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Inhibitory Efficiencies for Mechanism-Based Inactivators of Sialidases

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Sialidase; inactivation; catalytic efficiency; inhibitory efficiency; mechanism

Abstract

Here we describe the measurement of the inactivation rate constants for the mechanism-based inactivator 2,3-difluorosialic acid acting upon the sialidase from *Micromonospora viridifaciens*. Using double mixing stopped-flow experiments conducted in a 3-(*N*-morpholino)propanesulfonic acid buffer (100 mM, pH 7.00) at 25 °C, the derived kinetic parameters are $k_{\text{inact}}/K_i = (3.9 \pm 0.8) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and $K_i = 1.7 \pm 0.4 \text{ }\mu\text{M}$. We demonstrate that the inhibitory efficiency of the inactivation event is similar to the catalytic efficiency for this sialidase acting upon a typical substrate, 4-methylumbelliferone α -D-sialoside, $k_{\text{cat}}/K_m = (7.2 \pm 2.8) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$. Furthermore, we show that the catalytic efficiencies for inactivation and hydrolysis by the Kdnase from *Aspergillus fumigatus* are similar for the corresponding Kdn-analogues. We conclude that the deactivating effect of incorporating an axial 3-fluoro substituent onto the sialic acid scaffold is comparable to the enhanced activation that occurs when the 4-methylumbelliferone leaving group is changed to the more nucleofugal fluoride ion.

Introduction

The fundamental biological importance of a family of carbohydrates known as sialic acids—a group of nine carbon keto acids (non-2-ulosonic acids)—has piqued the interest of scientists and motivated them to identify and modulate the structures of these sugars.¹⁻⁴ Sialic acids are often situated at the non-reducing termini of glycoconjugates where they can modulate biological functions such as glycoprotein recognition. For example, the sialic acid *N*-acetylneuraminic acid **1** located at the termini of erythrocyte glycans regulates the half life of these blood cells; serum sialidases can hydrolyze these sialic acids thereby removing the end unit and unmasking the next sugar of the carbohydrate, a galactose residue. Upon sialic acid removal, these "asialoglycans" bind to hepatic Ashwell-Morell receptors, causing the erythrocyte to be internalized and degraded in the liver.^{5, 6} Sialic acids also play important roles in microbial pathogenesis.⁷⁻¹⁰ Less is known about the biological importance of Kdn (**2**, formerly known as 2-keto-3-deoxynonoic acid), a sialic acid that has a hydroxyl group in place of the C-5 *N*-acetyl group in **1**.^{11, 12}

Sialidases hydrolyze sialic acids; these enzymes have been identified in mammals, viruses, bacteria and fungi.^{3, 13-15} All sialidases are glycoside hydrolases (they belong to GH families 33, 34, and 89) that hydrolyze α -linked sialosides with net retention of anomeric configuration.^{16, 17} The active site of all sialidases contain key amino acid residues, including aspartic and glutamic acids, a nucleophilic tyrosine, and an arginine triad. Scheme 1 shows the first step of the currently accepted mechanism of action for sialidases, which involves the formation of a sialosyl-enzyme intermediate that undergoes hydrolysis to yield α -sialic acid as the initial product.¹⁸⁻²¹

SCHEME 1

Numerous compounds have been developed to modulate the activity of sialidases; most are either reversible inhibitors, which bind competitively to the enzymatic active site, or inactivators, which covalently bind to an active site residue and destroy the catalytic activity. Most reversible sialidase inhibitors are analogues of the quintessential sialidase inhibitor Neu2en5Ac (**3**).²²⁻²⁵

Withers and co-workers have developed fluorinated sugar analogues as mechanism-based glycosidase inactivators.²⁶ These molecules generally incorporate a good leaving group (e.g., fluoride ion) at the anomeric centre and either replace a sugar hydroxyl group with a fluorine atom (e.g., 2,4-dinitrophenyl 2-deoxy-2-fluoro- β -D-glucopyranoside (**4**)²⁷) or incorporate a second acetal centre by addition of a fluorine atom adjacent to the ring oxygen (e.g., 5-fluoro- α -D-galactopyranosyl fluoride (**5**)²⁸). Both are extremely effective strategies for the development of chemical tools for use as affinity probes in biological chemistry.²⁶ Interestingly, Watts et al. noted that the time-dependent inactivation of the sialidase from *Trypanosoma rangeli* by 2,3-difluoro-*N*-acetyl-neuraminic acid (**6**) could not be monitored because the rate of intermediate formation was too fast to be measured using non-stopped flow techniques.¹⁹ Indeed, measurement of kinetic parameters (k_{inact}/K_i) for inactivation by **6** has been reported for only a limited number of sialidases, in particular those with aglycone recognition sites, e.g., the Human NEU2 enzyme,^{29, 30} and the *trans*-sialidases from *Trypanosoma* spp., or those with moderate catalytic efficiencies, such as the influenza viral enzymes.^{31, 32} Measurement of k_{inact}/K_i values for more active sialidases has therefore required the use of the lower affinity mechanism-based inactivator 2,3-difluoro-Kdn (**7**).¹⁹

