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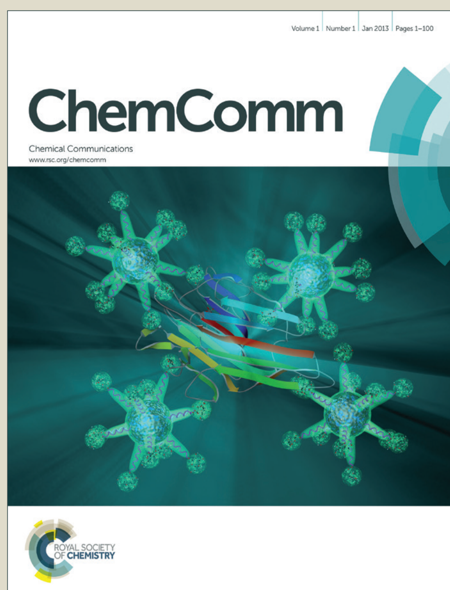

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Fluoro-glycosyl acridinones are ultra-sensitive active site titrating agents for retaining *β*-glycosidases†

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Novel fluorogenic 2-deoxy-2-fluoroglycosyl acridinone active site titrating reagents were synthesised and kinetic parameters determined for their inactivation of two retaining β-glucosidases, a β-galactosidase, a β-xylosidase and several cellulases. Fluorescence-monitored active site titration using this class of reagents reliably measured active enzyme concentrations down to 3 nM.

Glycosidases, or glycoside hydrolases, comprise an average of 1% of the entire genome of any given organism and play key roles in carbohydrate metabolism as well as glycoconjugate modification.[1] These enzymes are heavily used in a variety of industrial processes, including: brewing and food preparation, pulp and paper, pharmaceuticals and bioethanol generation.[2] Consequently there is considerable interest in identifying and improving glycosidases for a range of commercial applications, as well as for academic purposes. The identification of new enzymes such as glycosidases is being revolutionised by the development of robust methods for screening (meta)genomic libraries, from which hits provide ideal starting points for enzyme optimisation through directed evolution.[3]

Both of these paradigm-shifting techniques are reliant on high throughput plate-based heterologous expression and characterisation of enzymes.[4] A major limitation of these plate-based approaches is that the enzyme activity measured in each well is a product of both the inherent activity of the enzyme and the quantity of that enzyme expressed. In order to select enzymes of improved inherent activity, rather than those that are expressed at higher levels, it would be useful to have a rapid means for measuring the quantity of enzyme present in each well. This would speed up the selection process. Since kinetic parameters for each enzyme are unknown, it is not possible to quantify enzyme from activity alone. Unfortunately reagents that stoichiometrically tag the enzyme with a fluorescent probe are of limited use in a high-throughput format, since the enzyme would need to be separated from excess probe prior to quantitation.[5] Rather, a probe is needed that undergoes a stoichiometric reaction with the enzyme to release a sensitive reporter molecule. In this paper we describe a new class of highly sensitive fluorogenic active site titrating agents for use in direct quantitation of specific retaining glycosidases in complex mixtures.

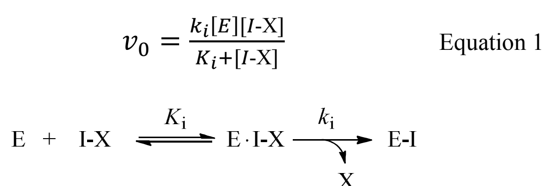
An active site titrating agent is a reagent that reacts specifically and stoichiometrically with the enzyme of interest and in doing so blocks further enzyme action and generates one molar equivalent of a quantifiable reporter molecule, typically a chromophore or fluorophore, allowing back-calculation of the enzyme concentration. This is typically achieved using a chromogenic or fluorogenic reagent that releases one equivalent of a chromophore or fluorophore upon covalent enzyme modification, but other strategies in which a quencher is released have also been used. Active site titrating agents were first devised for proteases[6] and have subsequently been developed for a variety of enzymes including lipases, aminoacyl-tRNA synthetases and retaining glycosidases.[7, 8] The latter are typically two step, double-displacement enzymes and their titrating agents are typically chromogenic substrates that have been designed to form a covalent adduct in the first step, with expulsion of a chromophore. This intermediate only very slowly undergoes the second hydrolysis step. Activated 2-deoxy-2-fluoro-glucopyranosides that release dinitrophenol or fluoride were earlier developed as active site titrating agents of *β*-glucosidases (Figure 1).[8, 9] The electron-withdrawing fluorine adjacent to the anomeric centre destabilises both the glycosylation and deglycosylation transition states of the reaction, slowing both steps, while the presence of a good leaving group accelerates the first step, ensuring

