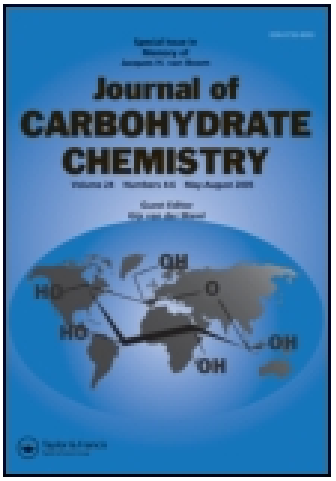

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Chromogenic Carbamate and Acetal Substrates for Glycosaminidases

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The enzyme Dispersin B is a medicinally relevant β-hexosaminidase with potential for use in the treatment of biofilm-related infections. Here we describe the synthesis and evaluation of a *p*-nitrophenyl glycosyl carbamate and a *p*-nitrophenyl glycosyl acetal as improved substrates of Dispersin B. The *p*-nitrophenyl glycosyl carbamate shows substantially improved analytical signal in continuous assays and superior kinetics (k_{cat}/K_m 6.1 mM^{-1} s^{-1}) when compared to the benchmark substrate 4-nitrophenyl *N*-acetyl-β-D-glucosaminide (k_{cat}/K_m 0.021 mM^{-1} s^{-1}) in measuring Dispersin B activity. The improvements observed are compared to the activity of jack bean β-hexosaminidase toward these substrates.

Keywords Glycosaminidase; Dispersin B; Substrate; Chromophore

INTRODUCTION

Bacterial biofilms have been estimated to be a factor in 65% of human infections.[1] One emerging treatment strategy for biofilm-related infections is to enzymatically degrade the extracellular matrix supporting the biofilm.[2] Dispersin B (DspB) is a candidate enzyme for treatment of a wide variety of biofilm-related infections as it specifically hydrolyzes the 1→6-linked β-*N*-acetylglucosamine exopolysaccharide found in many bacterial biofilms.[3–5] DspB is a family 20 glycoside hydrolase, which includes exo-hexosaminidases that use the 2-acetamido substituent as a neighboring participating group in glycoside hydrolysis.[6] The exo-activity of DspB and specificity of the enzyme has been confirmed by HPLC studies on synthetic oligosaccharides.[7,8]

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The substrate specificity of DspB leads to challenges in the development assays to monitor DspB activity. DspB has poor activity toward common aryl glycoside substrates, which makes assays with these substrates in dilute solutions or complex biological media challenging. For example, the most common hexosaminidase substrate is 4-nitrophenyl *N*-acetyl-β-D-glucosaminide (pNP-GlcNAc), which, upon enzymatic cleavage of the glycosidic bond, releases 4-nitrophenolate (pNP). Jack bean β-hexosaminidase, another family 20 exo-hexosaminidase, has a K_{cat}/K_m value of 13 mM^{-1} s^{-1} for pNP-GlcNAc, while the DspB enzyme has a value of only 0.021 mM^{-1} s^{-1}.

In an attempt to find an improved DspB substrate, we have synthesized a glycosyl acetal **1** and a glycosyl carbamate **2**, which may better mimic the 1→6 linkage in the native DspB substrate. Both substrates present less steric bulk around the glycosidic linkage than aryl glycosides but are still competent to release a chromophore upon glycosidic bond cleavage (Fig. 1).

Substrates containing *p*-nitroanilide are commonly used in peptide-based substrates for proteases; however, this is the first example of a *p*-nitroanilide-containing glycosidase substrate. Glycosylcarbamates have been synthesized as prodrugs and as enzymatically cleavable protecting groups, but kinetic analyses of these substrates have not been reported.[9–12] Although many alkyl glycosyl acetals have been synthesized, their activity as glycosidase substrates has not been reported.

