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Substituted Glycals as Probes of Glycosidase Mechanisms

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Abstract—D-Glucal and a series of substituted derivatives have been tested as substrates, inhibitors and inactivators of the *Agrobacterium faecalis* β-glucosidase in order to probe structure/function relationships in this enzyme. D-Glucal is shown to be a substrate ($k_{cat}=2.3$ min^{-1}, $K_m=0.85$ mM) undergoing hydration with stereospecific protonation from the α-face to yield 2-deoxy-β-D-glucose. 1-Methyl-D-glucal surprisingly serves as only a poor substrate ($k_{cat}=0.056$ min^{-1}, $K_m=57$ mM), also undergoing protonation from the α-face. 2-Fluoro-D-glucal, however, is completely inert, as a result of inductive destabilisation of the oxocarbenium ion-like transition state for protonation, and functions only as a relatively weak ($K_i=24$ mM) inhibitor. Similar behaviour was seen with almond β-glucosidase and yeast α-glucosidase and for the interaction of 2-fluoro-D-galactal with *Escherichia coli* β-galactosidase. A series of of α,β-unsaturated glucal derivatives was also synthesised and tested as potential substrates, inhibitors or inactivators of *A. faecalis* β-glucosidase. Of these only 1-nitro-D-glucal functioned as a time dependent, irreversible inactivator ($k_i=0.011$ min^{-1}, $K_i=5.5$ mM), presumably acting as a Michael acceptor. Electrospray mass spectrometric analysis revealed mutliple labeling of the enzyme by this inactivator, lessening its usefulness as an affinity label. Less reactive Michael acceptor glycals which might have been more specific (1-cyano-, 2-cyano-, 1-carboxylic acid, 1-carboxylic acid methyl ester) unfortunately did not function as inactivators or substrates, only as relatively weak reversible inhibitors ($K_i=3$–96 mM). Copyright © 1996 Elsevier Science Ltd

Introduction

Glycosidases are a class of enzymes which catalyse the hydrolysis of glycosidic linkages. Depending upon the enzyme, hydrolysis occurs with either net retention or net inversion of anomeric configuration, and likely mechanisms for these two processes are shown[1–3] in Figure 1. Both classes of enzyme contain a pair of carboxylic acids in their active sites in most cases. In the inverting glycosidases, one of these acts as a general acid and the other as a general base, reaction occurring by a direct displacement. In retaining glycosidases reaction occurs via a double-displacement mechanism in which a glycosyl-enzyme intermediate involving one of the carboxyl groups is formed and then hydrolysed with general acid/base assistance from the other group. Transition states for the glycosyl transfer steps in both cases involve considerable oxocarbenium ion character, just as is seen for the non-enzymatic reaction.[4]

Considerable interest has been expressed in these enzymes, not only academically, but also commercially. Such commercial interest rests in the enzymes themselves and in inhibitors thereof, there being a number of possible applications of glycosidase inhibitors.[5,6] Potential therapeutic uses include the control of blood glucose levels through inhibition of digestive glycosidases, as is currently achieved using acarbose,[7] or control of viral diseases such as AIDS via interference with the correct processing of viral glycoproteins and other viral recognition events.[8–11] A similar strategy is being developed for the control of cancer metastasis through inhibition of mannosidase II.[12] Design of new inhibitors depends upon a knowledge of the reaction mechanism and of the carbohydrate/protein interactions present in the complex. It is therefore of value to develop reagents which can provide insights into the mechanism, and which might be used to identify active site residues through affinity labeling and subsequent identification of labeled peptides.

One class of glycosidase inhibitors which has received relatively little attention, but which was discovered some while ago,[13] is that of the glycals, 1,2-unsaturated enol ether derivatives of sugars. D-Galactal was found to be an excellent inhibitor of the retaining enzyme *E. coli* β-galactosidase, the tight binding initially being assigned to the structural resemblance of the inhibitor to the oxocarbenium ion-like transition state. However, subsequent analysis revealed that D-galactal was functioning as a substrate, being hydrated by the enzyme to 2-deoxy-D-galactose.[14] The potent inhibition was a consequence of the accumulation of a 2-deoxygalactosyl-enzyme intermediate which hydrolysed relatively slowly, rather than any specific resemblance to the reaction transition state. Indeed, reversible binding of D-galactal itself to the enzyme was found to be very weak, presumably because the absence of the 2-hydroxyl group results in considerable loss of binding free energy. Interactions at the 2-position in this enzyme have been shown to contribute at least 8 kcal mol^{-1} to transition state binding.[14] ^{1}H NMR analysis of the products formed upon hydration of a number of glycals by their glycosidases in D_2O revealed that proton donation had occurred from the bottom face of the sugar ring.[15–19] On that basis the reaction mechanism shown in Figure 2 was proposed in which formation of the 2-deoxygalactosyl-enzyme intermediate arises from a concerted addition of the proto-

nated catalytic nucleophile, since identified[20] as Glu537, across the double bond. Similar outcomes have been observed for a number of other glycosidases.[15–19]

Modified glycals have the potential to be useful probes of glycosidase activity in many ways. Firstly it should be possible to increase the affinity of these glycals considerably if the 'normal' 2-substituent could be incorporated into the structure. Indeed this has proved to be the case for *N*-acetyl hexosaminidases where the incorporation of an *N*-acetyl group at C-2 resulted in a very good inhibitor of this class of enzymes, but one which is nonethless hydrated, in that case to *N*-acetylglucosamine.[21] Unfortunately it is not possible to incorporate a hydroxyl group at that position since this species, a glucoseen, rapidly ketonises and decomposes. An alternative substituent would be a fluorine which not only has a polarity more closely resembling that of the hydroxyl, but also has some capacity for hydrogen bonding. Furthermore, the presence of the electronegative fluorine substituent would likely render protonation of the enol ether impossible, thus providing a stable glycal which could bind well but not suffer hydration. Secondly it should prove possible to synthesise reactive derivatives of glycals which might function as alkylating agents by incorporation of a suitably conjugating group at C-1 or C-2, thereby producing a series of Michael acceptors. Such derivatives might