that the intermediate accumulates. While useful in quantifying relatively high concentrations (1 μM; ~ 50 μg/mL) of purified enzyme, these reagents are not sensitive enough for screening crude cell lysates, as needed for high throughput plate-based assays.

Given the generally greater sensitivity of fluorescence detection, a fluorogenic active site titrating agent for glycosidases could be useful. Any fluorogenic moiety in this class of molecule must also function as a sufficiently activated leaving group in order to ensure an accumulation of the covalent glycosyl-enzyme intermediate. Previous studies have shown that the phenolic leaving group attached to a 2-fluorosugar should have a pK_a below 6, and ideally 5 or lower in order to function effectively in trapping the intermediate.[8] *9H*-1,3-Dichloro-7-hydroxy-9,9-dimethylacridine-2-one (DDAO) is a promising fluorophore candidate for such a purpose, as it has a relatively low pK_a (~5) and other favourable properties including functional water solubility and excitation and emission at long wavelengths (λ_{ex} = 600 nm, λ_{em} = 656 nm).[10] This pK_a is sufficiently low that the fluorophore, which is optimally detectable in its deprotonated state, will enjoy a high fluorescence extinction coefficient under a broader range of assay conditions, down to approximately pH 5. The absorbance wavelength range for the anion is large and almost constant from 600 nm to 650 nm, thus this fluorophore can be stimulated by a large range of excitation wavelengths so that the excitation and emission wavelengths can be chosen to reduce interference by Rayleigh scattering.

We synthesised DDAO according to the procedure of Corey *et al.*[11] and confirmed that its absorbance spectra (Supplementary Figure 1) show maxima in the visible range at 414 nm for the protonated species (pH 2.0) and at 650 nm for the phenolate (pH 12.0). We also titrated it using fluorescence as a read-out to determine a pK_a of 5.34 ± 0.02 (fluorescence titration) as previously found.[12] An extinction coefficient value (ε) of 32100 ± 900 M^{-1} cm^{-1} was found at 600 nm in 50 mM phosphate buffer, pH 7.0. Deprotonated DDAO is fluorescent at 656 nm with a fluorescence response that is linear up to concentrations of 5 μM, which is suitable for the determination of most practical enzyme concentrations. Absorbance in the visible region can be used at higher enzyme concentrations. DDAO was coupled to four different 2-deoxy-2-fluorosugars *via* the Koenigs-Knorr glycosylation as shown in Scheme S1, yielding the four DDAO 2-deoxy-2-fluoroglycosides depicted in Figure 2. (See Supplementary methods for synthetic details and characterisation data.)