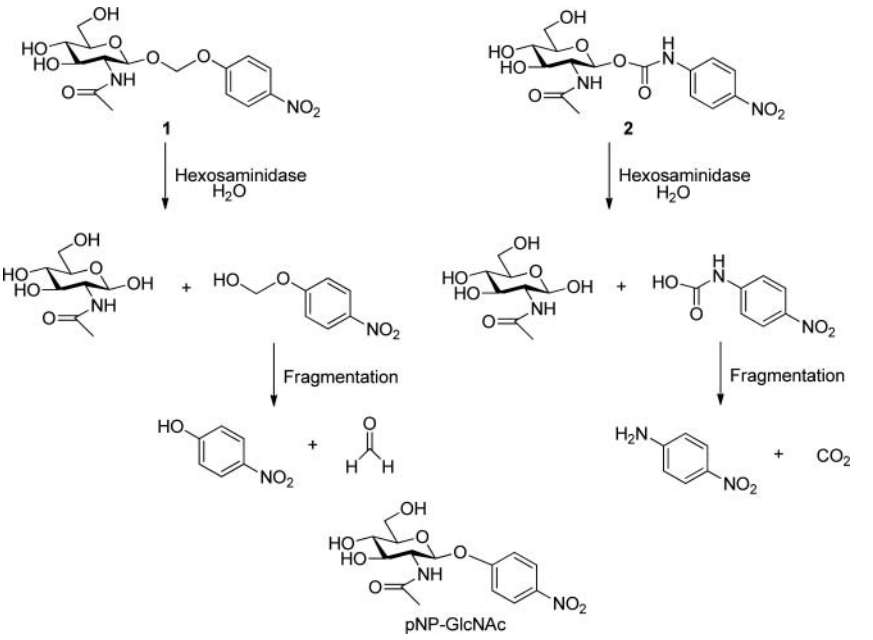
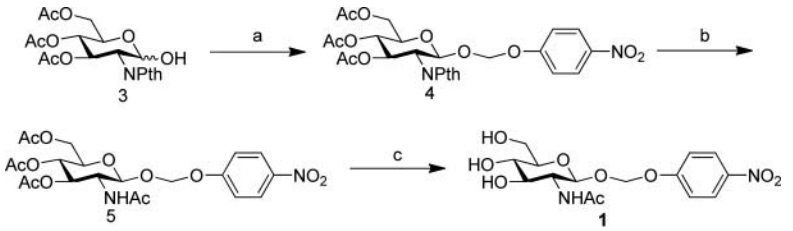


Figure 1: Carbamate and glycosyl acetal hexosaminidase substrates.

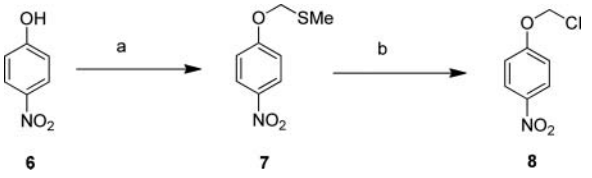
RESULTS/DISCUSSION

Synthesis of Substrates

Synthesis of the target compound **1** (Sch. 1) began with known 3,4,6-triacetyl-2-deoxy-2-phthalimido-D-glucose (**3**).[13] 1-Chloromethoxy-4-nitrobenzene (**8**) was readily synthesized in two steps from *p*-nitrophenol and chloromethylthioether (Sch. 2). Compound **3** was alkylated using (**8**) in acetonitrile with catalytic NaI and K_2CO_3 as a base.[14] This gave solely β-glycoside product in moderate yield (40%) (Sch. 1). The phthalimido group of **4** was then removed with a solution of ethylenediamine in butanol and the resulting aminol was acetylated with acetic anhydride in pyridine to give compound **5** in a 78% yield. The final deprotection of **5** to give **1** was carried out in the presence of NaOMe to afford compound **1** in 90% yield.

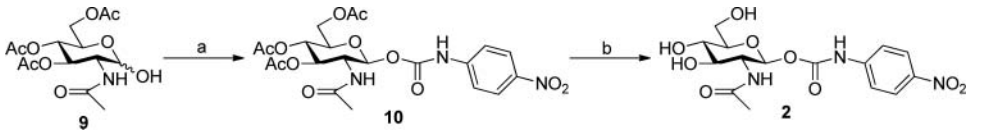


Scheme 1: (a) NaI, K_2CO_3, **8,** CH_3CN. (b) i. $NH_2CH_2CH_2NH_2$, butanol, 120°C. ii. Py, Ac_2O. (c) MeOH, NaOMe.