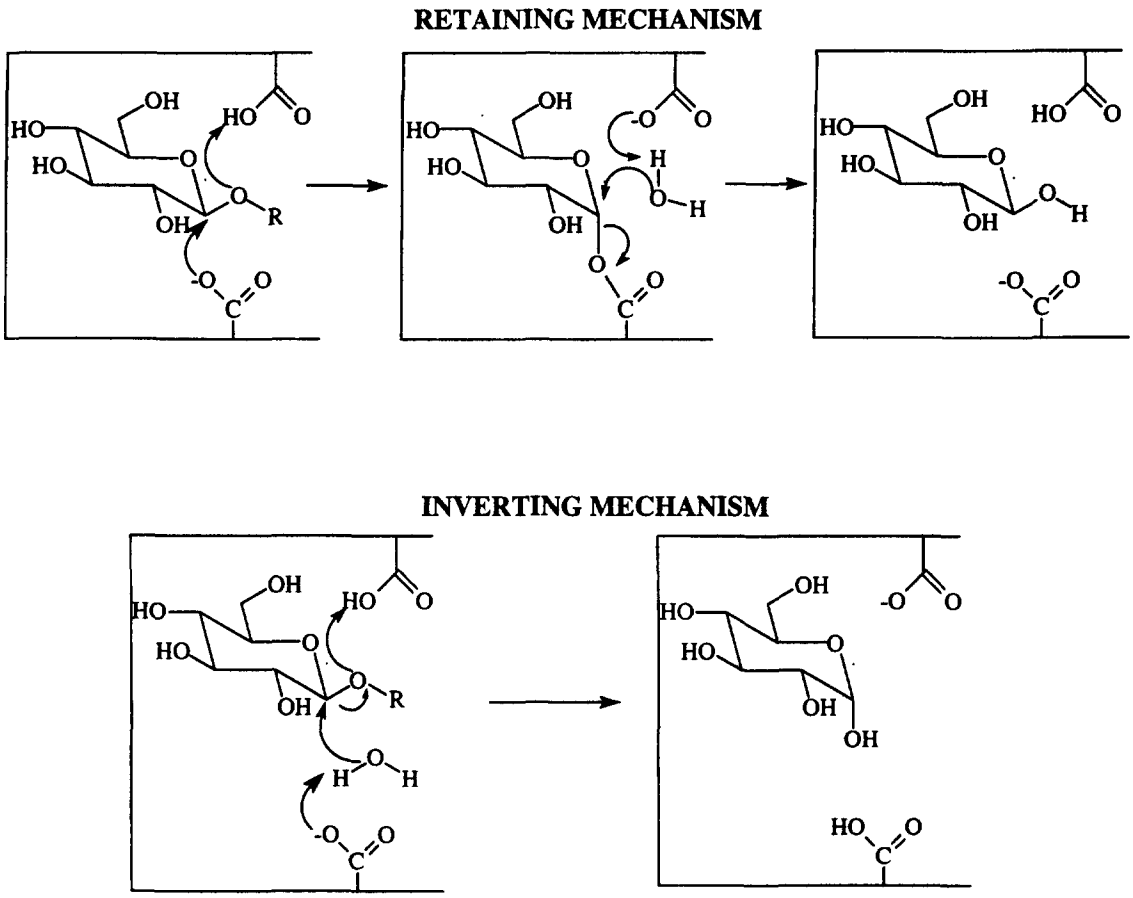


Figure 1. Mechanisms of inverting and retaining glycosidases.

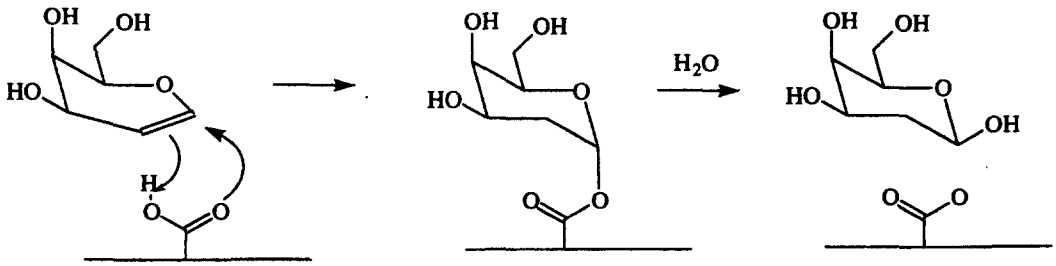


Figure 2. Postulated mechanism of hydration of D-galactal by *E. coli* β-galactosidase.

function as specific affinity labels by virtue of their resemblance to the substrate and might be used to identify amino acid residues at the active site, particularly those which interact with C-1 and C-2 of the substrate. We describe the synthesis and testing of such a series of substituted glycals.

Results and Discussion

Synthesis of substituted glycals

Syntheses of the protected glycal derivatives were carried out essentially according to literature procedures as described in the Experimental. Deprotection was generally achieved either by use of ammonia in dry methanol or sodium methoxide in dry methanol. All such inhibitors provided satisfactory elemental analyses and ^{1}H NMR spectra.