Herein, we use a double mixing stopped-flow instrument to monitor the rapid inactivation of the *Micromonospora viridifaciens* sialidase (*MvS*)—a catalytically efficient enzyme from a soil bacterium—by the mechanism-based glycosidase inactivator **6**. We report the kinetic

parameters for the rapid inactivation of *MvS* by **6**, as well as the inactivation of *MvS* and the *Aspergillus fumigatus* Kdnase (*AfK*) with **7**. We then compare the calculated inactivation efficiencies and the corresponding catalytic efficiencies for these enzymes with substrates containing an aryloxy leaving group (**8** and **9**).

Results and discussion

First, we synthesized the two mechanism-based inactivators, **6** and **7** (Supplementary Methods and Figures S1–6), by the synthetic route developed by Watts et al.³³ with the minor modification of using the fluorinating reagent XtalFluor-E (diethylaminodifluorosulfonium tetrafluoroborate),³⁴ which is less sensitive to moisture than DAST (diethylaminosulfur trifluoride), to incorporate the anomeric fluorine atom (Scheme 2).

SCHEME 2

We determined that no significant enzymatic activity could be detected in a sample taken by rapid removal of an aliquot of a freshly made solution of **6** (we tested both 30 and 0.5 μM) and *MvS* when mixed with substrate **8**. We therefore performed a series of double-mixing stopped-flow experiments in order to evaluate the kinetic parameters for this rapid inactivation reaction. In these experiments, a pneumatically mixed enzyme and inactivator solution is aged in a 'delay loop' (Supplementary Figure S7) and following a predetermined incubation time, this solution is rapidly mixed (in milliseconds) with substrate and the resulting enzymatic activity determined. We tested the inactivation of *MvS* by **6** at a pH of 7 rather than at its pH optima of ~ 5.5 ,¹⁸ in order to avoid artifacts in the stopped-flow experiment that could arise from mixing inactivation buffer with analysis buffer (higher pH gives a more sensitive assay) if these two buffers differ in their pH values. Using this non-optimal pH for enzyme activity, the inactivation

was nevertheless fast ($t_{1/2} < 350$ ms for **6**) = 1 μ M). Figure 1 shows typical activity traces obtained after the addition of substrate **8** to pre-incubated mixture of *MvS* and **6**.

FIGURE 1

We fit these initial rate measurement data (int/s) to a standard exponential equation to obtain values for the pseudo-first-order inactivation rate constant (k_{obs}); a typical data set is shown in Figure 2.

FIGURE 2

We then fit these k_{obs} values for inactivation of *MvS* by **6** to a Michaelis-Menten type equation (eqn. 1); the resultant plot is shown in Figure 3a.

$$k_{obs} = \frac{k_{inact}[I]}{K_i + [I]} \quad (1)$$

FIGURE 3

To determine the differences between the inhibitors difluorosialic acid and difluoroKdn, we measured rate constants for inactivation of *MvS* and *AfK* by **7**. The resultant plots are shown in Figures 3b and 3c. Interestingly, the plot of k_{obs} versus **[7]** for inactivation of *AfK* was linear up to an inactivator concentration of 0.4 mM, and as a result, we could estimate a value for k_{inact}/K_i but not for the separate parameters (k_{inact} and K_i). The calculated kinetic parameters for these inactivation experiments are listed in Tables 1 and 2.

***Micromonospora viridifaciens* Sialidase – Catalytic and Inhibitory Efficiencies and Proficiencies**

Although we compared reactions at different temperatures (i.e., enzyme-catalyzed inactivation at 25 °C, hydrolysis at 37 °C, and the corresponding spontaneous hydrolyses at 50 °C), a few general trends are apparent in the data listed in Tables 1 and 2. As an example, for *MvS* and *AfK* there are striking similarities between the catalytic efficiencies (k_{cat}/K_m) for

hydrolysis of 4-methylumbelliferyl glycosides and the inhibitory values (k_{inact}/K_i) for the corresponding 3-fluoroglycosyl fluorides. In addition, the calculated enzymatic proficiencies for the *MvS*-catalyzed reactions with **6** and **8** were similar (Table 1). Thus, based on k_{uncat} values (Table 1) we conclude that the intrinsic deactivating effect of an axial 3-fluoro substituent in place of a hydrogen atom is comparable to the inherent activation that results from changing the leaving group from an aryloxy group ($\text{p}K_{\text{a}}(4\text{-methylumbelliferone}) = 7.8$)³⁵ to a fluoride ion ($\text{p}K_{\text{a}}(\text{HF}) = 3.2$). Furthermore, we conclude that these intrinsic effects also occur in the sialidase-catalyzed reactions because it is likely that the C3 fluorine in **6** and the axial C3-H in **8** do not have significant interactions with the enzyme.

