Natural sialoside analogues for the determination of enzymatic rate constants

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Received 25th September 2006, Accepted 10th October 2006
First published as an Advance Article on the web 30th October 2006
DOI: 10.1039/b613909d

Two isomeric 4-methylumbelliferyl-α-D-*N*-acetylneuraminylgalactopyranosides (**1** and **2**) were synthesised. These compounds contain either the natural α-2,3 or α-2,6 sialyl–galactosyl linkages, as well as an attached 4-methylumbelliferone for convenient detection of their hydrolyses. These compounds were designed as natural sialoside analogues to be used in a continuous assay of sialidase activity, where the sialidase-catalysed reaction is coupled with an *exo*-β-galactosidase-catalysed hydrolysis of the released galactoside to give free 4-methylumbelliferone. The kinetic parameters for **1** and **2** were measured using the wild-type and nucleophilic mutant Y370G recombinant sialidase from *Micromonospora viridifaciens*. Kinetic parameters for these analogues measured using the new continuous assay were in good agreement with the parameters for the natural substrate, 3′-sialyl lactose. Given the selection of commercially available *exo*-β-galactosidases that possess a variety of pH optima, this new method was used to characterise the full pH profile of the wild-type sialidase with the natural sialoside analogue **1**. Thus, use of these new substrates **1** and **2** in a continuous assay mode, which can be detected by UV/Vis or fluorescence spectroscopy, makes characterisation of sialidase activity with natural sialoside linkages much more facile.

Introduction

A critical task involved for complete characterisation of an enzyme entails the careful monitoring of its kinetic behaviour in the presence of substrates and/or inhibitors. Such studies of biological activity should ideally involve the use of natural substrates, if experimentally practical. Nonetheless, many enzymatic processes are monitored using non-natural substrates that upon reaction produce changes in either the UV-visible absorption spectrum or the fluorescence emission intensity. Spectroscopic techniques such as these allow researchers to monitor reaction rates using low enzyme concentrations and small quantities of substrates, which often must be synthesised. An unwelcome consequence of using chromophoric substrates is that these materials often possess a greater intrinsic reactivity than do the corresponding natural substrates. For instance, with regard to chemical models of proteinases and esterases the use of activated esters, such as *p*-nitrophenyl acetate, generally results in the observation of large rate accelerations. This occurs because nucleophilic attack is rate-limiting, while for unactivated esters and amides breakdown of the tetrahedral intermediate is at least partially rate-determining. This effect has been labelled as "the *p*-nitrophenyl ester syndrome".[1]

In addition with respect to the corresponding enzymatic systems, it has been reported that *p*-nitrophenyl acetate initially acylates α-lytic protease on its active site histidine residue, and that this acylated imidazole species undergoes a subsequent N–O acyl transfer to give the expected acyl-enzyme intermediate.[2]

In the case of sialidases, two separate papers appeared in 2003 in which it was proposed that the *exo*-sialidase family of glycosidase and *trans*-glycosidases react *via* a double displacement mechanism and that a tyrosine residue acts as a nucleophile during the enzymatic reaction to give a transient sialosyl-enzyme intermediate.[3,4] Before this time, doubts concerning the identity of the nucleophile in sialidase-catalysed reactions resulted, in part, from contradictory kinetic data on the activity of tyrosine-mutant containing enzymes against activated substrates. For example, Chien *et al.* reported for a sialidase from *C. pefringens* that: "The mutation of $Tyr^{347} \rightarrow Ile$ did not change the activity of NanH significantly",[5] whereas Ghate and Air reported that the $Tyr^{409} \rightarrow Phe$ mutant of the influenza B sialidase displayed no observable activity.[6] Also, Wang *et al.* reported that mutation of the conserved tyrosine, to a cysteine, in the human membrane-associated sialidase gave about a 10-fold reduction in activity against both activated and natural substrates.[7] Such apparent paradoxical data likely results from the enzymatic rate-determining steps for k_{cat} and/or k_{cat}/K_m not involving cleavage of the glycosidic bond. That is, even if mutation of an active site residue dramatically reduces the rate of glycosidic bond cleavage, this effect can be masked if either a conformational change or deglycosylation is the rate-determining step for the wild-type enzyme; a conclusion that is supported by several kinetic studies on sialidase mutant enzymes.[8,9,10,11] Therefore, it is critical to measure the rate of enzyme-catalysed cleavage of both activated and natural substrates using both wild-type and mutant enzymes before drawing any mechanistic conclusions about the catalytic role of the residue in question.

The current assay used to monitor the hydrolysis of natural sialoside substrates, such as sialyl lactose, involves the discontinuous monitoring of the reaction mixture for the presence of sialic acid using *N*-acetylneuraminate lyase (EC 4.1.3.3) and coupling the pyruvate formed to either NADH oxidation using lactate dehydrogenase (LDH)[12] or to the formation of H_2O_2 using pyruvate oxidase.[13] An alternative method is based on an analysis of the released lactose.[14]

The current report details a simplified procedure for monitoring the enzymatic hydrolysis of natural sialoside linkages by coupling

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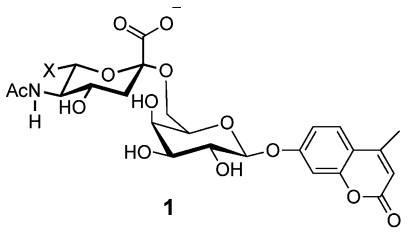

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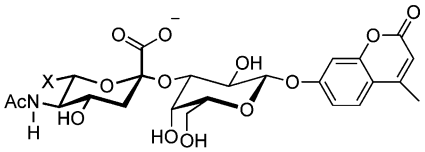

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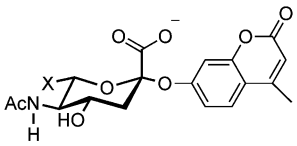

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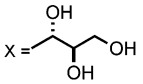
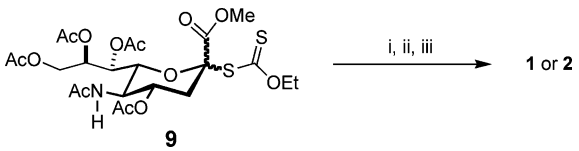


the first hydrolytic event, cleavage of a sialosyl–galactoside bond, to a second hydrolytic enzyme—an *exo*-β-galactosidase—to give a large change in either fluorescence intensity or UV-visible absorbance. Thus, two isomeric 4-methylumbelliferyl-α-D-*N*-acetylneuraminylgalactopyranosides (**1** and **2**) were synthesised and the biological activity of these compounds were compared to that of a natural substrate, sialyl lactose, as well as the standard activated substrate 4-methylumbelliferyl-α-D-*N*-acetylneuraminide (**3**) using wild-type and the Y370G mutant *Micromonospora viridifaciens* sialidases.