D-Glucal as a substrate

D-Glucal was first shown to be a substrate for *A. faecalis* β-glucosidase (Abg) by TLC analysis of reaction mixtures. Michaelis–Menten parameters for the hydration reaction of $k_{cat}=2.3$ min^{-1}, $K_m=0.85$ mM were then determined by monitoring the release of 2-deoxy-D-glucose using a coupled assay system involving hexokinase and glucose-6-phosphate dehydrogenase. This relatively low K_m value relative to the K_i for D-glucose ($K_i=6.4$ mM[22]) suggests either that D-glucal is binding somewhat more tightly because of its half chair conformation, or that the second step in catalysis, hydrolysis of the 2-deoxyglucosyl-enzyme intermediate, is rate-limiting resulting in accumulation of this intermediate thus lowering of K_m values. Interestingly, kinetic parameters for the Abg-catalysed hydrolysis of *p*-nitrophenyl 2-deoxyglucoside of $k_{cat}=1.5$ min^{-1}, $K_m=0.015$ mM were recently reported[23], the k_{cat} values being very similar to that observed for D-glucal. Pre-steady state kinetic analysis revealed that the deglycosylation step is rate-limiting for hydrolysis of *p*-nitrophenyl 2-deoxyglucoside also. Therefore, since the deglycosylation step in that case (hydrolysis of a 2-deoxyglucosyl-enzyme) is identical to that for hydration of D-glucal, and since very similar rate constants were determined in the two cases, it would appear that deglycosylation is also rate-limiting for the turnover of D-glucal. The K_m value determined for D-glucal cannot therefore be equal to the true dissociation constant, and must rather be regarded as providing a lower limit for the true dissociation constant.

The stereochemical course of the hydration reaction was determined by incubation of the enzyme with glucal in D_2O buffer and monitoring the reaction by ^{1}H NMR spectrometry. Upon incubation new methylene resonances at δ 1.46 (triplet) and δ 1.67 (double doublet) appeared with time due to the C-2 proton. The chemical shift and coupling constant of this proton identify it as the axial proton of 2-deoxy-D-glucose, thus the deuteron must have been added from the α-face of the glucal. This is the same stereochemical outcome as was seen with *E. coli* β-galactosidase, thus a mechanism equivalent to that shown in Figure 2 can be assumed.

2-Fluoro-D-glucal

2-Fluoro-D-glucal was first tested as a substrate for the *A. faecalis* β-glucosidase by incubation at a relatively high concentration (10 mM) for 16 h in the presence of an extremely high concentration (0.8 mM) of the enzyme, monitoring the reaction occasionally by ^{19}F NMR spectrometry. While formation of product should be readily apparent since 2-fluoro-D-glucal has a resonance at δ 170 whereas 2-deoxy-2-fluoro-D-glucose resonates at around δ 195, no evidence of conversion was obtained. An aliquot of this reaction mixture was then taken, and its enzyme activity assayed in order to ensure that no inactivation of the enzyme had taken place, caused either by the 2-fluoro-D-glucal or by a contaminant therein. No significant loss of activity could be detected thereby showing that 2-fluoro-D-glucal is neither a substrate nor an inactivator of *A. faecalis* β-glucosidase. Similar experiments were performed with both almond β-glucosidase and yeast α-glucosidase and in neither case was there any evidence of inactivation or substrate activity. 2-Fluoro-D-glucal was then tested as a reversible inhibitor of *A. faecalis* β-glucosidase, and competitive inhibition observed with $K_i=24$ mM, as shown in Table 1. Similar competitive inhibition ($K_i=6$ mM) was seen with the almond β-glucosidase, an enzyme which has also been shown to hydrate D-glucal.

A similar series of experiments was performed with 2-fluoro-D-galactal and the β-galactosidases from *E. coli* and *Aspergillus oryzae*. Once again, no substrate activity could be detected in either case, nor did the glycal function as a time-dependent inactivator of either enzyme. It did, however, function as a competitive inhibitor with K_i values of 19 and 0.2 mM, respectively. It should be noted, however, that samples of 2-fluoro glycals which were not *scrupulously* purified did inactivate their corresponding glycosidases due to the presence of minute quantities of a powerful inactivator whose identity remains unknown.

1-Methyl glucal

Methyl glucal (2,6-anhydro-1,3-dideoxy-D-*arabino*-hept-2-enitol) was investigated initially as a substrate for

Table 1. Inhibition constants for substituted glycals with *A. faecalis* β-glucosidase

Glycal	Reversible $K_{i\,(mM)}$
2-Fluoroglucal	24
1-Cyanoglucal	9
2-Cyanoglucal	26
1-(Methylcarboxylate) glucal	3.2
1-Carboxylate glucal	96

β-glucosidase under the usual conditions, but its rate of phosphate buffer-catalysed decomposition was found to be unacceptably high. The phosphate buffer ordinarily employed was therefore replaced with 10 mM HEPES–NaOH (pH 7.0) in which the non-enzymatic hydration of methyl glucal was found to be much slower. Kinetic parameters for hydrolysis of *p*-nitrophenyl glucoside were then determined under these conditions and found to be essentially identical to those in phosphate buffer. Kinetic parameters for the enzyme-catalysed hydration of methyl glucal were then determined by ^{1}H NMR spectrometry, relying upon the substantial difference in resonances due to the methyl group in the substrate (δ 1.74) and product 1,3-dideoxy-D-*gluco*-heptulose (δ 1.43). Integration of these resonances along with the equivalent resonances in a control sample containing no enzyme allowed determination of rates at each of the substrate concentrations investigated. After subtracting contributions from non-enzymatic hydrolysis, values of $k_{cat} = 0.06$ min^{-1} and $K_m = 60$ mM were obtained (Table 2).