***Aspergillus fumigatus* – Catalytic and Inhibitory Efficiencies**

Clearly, the Kdnase from *A. fumigatus*—the closest enzyme in structure to the sialidase from *M. viridifaciens*³⁶—displays a similar reactivity profile for the mechanism-based inactivator (**7**) and its substrate (**9**) to that of *MvS* and the corresponding compounds **6** and **8** (Table 2). As with *MvS*, the catalytic and inhibitory efficiencies of the *A. fumigatus* Kdnase suggest that similar reactivity changes result from the incorporation of a C3-fluorine atom and a fluoride leaving group into the inhibitor structure, and that the organism-specific binding pockets for the C-5 substituents have similar effects on catalysis and inactivation.

Comparison with the Catalytic and Inhibitory Proficiencies of Other Glycosidases

A comparison of the reported catalytic and inhibitory proficiencies for a β -glucosidase, which also uses a substrate that contains an equatorial aglycone, with those for *MvS* and *AfK* described herein shows that in contrast to the very similar catalytic proficiency (CP) and inhibitory proficiency (IP) values for the sialidase (Table 1) and Kdnase (Table 2), the calculated CP and IP values for the β -glucosidase varied by eight orders of magnitude (Table 3).

Specifically, the *Agrobacterium faecalis* β -glucosidase has a CP value ($k_{\text{cat}}/K_{\text{m}} \times 1/k_{\text{uncat}}$) of $3.3 \times 10^{16} \text{ M}^{-1}$ for the catalyzed hydrolysis of 4-nitrophenyl β -D-glucopyranoside ($\text{p}K_{\text{a(4-nitrophenol)}} = 7.2$), while the corresponding IP value for 2-deoxy-2-fluoro- β -D-glucopyranosyl fluoride ($k_{\text{inact}}/K_{\text{i}} \times 1/k_{\text{uncat}}$) was $1.6 \times 10^8 \text{ M}^{-1}$.

The similarity in the $k_{\text{cat}}/K_{\text{m}}$ and $k_{\text{inact}}/K_{\text{i}}$ values measured for catalysis by *MvS* with **8** and **6** (Table 1) and that for *AfK* with **9** and **7** (Table 2) is in contrast to the approximate 10^4 -fold difference (between $k_{\text{cat}}/K_{\text{m}}$ and $k_{\text{inact}}/K_{\text{i}}$) for *A. faecalis* β -glucosidase-catalyzed reactions of **10** and **11**. This discrepancy is consistent with the replacement of the natural C2 hydroxyl group in the substrate (**10**) with a C2 fluorine atom (**11**) reducing the transition state stabilization for the inactivation reaction by a factor of 10^3 – 10^4 ,^{37, 38} if the change in leaving groups (**10** vs. **11**) has the same rate accelerating effect to that noted above (**8** vs. **6**).

Moreover, removal of an electron withdrawing C2 hydroxyl group in glycosides generally results in the corresponding 2-deoxy glycoside being hydrolyzed around 10^3 -fold more rapidly.^{39,}

⁴⁰ Based on the similar rate constants for the spontaneous reactions of **6** and **8**, we proposed that if **10** and **11** hydrolyze via comparable transition states then the reactivity difference for their spontaneous reactions would be 10^3 -fold. Therefore, we conclude that 2-deoxy-2-fluoro- β -D-glucopyranosyl fluoride reacts in aqueous solutions via a concerted $\text{S}_{\text{N}}1$ -like TS while 4-nitrophenyl β -D-glucopyranoside reacts via a short-lived oxacarbenium ion intermediate ($\text{S}_{\text{N}}2$, $D_{\text{N}}^*A_{\text{N}}$).^{41, 42}

Hydrolysis of the 3-Fluorosialosyl-Enzyme Intermediate

We were unable to measure the reactivation of *MvS* by removing excess **6** and then monitoring for an increase in enzymatic activity because hydrolysis of the 3-fluorosialosyl-*MvS* intermediate is rapid. Instead, we measured this event by taking an inactivated stock solution of