$$v_0 = \frac{k_i[E][I\text{-X}]}{K_i + [I\text{-X}]} \qquad \text{Equation 1}$$



Initial attempts at monitoring reaction of *gluco*-derivative **1** with the well-studied model *β*-glucosidase Abg[13] from *Agrobacterium sp.* by monitoring time-dependent loss of activity were rendered challenging by the extremely rapid inactivation observed, even at low concentrations of reagent. Indeed, the highest concentration that could be tested was 39 nM since above that value the enzyme was almost completely inactivated at the shortest assay time physically possible. Kinetic parameters were therefore determined by directly monitoring the release of DDAO using a stopped flow apparatus. Reaction of Abg with different concentrations of **1,** each in excess of the Abg concentration**,** resulted in a series of curves reflecting pseudo-first order release of DDAO. Pseudo-first order rate constants for inactivation at each concentration of **1** were derived by fitting the curves to a first order function and then fitted to Equation 1 to yield an inactivation rate constant k_i of 7800 min^{-1} and a dissociation constant K_i of 6 μM (Table 1). Compound **1** therefore binds to Abg approximately 40-fold more tightly than does 2,4-dinitrophenyl 2-deoxy-2-fluoro-*β*-D-glucopyranoside (DNP-2FGlc), indicating that Abg accommodates large aromatic aglycones very well. Further, the inactivation rate constant k_i is almost 30-fold greater than that for DNP-2FGlc, with the net result that the second order rate constant for inactivation of Abg by **1** (k_i/K_i) is over 1000-fold higher than that for DNP-2FGlc. Indeed the value of k_i/K_i = 1.3×10^9 M^{-1} min^{-1} is approaching diffusion-control: the reaction could not really be much faster! Since Abg, like most other GH1 glycosidases, has a broad *gluco*/*galacto* specificity, DDAO-2-deoxy-2-fluoro-*β*-D-galactopyranoside (DDAO-2FGal, **2**) was also tested as an inactivator and found to be highly effective, with a second order rate constant (k_i/K_i) of 6.18×10^6 M^{-1} min^{-1}, exceeding that of 2-deoxy-2-fluoro-β-D-galactopyranosyl fluoride (2FGalF) by over 7000 fold. DDAO-2FGal (**2**) and particularly DDAO-2-deoxy-2-fluoro-*β*-D-glucopyranoside (DDAO-2FGlc, **1**) are therefore ideal active site titration reagents, reacting extremely rapidly and releasing a readily detectable aglycone.

Given this success, **1** was also tested as an inactivator of human *β*-glucocerebrosidase (GCase), the GH 30 glycosidase whose deficiency leads to Gaucher disease. Since the natural substrate of GCase is a glycolipid, it was possible that the hydrophobic aglycone might be well accommodated. This was partially true as, based on relative k_i/K_i values, GCase was inactivated twice as efficiently by **1** as by the known inactivator 2-deoxy-2-fluoro-*β*-D-glucopyranosyl fluoride (2FGlcF)[14], and four times as efficiently as by DNP-2FGlc[15]though the limited aqueous solubility of **1** (approximately 700 μM), precluded accurate determination of individual values of k_i and K_i. Compound **1** can therefore function as a sensitive active site titrating agent for GCase if incubated for sufficient time.

The other DDAO 2-deoxy-2-fluoroglycopyranosides synthesised were likewise found to be inactivators of corresponding retaining *β*-glycosidases (Table 1). Indeed DDAO-2FGal (**2**) was found to be a remarkably effective inactivator of the eponymous *E. coli* lac *z β*-galactosidase, reacting over 800-fold faster than does DNP-2FGal[16], largely as a consequence of tighter binding. Since the lac *z β*-galactosidase is widely employed as a reporter enzyme in biological systems, this probe could easily find use in accurate measurement of its expression levels in a variety of systems.[12] In the case of the GH52 *exo-β*-xylosidase Bhx from *Bacillus halodurans*[17], DDAO-2-