The stereochemical course of the protonation event was again investigated by ^{1}H NMR analysis of the enzyme-catalysed reaction in D_2O buffer, along with parallel studies of the non-enzymic reaction. A sample of fully protiated 1,3-dideoxy-D-*gluco*-heptulose product was first obtained by incubation of methyl glucal for 8 days in H_2O-containing phosphate buffer. ^{1}H-NMR analysis of this sample allowed assignment of the resonances due to the axial (δ 1.56, t, $J = 12$ Hz) and equatorial (δ 2.10, dd, $J = 12$, 5 Hz) protons at the new methylene position. Analysis of the product in the D_2O sample containing enzyme revealed resonances due to both axial *and* equatorial protons at that position in a ratio of 3:2. This product ratio reflects the combination of both an enzyme-catalysed and a spontaneous process. Analysis of the sample from the parallel reaction with no enzyme also revealed a mixture of products, but this time in a ratio of 1:1. This, therefore, clearly indicates that the product of the enzymatic reaction is that in which proton addition has occurred from the α-face of the substrate. This is the same stereochemical course seen with glucal, indicating that even though the rate is reduced as a consequence of the presence of the anomeric methyl group, the mechanism is not significantly altered.

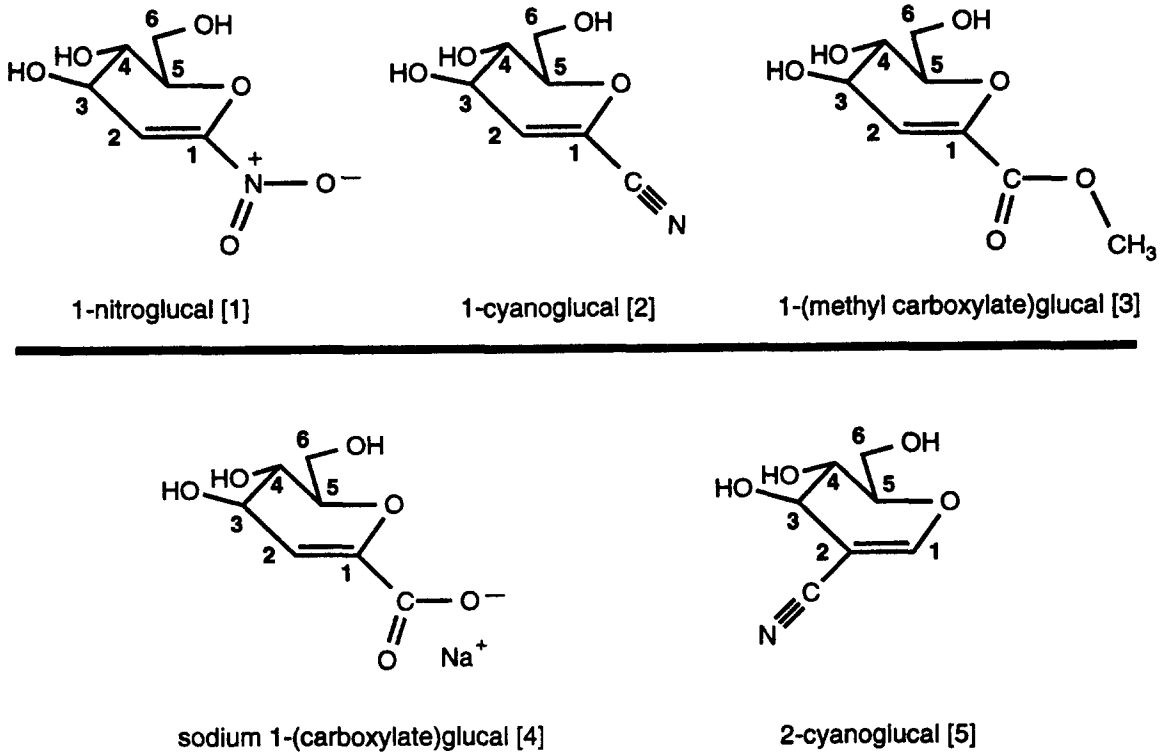
Table 2. Kinetic parameters for *A. faecalis* β-glucosidase with glycal substrates and inactivators

Glycal	k_{cat} (min^{-1})	K_m (mM)
Glucal	2.3	0.85
1-Methylglucal	0.06	60
1-Nitroglucal[a]	0.011[a]	5.5[a]

[a] Inactivation parameters, k_i and K_i.

1-Nitroglucal

1-Nitroglucal (1,5-anhydro-2-deoxy-1-nitro-D-*arabino*-hex-1-enitol; also named as 1,2-dideoxy-1-nitro-D-*arabino*-hex-1-enopyranose,[24] see Scheme 1) and kindly provided by Professor A. Vasella of the E.T.H., Zurich was first tested as an inactivator of *A. faecalis* β-glucosidase. Time-dependent inactivation according to pseudo-first-order kinetics was observed, as shown in



Scheme 1.

logarithmic form in Figure 3(a). A reciprocal replot of the pseudo-first order rate constants obtained from the slopes of the lines in Figure 3(a) is shown in Figure 3(b). This yielded inactivation parameters of $K_i = 5.5 \pm 0.9$ mM, and $k_i = \{1.1 \pm 0.1\} \times 10^{-2}$ min^{-1} (Table 2). This inactivation reaction was shown to most likely be the consequence of labelling of an active site residue by demonstrating that a reversible, competitive inhibitor, 1-deoxy-β-D-glucosyl benzene ($K_i = 3.4$ mM[25]) protects the enzyme against inactivation by nitroglucal. As seen in Figure 3, addition of 9.9 mM 1-deoxy-β-D-glucosyl benzene to an inactivation mixture containing 7.8 mM nitroglucal reduced the inactivation rate from 0.0069 to 0.0036 min^{-1}, exactly as predicted if both bind to the same site.

