydrate mimics that contain an endocyclic amino group, the so-called "imino-sugars" (4), a notable example of which is 1-deoxynojirimycin (DNJ, **1**) a compound that has the same hydroxyl configuration as 1-deoxyglucose (5). In general, imino-sugars are good inhibitors of glycosidase enzymes, probably due to protonation at physiological pH of the secondary amine of the imino-sugar, thus generating a cation mimic of the glucosyl oxacarbenium ion (**2**) which emulates the glycosylation transition state structure (3, 6).

New glycosidase inhibitors isolated from natural sources are often prototypes for the design and synthesis of analogues as potential therapeutic agents. For example,

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K.S.E. Tanaka,[1] J. Zhu, X. Huang,[2] and A.J. Bennet.[3] Department of Chemistry, Simon Fraser University, Burnaby, British Columbia, V5A 1S6, Canada.
F. Lipari.[4] McGill Cancer Centre, McGill University, 3655 Drummond Street, Montréal, QC H3G 1Y6, Canada.

[1]Current address: Department of Biochemistry, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, U.S.A.
[2]Current address: Conjuchem, 225 President-Kennedy Avenue, Montréal, QC H2X 3Y8, Canada.
[3]Author to whom correspondence may be addressed. Telephone: (604) 291-3532. Fax: (604) 291-3765. e–mail: bennet@sfu.ca
[4]Current address: Boehringer Ingelheim (Canada) Ltd. BioMéga Research Division, 2100 Cunard Street, Laval, QC H7S 2G5, Canada.

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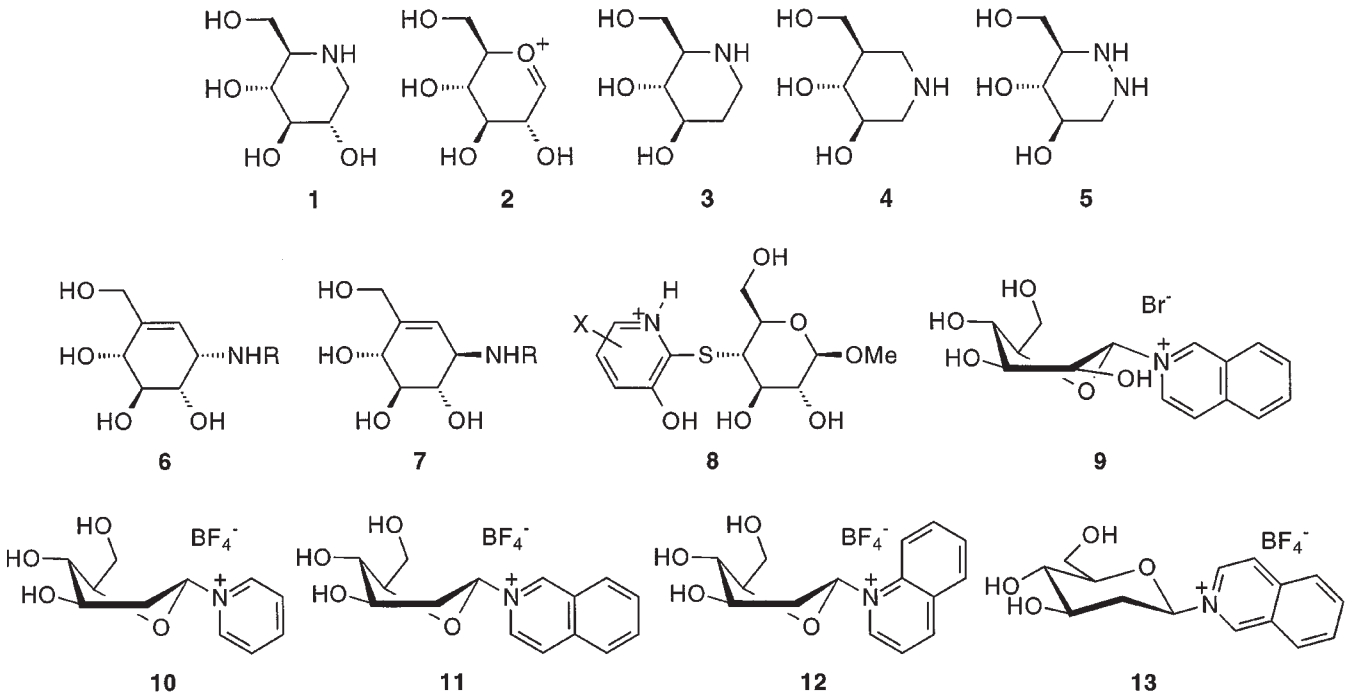
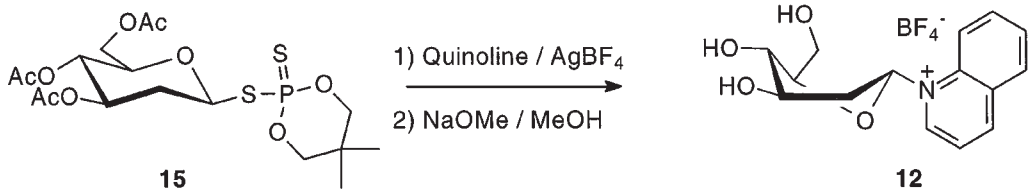