Results and discussion

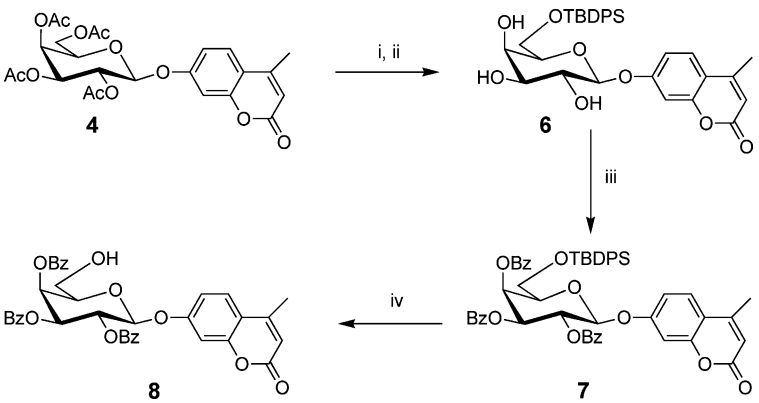
The syntheses of the two galactosyl acceptors **6** and **8** are shown in Scheme 1. Whilst formulating the synthetic route to **1** and **2**, several different sialosyl donor and promoter pairs were tried, and these included: (i) sialosyl chloride with one of silver carbonate,[15] silver salicylate,[16] or silver zeolite;[17] (ii) sialosyl xanthate and NIS with TMSOTf as a catalyst;[18] and (iii) sialyl hemiketal with Ph_2SO and Tf_2O.[19] In the current system, the best reagent combination found was the sialosyl xanthate **9** using TMSOTf and NIS as the activators. In all other cases studied, the undesired 2,3-didehydrosialic acid was isolated as the major product. Thus, coupling of **9** with **8** and **6** proceeded in acceptable yields to give after column chromatography the fully protected sialyl lactose analogues **10** and **11**, respectively. Subsequently, two step deprotection gave the required analogues **1** and **2** in yields of 76 and 47%, respectively (Scheme 2). Of note, it had been previously shown that for a 6-monoprotected β-galactopyranoside that the 2,3-isomer predominates.[20]



Scheme 2 *Reagents and conditions*: (i) **6** or **8**, NIS, cat TMSOTf, −40 °C (ii) MeOH, cat. NaOMe, rt, 0.5 h; (iii) LiOH, H_2O–THF (1 : 1 v/v), 0 °C, 0.5 h.

For the current kinetic protocols to be an effective tool for monitoring the rate of sialidase-catalysed reactions, the second enzymatic process must not be, even partially, rate-limiting (Scheme 3). In order to demonstrate that this condition was fulfilled, the kinetic data acquired at the highest and lowest concentrations of **1** (or **2**) were repeated using twice the quantity of β-galactosidase. In all cases, the observed rate for formation of 4-methylumbeliferone was unchanged.

In order for **1** and **2** to be considered good analogues of their respective natural substrates, it is important to verify that when the sialidase-catalysed reactions of **3** proceed at much greater rates than do the reactions of sialyl lactose, then the observed rates



Scheme 1 *Reagents, conditions and yields*: (i) MeOH, cat NaOMe, r.t., 95%; (ii) TBDPSCl, imidazole, DMF, 0 °C, 87%; (iii) BzCl, DCM, pyridine, cat. DMAP, 84%; (iv) 3% HCl in MeOH, 48 h, r.t. 82%.

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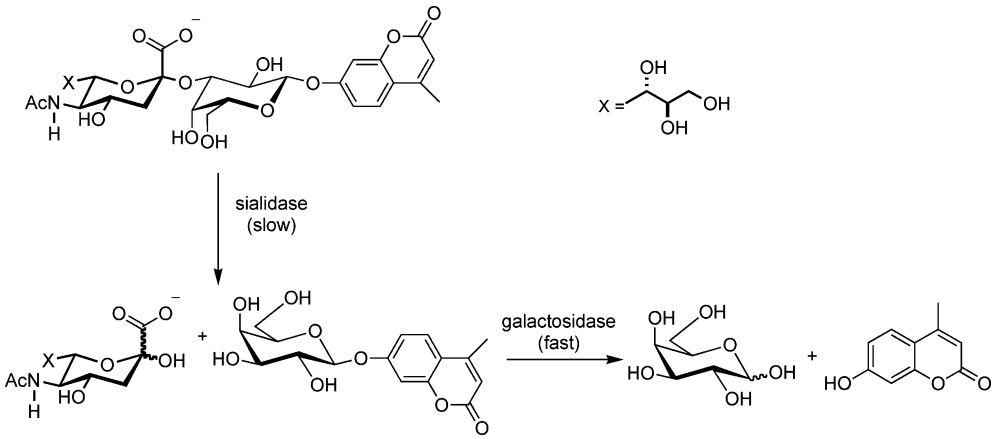

Scheme 3

Table 1 Michaelis–Menten kinetic parameters for the Y370G mutant *M. viridifaciens* sialidase-catalysed hydrolysis of **1**, **2**, **3** and 3′-sialyl lactose at pH 5.25 and 37 °C

Substrate	k_{cat}/s^{-1}	$(k_{cat}/K_m)/M^{-1}\ s^{-1}$	K_m/mM
1	0.069 ± 0.003	290 ± 50	0.24 ± 0.03
2	0.20 ± 0.02	530 ± 150	0.38 ± 0.07
3	44 ± 0.1	(1.3 ± 0.2) × 10^6	0.035 ± 0.006
3′-sialyl lactose	0.012[a]	12.6[a]	0.95 ± 0.27

[a] Data taken from ref. 10.

for **1** and **2** are similar to those for sialyl lactose. Table 1 lists the kinetic parameters for the Y370G mutant *M. viridifaciens* sialidase-catalysed hydrolysis of **3**, 3′-sialyl lactose and the two new substrates (**1** and **2**).