The irreversibility of the inactivation reaction was demonstrated by dialysing a sample of inactivated enzyme overnight to remove excess inactivator, then assaying the enzyme sample. No activity could be detected, nor was activity regained upon further incubation of this dialysed sample over 48 h at 37 °C, even when incubated in the presence of 21 mM cellobiose or 1-deoxy-β-D-glucosyl benzene, ligands which

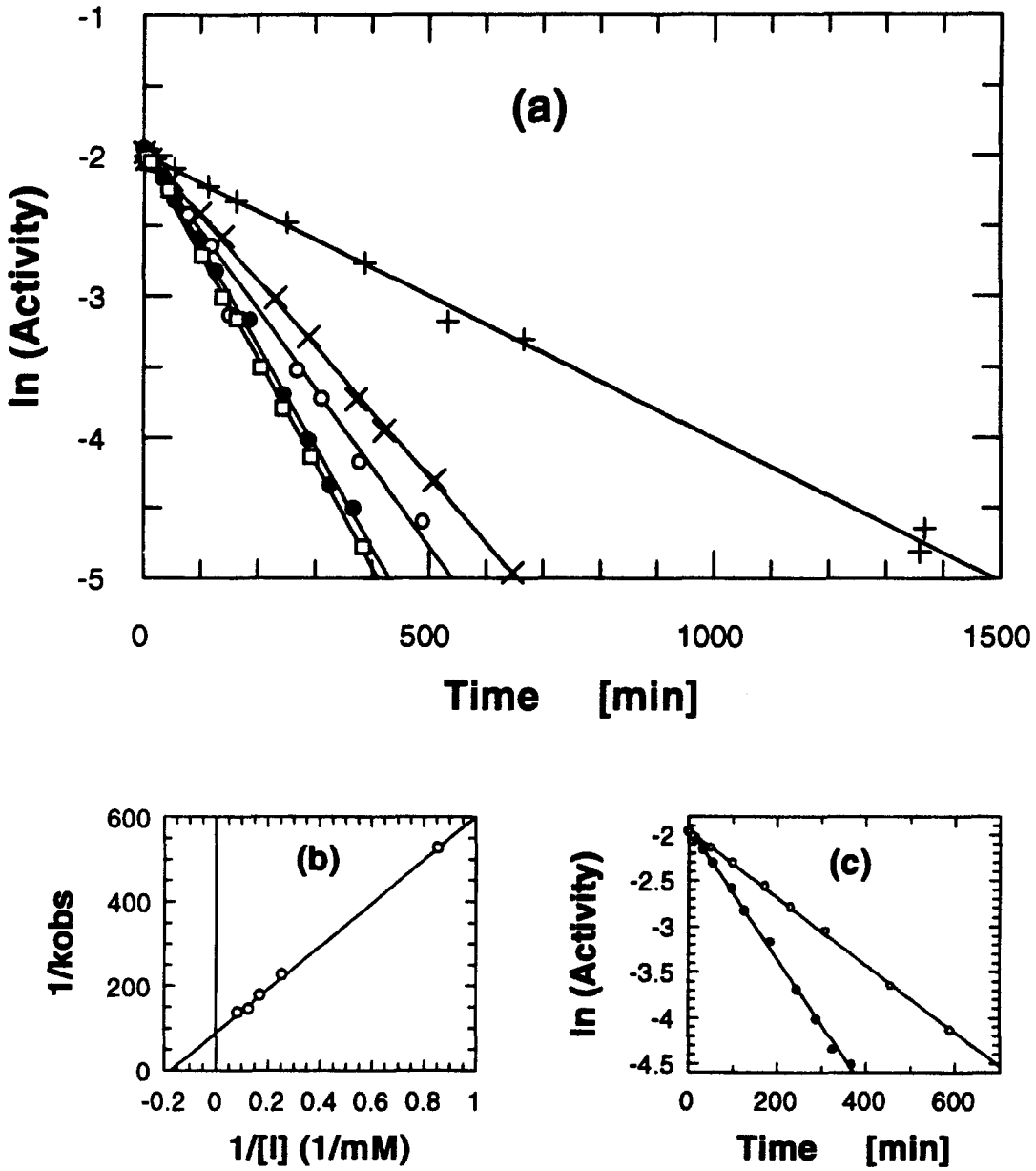


Figure 3. Determination of kinetic parameters for the inactivation of pABG5 by nitroglucal. (a) Time-dependent decrease in Abg activity with increasing concentrations of nitroglucal. Inactivation reactions were performed at 37 °C in 50 mM sodium phosphate buffer (pH 6.8.) with a fixed concentration of Abg and 0.1% BSA. Residual activity assays were performed as described in the text. The concentrations of nitroglucal in the inactivation reactions were 1.2 (+), 3.9 (x), 5.9 (o), 7.8 (●), and 11.7 (□) mM, and the values of k_{obs} were 0.0019, 0.0044, 0.0056, 0.0069, and 0.0073 min^{-1}, respectively. (b) A double-reciprocal plot of the k_{obs} values from (a). (c) Protection against nitroglucal inactivation by glucosyl benzene. Reactions were carried out with 7.8 mM nitroglucal and 0 (●) or 9.9 (○) mM glucosyl benzene: values of k_{obs} obtained were 0.0069 or 0.0036 min^{-1}, respectively.

have previously reactivated enzymes which had been inactivated by the trapping of an intermediate.[26] The fact that the enzyme could not be reactivated by transglycosylation suggests that the inactivation is not a consequence of accumulation of a relatively stable glycosyl–enzyme intermediate. In order to determine the stoichiometry of the inactivation process, a sample of the inactivated enzyme was subjected to analysis by electrospray mass spectrometry. While a mass for the unreacted enzyme of 51,205($\pm$8) Da was readily determined, the nitroglucal-inactivated sample yielded a complex mass spectrum indicative of a highly heterogeneous enzyme sample. This demonstrates that the enzyme has reacted with nitroglucal at a number of different sites, limiting the usefulness of this reagent as a tool for identifying key active site residues. Attempts to limit the extent of non-specific labeling while maintaining useful inactivation behaviour by adjusting times of reaction and concentrations of reagent were unsuccessful. Attention, therefore, turned to the synthesis and testing of less reactive Michael acceptors.