Scheme 1.



isofagomine (**4**) an analogue of the natural product fagomine (**3**) isolated from buckwheat seeds (7) has recently been synthesized and shown to be a potent inhibitor (K_i = 0.11 μM) of the almond-emulsion β-glucosidase (8). Yet another related compound, 1-azafagomine (**5**) is a good inhibitor of both yeast α-glucosidase (K_i = 3.9 μM) and almond-emulsion β-glucosidase (K_i = 0.65 μM) (9).

A separate class of nitrogen-containing natural products is the "amino-sugars" which possess an exocyclic rather than endocyclic amino group (4). One such family of amino-sugar glycosidase inhibitors is the validamycins. which contain the critical valienamine structural motif (**6**). An example of this type of glycosidase inhibitor is the tight binding natural product acarbose (10, 11). Various epimeric analogues of valienamine, i.e., epi-valienamine (**7**) have also been synthesized and tested as potential β-glycosidase inhibitors (12).

Using an alternative strategy, Knapp et al. (13) explored the effectiveness of substituted 3-hydroxypyridines (**8**) rather than the more customary carbohydrate-based compounds as glycosylation TS mimics. The tightest binding of these aromatic compounds (**8**, X = H) was shown to be a weak competitive inhibitor (K_i = 0.8 mM) of *Agrobacterium* β-glucosidase (13).

Natural substrates generally are bound much more weakly to enzymes than the corresponding natural inhibitors (14), and as a result of this, the design of potential reversible glycosidase inhibitors rarely, if ever, exploit the modification of a natural substrate structure. Most glycosidase-catalyzed reactions are, however, monitored using nonnatural substrates that contain an aromatic aglycon leaving group. In 1985, Hosie and Sinnott (15) reported that yeast α-glucosidase efficiently catalyzes the hydrolysis of α-D-glucopyranosyl pyridinium bromides with an apparent binding constant (K_m) for the isoquinolinium substrate (**9**) of 6.6 μM. Thus, this man-made substrate binds to yeast α-glucosidase more tightly than the naturally occurring inhibitor DNJ (**1**) (K_i = 13 μM) (16).

Given that the C-2 hydroxyl-group of glycosides is critical for efficient glycosidase-catalyzed hydrolysis of carbohydrate substrates (17), we decided to test whether 2-deoxyglucopyranosyl pyridinium salts would retain the tight binding interactions exhibited by their 2-hydroxy counterparts, while simultaneously becoming more resistant to enzyme-catalyzed hydrolysis. Accordingly, the 2-deoxy-α- and β-glucopyranosyl pyridinium salts **10**, **11**, **12**, and **13** were synthesized, and their binding affinities and hydrolytic stabilities were evaluated for various glucosidase and mannosidase enzymes.

Results and discussion

The route used for the synthesis of **12** is shown in Scheme 1. This synthetic scheme involves a silver-promoted S_N2 reaction and is similar to the synthetic route employed for the production of both **10** and **11** (18).