Scheme 2: (a) $ClCH_2SCH_3$, NaI, NaH, DMF. (b) $SOCl_2$, DCM.

Compound **2** was synthesized in four steps from commercially available *N*-acetylglucosamine. Following peracetylation and selective anomeric deacetylation with benzyl amine,[15] the free hemiactal **9** was treated with *p*-nitrophenyl isocyanate in toluene in the presence of a catalytic amount of triethylamine (Sch. 3). The resulting protected glycosyl carbamate (**10**) precipitated directly from the reaction mixture, giving only the β-carbamate. These results are consistent with observations by Scheeren et al., where glucose derivatives were reacted with various alkyl and aryl isocyanates, yielding only the β-carbamates in the presence of a catalytic base.[16] Deprotection of the remaining acetyl groups was challenging given the acid and base sensitivity of the glycosyl carbamate **10**. Zemplen conditions yielded a return of *N*-acetyl-glucosamine and



Scheme 3: (a) pNP-NCO, cat TEA, Tol, rt, 91%. (b) $NH_2NH_2 \bullet H_2O$, MeOH, rt, 48%.

the *p*-nitrophenyl isocyanate, likely from deprotonation of the carbamate nitrogen followed by elimination of the hemiacetal. HCl in methanol also afforded *N*-acetyl-glucosamine. Careful addition of 3.3 equivalents of hydrazine monohydrate in methanol and monitoring the reaction over 6 h yielded the final product **2** along with some carbamate cleavage product. Crude NMR analysis revealed 62% yield of the desired product **2** by comparison of the integration of anomeric signals; however, 48% was isolated via flash chromatography.

Substrate Activity

Initial enzymatic cleavage of substrate **1** was followed by absorbance spectroscopy. No increase in A_{405} indicative of glycosyl bond cleavage was observed under standard assay conditions (5 mM **1**, 0.1 μM DspB, 50 mM phosphate, 100 mM NaCl, pH 6) over a 30-min incubation. Further experiments were carried out to determine if the glycosidic bond was being cleaved but the acetal failed to fragment. Products of the enzyme incubation were analyzed by ^{1}H NMR and showed that no glycosidic bond cleavage was occurring over the course of the incubation. Similar assays using substrate **1** were also carried out with the jack bean β-hexosaminidase (**1** 5 mM, jack bean β-hexosaminidase 25 nM, sodium citrate buffer 50 mM, pH 5.5). No cleavage of the substrate was observed spectrophotometrically or by ^{1}H NMR analysis. To determine if compound **1** is an inhibitor of the enzymes, crude assays were carried out with pNP-GlcNAc (0.5 mM) in the presence or absence of **1** (5 mM). No difference in the pNP-GlcNAc hydrolysis rate in the presence or absence of **1** was observed, suggesting the enzymes showed little or no recognition of **1**.

It is surprising that no affinity for substrate **1** toward DspB or jack bean β-hexosaminidase was observed. Although it is unclear as to the reason for the reduced recognition of substrate **1** by the enzymes, we hypothesized that this may be due to a strong conformational bias in the substrate, preventing recognition. From the ^{1}H NMR spectra of **1** the acetyl protons resonate at 1.46 ppm, significantly upfield from the region of 1.9 to 2.1 ppm commonly observed for acetyl protons in *N*-acetylglucosamine. This suggests that the anisotropic effect of the aryl ring may be sufficiently close to the acetyl proton to perturb their resonance. 2-Phenethyl glycosides have been crystallized and subjected to modeling; these compounds show the phenyl ring folded back toward the

Table 1: Kinetic characterization of substrate **2** and comparison with pNPGlcNAc