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deoxy-2-fluoro-*β*-D-xylopyranoside (DDAO-2FXyl, **3**) was found to function as an inactivator, but with a 5-fold lower inactivation efficiency (k_i/K_i) than that for 2,4-dinitrophenyl 2-deoxy-2-fluoro-*β*-D-xylopyranoside (DNP-2FXyl), most of the difference residing in the better binding of DNP-2FXyl (K_i = 25 ± 5 μM) compared to **3** (K_i = 110 ± 30 μM). Nonetheless **3** remains an efficient inactivator of Bhx ($t_{1/2}$ = 20 sec at saturation), thus can be used in accurate quantitation. Finally, the cellobioside derivative DDAO-2-deoxy-2-fluoro-*β*-cellobioside (DDAO-2FCel, **4**) was tested as an inactivator of two enzymes with cellulase activity, the GH10 *endo*-glycanase Cex from the soil bacterium *Cellulomonas fimi*[18] and the commercially important fungal *endo*-glucanase EG I from *Trichoderma reesei.*[19] **4 was** found to be a 3-fold better inactivator of Cex than the previously tested 2,4-dinitrophenyl 2-deoxy-2-fluoro-*β*-cellobioside (DNP-2FCel), based on k_i/K_i values, [18] and proved to be the superior reagent for inactivating the fungal enzyme EG I with a k_i/K_i value 20-fold greater than that measured for DNP-2FCel.[20] Given current interest in cellulase–mediated biomass conversion processes, these compounds have potential applications in monitoring and quantitating the enzymes used.

In order to determine practical limits of sensitivity of DDAO 2-deoxy-2-fluoroglycosides as active site reagents we used Abg as our model system, with DDAO-2FGlc (**1**) as the titrant. Initial studies were performed by simply measuring endpoint absorbances in a standard UV-Visible spectrophotometer (600 nm) after allowing various concentrations of Abg to react with an excess of **1.** Results are shown in Fig 3A as a plot of released DDAO concentration versus concentration of Abg employed in each experiment. The concentrations of Abg employed were determined using the extinction coefficient of $\varepsilon_{280}^{0.1\%}$ = 2.20 cm^{-1} previously determined by quantitative amino acid analysis.[13] The slope of 1.02 ± 0.02 confirms the validity of the method. Parallel titration of Abg by DNP-2FGlc likewise yielded a plot with a slope of 1.01 ± 0.04. Using this UV-Vis methodology, concentrations of Abg down to 70 nM (25 pmol in 0.25 mL) could be reliably determined; however, measurement of amounts under 50 nM was accompanied by considerable noise. Use of a fluorescent read-out significantly improved sensitivity. Active site titrations with **1** were monitored either by stopped flow (Applied Photophysics) or by using a standard fluorimeter (Cary Eclipse) at excitation and emission wavelengths of 600 nm and 656 nm, respectively. Use of stopped flow allows the time course for the reaction between enzyme and inactivator to be monitored, as seen in Fig. 3C. However, the small volumes used (150 μL), and associated errors again limited accurate measurement to concentrations above 50 nM. Much greater (ten-fold) sensitivity was obtained by fluorometric measurement of end points in a 500 μL cell using a standard fluorimeter to increase the precision of the measurement. Abg concentrations as low as 3 nM, or 2 pmol per 0.5 mL, were accurately determined as shown in Fig 3B. This is a satisfactory range for use in the detection of enzyme concentrations during plate-based high-throughput screening processes.

In conclusion, we have developed a new class of highly sensitive fluorogenic active site titrants for quantitation of retaining glycosidases. Importantly, all enzymes tested within this study were inactivated, thus the bulky nature of the aglycone does not seem to be an impediment to general application. Further, the sensitivity of the reagent is sufficient to allow quantitation of glycosidases at typical protein expression levels. We plan to use these reagents to monitor expressed enzyme levels within screens for directed evolution and metagenomic studies, thereby allowing us to directly determine specific activities of the enzymes so discovered. This class of reagent will however likely fulfil many other applications.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Experiment details and compound characterisation. See DOI: 10.1039/c000000x/

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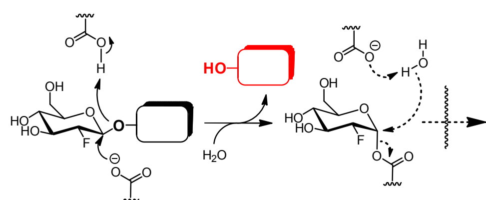

Figure 1. Mechanism of action of an active site titrating agent upon a retaining β-glucosidase. One equivalent of a fluorescent leaving group (shown in red) is released per enzyme molecule.