Presented in Table 1 are the inhibition constants (K_i) measured for the interaction of **10**, **11**, **12**, and **13** with several

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Table 1. Dissociation constants (μM) measured for the binding of 2-deoxyglucopyranosyl pyridinium salts to various glycosidases.[a]

Enzyme and Source	**10**	11	12	13
α-Glucosidase — yeast[b]	107 (8)	2.9 (0.5)	800[c]	—
α-Glucosidase — rice[d,e]	141 (52)	44 (7)	NI[f]	—
β-Glucosidase — almonds[b]	—	—	—	7400 (800)
α-Mannosidase — almonds[b]	31000[g]	8000[h]	—	–
α-Mannosidase — yeast[d]	NI[i]	NI[j]	—	—

[a]Data were analyzed using nonlinear least-squares regression; estimate of standard error in brackets.
[b]Temperature = 25°C.
[c]Determined as a K_m value that was estimated using five concentrations of **12** between 0.112 mM and 2.52 mM, estimated error 25%.
[d]Temperature = 37°C.
[e]Slow onset to maximal binding.
[f]No inhibition detected at 1.1 mM.
[g]Value estimated using two concentrations of **10** (3.5 and 8.4 mM); estimated error 15%.
[h]Value estimated using two concentrations of **11** (1.3 and 2.5 mM); estimated error 40%.
[i]No inhibition detected at 5.0 mM.
[j]No inhibition detected at 1.0 mM.

Table 2. Rate constants (k_{cat} and k_{hyd}) for the glycosidase-catalyzed and the spontaneous hydrolyses of 2-deoxyglucopyranosyl pyridinium salts at 25°C.

	9	11	12
Yeast α-glucosidase (k_{cat}) s^{-1}	0.52[a]	7.1×10^{-3}[b]	5.5[c]
Spontaneous hydrolysis (k_{hyd}) s^{-1}	2.6×10^{-10} [d]	4.58×10^{-8} [e]	3.14×10^{-5} [f]
Rate acceleration (k_{cat}/k_{hyd})	2.0×10^{9}	1.6×10^{5}	1.8×10^{5}

[a]Value taken from ref. 15.
[b]Calculated from relative k_{cat} values measured in comparison to compound **9** (15), estimated error 5%.
[c]Calculated from relative k_{cat} values measured in comparison to compound **9** (15), estimated error 25%.
[d]Extrapolated from a $pK_a(B–H^+)$ value of 5.38 and eq. [3] taken from ref. 22*a*.
[e]Value extrapolated from kinetic data measured between 55–95°C.[5]
[f]Value measured directly at 25°C.[5]

glycosidases. A Lineweaver–Burk plot of the kinetic data for yeast α-glucosidase-catalyzed hydrolysis of 4-nitrophenyl α-D-glucopyranoside in the presence of various concentrations of **11** is illustrated in Fig. 1. When detailed kinetic measurements were possible (K_i < 200 μM), only competitive inhibition of the glycosidases by the test compounds was observed. Consequently, these compounds are binding in a reversible manner to the enzyme active site. Table 2 presents the measured k_{cat} values for yeast α-glucosidase-catalyzed hydrolysis of **9**, **11**, and **12**. Also given in Table 2 are the corresponding spontaneous first-order reaction rates (k_{hyd}).

Hosie and Sinnott (15) proposed that yeast α-glucosidase-catalyzed hydrolysis of glucopyranosyl pyridinium salts occurs via the mechanism depicted in Scheme 2, a process in which glucosylation (k_{gluc}) is rate-limiting for k_{cat}, and a kinetically significant nonchemical step (k_2) is rate-limiting for k_{cat}/K_m.

The same scheme must hold for the reactions of the 2-deoxy salts, since: (*i*) k_{cat} for **12** is about 800–fold greater than k_{cat} for **11** (Table 2), thus k_{degluc} cannot be rate limiting (i.e., $k_{degluc} > k_{gluc}$); and (*ii*) k_{cat}/K_m for **11** and **12** are within a factor of three of each other and consequently, $_{kgluc}$ is not the major rate limiting step for k_{cat}/K_m. The apparent K_m for an enzyme-catalyzed reaction is given by eq. [1], where Σ[ES] is the sum of all enzyme bound species (14). Thus, the only two enzyme-bound species which contribute to the apparent K_m or K_i values are ES and ES′ (Scheme 2).

$$[1] \qquad K_m = \frac{[E][S]}{\sum[ES]}$$

Notably, the apparent dissociation constants measured for binding of **11** (K_i = 2.9 μM) and its 2-OH analogue [K_m = 6.6 μM (15); K_m = 7.7 μM (19)] to yeast α-glucosidase indicate that removal of the 2-hydroxyl moderately enhances the free energy for association by about 2 kJ mol^{-1} at 25°C.