Enzyme/ Substrate	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($mM^{-1}\ s^{-1}$)	Literature k_{cat}/K_M ($mM^{-1}\ s^{-1}$)
DisB/**2**	2.3 ± 0.2	14.0 ± 0.1	6.1	—
DisB/pNP-GlcNAc	46 ± 2	1.0 ± 0.1	0.021	0.02[3]
Jack bean/**2**	0.43 ± 0.02	22 ± 2	51	—
Jack bean/pNPGlcNAc	0.58 ± 0.03	7.9 ± 0.3	13.6	35.6[18]

pyranoside,[17] and similar shifts in neighboring acetyl protons have been observed in other large flexible aromatic glycosides, such as naphthylmethyl N-acetyl glucosamine glycosides.[18] Unfortunately, our hypothesis could not be supported by NOE experiments as no through-space interactions between the aryl protons and the acetyl group were observed.

Analysis of substrate **2** with DspB and jack bean β-hexosaminidase yielded promising results under the same conditions as substrate **1**. With DspB a 20-fold improved K_m and a 200-fold improved k_{cat}/K_m were observed when compared to the benchmark pNP GlcNAc substrate (Table 1). Interestingly, when substrate **2** was studied with the jack bean β-hexosaminidase, only a three-fold improvement in k_{cat} was observed and the K_m remained similar to pNP-GlcNAc substrate. The values of K_m and k_{cat} for pNP-GlcNAc and jack bean β-hexosaminidase agree well with previous literature values.[19]

The improved K_m for **2** observed for DspB supports our hypothesis that reduced steric congestion around the glycosidic linkage would improve recognition of the substrate. As the jack bean β-hexosaminidase is promiscuous with respect to the recognition of the aglycon, no substantial differences in K_m between substrate **2** and pNP-GlcNAc were observed. Both enzymes showed improved turnover of the glycosylcarbamates in comparison to pNPGlcNAc, demonstrating that glycosyl carbamates are good substrates for family 20 glycoside hydrolases.

The improved analytical signal obtained for substrate **1** over pNP-GlcNAc is shown in Figure 2 upon hydrolysis with DspB. The increase in analytical signal results from both the enzyme having improved kinetics as well as *p*-nitroaniline having a higher extinction coefficient under the assay conditions than *p*-nitrophenol. The combination of improved kinetics and improved extinction coefficient gives an overall sensitivity enhancement of approximately 35 times under the standard assay conditions (5 mM substrate).

In conclusion, we have demonstrated that the *p*-nitrophenyl glycosyl acetal (**1**) is a poor substrate for two family 20 glycosidases. Further experiments are required to determine the reason these glycosides are so poorly recognized but the conformation of these substrates is likely playing a role. Glycosyl carbamates have been shown to be effective substrates for DspB and jack bean

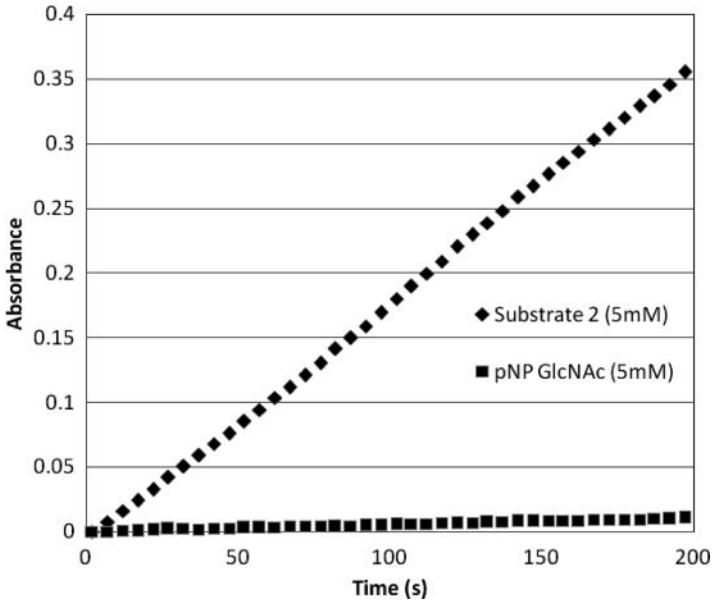


Figure 2: Hydrolysis of substrate **2** (♦) monitored at 410 nm and hydrolysis of pNP-GlcNAc (■) monitored at 405 nm over time in the presence of 50 nM DisB, 50 mM phosphate (pH 5.8), and 50 mM NaCl.

