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A Strategy for Functional Proteomic Analysis of Glycosidase Activity from Cell Lysates[**]

David J. Vocadlo[†*] and Carolyn R. Bertozzi[*]

General Methods

All chemicals were obtained from commercial suppliers and were used as received unless otherwise noted. The silica gel used in column flash chromatography was E. Merck No. 9385 60 Å, 230-400 mesh. Analytical thin layer chromatography (TLC) was conducted on Analtech Uniplate silica gel plates with detection by ceric ammonium molybdate, successive treatment with triphenyl phosphine and ninhydrin, and/or UV light. All solvents used in chemical reactions were distilled under N_2 atmosphere prior to usage. THF was dried and deoxygenated over Na[O] and benzophenone, and CH_2Cl_2 and CH_3CN were dried over CaH_2. Unless otherwise specified, all solvents were removed with a rotary vacuum evaporator. The ^{1}H-NMR, ^{13}C-NMR and ^{31}P-NMR spectra at 500, 126 and 400 MHz, respectively, with DRX-500 and AMX-400 spectrometers. Chemical shifts are reported in δ values downfield from tetramethylsilane and *J* coupling constants are reported in Hz. Low and high resolution fast atom bombardment (FAB^+) or electrospray ionization (ESI) mass spectra were obtained at the University of California

at Berkeley Mass Spectrometry Laboratory. Elemental analyses were obtained at the University of California at Berkeley Microanalytical Laboratories.

para-Nitrophenyl 6-*O*-tosyl-β-D-galactopyranoside.

To a solution of *para*-nitrophenyl-β-D-galactopyranoside (Sigma, **1**, 1.4 g, 4.6 mmol) in dry pyridine (25 mL) at 0 °C was added, with stirring over 30 min, a solution of tosyl chloride (1.05 g, 5.50 mmol) in dry pyridine (25 mL). The reaction mixture was slowly warmed from 0°C to RT over a period of 2 h and then maintained for 2 h at RT. After completion of the reaction as judged by TLC, brine was added (3x mL) and the aqueous phase was extracted three times with ethyl acetate. The combined organic phases were dried over $MgSO_4$, filtered, and concentrated in vacuo to yield a white amorphous solid. Gradient flash column silica gel chromatography (2:1; ethyl acetate: hexanes to ethyl acetate) afforded the desired product as a crystalline white solid (**2**, 1.24 g, 2.71 mmol, 59 %). Recrystallization of this material could be readily accomplished from cold absolute ethanol. ^{1}H NMR (400 MHz, CD_3OD) δ: 8.16 (2 H, AB, J = 8.8), 7.72 (2 H, AB, J = 8.4), 7.29 (2 H, J = 8.4), 7.16 (2 H, AB, J = 8.8), 5.02 (1 H, d, J = 7.6), 4.35-4.27 (2 H, m), 4.10-4.07 (1 H, m), 3.82 (1 H, dd, J = 9.6, 8.0), 3.66 (1 H, dd, J = 9.6,3.2), 2.37 (3 H, s). ^{13}C NMR (125.77 MHz, CD_3OD) δ: 163.51, 246.74, 143.63, 133.72, 130.96, 128.87, 126.58, 117.62, 101.37, 74.19, 74.09, 71.52, 70.87, 21.56. +FAB (LiCl): *m/z* 462 $(M + Li)^+$; Anal. Calcd for $C_{19}H_{21}N_1O_{10}S$; C, 50.09; H, 4.65; N, 3.08; Exp. C, 50.24; H, 4.89; N, 2.91. mp 97 ˚C (decomp).

para-Nitrophenyl 6-azido-6-deoxy-β-D-galactopyranoside

A solution of *para*-nitrophenyl 6-*O*-tosyl-β-D-galactopyranoside (**2**, 1.1 g, 2.4 mmol) and sodium azide (0.92 g, 14 mmol) in dry DMF (25 mL) was heated to 70 °C overnight after which time the reaction was judged complete by TLC analysis. The solvent was removed *in vacuo* and the residue was dissolved in ethyl acetate to yield a suspension. Solid materials were removed by filtration through a bed of Celite™ and the filtrant was concentrated in vacuo to yield a pale tan solid. Gradient flash column silica gel chromatography (2:1; ethyl acetate: hexanes to 5:1 ethyl acetate; hexanes) afforded the desired product as a crystalline white solid (**3**, 0.68 g, 2.9 mmol, 87 %). ^{1}H NMR (400 MHz, CD_3OD) δ: 8.16 (2 H, AB, *J* = 8.8), 7.72 (2 H, AB, *J* = 8.4), 7.29 (2 H, *J* = 8.4), 7.16 (2 H, AB, *J* = 8.8), 5.02 (1 H, d, *J* = 7.6), 4.35-4.27 (2 H, m), 4.10-4.07 (1 H, m), 3.82 (1 H, dd, *J* = 9.6, 8.0), 3.66 (1 H, dd, *J* = 9.6,3.2), 2.37 (3 H, s). ^{13}C NMR (100.6 MHz, CD_3OD) δ: 163.72, 150.22, 143.89, 126.60, 117.66, 101.88, 75.89, 74.43, 71.74, 70.49, 52.53. Anal. Calcd for $C_{12}H_{14}N_4O_7$; C, 44.18; H, 4.33; N, 17.17; Exp. C, 44.14; H, 4.39; N, 16.96. mp 199 ˚C (decomp).