The orientation of the aromatic ring is a critical variable for binding affinity of these compounds to the yeast enzyme. Specifically, fusion of a second aromatic ring onto the pyridinium compound (**10**) gives **11** and **12**, and this change causes either an increase of 8.9 kJ mol^{-1} (**11**) or a decrease of 5.0 kJ mol^{-1} (**12**) in the free energy of binding to the enzyme. An identical trend in K_i values (**11** < **10** < **12**) is also apparent for inhibition of the rice α-glucosidase, despite the attenuated potency of these compounds with this enzyme (Table 1).

It is presumed that the origin of the tight binding between the yeast enzyme and **11** results from strong hydrophobic interactions between the active-site and the isoquinoline ring. Lemieux (20) has stressed the important contribution to binding free energy that occurs as ordered water molecules

[5]J. Zhu and A.J. Bennet. Unpublished data.

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Scheme 2.

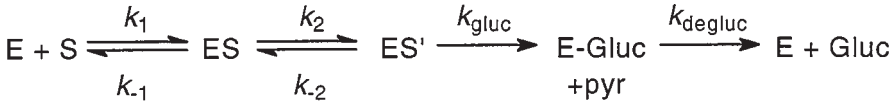
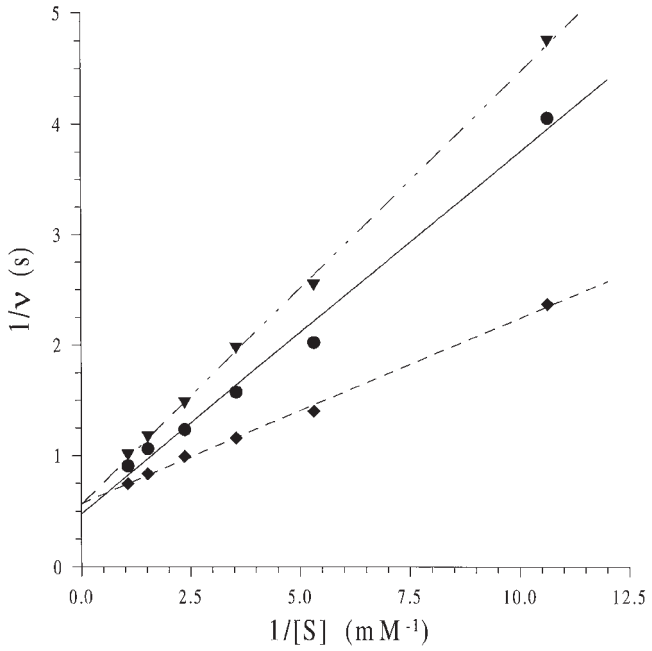


Fig. 1. Lineweaver–Burk plot for the inhibition of yeast α-glucosidase-catalyzed hydrolysis of 4-nitrophenyl α-D-glucopyranoside (**S**) by 2-deoxy-α-D-glucopyranosyl isoquinolinium tetrafluoroborate; symbols represent different concentrations of **11**: ◆ 1.57 μM, ● 3.77 μM, and ▼ 6.91 μM. Kinetic data measured at concentrations of **11** of 0.00 and 0.63 μM have been omitted for clarity.