β-hexosaminidase. DspB displays substantially improved kinetics toward the glycosyl carbamate **2** over the standard pNPGlcNAc substrate.

EXPERIMENTAL

General

Flash chromatography was performed on Silica-P Flash Silica Gel 60 (40–63 μm particle size, Silicycle). Reactions were monitored by TLC using Silica Gel 60 F254 (EMD Science) with detection by quenching of fluorescence and/or by visualization with phosphomolybdic acid in ethanol (0.5% w/v), methanolic H_2SO_4 (10% v/v), or ninhydrin in ethanol (0.2% w/v). Reagents were obtained from Sigma-Aldrich or Acros Organics and were used without further purification. Jack bean β-hexosaminidase was obtained from Sigma-Aldrich as a solution concentration of 1.5 mg/mL. A molecular weight of 100 KDa was used to calculate the jack bean β-hexosaminidase concentration. Dispersin B (42 KDa, $\varepsilon = 58670\ M^{-1}\ cm^{-1}$) was obtained from Kane Biotech. ^1H and ^{13}C NMR spectra were recorded at 25°C with a Mercury 300 MHz or a Varian 400 MHz. High-resolution mass spectra were obtained from an ABI/Sciex QStar mass spectrometer (ESI). Absorption spectra were measured on a Schimadzu UV-2401PC spectrophotometer.

Synthesis

4-Nitrophenoxymethoxy 2-phthalimido-3,4,6-tri-O-*acetyl-2-deoxy-β-D-glucopyranoside (4)*

3,4,6-Triacetyl-2-deoxy-2-phthalimido-D-glucose (**3**) (500 mg, 1.15 mmol) was dissolved in acetonitrile (5 mL) and to it K_2CO_3 (238 mg, 1.72 mmol) was added followed by the addition of 4-nitrophenoxymethoxychloride (430 mg, 2.3 mmol) and NaI (258 mg, 1.72 mmol). The reaction was stirred for approximately 16 h at rt. TLC analysis showed generation of a new spot (EtOAC:*n*-pentane, 1:2, R_f 0.5). Solvent was evaporated and the crude mixture was diluted with dichloromethane (25 mL) and washed with water (50 mL); the organic phase was separated, dried over $MgSO_4$, filtered, and evaporated to syrup in vacuo. The crude product thus obtained was purified by flash chromatography eluting with (EtOAC:*n*-pentane, 1:3) to furnish 4-nitrophenoxymethoxy 2-phthalimido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside **4** (269 mg, 40%) as colorless amorphous solid. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 7.68–7.53 (m, 6H), 6.79–6.72 (m, 2H), 5.82 (dd, 2H, J = 9.2 Hz, J = 9.2 Hz,), 5.71 (d, 1H, J = 8.6 Hz), 5.44–5.37 (m, 2H), 5.18 (t, 1H, J = 9.1 Hz), 4.39–4.28 (m, 2H), 4.22 (dd, 1H, J = 2.1 Hz, 10.2 Hz), 3.97–3.92 (m, 1H), 2.13 (s, 3H), 2.02 (s, 3H), 1.81 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 170.6 (2), 170 (2), 169.4, 160.1, 141.6, 134 (2), 125.3 (2), 123.3 (4), 115.2 (2), 94.1, 88.1, 72.2, 70.1, 68.5, 61.7, 54, 20.7, 20.6, 20.4. HRMS calcd for $C_{27}H_{26}N_2O_{13}Na(M+Na)^+$: 609.1333; found 609.1337.