MvS (in the presence of excess **6**) and diluting it 50-fold into a solution containing a saturating solution of substrate ($\sim 12 \times K_m$). Figure 4 shows a change in activity trace ($\text{abs}_{(t=x+1)} - \text{abs}_{(t=x)}$) as a function of time in seconds after the addition of inactivated *MvS* to a saturating concentration of substrate. The derived reactivation rate constant is $(1.2 \pm 0.1) \times 10^{-2} \text{ s}^{-1}$. Given that deglycosylation is rate-limiting for hydrolysis of natural substrates by *MvS*¹⁸ we conclude that incorporation of an axial fluorine atom slows deglycosylation down by a factor of $\sim 2.3 \times 10^4$ ($277^{43} \div 0.012$).

Figure 4

Experimental

General procedures and materials

All chemical reagents were analytical grade or better, purchased from Sigma-Aldrich unless stated otherwise, and used without further purification. Solvents for anhydrous reactions were either HPLC grade or dried and distilled prior to use. Dichloromethane and methanol were dried over calcium hydride and magnesium methoxide, respectively. For anhydrous reactions all glassware was dried overnight at 100–150 °C in an oven prior to use. Thin layer chromatography (TLC) was performed on aluminum sheet TLC plates (0.25 mm) pre-coated with Merck silica gel 60 F₂₅₄. Compounds were visualised using para-anisaldehyde. Flash chromatography was conducted on Merck silica gel 60. Melting points were recorded on an OptiMelt apparatus and are uncorrected. Optical rotations were measured on a Perkin-Elmer 341 polarimeter and units are reported in $\text{deg cm}^2 \text{ g}^{-1}$ (concentration units are $\text{g}/100 \text{ cm}^3$). ¹H and ¹³C NMR spectra were recorded on a Bruker NMR spectrometer (AVAVG III 400, 500 or TCI 600 MHz instrument). Deuterated chloroform (CDCl₃) and water (D₂O) were used as solvent and internal reference, chemical shifts (δ) are reported in parts-per-million (ppm) downfield from tetramethylsilane, and

coupling constants (J) are listed in Hz. ^{19}F spectra were acquired on either an AVAVG III 400 or 500 Hz instrument. CDCl_3 and D_2O were used as solvent, spectra were referenced to an external trifluorotoluene standard ($\delta = -63.72$ ppm; on this scale $\text{CFCl}_3 = 0$ ppm and trifluoroacetic acid resonates at -76.55 ppm). *Milli-Q Water* ($18.2 \text{ M}\Omega \text{ cm}^{-1}$) was used for all kinetic experiments. pH values were measured using a VWR pH meter attached to a standard pH electrode. All kinetic data were fit using a computer program to the appropriate non-linear least squares equation.

Synthesis of difluorosialic acid (6)

Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-3-fluoro-D-erythro- β -L-manno-non-2-ulopyranosyl fluoride **12**

Diethylaminodifluorosufinium tetrafluoroborate (XtalFluor-E) (1.3 g, 6.1 mmol) and hemiacetal **S1-4** (1.03 g, 2.03 mmol)³³ were added to a mixture of triethylamine trihydrofluoride ($\text{Et}_3\text{N} \cdot 3\text{HF}$) (1.4 mL, 8.1 mmol) and anhydrous dichloromethane (10 mL). The resulting mixture was left to stir at rt for 4 h. After the reaction was complete (monitored by TLC with EtOAc/Hex (8.5/1.5, v/v as eluent), an aqueous solution of sodium bicarbonate (~35 mL, 5%) was added to neutralize the reaction and this mixture was then extracted with dichloromethane (3×50 mL). The combined organic layers were washed with brine (100 mL) and dried over anhydrous sodium sulphate (Na_2SO_4) and passed through a pad of silica, which was washed with EtOAc/Hexane (8.5:1.5, v/v). Evaporation of the volatile components under reduced pressure gave a mixture of difluorosialic acid **12** and a second isomer (919 mg, 89%). The ratio of the two major isomers of per-*O*-acetylated-2,3-difluorosialic acid was measured using ^{19}F NMR spectroscopy to be 2:1 (β : α). Pure per-*O*-acetylated-2,3-difluorosialic acid **12** was obtained by crystallization from ethyl acetate and *tert*-butyl methyl ether to give long colourless needles (512 mg, 50%), mp = $66\text{--}69^\circ\text{C}$ (lit. $68\text{--}70^\circ\text{C}$),³³ ^1H NMR (500 MHz, CDCl_3) 5.49 (br dd, 1H, $J_{4,\text{F}3} = 25.5$, $J_{4,5} = 10.8$,