The data in Table 1 reveals that both **1** and **2** display similar reactivity to that of 3′-sialyl lactose with the Y370G *M. viridifaciens* mutant enzyme. More importantly, for the enzyme-catalysed hydrolyses of **1** and **2**, full Michaelis–Menten curves were obtained. In contrast, because of the complexity of using a discontinuous assay the reported rate constant values for the hydrolysis of 3′-sialyl lactose were calculated based on the measured rate at a single high concentration of substrate and a binding affinity that was measured in a competitive assay.[10]

An additional advantage of the current methodology over the previous discontinuous assays is the commercially availability of *exo*-β-galactosidases that possess very different pH optima. Thus, it is relatively easy to probe the catalytic activity of sialidases against these natural substrate analogues at pH values between 3.8 and 8.9 (Table 2). Shown in Fig. 1 and 2 are the pH-rate profiles for the *M. viridifaciens* sialidase-catalysed hydrolysis of **1** and **3**.[4]

As can be seen from the data shown in Fig. 1 and 2 the pH-rate profiles for the sialidase-catalysed hydrolysis of **1** and **3** are remarkably similar. This observation is consistent with the rate-limiting steps for both the activated and natural substrate analogue being the same at each pH value. In the case of the *M. viridifaciens* sialidase the rate-determining steps, at a pH of 5.25, have been proposed for the two kinetic terms k_{cat}/K_m and k_{cat} to be a conformational change of the initial Michaelis complex and deglycosylation, respectively.[11] Furthermore, this impediment to the measurement of full Michaelis–Menten curves using natural substrates is undoubtedly the reason that previous pH-activity profiles have been made using a single substrate concentration.[21,22,23]

Table 2 Michaelis–Menten kinetic parameters for the *M. viridifaciens* sialidase-catalysed hydrolysis of **1** at 37 °C[a]

pH	k_{cat}/s^{-1}	$10^6 \times (k_{cat}/K_m)/M^{-1}\ s^{-1}$	K_m/μM
3.83	60.1 ± 5.7	3.2 ± 1.2	19.0 ± 5.5
4.25	90.4 ± 2.4	6.11 ± 0.80	14.8 ± 1.5
4.78	83.5 ± 2.0	5.76 ± 0.53	14.5 ± 1.0
5.25	46.7 ± 3.4	1.60 ± 0.44	29.2 ± 5.8
6.14	22.7 ± 0.9	1.73 ± 0.31	13.1 ± 1.8
7.15	7.52 ± 0.36	1.51 ± 0.29	4.97 ± 0.73
8.03	1.75 ± 0.02	1.93 ± 0.09	0.91 ± 0.03
8.92	0.553 ± 0.005	0.61 ± 0.02	0.91 ± 0.02

[a] The enzyme stock used in these experiments is the same one as that used for the hydrolysis of **3**.[10]

Conclusions

In summary, two new sialidase substrates have been synthesised that contain the natural sialyl–galactose linkage, yet have the advantage of being detected in a continuous assay mode when coupled with an *exo*-β-galactosidase to release the chromophore 4-methylumbelliferone. Monitoring of sialidase activity by this method allows for the complete characterisation of natural sialoside activity, using a continuous assay over a broad pH range and is amenable to a variety of UV or fluorescence detection methods.

Experimental

All pH values were measured using a Radiometer pHM82 standard pH meter and a standard combination glass electrode standardised with Fisher certified buffers (pH = 4.0, 7.0 and 10.0). NMR spectra were acquired on Varian Unity-500 spectrometer. Chemical shifts (δ_H and δ_C) are in ppm downfield from signals for TMS. The residual signal from deuterated chloroform and external TMS salt (D_2O) were used as ^1H NMR references; for ^{13}C NMR spectra, natural abundance signals from $CDCl_3$ and external TMS salt (D_2O) were used as references. Coupling constants (J) are given in Hz. Melting points were determined on a Gallenkamp melting point apparatus and are not corrected. Optical rotations were measured on a Perkin-Elmer 341 polarimeter.

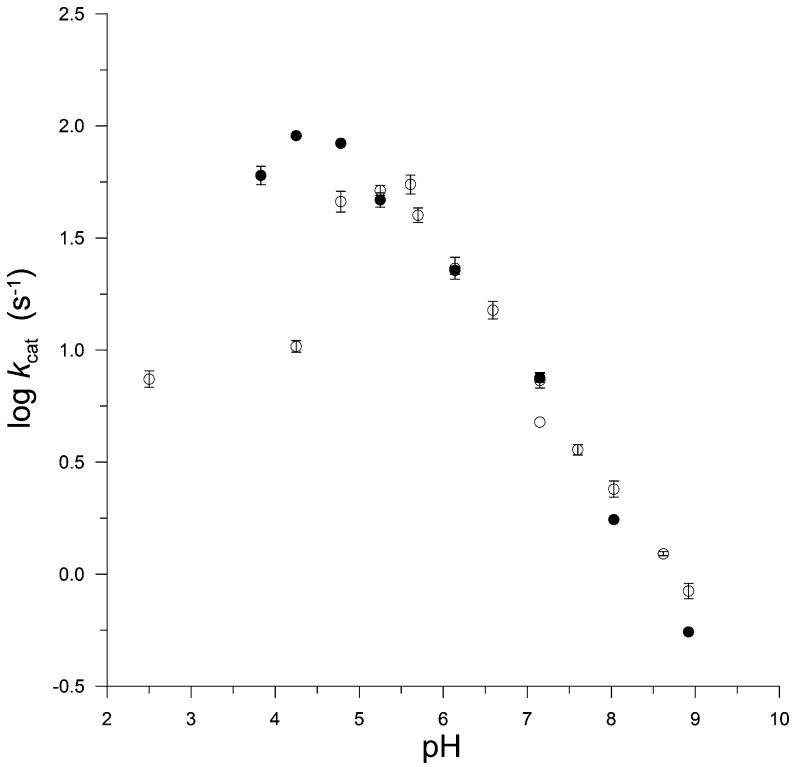

Fig. 1 Effect of pH on k_{cat} values for the wild-type *Micromonospora viridifaciens* sialidase-catalysed hydrolysis of **1** (●) and **3** (○). All kinetic parameters were determined at 37 °C. Data for the wild-type enzyme adapted with permission from reference 4. Copyright 2003 American Chemical Society.