Kinetic studies using other α β-unsaturated glucals

The following α β-unsaturated glycals were first examined as potential inactivators of Abg by incubating them with the enzyme, at the concentrations indicated in parentheses, for up to 24 h at 37 °C. 1-Cyanoglucal (33 mM), 2-cyanoglucal (33 mM), 1-(methyl carboxylate)glucal (10 mM) and sodium 1-(carboxylate)glucal (11 mM). At various time intervals small aliquots were removed and residual enzyme activity was assayed using the substrate βGlcPNP. No time-dependent inactivation of Abg was detected during these tests when compared with parallel control reactions. Furthermore, all of these α β-unsaturated derivatives of glucal remained unchanged (as determined by TLC) throughout their respective incubation periods, indicating that none acted as substrates of the enzyme.

The compounds were then tested as reversible inhibitors and the K_i values shown in Table 1 obtained. None of these bound as well as glucal apparently does. However, it must be remembered that the K_m value for glucal is significantly lower than its true K_d, thus such comparisons are not meaningful. Interestingly 2-fluoroglucal and 2-cyano-glucal bind with approximately equal affinities despite the considerable difference in the sizes of their 2-substituents. Possibly repulsive dipolar interactions are responsible for weakening of binding, particularly in the case of the fluorine substituent.

Binding of the glycals substituted at the 1-position is very dependent on the nature of the substituent. The fact that 1-carboxyglucal binds so poorly is unsurprising in light of the recent demonstration[27] that substrates bearing negatively charged substituents bind very poorly to Abg due to destabilising electrostatic interactions with the active site carboxyl groups. This is corroborated by the fact that the methyl ester binds reasonably well. However, methyl glucal, with only a methyl substituent at that position binds very poorly. This would suggest that the improved binding observed with the 1-methyl ester and the 1-cyano-glucal is a consequence of favourable polar interactions with the substituent itself, something in which the methyl substituent cannot participate.

Conclusions

While D-glucal has proved to be a useful probe of the active site, allowing insights into protonation and hydrolysis modes, the substituted glycals have been less valuable. The poor binding and very slow hydrolysis of the otherwise more reactive 1-methyl glucal suggest considerable steric hindrance between the enzyme and substrate at the active site of the enzyme, not only in the ground state, as reflected by its K_m value, but even more so at the transition state for protonation/formation of the glycosyl-enzyme intermediate as revealed by the 3000-fold reduction in k_{cat}/K_m values relative to D-glucal. The even more severe reduction in rate observed for 2-fluoro glucal cannot be due to steric effects, but is likely a consequence of a combination of two factors. Firstly, the electronegative fluorine will inductively disfavour protonation. Secondly, due to the limited hydrogen bonding capability of this substituent, crucial stabilising interactions between the substrate 2-hydroxyl and the enzyme are not developed. The combination of these factors is clearly sufficient to render the glycal inert. The potential of α,β-unsaturated glycals to act as affinity labels via 1,4-conjugate addition reactions was also not realised, despite the early promise of 1-nitro glucal which unfortunately proved too reactive to be of use in this system. Less reactive Michael acceptors, which might have been more specific, did not function as affinity labels, but only as relatively poor reversible inhibitors.

Experimental

Synthesis

Materials and general methods. Thin-layer chromatography (TLC) was performed using analytical plates (silica gel 60 F_{254}, Merck); compounds were visualized under UV light or after charring with 10% H_2SO_4 in methanol. Column chromatography was performed using Kieselgel 60 (230–400 mesh) silica gel. If possible, tetramethylsilane (TMS) was used as an external reference in ^{1}H NMR; spectra obtained in D_2O were referenced externally to 2,2-dimethyl-2-silapentane-5-sulphonate. ^{13}C NMR spectra were recorded with proton decoupling. ^{19}F NMR chemical shifts were measured against external trifluoroacetic acid but reported relative to $CFCl_3$. Trifluoroacetic acid resonates 76.53 ppm upfield from $CFCl_3$.

D-Glucal (1,5-anhydro-2-deoxy-D-arabino-hex-1-enitol) was obtained by deacetylation of 3,4,6-tri-*O*-acetyl-glucal (Sigma Chemical Co.) using NaOMe/MeOH as described.[28] 1-Nitro-D-glucal was kindly provided by Professor A. Vasella of the University of Zurich.

Sodium 1-(carboxylate)-D-glucal and 1-(methyl carboxylate)-D-glucal were prepared by Dr Bill Stirtan.[29]

1-Methyl-D-glucal(anhydro-1,3-dideoxy-D-arabino-hept-2-enitol). 3,4,6-Tri-*O*-(*t*-butyldimethylsilyl)methylglucal was prepared as described.[30] Deprotection using TBAF {tetra(*n*-butyl)ammonium fluoride} in THF yielded 1-methylglucal. Column chromatography (4:1:2 $CHCl_3$:MeOH:hex) gave a crude product, which was crystallised from ether, MeOH, then petroleum ether to yield the final product (mp 100–102 °C) in 27% yield (from persilylated glucal). ^{1}H NMR (300 MHz, D_2O): δ 4.58 (dd, 1H, $J_{3,4}=2.9$ Hz, $J_{1,3}=1.0$ Hz, H-3); 4.14 (ddd, 1H, $J_{1,4}=1.7$ Hz, $J_{3,4}=2.9$ Hz, $J_{4,5}=6.7$ Hz, H-4); 3.88 (ddd, 1H, $J_{6,7}=3.3$ Hz, $J_{6,7'}=4.8$ Hz, $J_{5,6}=8.4$ Hz, H-6); 3.82 (m, 2H, H-7, H-7′); 3.60 (dd, 1H, $J_{4,5}=6.7$ Hz, $J_{5,6}=8.4$ Hz, H-5); 1.73 (dd, 3 H, $J_{1,4}=1.7$ Hz, $J_{1,3}=1.0$ Hz, H-1). DCI MS: *m/z* 178 ($M+NH_4^+$). Anal. calcd for $C_7H_{12}O_4$ (160.2): C, 52.49; H, 7.55 Found: C, 52.29 H, 7.76.