H-4), 5.42–5.35 (m, 2H), 5.29 (m, 1H, H-7), 5.13 (dt, 1H, $J_{3,F3} = 50.7$, $J_{3,4} = 2.6$, H-3), 4.31–4.40 (m, 2H, H-6, H-9'), 4.19 (dd, 1H, $J_{9,9'} = 12.6$, $J_{9,8} = 5.1$, H-9), 4.01–4.09 (m, 1H, H-5), 3.91 (s, 3H, OCH₃), 2.17, 2.12, 2.02, (4 s, 12H, OAc), 1.94 (s, 3H, NHCOCH₃). ¹³C NMR (151 MHz, CDCl₃): 20.77, 20.86, 20.90, 21.06 (OAc), 23.48 (NHAc), 45.96 (C-5), 53.94 (OMe), 62.04 (C-9), 67.26 (C-7), 68.61 (dd, $J_{4,F3} = 17.4$, $J_{4,F2} = 6.0$, C-4), 69.09 (C-8), 71.95 (d, $J_{6,F3} = 3.9$ Hz, C-6), 85.71 (dd, $J_{3,F2} = 19.4$, $J_{3,F3} = 194.6$ Hz, C-3), 104.77 (dd, $J_{2,F3} = 17.4$, $J_{2,F2} = 225.6$ Hz, C-2), 164.43 (br d, $J_{1,F2} = 29.8$ Hz, C-1), 169.91, 170.43, 170.49, 170.67, 170.71 (s, C=O). ¹⁹F (471 MHz, CDCl₃) –216.93 (ddd, $J_{F3,H3} = 50.5$, $J_{F3,H4} = 25.5$, $J_{F3,F2} = 11.2$, F-3), –123.36 (d, $J_{F2,F3} = 11.3$, F-2). HRMS-FAB (m/z): [M + H⁺] calcd for C₂₀H₂₇F₂NO₁₂, 512.1580; Found, 512.1582. The above NMR spectral data match those reported in the literature.³³

Methyl 5-acetamido-3,5-dideoxy-3-fluoro-D-erythro-β-L-manno-non-2-ulopyranosonyl fluoride 13

To a solution of **12** (189.4 mg, 0.37 mmol) in dry methanol (10 mL) at 0 °C was added sodium methoxide (20 mg, 0.37 mmol) and this mixture was stirred for 6 h. The reaction mixture was neutralized by addition of Amberlite IR-120+ resin (H⁺ form). This solution was then filtered and the resin was washed with dry methanol. The combined filtrate was concentrated to afford the desired product as a colorless oil **13** (109.1 mg, 86%). The resultant product was used in the next step without further purification. ¹H NMR (400 MHz, D₂O) 5.22 (dt, 1H, $J_{3,F3} = 51.4$, $J_{3,F2} = 2.6$, $J_{3,4} = 2.6$, H-3), 4.29 (t, 1H, $J_{5,4} = 10.3$, H-5), 4.17 (ddd, 1H, $J_{4,F3} = 26.4$, $J_{4,5} = 10.7$, $J_{4,5} = 2.04$, H-4), 3.84–3.90 (m, 2H, H-6, H-8), 3.67–3.60 (m, 1H, H-9), 3.56 (d, 1H, $J = 8.9$, H-7), OCH₃), 2.05 (s, 3H, NHCOCH₃). ¹⁹F NMR (471 MHz, CDCl₃) –216.93 (ddd, $J_{F3,H3} = 50.5$, $J_{F3,H4} = 25.5$, $J_{F3,F2} = 11.2$, F-3), –123.36 (d, $J_{F2,F3} = 11.3$, F-2).

5-Acetamido-3,5-dideoxy-3-fluoro-D-erythro-β-L-manno-non-2-ulopyranosonyl fluoride 6