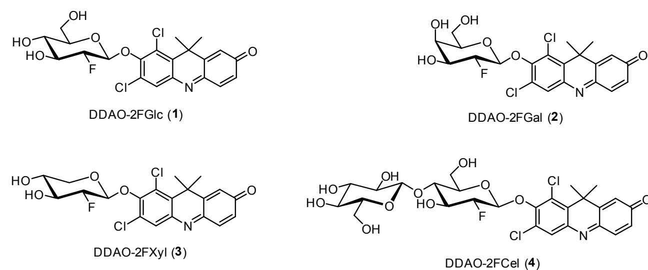

Figure 2: The structures of DDAO 2-deoxy-2-fluoroglycopyranoside active site titrating agents**.**

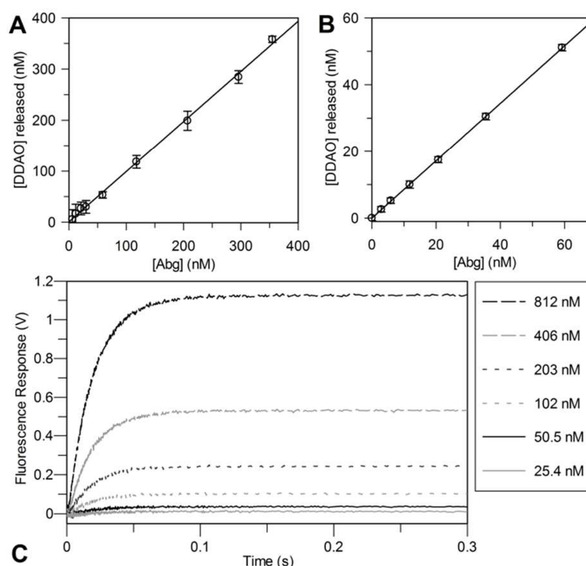

Figure 3. A) Active site titration of Abg by 1 monitored by absorbance at 600 nm and plotted as a function of Abg concentration. B) Active site titration of Abg by 1 on the Cary Eclipse fluorimeter. Fluorescence response monitored at excitation/emission wavelengths of 600 nm/656 nm at 600 V. C) Burst measurement by fluorescence on a stopped flow instrument.

Table 1. Summary of inactivation constants.

Enzyme	Inactivator	k_i (min^{-1})	K_i (μM)	k_i/K_i (mM^{-1} min^{-1})
Abg	DDAO-2FGlc	7800 ± 500	6 ± 1	$(1.3 \pm 0.2)\times10^6$
	DNP-2FGlc[21]	283	245	1160
	DDAO-2FGal	--	--	6180 ± 80
	2FGalF[9]	2.6	3200	0.81
GCase	DDAO-2FGlc	0.042 ± 0.008	900 ± 200	0.05 ± 0.01
	DNP-2FGlc[15]	0.030	2.5	0.012
	2FGlcF[14]	--	--	0.023
LacZ	DDAO-2FGal	2.58 ± 0.01	9.8 ± 0.9	280 ± 30
	DNP-2FGal[16]	2.4	7100	0.34
Bhx	DDAO-2FXyl	2.0 ± 0.4	110 ± 30	18 ± 5
	DNP-2FXyl	2.4 ± 0.2	25 ± 5	100 ± 20
Cex	DDAO-2FCel	0.17 ± 0.05	90 ± 40	2.0 ± 0.2
	DNP-2FCel[18]	0.067	110	0.612
EG I	DDAO-2FCel	0.033 ± 0.002	220 ± 30	0.15 ± 0.02
	DNP-2FCel[20]	0.022	3100	0.0071

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