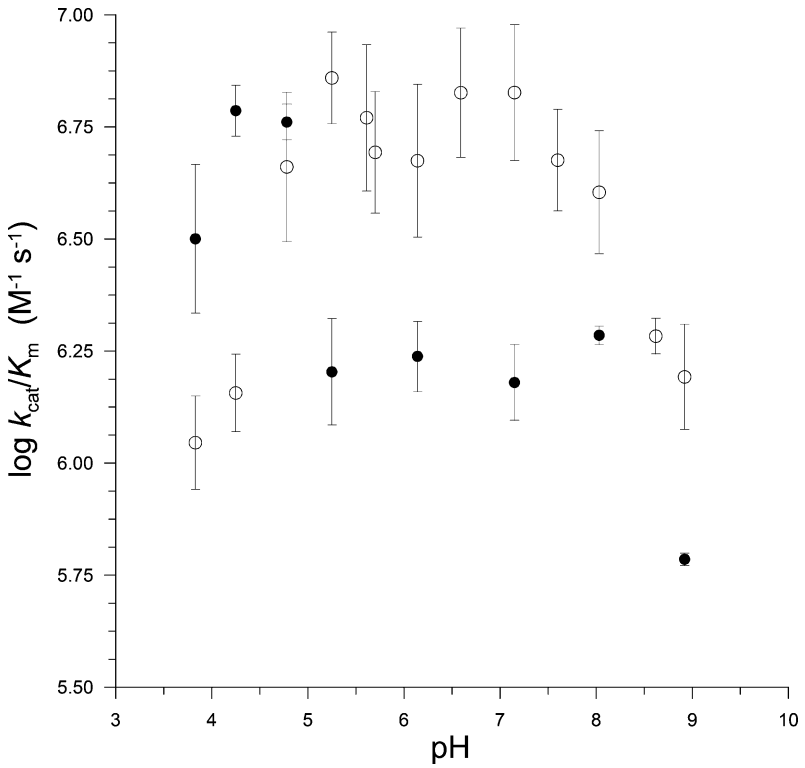

Fig. 2 Effect of pH on k_{cat}/K_m values for the wild-type *Micromonospora viridifaciens* sialidase-catalysed hydrolysis of **1** (●) and **3** (○). All kinetic parameters were determined at 37 °C. Data for the wild-type enzyme adapted with permission from reference 4. Copyright 2003 American Chemical Society.

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4-Methylumbelliferyl-β-D-galactopyranoside (5)[24]

A solution of 4-methylumbelliferyl-2,3,4,6-tetra-*O*-acetyl-β-D-galacto-pyranoside (**4**,[25] 20.0 g, 0.039 mol) in methanol (250 cm^3) that contained a catalytic amount of sodium methoxide was allowed to stir at rt for 0.5 h. Part of the product precipitated from the solution and it was filtered and washed thoroughly with methanol. The filtrate was neutralised by the addition of Dowex 50 (H^+) ion-exchange resin, filtered and concentrated to obtain a second crop of crystalline **5**. Total yield (12.7 g, 95%). The ^1H NMR spectrum is identical to that reported in the literature.[24]

4-Methylumbelliferyl-6-*O*-(*tert*-butyldiphenylsilyl)-β-D-galactopyranoside (6)[24]

tert-Butyldiphenylsilyl chloride (2.4 cm^3, 8.9 mmol) was added, over a period of 5 min, to a cooled solution of 4-methylumbelliferyl-β-D-galactopyranoside (**5**, 3 g, 8.9 mmol) and imidazole (1.2 g, 17.7 mmol) in dry DMF (30 cm^3) at 0 °C. The resultant solution was stirred overnight at rt. Subsequently, the solution was diluted with diethyl ether (500 cm^3), washed with water (500 cm^3) and brine (500 cm^3) and then dried over anhydrous Na_2SO_4. A pale yellow solid was obtained after evaporation of the solvent and this was purified by flash column chromatography using ethyl acetate as the eluent to afford **6** as a white solid (4.5 g, 87%): mp 168–169 °C. $[\alpha]_D^{20}$ −70.6 (*c* 0.3, CH_3CN), ^1H NMR (500 MHz, $CDCl_3$) δ: 1.07 (s, 9 H, *t*-Bu), 2.40 (s, 3 H, CH_3), 3.69–3.72 (m, 2 H, H-3, H-6a), 3.97–4.00 (m, 3 H, H-2, H-5, H-6b), 4.17 (d, 1 H, $J_{4,3} = 3.0$, H-4), 4.94 (d, 1 H, $J_{1,2} = 7.5$, H-1), 6.18 (d, 1 H, $J_{3',Me} = 1.0$, H-3′), 6.94 (d, 1 H, $J_{8',6'} = 2.5$, H-8′), 7.00 (dd, 1 H, $J_{6',5'} = 9.0$, $J_{6',8'} = 2.5$, H-6′), 7.28–7.67 (m, 11 H, H-5′ and Ar–H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 18.9, 19.4, 27.0, 63.5, 69.2, 71.5, 73.8, 75.1, 100.8, 104.5, 112.9, 113.8, 115.2, 125.8, 128.0 (2 × C), 130.1, 132.8, 133.0, 135.8 (2 × C), 152.7, 154.8, 159.9, 161.2. Anal. calcd for $C_{32}H_{36}O_8Si$: C, 66.6, H, 6.3; found: C, 66.3, H, 6.45.