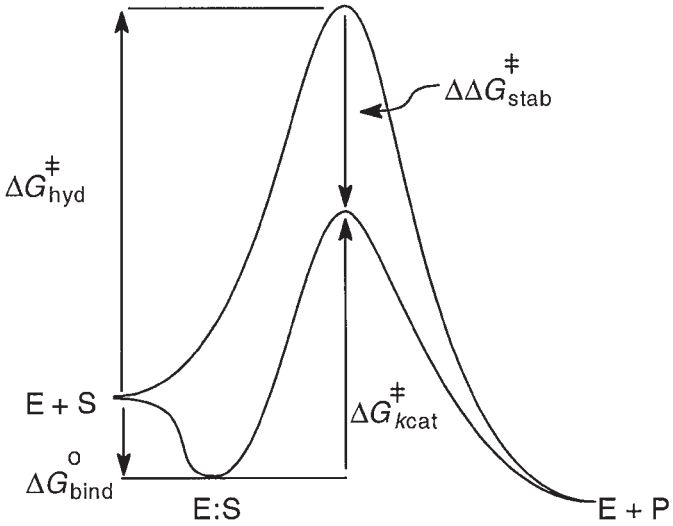
surrounding nonpolar surfaces in solution are liberated into bulk solution when a hydrophobic moiety binds to a biological receptor. Carbohydrate–protein interactions of this nature have been discussed by Wong (21) in terms of providing a favourable entropic contribution towards the free energy for binding of hydrophobic groups.

Another potential source of the tight binding of these compounds may be that their ground state conformations are $^1S_3$ skew-boats rather than the usual $^4C_1$ chair (18, 22), and thus, if these α-glucosidase enzymes bind substrates in non-chair conformations, it is to be expected that conformationally biased compounds such as **9**, **10**, and **11** will display lower binding constants (K_ms) than the natural substrates.

The β-epimer of **11**, 2-deoxy-β-D-glucopyranosyl isoquinolinium bromide (**13**), is a mediocre inhibitor of almond-emulsion β-glucosidase (K_i = 7.4 mM). Therefore, it is clear that the attachment of an extended aromatic ring onto the anomeric carbon of a carbohydrate analogue will not guarantee an increase in binding affinity of glucosidase inhibitors.

Both mannosidases investigated, namely the retaining enzyme from almond and the inverting enzyme from yeast, showed either weak or no affinity for the 2-deoxyglucopyranosyl pyridinium salts, suggesting that the 2-OH group is important for binding to α-mannosidases. However, since kinetic studies using the parent α-mannopyranosyl pyridinium

Scheme 3.



salts have not been reported, no definite conclusions concerning the mannosidase enzymes can be made at this time.

The yeast α-glucosidase hydrolysis of compound **11** is roughly 73 times less than for the glucosyl salt **9** (Table 2), a rate difference that corresponds to a $\Delta\Delta G^{\ddagger}_{kcat}$ (Scheme 3) of around 10.6 kJ mol^{-1}. A similar $\Delta\Delta G^{\ddagger}_{kcat}$ of 9.7 kJ mol^{-1} is observed for barley α-glucosidase-catalyzed hydrolysis of maltose (23). This similarity is not too surprising given that both enzymes are members of glycosidase family 31 (24). For members of other glycosidase families, the removal of a 2-OH group from the substrate can lead to larger differences in $\Delta\Delta G^{\ddagger}_{kcat}$. For example, the β-glucosidase from *Agrobacterium* gives a value for $\Delta\Delta G^{\ddagger}_{kcat}$ of 22 kJ mol^{-1} for the hydrolysis of 4-nitrophenyl β-D-glucopyranoside when compared to its 2-deoxy derivative (25).

At the present time, a more detailed analysis of the magnitude of $\Delta\Delta G^{\ddagger}_{kcat}$ is unwarranted since X-ray crystallographic 3-D structure analysis has not been reported for any member of the α-glucosidase family 31.

The spontaneous hydrolysis of **11** occurs approximately 176-fold more rapidly than that of the corresponding glucoside (Table 2) (18, 22*a*). From this, it can be concluded that removal of the 2-OH group reduces $\Delta\Delta G^{\ddagger}_{stab}$ by approximately 23.5 kJ mol^{-1} (i.e., 176 × 79–fold) for the yeast enzyme-catalyzed process. Furthermore, yeast α-glucosidase accelerates the hydrolysis of both **11** and **12** (the best and worst inhibitors, respectively) by similar amounts (Table 2). Therefore, the differential hydrophobic interactions that occur in the enzyme-substrate complexes for **11** and **12** are maintained at their respective α-glucosidase-catalyzed hydrolysis TSs, and the free energy differences associated with the measured k_{cat}/k_{hyd} rate accelerations ($\Delta G^{\ddagger}_{kcat} - \Delta G^{\ddagger}_{hyd}$, Scheme 3) are indistinguishable. In other words, the nonspe-