4-Nitrophenoxymethoxy 2-acetamido-3,4,6-tri-O-*acetyl-2-deoxy-β-D-glucopyranoside (5)*

Compound **4** (200 mg, 0.34 mmol) was dissolved in butanol (5 mL) under nitrogen and to it ethylenediamine (1 mL) was added. The solution was heated to 120°C for 20 h, after which time the solvent was evaporated and the crude oil was dried under vacuum. To the crude oil, pyridine (3 mL) and acetic anhydride (2 mL) were added and the solution was stirred at rt overnight. The solvent was evaporated and the crude mixture was diluted with dichloromethane (25 mL) and washed with 1N HCl (25 mL), water (50 mL), and sodium bicarbonate solution (25 mL); the organic phase was separated, dried over $MgSO_4$, filtered, and evaporated to syrup in vacuo. The crude product thus obtained was purified by flash chromatography eluting with (EtOAC:*n*-pentane, 1:1) to furnish 4-nitrophenoxymethoxy 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside **5** (132 mg, 78%) as colorless oil. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 8.16–8.11(m, 2H), 7.07–7.02 (m, 2H), 5.44 (d, 1H, J = 7.6 Hz,), 5.34 (d, 1H, J = 7.6 Hz), 5.23 (t, 1H, J = 8.9 Hz), 5.13–5.05 (m, 2H), 4.85 (d, 1H, J = 8.5 Hz), 4.23 (dd, 1H, J = 4.5 Hz, 12 Hz), 4.12 (dd, 1H, J = 2.3 Hz, 10 Hz), 3.97–3.93 (m, 1H), 3.70–3.66 (m, 1H), 2.04 (s, 3H), 1.96 (s, 3H), 1.94 (s, 3H), 1.46 (s, 3H).

^{13}C NMR (75 MHz, $CDCl_3$) δ: 171, 170.7, 169.8, 161.6, 142.5, 125.7 (2), 116.1 (2), 97.3, 89.1, 72.2, 72.0, 68, 61.7, 53.9, 22.8, 20.7, 20.6 (2). HRMS calcd for $C_{21}H_{26}N_2O_{12}Na(M+Na)^+$: 521.1383; found 521.1386.

4-Nitrophenoxymethoxy 2-acetamido-2-deoxy- β-D-glucopyranoside (1)

Compound **5** (100 mg, 0.2 mmol) was dissolved in MeOH (5 mL) followed by addition of NaOMe (0.5 M in MeOH, 0.1 mL). The solution was allowed to stir at rt for 6 h. Then the solution was neutralized with Amberlite IR 120 H^+ resin and filtered. The filtrate was evaporated to afford pure compound 4-nitrophenoxymethoxy 2-acetamido-2-deoxy- β-D-glucopyranoside **1** (67 mg, 90%) as amorphous white solid. ^{1}H NMR (MeOD, 300 MHz) δ: 8.24–8.19 (m, 2H), 7.21–7.15 (m, 2H), 5.55 (d, 1H, J = 7.6 Hz), 5.46 (d, 1H, J = 7.6 Hz), 4.75 (d, 1H, J = 8.5 Hz), 3.89 (dd, 1H, J = 1.7 Hz, 10.4 Hz), 3.72–3.63 (m, 2H), 3.47–3.41 (ddd, 1H, J = 8.5 Hz, 1.5 Hz, 8.7 Hz), 3.36–3.27 (m, 3H), 1.49 (s, 3H). ^{13}C NMR (75 MHz, MeOD) δ: 173.5, 163.6, 143.7, 126.6 (2), 117.6 (2), 99.3, 90.5, 78.4, 75.5, 72, 62.8, 57, 22.7. HRMS calcd for $C_{15}H_{20}N_2O_9Na(M+Na)^+$: 395.1067; found 395.1064.