4-Methylumbelliferyl-2,3,4-tri-*O*-benzoyl-6-*O*-(*tert*-butyldiphenylsilyl)-β-D-galactopyranoside (7)

A solution of 4-methylumbelliferyl 6-*O*-(*tert*-butyldiphenylsilyl)-β-D-galactopyranoside (**6**, 1.0 g, 1.7 mmol) in pyridine (15 cm^3) and dichloromethane (10 cm^3) was cooled to 0 °C. Then benzoyl chloride (1.0 cm^3, 8.7 mmol) and a catalytic amount of *N*,*N*-dimethylaminopyridine were added and the mixture was stirred overnight. The resulting solution was diluted with dichloromethane (50 cm^3), washed with 1 M HCl (50 cm^3), saturated $NaHCO_3$ (100 cm^3), water (100 cm^3) and brine (100 cm^3) and dried over anhydrous Na_2SO_4. The crude product obtained after evaporation of the solvent was purified by flash column chromatography using ethyl acetate–hexane (1 : 2) as the eluent to afford **7** as a white solid (1.3 g, 84%): mp 246–247 °C. $[\alpha]_D^{20}$ +111.4 (*c* 0.5, $CHCl_3$), ^1H NMR (500 MHz, $CDCl_3$) δ: 1.02 (s, 9 H, *t*-Bu), 2.36 (s, 3 H, CH_3), 3.86 (dd, 1 H, $J_{6a,6b} = 10.5$, $J_{6a,5} = 7.0$, H-6a), 3.90 (dd, 1 H, $J_{6b,6a} = 10.5$, $J_{6b,5} = 7.0$, H-6b), 4.24 (t, 1 H, $J_{5,6b} + J_{5,6a} = 14.0$, H-5), 5.41 (d, 1 H, $J_{1,2} = 8.0$, H-1), 5.70 (dd, 1 H, $J_{3,2} = 10.5$, $J_{3,4} = 3.5$, H-3), 6.00 (dd, 1 H, $J_{2,1} = 8.0$, $J_{2,3} = 10.5$, H-2), 6.08 (d, 1 H, $J_{4,3} = 3.5$, H-4), 6.17 (d, 1 H, $J_{3',Me} = 1.0$, H-3′), 6.95–8.03 (m, 28 H, Ar–H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 18.7, 19.0, 26.7, 61.5 (C-6), 67.6 (C-4), 69.5 (C-2), 71.7 (C-3), 74.7(C-5), 99.5 (C-1), 104.7 (C-3′), 113.2, 113.8, 115.5, 125.7, 127.7, 127.8, 128.3, 128.5, 128.6, 128.8, 129.0, 129.3, 129.7, 129.9, 130.0, 132.4, 132.7, 133.3, 133.4, 135.5, 135.6, 152.0, 154.7, 159.4, 160.8, 165.2, 165.3, 165.5. Anal. calcd for $C_{53}H_{48}O_{11}Si$: C, 71.6, H, 5.4; Found: C, 71.4, H, 5.6.

4-Methylumbelliferyl-2,3,4-tri-*O*-benzoyl-β-D-galactopyranoside (8)

4-Methylumbelliferyl-2,3,4-tri-*O*-benzoyl-6-*O*-(*tert*-butyldiphenylsilyl)-β-D-galactopyranoside (**7**, 1.0 g, 1.1 mmol) was added into a round-bottomed flask containing methanol (35 cm^3) and diethyl ether (35 cm^3). To this mixture acetyl chloride (1.5 cm^3) was added dropwise over a period of 5 min and the solution was then stirred for 2 d at rt. After removal of the solvent under vacuum the resultant residue was purified by flash column chromatography using ethyl acetate–hexane (5 : 3) as the eluent to afford **8** as a white solid (0.6 g, 82%): mp 264–265 °C. $[\alpha]_D^{20}$ +169.4 (*c* 0.5, $CHCl_3$), ^1H NMR (500 MHz, $CDCl_3$) δ: 2.39 (s, 3 H, CH_3), 3.72 (dd, 1 H, $J_{6a,6b} = 12.0$, $J_{6a,5} = 7.0$, H-6a), 3.89 (dd, 1 H, $J_{6b,6a} = 12.0$, $J_{6b,5} = 7.0$, H-6b), 4.23 (t, 1 H, $J_{5,6b} + J_{5,6a} = 14.0$, H-5), 5.48 (d, 1 H, $J_{1,2} = 8.0$, H-1), 5.70 (dd, 1 H, $J_{3,2} = 10.5$, $J_{3,4} = 3.5$, H-3), 5.92 (d, 1 H, $J_{4,3} = 3.5$, H-4), 6.14 (dd, 1 H, $J_{2,1} = 8.0$, $J_{2,3} = 10.5$, H-2), 6.19 (s, 1 H, H-3′), 6.96 (dd, 1 H, $J_{6',5'} = 8.5$, $J_{6',8'} = 2.5$, H-6′), 7.02 (d, 1 H, $J_{8',6'} = 2.5$, H-8′), 7.26–8.15 (m, 16 H, H-5′ and Ar–H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 18.6, 60.5, 68.6, 69.5, 71.6, 74.7, 99.5, 104.7, 113.3, 113.7, 115.6, 125.8, 128.4, 128.5, 128.7, 129.7, 130.1, 133.5, 134.0, 152.1, 154.8, 159.3, 160.8, 165.3, 165.5, 166.7. Anal. calcd for $C_{37}H_{30}O_{11}$: C, 68.3, H, 4.65; found: C, 68.3, H, 4.7.