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cific, hydrophobic interactions of the quinoline (and isoquinoline) moiety with the yeast enzyme's active site are of a similar magnitude in both the enzyme–substrate complex and the rate limiting glucosidation transition state, and as a consequence, the observed rate acceleration must be caused by an increase in binding of the 2-deoxyglucose unit to the glucosidase-catalyzed transition state relative to the E–S complex.

Materials and methods

All reagents and procedures used for the measurement of enzymatic binding constants with compounds **10**, **11**, and **13** were identical to those described previously (26, 27). Quinoline was purchased from Aldrich and purified by recrystallization of its hydrogen sulfate salt from HOAc–Et_2O, followed by neutralization and fractional distillation at atmospheric pressure.

All new compounds were fully characterized using NMR spectroscopy. All observed resonances in the NMR spectra were fully assigned using ^{1}H–homonuclear (28) and ^{1}H–^{13}C-heteronuclear (29) chemical shift correlated NMR spectroscopy techniques. All coupling constants (J) are listed in hertz (Hz). Synthesis of the substrate α-D-glucopyranosyl 4′-bromoisoquinolinium bromide (19) and the 2-deoxy compounds **10** (18), **11** (18), and **13** (30) were accomplished according to published procedures.

3,4,6-Tri-*O*-acetyl-2-deoxy-α-D-*arabino*-hexopyranosyl quinolinium tetrafluoroborate (14)

Silver tetrafluoroborate (0.63 g, 3.1 mmol) was added to a solution of the 2-thiono-1,3-dioxa-2-phosphorinane **15** (18) (1.50 g, 3.1 mmol) in quinoline (2 mL) and dichloromethane (1.5 mL). After the solution had stirred at rt for 2 h, the silver salts were precipitated by the addition of methanol (250 mL). Following filtration, the solvent was removed under reduced pressure. The resulting residue was dissolved in a minimum volume of methanol and subsequent addition of diethyl ether (500 mL) resulted in precipitation of the product. This procedure was repeated and the final colourless powder was crystallized from methanol–ether to give **14** (0.83 g, 53%), mp 111–113°C. ^{1}H NMR (400 MHz, D_2O) δ: 2.00, 2.17, 2.26 (s, 9 H, 3 × CH_3), 2.86 (ddd, 1 H, $J_{2a,1}$ = 4.2, $J_{2a,2e}$ = 15.4, $J_{2a,3}$ = 7.3, H-2a), 3.02 (ddd, 1 H, $J_{2e,1}$ = 6.2, $J_{2e,3}$ = 4.2, H-2e), 4.23 (dd, 1 H, $J_{6a,5}$ = 3.0, $J_{6a,6b}$ = 12.6, H-6a), 4.29 (ddd, 1 H, $J_{5,4}$ = 6.5, $J_{5,6b}$ = 6.7, H-5), 4.66 (dd, 1 H, H-6b), 5.21 (t, 1 H, $J_{4,3}$ + $J_{4,5}$ = 12.4, H-4), 5.53 (ddd, 1 H, $J_{3,4}$ = 5.6, H-3). 7.28 (dd, 1 H, H-1), 8.07 (ddd, 1 H, $J_{5',6'}$ = 7.9, $J_{6',7'}$ = 7.0, $J_{6',8'}$ = 0.7, ArH-6′), 8.19 (dd, 1 H, $J_{3',2'}$ = 6.2, $J_{3',4'}$ = 8.2, ArH-3′), 8.30 (ddd, 1 H, $J_{5',7'}$ = 1.4, $J_{7',8'}$ = 8.7, ArH-7′), 8.43 (dd, 1 H, $J_{2',4'}$ = 1.4, ArH-4′), 8.50 (brd, 1 H, ArH-5′), 9.26 (brd, 1 H, ArH-8′), 9.52 (dd, 1 H, ArH-2′). Anal. calcd. for $C_{21}H_{24}BF_4NO_7$: C 51.52, H 4.94, N 2.86; found: C 51.50, H 4.87, N 2.72.