2-Fluoro-D-glucal (1,5-anhydro-2,2-dideoxy-2-fluoro-D-arabino-hex-1-enitol). 3,4,6-Tri-*O*-acetyl-2-fluoro-D-glucal was synthesised as described.[31] Deprotection (0.1 M NaOMe in anhydrous MeOH for 20 min), neutralisation with Dowex 50W-X8 (H+) resin, and flash chromatography (silica gel; EtOAc) yielded 2-fluoroglucal as a gum that resisted crystallisation. ^{1}H NMR (400 MHz, D_2O): δ 6.74 (d, 1H, $J_{1,F}=4.8$ Hz, H-1); 4.36 (dd, 1H, $J_{3,F}=1.8$ Hz, $J_{3,4}=5.5$ Hz, H-3); 3.93, 3.84–3.74 (4 H, H-4,5,6,6′). ^{19}F NMR (254 MHz, D_2O): δ 169.57 (dd, $J_{F,1}=5$ Hz, $J_{F,3}=2$ Hz, F-2). Anal. calcd for $C_6H_9O_4F$: C, 43.91; H, 5.53. Found: C, 44.12; H, 5.70.

3,4,6-Tri-*O*-acetyl-2-fluoro-D-galactal. Was obtained as a crystalline product (mp 67–68 °C) from 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-α-D-galactopyranosyl bromide as described.[32] ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.75 (d, 1H, $J_{1,F}=5$ Hz, H-1); 5.88 (dd, 1H, $J_{3,F}=1$ Hz, $J_{3,4}=4$ Hz, H-3); 5.42, 4.35–4.15 (m, 4H, H-4,5,6,6′); 2.08, 2.13 (2s, 9H, 3 × OAc). Anal. calcd for $C_{12}H_{15}O_7F$: C, 49.70; H, 5.20. Found: C, 49.67; H, 5.30.

2-Fluoro-D-galactal. Deacetylation of 3,4,6-tri-*O*-acetyl-2-fluoro-D-galactal to yield 2-fluorogalactal was performed as described in the synthesis of 2-fluoroglucal. Crystals (mp 132–133 °C) were obtained from MeOH/ether. ^{1}H NMR (400 MHz, D_2O): δ 6.74 (d, 1H, $J_{1,F}=5.2$ Hz, H-1); 4.68 (d, 1H, $J_{3,4}=5.2$ Hz, H-3); 4.07–3.71 (m, 4 H, H-4,5,6,6′). ^{19}F NMR (254 MHz, D_2O) δ 170.9 (m, F-2). Anal. calcd for $C_6H_9O_4F$: C, 43.91; H, 5.53. Found: C 44.20; H, 5.42.

1-Cyano-D-glucal (2,6-anhydro-3-deoxy-D-arabino-hept-2-enononitrile). 3,4,6-Tri-O-acetyl-1-cyanoglucal was prepared by reductive elimination of the α-bromo-β-D-glucosylcyanide using zinc and one equivalent of pyridine as described.[33] Deacetylation using NH_3-satd MeOH[34] gave the crude product, which was purified further by column chromatography (27:2:1 EtOAc: MeOH:H_2O) and recrystallised from acetone, ether and hexane to give the final product (mp 95–97 °C) in 70% yield. ^{1}H NMR (200 MHz, D_2O): δ 5.80 (d, 1H, $J_{2,3}=3$ Hz, H-2); 4.30 (dd, 1H, $J_{2,3}=3$ Hz, $J_{3,4}=7$ Hz, H-3); 4.05 (ddd, 1H, $J_{4,5}=9$ Hz, $J_{5,6}=2.8$ Hz, $J_{5,6'}=4$ Hz, H-5); 3.88 (d, 1H, $J_{5,6}=2.8$ Hz, H-6); 3.86 (d, 1 H, $J_{5,6'}=4$ Hz, H-6′); 3.71 (dd, 1H, $J_{3,4}=7$ Hz, $J_{4,5}=9$ Hz, H-4). ^{13}C NMR (50 MHz, D_2O): δ 129.04 (s, C-1); 118.98 (s, C-2); 114.72 (s, CN); 81.03, 68.68, 68.02, 60.34 (s, C-3–6). DCI MS: *m/z* 189 ($M+NH_4^+$). Anal. calcd for $C_7H_9NO_4$ (171.15): C, 49.12; H, 5.30; N, 8.18. Found: C, 49.42; H, 5.29; N, 8.26.

2-Cyano-D-glucal (1,5-anhydro-2-deoxy-2-cyano-D-arabino-hex-1-enitol). 3,4,6-Tri-*O*-acetyl-2-cyanoglucal was synthesised from per-*O*-acetylated D-glucal using chlorosulphonyl isocyanate and subsequent treatment with triethylamine.[35] Deacetylation[34] with NH_3-satd MeOH yielded the crude product. Recrystallisation from acetone and hexane gave colorless needles (mp 123–125 °C) in 57% yield. ^{1}H NMR (200 MHz, D_2O): δ 7.30 (d, 1H, $J_{1,3}=1$ Hz, H-1); 4.29 (dd, 1H, $J_{1,3}=1$ Hz, $J_{3,4}=7$ Hz, H-3); 4.12 (ddd, 1H, $J_{5,6}=3$ Hz, $J_{5,6'}=4$ Hz, $J_{4,5}=9$ Hz, H-5); 3.89 (d, 1 H, $J_{5,6}=3$ Hz, H-6); 3.86 (d, 1H, $J_{5,6'}=4$ Hz, H-6′); 3.73 (dd, 1H, $J_{3,4}=7$ Hz, $J_{4,5}=9$ Hz, H-4). ^{13}C NMR (50 MHz, D_2O): δ 159.11 (s, C-1); 118.70 (s, CN); 91.10 (s, C-2); 80.89, 67.55, 67.39 (s, C-3, C-4, C-5), 60.25 (C-6). IR λ_{max} cm-1: 2220 (conj. CN); 1627 (conj. C=C). DCI MS: *m/z* 189 ($M+NH_4^+$). Anal. calcd for $C_7H_9NO_4$ (171.15): C, 49.12; H, 5.30; N, 8.18. Found: C, 49.08; H, 5.38; N, 8.12.