4-Methylumbelliferyl[methyl(5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galactonon-2-ulopyranosyl)onate]-(2→6)-2,3,4-tri-*O*-benzoyl-β-D-galactopyranoside 10

A mixture of donor **9**[26] (500 mg, 0.84 mmol), acceptor **8** (819 mg, 1.26 mmol) and powdered 3 Å molecular sieves (2 g) in dry CH_2Cl_2 (15 cm^3) was stirred at rt in an atmosphere of N_2 for 15 h. The mixture was cooled to −40 °C and a solution of NIS (378 mg, 1.68 mmol) and TMSOTf (0.03 cm^3, 0.17 mmol) in dry CH_3CN (5 cm^3) was added. The mixture was allowed gradually to warm to room temperature over a period of 3 h. The resulting mixture was diluted with CH_2Cl_2 (10 cm^3) and filtered through a pad of celite. The celite pad was thoroughly washed with CH_2Cl_2 (30 cm^3). The combined filtrates were washed successively with 10% (w/v) aqueous $Na_2S_2O_3$ (50 cm^3), sat. $NaHCO_3$ (50 cm^3), water (50 cm^3), brine (50 cm^3), dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by flash column chromatography using $CHCl_3$–CH_3OH (25 : 1) to afford an α–β mixture and the elimination product. This mixture was separated on a chromatotron using $CHCl_3$–CH_3OH (100 : 1 v/v) as the eluent. Yield: **10** (α-isomer) 318 mg, 34%. β-Isomer: 106 mg, 11%. Data for **10**: mp 142–145 °C. $[\alpha]_D^{20}$ +70.2 (*c* 0.2, $CHCl_3$), ^1H NMR (500 MHz, $CDCl_3$) δ: 1.83, 1.84, 2.23, 2.24, (4 × s, 12 H, CH_3), 1.88–1.94 (m, 4 H, H-3″a, CH_3), 2.31 (s, 3 H, CH_3), 2.42 (dd, 1 H, $J_{3''e,3''a} = 13.0$, $J_{3''e,4''} = 4.5$, H-3″e), 3.61 (s, 1 H, CH_3), 3.65–3.71 (m, 2 H, H-6a, H-6b), 3.98 (dd, 1 H, $J_{9''a,9''b} = 12.5$, $J_{9''a,8''} = 7.5$, H-9″a), 4.04 (q, 1 H, $J_{5,\text{“}4\text{”}} + J_{5,\text{“}6\text{”}} + J_{5,\text{“}NH} = 30.0$, H-5”), 4.14 (dd, 1 H, $J_{6,\text{“}5\text{”}} = 10.0$, $J_{6,\text{“}7\text{”}} = 2.0$, H-6″), 4.42 (dd, 1 H, $J_{9''b,9''a} = 12.5$, $J_{9''b,8''} = 3.0$, H-9″b), 4.63 (t, 1 H, $J_{5,6a} + J_{5,6b} = 16.0$, H-5), 4.76 (dt, 1 H,

$J_{4,“3”a} + J_{4,“5”} = 20.0$, $J_{4,“3”e} = 4.5$, H-4″), 5.03 (d, 1 H, $J_{NH,5''} = 10.0$, N–H), 5.21 (dd, 1 H, $J_{7,“8”} = 10.0$, $J_{7,“6”} = 2.0$, H-7″), 5.55 (ddd, 1 H, $J_{8,“7”} = 10.0$, $J_{8,“9”a} = 7.5$, $J_{8,“9”b} = 3.0$, H-8″), 5.64 (d, 1 H, $J_{1,2} = 8.0$, H-1), 5.82 (dd, 1 H, $J_{3,2} = 10.5$, $J_{3,4} = 3.5$, H-3), 5.95 (dd, 1 H, $J_{2,3} = 10.5$, $J_{2,1} = 8.5$, H-2), 6.06 (d, 1 H, $J_{4,3} = 3.5$, H-4), 6.09 (s, 1 H, H-3′), 6.94 (dd, 1 H, $J_{6',5'} = 8.5$, $J_{6',8'} = 2.5$, H-6′), 7.18–8.03 (m, 17 H, H-5′, H-8′, Ar–H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 18.9 (CH_3), 21.0, 21.1 (2 × C), 21.3, 23.4, 29.9, 38.1 (C-3″), 49.5 (C-5″), 53.2 (–OCH_3), 63.5 (C-6), 63.8 (C-9″), 67.5 (C-7″), 67.8 (C-8″, C-4), 68.8 (C-4′), 69.8 (C-2), 71.9 (C-3), 72.6 (C-5), 73.1 (C-6″), 98.9 (C-1), 100.1 (C-2″), 104.4, 113.1, 114.4, 115.4, 125.7, 128.5, 128.6, 128.8, 129.3, 129.5, 129.8, 130.0 (2 × C), 130.2, 133.3, 133.5 (2 × C), 152.4, 155.3, 159.9, 161.2, 165.4, 165.5 (2 × C), 168.1, 170.3, 170.8, 171.2 (2 × C), 171.3. Anal. calcd for $C_{57}H_{57}NO_{23}$: C, 60.9, H, 5.1, N, 1.25; found: C, 60.7, H, 5.3, N, 1.5.

4-Methylumbelliferyl(5-acetamido-3,5-dideoxy-D-glycero-α-D-galactonon-2-ulopyranosylonic acid]-(2→6)-β-D-galactopyranoside (1)