2-Deoxy-α-D-*arabino*-hexopyranosyl quinolinium tetrafluoroborate (12)

A solution of sodium methoxide (4 equiv) in methanol (10 mL) was added in one portion, with stirring, to an ice-cold solution of **14** (0.12 g, 2.5 mmol) in methanol (15 mL) that was maintained under an inert atmosphere. After stirring for 15 min, the resulting solution was neutralized by the addition of I-120 Amberlite resin (H^+-form) (2.5 g). After an additional 3 min, a solution of HBF_4 in ether (35% w/v, 1 mL) was added and the resultant acidic solution was filtered directly into cold anhydrous ether (–78°C, 250 mL). The ensuing cloudy solution was kept in a freezer (–16°C) for 24 h. After decanting the solvent, addition of acetonitrile (5 mL) to the solid residue gave a clear solution. Subsequent addition of anhydrous ether (250 mL) gave a cloudy solution and this suspension was placed into a freezer (–16°C). After 48 h, the resultant colourless, hygroscopic solid was filtered and dried to give an analytically pure sample of **12** (0.65 g, 71%). ^{1}H NMR (400 MHz, D_2O) δ 2.63 (ddd, 1 H, $J_{2a,1}$ = 5.3, $J_{2a,2e}$ = 15.4, $J_{2a,3}$ = 9.9, H-2a), 2.92 (dt, 1 H, $J_{2e,1}$ + $J_{2e,3}$ = 8.3, H-2e), 3.41 (ddd, 1 H, $J_{5,6a}$ = 2.3, $J_{5,6b}$ = 5.7, $J_{5,4}$ = 8.6, H-5), 3.67 (dd, 1 H, $J_{6a,6b}$ = 12.5, H-6a), 3.71 (t, 1 H, $J_{4,3}$ + $J_{4,5}$ = 16.0, H-4), 3.82 (dd, 1 H, H-6b), 4.25 (ddd, 1 H, $J_{3,2e}$ = 4.3, $J_{3,4}$ = 7.6, H-3). 7.16 (brt, 1 H, $J_{2e,1}$ = 4.0, H-1), 7.99 (brt, 1 H, $J_{5',6'}$ + $J_{6',7'}$ = 15.4, ArH-6′), 8.07 (dd, 1 H, $J_{2',3'}$ = 6.2, $J_{3',4'}$ = 8.1, ArH-3′), 8.22 (brt, 1 H, $J_{6',7'}$ + $J_{7',8'}$ = 16.2, ArH-7′), 8.35 (brd, 1 H, $J_{5',6'}$ = 8.2, ArH-5′), 8.55 (brd, $J_{7',8'}$ = 9.2, ArH-8′), 9.16 (brd, 1 H, ArH-4′), 9.43 (brd, 1 H, ArH-2′). Anal. calcd. for $C_{15}H_{18}BF_4NO_4$: C 49.62, H 5.00, N 3.86; found: C 49.46, H 5.19, N 4.09.

Measurement of k_{cat} values

The k_{cat} value for yeast α-glucosidase-catalyzed hydrolysis of **11**, relative to that for **9**, was measured under saturation conditions ([**11**] = 303 μM and [**9**] = 110 μM) in 50 mM phosphate buffer (pH 6.8, 0.1% w/v BSA) at 25°C. The relative rate of formation of isoquinoline from the two substrates was monitored at 337 nm. Yeast α-glucosidase-catalyzed hydrolysis of **12** was monitored (using an identical enzyme concentration to that from the experiments with both **9** and **11**) at 320 nm in 50 mM phosphate buffer (pH 6.8, 0.1% w/v BSA) at 25°C. The kinetic parameters V_{max} and K_m were calculated using a nonlinear least-squares fit of the initial rate vs. concentration data. The relative k_{cat} value for α-glucosidase-catalyzed hydrolysis of **12** was calculated from the observed V_{max} values and the measured Δ(ε) values for **11** and **12** at the respective wavelengths used to monitor their hydrolysis reactions (**11**, Δ(ε) = 2010 at 337 nm; **12**, Δ(ε) = 2770 at 320 nm).

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