Enzyme kinetics

Sigma Chemical Co. supplied biological buffers, almond emulsin β-glucosidase (Type 1), *E. coli* β-galactosidase, and *A. oryzae* β-galactosidase. Cloned *Agrobacterium faecalis* β-glucosidase (Abg) was prepared as described.[25] The buffer and reaction temperature used with each enzyme were as follows: Abg, 50 mM sodium phosphate, pH 6.8, 37 °C; *E. coli* β-galactosidase, 100 mM sodium phosphate, 1 mM $MgCl_2$, pH 7.0, 25 °C; almond β-glucosidase, 50 mM sodium phosphate, pH 6.8, 30 °C; *A. oryzae* β-galactosidase, 50 mM NaOAc, 1 mM $MgCl_2$, pH 4.5, 30 °C.

Kinetic parameters for substrates. Values of K_m and V_{max} were determined by measuring initial rates using five to eight different concentrations of the substrate, which typically ranged between 0.3 and 5 K_m. Parameters were calculated by fitting data to a weighted nonlinear regression of the Michaelis–Menten equation using GraFit.[36] Rates of Abg-catalysed hydration of methylglucal were determined by ^{1}H NMR. Thus five reaction buffer mixes (0.5 ml) containing HEPES–NaOH (10 mM, pH 7.0), BSA (0.1%) and methylglucal were incubated at 37 °C, and reaction started by the addition of Abg (0.65 mg) to each solution. After 110 h the mixes were frozen and lyophilised and their ^{1}H NMR spectra measured. The concn of the reaction product was determined by integration of the product methyl hydrogen peak (δ = 1.43 ppm), and corrected for spontaneous hydration by subtracting

the integral of the same peak in the appropriate control.

Inhibition constants. Rates of reaction were determined as a function of both varying substrate and inhibitor concentration over the concentration ranges indicated below. Data obtained were analysed using the nonlinear regression analysis program GraFit.[36] Almond β-glucosidase: *p*-nitrophenyl β-D-glucopyranoside (K_m = 3 mM), 1.2–6.1 mM; 2-fluoro-D-glucal, 1–10 mM. *A. faecalis* β-glucosidase: *p*-nitrophenyl-β-D-glucopyranoside (K_m = 0.1 mM), 0.02–0.15 mM; 2-fluoro-D-glucal, 0–30 mM. *E. coli* β-galactosidase: *p*-nitrophenyl β-D-galactopyranoside (K_m = 0.03 mM), 0.045–0.3 mM; 2-fluoro-D-galactal, 0–45 mM. *A. oryzae* β-galactosidase: *p*-nitrophenyl β-D-galactopyranoside (K_m = 1.2 mM), 1.2–8 mM; 2-fluoro-D-galactal, 0–1 mM.

Inactivation studies. Samples of Abg in sodium phosphate buffer (50 mM, pH 6.8) containing 1% BSA and the desired concentration of potential inactivator were incubated at 37 °C and the residual enzyme activity was measured at various time intervals by removing a 10 μL aliquot of the inactivation reaction mixture and adding this to a fresh tube containing a much larger volume (1.00 mL) of a saturating concentration of the substrate.[25] In the case of nitroglucal this was repeated at each of a series of concentrations of inactivator (as shown in legend to Fig. 3). Pseudo-first order rate constants for inactivation at each concentration were determined either by the log plot shown, or by direct fit of the lines to a first order equation. Values of the inactivation parameters k_i and K_i were determined by reciprocal analysis.

Test for reactivation of nitroglucal-inactivated Abg. Abg (0.13 mg) was inactivated by treatment with 16 mM nitroglucal, dialysed extensively and the dialysate divided into three aliquots. To one aliquot was added BSA (0.1% final) in 50 mM phosphate buffer. To the second aliquot was added BSA (0.1% final) plus cellobiose (21 mM final) in 50 mM phosphate buffer. To the third aliquot was added BSA (0.1% final) plus glucosyl benzene (21 mM final) in 50 mM phosphate buffer. These 0.11 mL reactions were incubated at pH 6.8 at 37 °C and aliquots were removed at time intervals and assayed (1 mM βGlcPNP). Equivalent controls containing enzyme which had not been inactivated were also run.

Electrospray mass spectrometry. Ion-spray protein mass spectrometry was carried out on a PE-Sciex API III triple quadrupole instrument (Sciex, Thornhill, Ontario) in the laboratory of Professor R. Aebersold at the Biomedical Research Centre of the University of British Columbia. Spectra were collected in the LC-MS mode (single MS) by Dr S. C. Miao. Samples were loaded onto the spectrometer by HPLC using a 1.00 mm microbore PLRP-S column (Michrom Bioresources Inc.) eluting with the following solvent system: 20% solvent B in solvent A to 100% solvent B over 10 min, followed by 100% solvent B over 2 min. The composition of solvent A was 0.05% TFA, 2% acetonitrile in water. The composition of solvent B was 0.045% TFA, 80% acetonitrile in water.

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