To a solution of **10** (280 mg, 0.25 mmol) in dry methanol (10 cm^3) was added a methanolic sodium methoxide solution (25 cm^3) and stirred for 30 min at rt. Then the solution was neutralised by adding Amberlite IR-120 (H^+ form). The resulting solution was filtered and the resin was washed several times with methanol. The solutions were combined and evaporated to give a solid residue that was subsequently dissolved in an aqueous solution of LiOH (1 M, 15 cm^3) and THF (15 cm^3) that was maintained at 0 °C. The resultant solution was stirred for 30 min at rt. The solution was then neutralised by adding Amberlite IR-120 (H^+ form) and filtered. The resin was washed several times with methanol. The solutions were combined and evaporated to give a solid residue that was purified by flash column chromatography using ethyl acetate: methanol–water (10 : 2 : 1). The fractions that contained the product were combined and concentrated. The concentrated aqueous solution was then lyophilised to obtain a solid. (120 mg, 76%): mp 205 °C (decomp). $[\alpha]_D^{20}$ −80.8 (*c* 0.2, H_2O), ^{1}H NMR (500 MHz, D_2O) δ: 1.68 (t, 1 H, $J_{3''a,3''e} = J_{3''a,4''} = 12.0$, H-3″a), 1.98 (s, 1 H, CH_3), 2.39 (d, 3 H, $J_{Me,3'} = 1.0$, CH_3), 2.78 (dd, 1 H, $J_{3''e,3''a} = 12.0$, $J_{3''e,4''} = 4.0$, H-3″e), 3.49 (dd, 1 H, H-8″), 3.57 (dd, 1 H, $J_{9''a,9''b} = 12.5$, $J_{9''a,8''} = 6.5$, H-9″a), 3.63–3.72 (m, 4 H, H-4, “H-6”, H-5, H-6a), 3.78–3.85 (m, 4 H, H-9″b, H-7, “H-2, H-3), 3.95–4.02 (m, 3 H, H-6b, H-4, H-5”), 5.10 (d, 1 H, $J_{1,2} = 7.0$, H-1), 6.17 (d, 1 H, $J_{3',Me} = 1.0$, H-3′), 7.06 (d, 1 H, $J_{8',6'} = 2.5$, H-8′), 7.08 (dd, 1 H, $J_{6',5'} = 9.0$, $J_{6',8'} = 2.5$, H-6′), 7.66 (d, 1 H, $J_{5',6'} = 9.0$, H-5′). ^{13}C NMR (125 MHz, D_2O) δ: 18.2 (CH_3), 22.1 (CH_3), 40.5 (C-3“), 52.0 (C-4”), 62.7 (C-9″), 63.1 (C-6), 68.3 (C-8,“C-5), 68.5 (C-5”), 70.5 (C-3), 71.7 (C-7″), 72.5 (C-2), 72.7 (C-6″), 74.1 (C-4), 100.4 (C-1, C-2″), 103.6 (C-8′), 111.1 (C-3′), 114.1 (C-6′), 114.9 (C-4′), 126.6 (C-5′), 153.6 (C-9′), 156.0 (C-10′), 159.7 (C-7′), 164.2 (C-2′), 173.4 (C-1″), 175.1 (C=O, amide). HRMS (FAB) *m/z* (M–H$^+$), $C_{27}H_{34}NO_{16}$ requires 628.1883, found 628.1891.

4-Methylumbelliferyl[methyl(5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galactonon-2-ulopyranosyl)onate]-(2→3)-6-*O*-(*tert*-butyldiphenylsilyl)-β-D-galactopyranoside (11)

Donor **9**[26] (500 mg, 0.84 mmol) and the acceptor **6** (726 mg, 1.26 mmol) were coupled and purified as described in the synthesis of **10**. Yield: 308 mg, 35%: mp 129–130 °C. $[\alpha]_D^{20}$ −26.9 (*c* 0.5, $CHCl_3$), ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.05 (s, 9 H, $C(CH_3)_3$), 1.90, 2.00, 2.12, 2.16, (4 × s, 12 H, CH_3), 2.03–2.06 (m, 4 H, H-3″a, CH_3), 2.39 (s, 3 H, CH_3), 2.76 (bs, 1 H, OH), 2.82 (dd, 1 H, $J_{3''e,3''a} = 13.0$, $J_{3''e,4''} = 4.5$, H-3″e), 2.98 (bs, 1 H, OH), 3.76–3.81 (m, 4 H, H-6a, CH_3), 3.86–3.89 (m, 2 H, H-6b, H-4), 3.95 (dd, 1 H, $J_{5,6a} = 10.5$, $J_{5,6b} = 6.5$, H-5), 3.99–4.06 (m, 3 H, H-2, H-5″, H-9″a), 4.12 (dd, 1 H, $J_{6,“5”} = 11.0$, $J_{6,“7”} = 2.0$, H-6″), 4.25 (dd, 1 H, $J_{3,2} = 12.5$, $J_{3,4} = 2.5$, H-3), 4.29 (dd, 1 H, $J_{9''b,9''a} = 13.0$, $J_{9''b,8''} = 3.0$, H-9″b), 4.98 (ddd, 1 H, $J_{4,“3”a} = 12.0$, $J_{4,“5”} = 10.0$, $J_{4,“3”e} = 4.5$, H-4″), 5.22 (d, 1 H, $J_{NH,5''} = 10.0$, N–H), 5.24 (d, 1 H, $J_{1,2} = 8.0$, H-1), 5.34 (dd, 1 H, $J_{7,“8”} = 9.5$, $J_{7,“6”} = 2.0$, H-7″), 5.49 (m, 1 H, H-8″), 6.15 (d, 1 H, $J_{3',Me} = 1.0$, H-3′), 7.03 (d, 1 H, $J_{8',6'} = 2.5$, H-8′), 7.04 (dd, 1 H, $J_{6',5'} = 9.0$, $J_{6',8'} = 2.5$, H-6′), 7.25–7.65 (m, 11 H, H-5′, Ar–H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 18.9, 19.4, 21.0 (2 × C), 21.1, 21.5, 23.4, 26.9, 27.0, 29.9, 38.2 (C-3″), 49.7 (C-5″), 53.5 (C-6), 62.6 (C-9″), 63.0 (C-5), 67.1 (C-7″), 68.1 (C-8″, C-4), 68.7 (C-2), 68.9 (C-4″), 72.9 (C-6″), 74.2 (–OCH_3), 77.0 (C-3), 97.6 (C-2″), 100.2 (C-1), 104.3, 112.7, 114.2, 114.9, 125.6, 127.9, 128.0, 130.0, 130.1, 132.8, 133.0, 135.7, 135.8, 152.8, 155.0, 160.1, 161.5, 168.4 (C-1″), 170.2, 170.4, 170.6, 170.8, 171.1. Anal. calcd for $C_{52}H_{63}NO_{20}Si$: C, 59.5, H, 6.05, N, 1.3; found: C, 59.3, H, 5.9, N, 1.6.