Compound **13** (109.10 mg, 0.32 mmol) was dissolved in THF/H₂O (3/1 v/v) followed by the addition of LiOH.H₂O (13.5 mg, 0.32 mmol). The resultant mixture was stirred at 0 °C for 1 h after which it was neutralized by adding Amberlite IR-120+ resin (H⁺ form) and the reaction mixture was filtered to remove the resin. The resin was washed with dry methanol and then the combined filtrates were concentrated *in vacuo*. The remaining syrupy residue was lyophilized to afford the 2,3-difluorosialic acid **6** (92.7 mg, 88%) as a low melting solid, mp = 21–25 °C, $[\alpha]_D^{20} = -29$ (c = 0.1, H₂O), ¹H NMR (600 MHz, D₂O) 5.22 (dt, 1H, $J_{3,F3} = 51.4$, $J_{3,F2} = 2.6$, $J_{3,4} = 2.6$, H-3), 4.29 (t, 1H, $J_{5,4} = 10.6$, H-5), 4.17 (br dd, 1H, $J_{4,F3} = 28.4$, $J_{4,5} = 10.3$, H-4), 3.84–3.91 (m, 2H, H-8, H-9'), 3.82 (br d, 1H, $J_{5,4} = 10.6$, H-6), 3.62 (br dd, 1H, $J_{9,9'} = 11.8$, $J_{9,8} = 6.2$, H-9), 3.57 (br d, 1H, $J_{7,8} = 9.2$, H-7), 2.05 (s, 3H, NHCOCH₃). ¹³C NMR (151 MHz, D₂O) 21.48 (s, 1C, NHCOCH₃), 46.36 (d, $J_{5,F3} = 3.3$, C-5), 62.46 (C-9), 67.38 (C-7), 68.63 (br dd, $J_{4,F2} = 5.9$, $J_{4,F3} = 18.0$, C-4), 69.98 (C-8), 72.10 (d, 1C, $J_{6,F2} = 4.1$, C-6), 88.35 (br dd, 1C $J_{3,F3} = 183.9$, $J_{3,F2} = 18.5$, C-3), 106.07 (dd, 1C, $J_{2,F} = 219.5$, $J_{2,F3} = 14.9$, C-2), 168.80 (dd, $J_{1,F2} = 26.9$, C-1), 174.56 (s, C=O). ¹⁹F (376 MHz, D₂O) –217.95 (ddd, $J_{F3,H3} = 51.5$, $J_{F3,H4} = 28.5$, $J_{F3,F2} = 11.2$, F-3); –121.27 (d, $J_{F2,F3} = 11.6$, F-2). HRMS-FAB (m/z): [M + H⁺] calcd for C₁₁H₁₇F₂NO₈, 330.1001; Found, 330.0997. The above NMR spectral data match those reported in the literature.³³

Experimental measurement of the kinetic parameters for inactivation of *M. viridifaciens* sialidase by 6.

All kinetics experiments were performed at 25 °C in MOPS buffer (100 mM, pH 7.00) containing KCl (100 mM) and BSA (0.01 %) using an Applied Photophysics SX20 stopped-flow spectrophotometer that was interfaced to an external RMS 6 Lauda temperature controller. Specifically, the activity of *MvS* after incubation with various concentrations of **6** was determined by monitoring fluorescence intensity changes ($\lambda_{ex} = 365$ nm and $\lambda_{em} > 440$ nm) resulting from

the sialidase-catalyzed hydrolysis of 4-methylumbelliferyl 5-*N*-acetyl- α -D-neuraminide (4-MU- α Neu5Ac). The spectrometer was used in 'double' mixing mode (**Supplementary Figure S7**), which involves the use of two pneumatic drives and four syringes. The first pneumatic mixing combined equal volumes of buffered (200 mM, pH 7.00, KCl = 200 mM) *MvS* stock solution (0.23–1.80 μ M; syringe A) and the inactivator (2.0–160.0 μ M; syringe B) in mixer 1 (total volume 220 ± 10 μ L) and this resulted in a bolus of enzyme and inactivator solution filling delay loop D (**Supplementary Figure S7**). Following aging of this solution for various time intervals (10–3000 ms) a second pneumatic drives pushed equal volumes of 4-MU- α Neu5Ac (140 μ M; syringe C) and water (syringe D); where the solution from syringe D pushed the incubation mix to mixer 2 where it combined with substrate (total volume 180 ± 10 μ L). The residual activity of *MvS* (aged solution) was recorded for each concentration of **6** and at least three repeat measurements were averaged and the standard error calculated for each concentration and incubation time. The pseudo-first-order rate constants for inactivation were then calculated from the mean and standard deviation of the residual activity versus the incubation time by fitting the data to a standard first order exponential decay. The residual activity at each incubation time was calculated as the mean (and standard deviation) from several initial rate measurements at each inactivator concentration.

Control experiments were performed in which it was shown that: 1) when water was placed in syringe B, no decrease in activity of *MvS* was observed; and 2) the observed inactivation rate constant (k_{obs}) was independent of the concentration of *MvS* (data not shown).

The pseudo first order rate constants (k_{obs}) for inactivation of *MvS* by **7** and **6** and *A/K* by **7** were calculated by fitting the slopes of residual activity versus incubation time to a standard first-order exponential decay. The inactivation rate constants (k_{inact}) and the apparent dissociation constants

(K_i) were estimated by fitting the k_{obs} values and inactivator concentrations to a standard Michaelis-Menten equation.