*4-Nitrophenyl carbamoyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside (10)*

Compound **9** (200 mg, 0.58 mmol) was dissolved in toluene (10 mL) followed by the addition of a catalytic amount of triethylamine (0.05 mL) and *p*-nitrophenyl isocyanate (190 mg, 1.16 mmol). The solution was stirred at rt for 6 h, at which point a yellow solid had precipitated. The solution was chilled in an ice bath and pure 4-nitrophenyl carbamoyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside was isolated by filtration (253 mg, 86% yield) ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 8.22 (d, 2H, J = 9.2 Hz), 7.51 (d, 2H, J = 9.2 Hz), 5.61 (d, 1H, J = 8.8 Hz), 5.58 (bd, 1H, J = 5.2 Hz), 5.13–5.05 (m, 2H), 4.45 (d, 1H, J = 8.5 Hz), 4.35 (dd, 1H, J = 4.5 Hz, 12 Hz), 4.15 (dd, 1H, J = 2.3 Hz, 12 Hz), 3.86–3.72 (m, 1H), 2.06 (s, 3H), 2.04 (s, 3H), 2.03 (s, 3H), 1.98 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 171.4, 170.6, 169.8, 161.2, 148.5, 141.2, 130.7 (2), 116.1 (2), 102.3, 89.2, 73.1, 71.9, 68, 61.4, 53.7, 22.4, 20.8, 20.6 (2). HRMS calcd for $C_{21}H_{26}N_2O_{12}Na(M+Na)^+$: 534.1374; found 534.1379.

4-Nitrophenyl carbamoyl 2-acetamido-2-deoxy-β-D-glucopyranoside (2)

Compound **10** (200 mg, 0.4 mmol) was stirred as a slurry in MeOH (10 mL) and to it was added hydrazine monohydrate (0.05 mL, 1.3 mmol). After 4 h, the reaction mixture was evaporated under reduced pressure, and the remaining residue was purified by flash chromatography eluting with MeOH:DCM (1:20) to afford 4-nitrophenyl carbamoyl 2-acetamido-2-deoxy-β-D-glucopyranoside 2 (80 mg, 48% yield) as a faint yellow powder. ^{1}H NMR (MeOD, 400 MHz) δ: 8.22 (d, 2H, J = 9.2 Hz), 7.51 (d, 2H, J = 9.2 Hz), 5.61 (d, 1H, J = 8.8 Hz), 3.95 (m,

2H), 3.75 (dd, 1H, J = 1.7 Hz, 10.4 Hz), 3.62–3.31 (m, 3H), 2.04 (s, 3H). ^{13}C NMR (75 MHz, MeOD) δ: 173.5, 143.6, 141.7, 129.2 (2), 113.6 (2), 105.2, 90.5, 78.1, 75.2, 72.3, 62.4, 57.3, 22.4. HRMS calcd for $C_{15}H_{19}N_3O_9Na(M+Na)^+$: 408.1062; found 408.1068.

Enzyme Assays

Enzyme reactions were carried out in solutions containing 50 mM phosphate (pH 5.8); 50 mM NaCl, 25 nM DspB, or 50 mM citrate (pH 5.5); 10 nM jack bean β-hexosaminidase; and varying concentrations of **2** (0.1–2.5 mM) at 25°C. Due to substrate solubility constraints and background absorbance, 2.5 mM was used as a maximum concentration of substrate. Reactions were monitored in real time using the increase of absorbance at 410 nm resulting from the hydrolysis and release of pNA. Michaelis-Menten kinetic parameters were obtained from direct curve fitting of the data. A slow but measurable background hydrolysis rate was also monitored and subtracted from the enzyme-catalyzed reaction. A control experiment replacing DspB with bovine serum albumin (BSA, 1 μM) showed no increase in reaction rate above background. The assay was also performed with varying concentrations of pNPGlcNAc (5—1 mM) as a substrate for comparison measuring absorbance at 405 nm, in the same concentration range; however, Michaelis-Menten kinetic parameters were obtained from double reciprocal plots (1/v vs. 1/S) using first-order kinetics due to solubility limitations of pNPGlcNAc. All assays were performed in triplicate. Under the assay conditions the extinction coefficients of the chromophores were p-nitrophenol 7280 M^{-1} cm^{-1} at 405 nM and p-nitroaniline 5450 M^{-1} cm^{-1} at 410 nM.

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