4-Methylumbelliferyl(5-acetamido-3,5-dideoxy-D-glycero-α-D-galactonon-2-ulopyranosylonic acid]-(2→3)-β-D-galactopyranoside (2)

Compound **11** (250 mg, 0.24 mmol) was treated with sodium methoxide in methanol and then with LiOH (H_2O–THF) as described above for the synthesis of **1**. The crude product obtained was purified by flash column chromatography using ethyl acetate–methanol–water (10 : 2 : 1) as the eluent. The fractions that contained the product were combined and concentrated. The concentrated aqueous solution was then lyophilised to obtain a solid (70 mg, 47%): mp 215 °C (decomp.). $[\alpha]_D^{20}$ −14.5 (*c* 0.2, H_2O), ^{1}H NMR (500 MHz, D_2O) δ: 1.82 (t, 1 H, $J_{3'a,3''e} + J_{3''a,4''} = 25.0$, H-3″a), 2.01 (s, 1 H, CH_3), 2.41 (d, 3 H, $J_{Me,3'} = 1.0$, CH_3), 2.78 (dd, 1 H, $J_{3''e,3''a} = 12.5$, $J_{3''e,4''} = 5.0$, H-3″e), 3.57–3.62 (m, 2 H, H-8″, H-9″a), 3.64 (dd, 1 H, $J_{7'',8''} = 10.5$, $J_{7'',6''} = 2.0$, H-7″), 3.69 (ddd, 1 H, $J_{4'',3''a} = 12.5$, $J_{4'',5''} = 10.0$, $J_{4'',3''e} = 4.5$, H-4″), 3.77 (m, 2 H, H-6a, H-6b), 3.81–3.93 (m, 5 H, H-2, H-5, H-5″, H-6″, H-9″b), 4.04 (d, 1 H, $J_{4,3} = 3.0$, H-4), 4.24 (dd, 1 H, $J_{3,2} = 9.5$, $J_{3,4} = 3.0$, H-3), 5.24 (d, 1 H, $J_{1,2} = 8.0$, H-1), 6.21 (d, 1 H, $J_{3',Me} = 1.0$, H-3′), 7.07 (d, 1 H, $J_{8'',6'} = 2.5$, H-8′), 7.11 (dd, 1 H, $J_{6',5'} = 9.0$, $J_{6',8'} = 2.5$, H-6′), 7.71 (d, 1 H, $J_{5',6'} = 9.0$, H-5′). ^{13}C NMR (125 MHz, D_2O) δ: 18.0 (CH_3), 22.2 (CH_3), 39.8 (C-3″), 51.9 (C-5″), 60.8 (C-6), 62.6 (C-9″), 67.4 (C-4), 68.1 (C-8″), 68.5 (C-4″), 68.9 (C-2), 71.9 (C-6″), 73.0 (C-7″), 75.4 (C-5), 75.7 (C-3), 100.1 (C-1, C-2″), 103.3 (C-8′), 111.0 (C-3′), 114.1 (C-6′), 114.7 (C-4′), 126.5 (C-5′), 153.5 (C-10′), 155.9 (C-9′), 159.5 (C-7′), 164.1 (C-2′), 174.1 (C-1″),175.1 (C=O, amide). HRMS (FAB) *m/z* (M–H$^+$), $C_{27}H_{34}NO_{16}$ requires 628.1883, found 628.1872.

Kinetic protocols

Michaelis–Menten parameters were measured under identical conditions for both wild-type and the Y370G mutant. Each 0.4 cm^3 reaction was performed at 37 °C by equilibrating the buffer,

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Table 3 Wavelength and $\Delta\varepsilon$ values used to measure hydrolysis of **1** at various pH values and 37 °C[a]

pH	Wavelength/nm	$\Delta\varepsilon$/M^{-1} cm^{-1}
3.83	341	3284
4.25	342	3175
4.78	340	3336
5.25	357	549
6.14	340	3170
7.15	342	3962
8.03	Ex. 365/em. 450	6.05×10^{6}[a]
8.92	Ex. 365/em. 450	9.82×10^{6}[a]

[a] For pH 8.03 and 8.92, hydrolysis was detected by the change in fluorescence intensity, where the '$\Delta\varepsilon$' listed refers to the change in fluorescence observed using a 10 × 2 mm cell with a PMT voltage of 450 V.

substrate and *exo*-galactosidase in the cell block for 3 min prior to the addition of sialidase (0.05 cm^3). Kinetic parameters were determined from a minimum of seven initial rate measurements within a substrate concentration range of at least $K_m/5$ to $5K_m$.

To determine the effect of pH on catalysis, kinetic measurements were carried out over a pH range of 3.8–8.9. The buffers used were NaOAc–HOAc (pH range 3.8–5.7), 2-(*N*-morpholino)ethanesulfonic acid (MES-NaOH, pH range 5.6–7.2) and tris-(hydroxymethyl)aminomethane (Tris-HCl, pH range 7.1–8.9), ionic strength was maintained at 0.1 M (NaCl). The following β-galactosidases were used in the various assays: for the pH range 3.8–7.15 the *Aspergillus oryzae* enzyme (Sigma G-5160) and for the pH values 8.03 and 8.92 the *Escherichia coli* enzyme (Roche Diagnostics 10745731001). For the pH range 3.8–7.2, the progress of the reactions was continuously monitored for 10 min using a Cary 3E spectrophotometer equipped with a Peltier temperature controller. Extinction coefficient differences were calculated by measuring the change in absorbance of the substrate and that of the products released by enzymatic hydrolysis. For the pH values 8.03 and 8.92, the progress of the reactions was continuously monitored for 10 min using a Cary Eclipse fluorescence spectrophotometer equipped with a Peltier temperature controller. Wavelength and extinction difference values for hydrolysis of **1** at each pH are given in Table 3. Hydrolysis of **2** at pH 5.25 was monitored at 341 nm ($\Delta\varepsilon$ value of 2612 M^{-1} cm^{-1}). The rate *versus* substrate concentration data were fitted to the Michaelis–Menten equation using a standard nonlinear least-squares program (GraFit).

Acknowledgements

This work was supported by the Discovery Grant programme of the Natural Sciences and Engineering Research Council of Canada.

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