Evaluation of the rate constant for reactivation of inactivated *M. viridifaciens* sialidase

A stock solution of *MvS* (1.9 μM) was inactivated in acetate buffer (pH 5.25) using **6** (10 μM) at 25 °C for 10 min, at which time an aliquot (20 μL) of this solution was added to a cuvette containing MOPS buffer (980 μL , 100 mM, pH 7.00), KCl (100 mM), BSA (0.01 %) and 4-nitrophenyl 5-*N*-acetyl- α -D-neuraminide (200 μM ; $\sim 12 \times K_m$). The reactivation rate constant was calculated by averaging the absorbance signal over 1 second time intervals and fitting the change in absorbance per second vs time to a standard first order rate equation.

Supplementary material

Supplementary material is available with this article through the journal Web site at <http://nrcresearchpress.com>. The supplementary material includes full synthetic details, kinetic procedures for the measurement of kinetic parameters for inactivation of *MvS* and *AfK* by **7**, ^1H , ^{13}C , and ^{19}F NMR spectra of **6** and **7**, a schematic diagram of the double-mixing stopped-flow spectrometer set-up, and a full tabulation of the measured k_{obs} values for inactivation.

Acknowledgement

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Table 1. Kinetic parameters for the spontaneous (k_{uncat}) and the *Micromonospora viridifaciens* sialidase-catalyzed hydrolysis ($k_{\text{cat}}/K_{\text{m}}$) of MU α Neu5Ac (**8**) and MU α Kdn (**9**) and the corresponding rate constants ($k_{\text{inact}}/K_{\text{i}}$) for inactivation of this enzyme by 2,3-difluorosialic acid (**6**) and 2,3-difluoroKdn (**7**). Catalytic proficiency ($\text{CP} = k_{\text{cat}}/K_{\text{m}} \times 1/k_{\text{uncat}}$) and the inhibitory proficiency ($\text{IP} = k_{\text{inact}}/K_{\text{i}} \times 1/k_{\text{uncat}}$) are also listed.

Substrate/Inhibitor	k_{uncat} (s^{-1})	$k_{\text{cat}}/K_{\text{m}}$ or $k_{\text{inact}}/K_{\text{i}}$ ($\text{M}^{-1} \text{s}^{-1}$)	K_{i} (μM)	CP or IP (M^{-1})
MU α NeuAc (8)	$4.40 \times 10^{-6}{}^a$	$(7.2 \pm 2.8) \times 10^6{}^b$	—	$1.6 \times 10^{+12}$
2,3-Difluorosialic acid (6)	$1.67 \times 10^{-6}{}^c$	$(3.9 \pm 0.8) \times 10^6{}^d$	1.7 ± 0.4	$2.3 \times 10^{+12}$
MU α Kdn (9)		$(1.03 \pm 0.36) \times 10^3{}^e$	—	
2,3-DifluoroKdn (7)		$(2.92 \pm 0.89) \times 10^3{}^f$	40 ± 12	

^a Value extrapolated using the β_{lg} value from reference ⁴⁴ and the hydrolysis data for 4-nitrophenyl α NeuAc ($k_{\text{obs}} = 3.08 \pm 0.06 \times 10^{-5} \text{ s}^{-1}$, $T = 50^\circ\text{C}$, reference ⁴⁵). ^b Data taken from references ⁴⁶ and ⁴³ at pH 5.25 and $T = 37^\circ\text{C}$. ^c Hydrolysis reaction performed at a pH of 7.0 and $T = 50^\circ\text{C}$, references ³¹ and ³². ^d This study, conditions pH 7.00 and $T = 25^\circ\text{C}$, $k_{\text{inact}} = 6.7 \pm 0.3 \text{ s}^{-1}$. ^e Data taken from reference ⁴⁷ at pH 5.25 and $T = 37^\circ\text{C}$. ^f This study, conditions pH 5.25 and $T = 25^\circ\text{C}$, $k_{\text{inact}} = 0.19 \pm 0.02 \text{ s}^{-1}$.

Table 2. Kinetic parameters for the *Aspergillus fumigatus* Kdnase-catalyzed hydrolysis ($k_{\text{cat}}/K_{\text{m}}$) of MU α KDN (**9**) and the corresponding rate constants ($k_{\text{inact}}/K_{\text{i}}$) for inactivation of this enzyme by 2,3-difluoroKdn (**7**).

Substrate/Inhibitor	$k_{\text{cat}}/K_{\text{m}}$ or $k_{\text{inact}}/K_{\text{i}}$ ($\text{M}^{-1} \text{s}^{-1}$)
MU α Kdn (9)	47.7 ± 3.6^a
2,3-DifluoroKdn (7)	12.0 ± 0.2^b

^a Data taken from reference ⁴⁷ conditions pH 5.2 and T = 37 °C. ^b This study, conditions pH 5.25 and T = 25 °C. The best fit of the data was to a linear regression model, which necessitates that $K_{\text{i}} \gg 0.4 \text{ mM}$.

Table 3. Kinetic parameters for the *Agrobacterium faecalis* β -glucosidase-catalyzed hydrolysis ($k_{\text{cat}}/K_{\text{m}}$) of 4-nitrophenyl β -D-glucopyranoside (**10**) and the corresponding rate constants ($k_{\text{inact}}/K_{\text{i}}$) for inactivation of this enzyme by 2-deoxy-2-fluoro- β -D-glucopyranosyl fluoride (**11**).

Substrate/Inhibitor	k_{uncat} (s^{-1})	$k_{\text{cat}}/K_{\text{m}}$ or $k_{\text{inact}}/K_{\text{i}}$ ($\text{M}^{-1} \text{s}^{-1}$)	CP or IP (M^{-1})
10	$5.2 \times 10^{-11}^a$	$1.7 \times 10^6^b$	$3.3 \times 10^{+16}$
11	$1.6 \times 10^{-6}^c$	250^d	$1.6 \times 10^{+8}$

^a Data taken from reference ⁴⁸ conditions pH 5.2 and T = 37 °C. ^b Data taken from reference ³⁷. ^c Estimated by correcting the value for 2,4-dinitrophenyl β -D-glucopyranoside (reference ⁴⁰) with the difference in rate constants for β -D-galactopyranosyl fluoride and 2,4-dinitrophenyl β -D-galactopyranoside (reference ⁴⁹). ^d Data taken from reference ⁵⁰.

Scheme 1. General Mechanistic Scheme for the Sialidase-Catalyzed Formation of the Glycosyl-Enzyme Intermediate.

Scheme 2. Synthesis of Mechanism-Based Inactivators **6** and **7**. Reagents and conditions: (i) *E. coli* Neu5Ac aldolase, sodium 3-fluoropyruvate; (ii) methanol, H^+ -resin; (iii) Ac_2O , DMAP, pyridine; (iv) $NH_2NH_3^+ AcO^-$; (v) XtalFluor-E, $Et_3N \cdot 3HF$, CH_2Cl_2 ; (vi) NaOMe (cat), MeOH; (vii) $LiOH \cdot H_2O$, THF/ H_2O (3:1 v/v). Yields during the synthesis of **6**: (i) 81%; (ii) 94%; (iii) 81%; (iv) 91%; (v) 50%; (vi) 86%; (vii) 88%. Yields during the synthesis of **7**: (i) 83%; (ii) 85%; (iii) 87%; (iv) 74%; (v) 55%; (vi) 95%; (vii) 91%.

Figure 1: Representative traces for the changes in fluorescence intensity, after addition of 4-MU- α Neu5Ac (70 μM) following incubation of *MvS* with **6** (5 μM) for five different time intervals; T = 10 ms (red line); T = 50 ms (blue line) T = 100 ms (brown line); T = 250 ms (purple line); and T = 500 ms (black line). Reaction conditions, pH = 7.00 (100 mM MOPS buffer, 100 mM KCl) and T = 25 $^{\circ}C$.

Figure 2: Time dependent inactivation of *Micromonospora viridifaciens* sialidase by **6** (5 μM). Reaction conditions, pH = 7.00 (100 mM MOPS buffer, 100 mM KCl) and T = 25 $^{\circ}C$. The solid line is the best non-linear fit to a standard exponential rate equation.

Figure 3: Pseudo-first order rate constants for loss of enzymatic activity as a function of the of inactivator concentration: panel A *MvS* inactivation by **6**; panel B *MvS* inactivation by **7**; and panel C *AfK* inactivation by **7**. The solid lines are the best non-linear fits to the appropriate equations (see text for full details).

Figure 4: Time dependent reactivation of *Micromonospora viridifaciens* sialidase at pH = 7.00 (100 mM MOPS buffer, 100 mM KCl) and T = 25 $^{\circ}C$ (every 10th data point is shown). The solid line is the best non-linear fit to a standard exponential rate equation.

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Structures 1–9 and Structures 10–13

Scheme 1

Scheme 2

Figure 1

Figure 2

Figure 3

Figure 4