para-Chlorothiophenyl 2-deoxy-2-fluoro-β-D-galactopyranoside

To a cold (0 °C) solution of the crude bromide **4**[1] (4 g, ~ 10 mmol) in CH_2Cl_2 (75 mL), was added a cold (0 °C) mixture of tetrabutylammonium hydrogen sulfate (4.00 g, 11.8 mmol) and *para*-chlorothiophenol (3.30 g, 22.8 mmol) in 1 M NaOH (50 mL). The resulting brown mixture was allowed to warm to RT and stirred overnight after which the

reaction was judged to be complete by TLC analysis. The organic phase was collected, washed three times with 1 M NaOH, once with water, and then dried over $MgSO_4$. The solvent was removed in vacuo to yield an orange residue. Gradient flash column silica gel chromatography (2:1; diethyl ether: hexanes to 1:1 diethyl ether; hexanes) afforded the desired product as a crystalline white solid which was used in the next reaction. To a solution of *para*-chlorothiophenyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-β-D-galactopyranoside (2.1 g, 4.4 mmol) in dry methanol (40 mL) was added a catalytic quantity of 1 M NaOMe in methanol. The reaction was allowed to proceed at RT for fifty min at which time the reaction was judged complete by TLC analysis. A sufficient quantity of Amberlyst IR-20 (H^+ form) resin was added to the mixture to render the pH of the solution neutral. The resin was removed by filtration and the solvent removed *in vacuo* to yield a white crystalline solid (**5**, 1.32 g, 4.27 mmol, 67 % over two steps). ^{1}H NMR (400 MHz, CD_3OD) δ: 7.56 (2 H, AB, *J* = 8.8), 7.35 (2 H, AB, *J* = 8.8), 4.77 (1 H, dd, *J* = 9.6, 2.0), 4.35 (1 H, dt, *J* = 50.8, 8.8), 3.97 (1 H, t, *J* = 3.2), 3.83-3.75 (2 H, m, 3.84-3.77), 3.72 (1 H, dd, *J* = 11.2, 4.8), 3.67-3.64 (1 H, m). ^{13}C NMR (100.6 MHz, CD_3OD) δ: 134.84, 134.67, 132.94, 130.03, 90.58 (*J* = 183.1), 86.07 (*J* = 24.1), 80.79, 74.24 (*J* = 18.1), 70.95 (*J* = 9.0), 62.35. Anal. Calcd for $C_{12}H_{14}ClFO_4S$; C, 46.68; H, 4.57; Exp. C, 46.74; H, 4.64.

para-Chlorothiophenyl 6-azido-2,6-dideoxy-2-fluoro-β-D-galactopyranoside

To a cold (0 °C) solution of *para*-chlorothiophenyl 2-deoxy-2-fluoro-β-D-galactopyranoside (**5**, 500 mg, 1.65 mmol) in dry pyridine (5 mL) was added, with

stirring over 30 min, a solution of tosyl chloride (343 mg, 1.8 mmol) in dry pyridine (5 mL). The reaction mixture was slowly warmed from 0 °C to RT over a period of 2 h and then maintained for 2 h at RT. After completion of the reaction as judged by TLC, brine was added (3 x mL) and the aqueous phase was extracted three times with ethyl acetate. The combined organic phases were dried over $MgSO_4$, filtered, and concentrated in vacuo to yield a white amorphous solid. Gradient flash column silica gel chromatography (2:1; ethyl acetate: hexanes to pure ethyl acetate) afforded *para*-chlorothiophenyl 2-deoxy-2-fluoro-6-*O*-tosyl-β-D-galactopyranoside **6** as a crystalline white solid. This intermediate was used in the next step without further purification or characterization. A solution containing *para*-chlorothiophenyl 2-deoxy-2-fluoro-6-*O*-tosyl-β-D-galactopyranoside (**6**, 642 mg, 1.35 mmol) and sodium azide (900 mg, 13.8 mmol) in DMF (10 mL) was heated to 70 °C overnight after which time the reaction was judged complete by TLC analysis. The solvent was removed in vacuo and the residue was dissolved in ethyl acetate to yield a suspension. Solid materials were removed by filtration through a bed of Celite™ and the filtrant was concentrated in vacuo to yield a pale tan solid. Gradient flash column silica gel chromatography (1:2; diethyl ether: hexanes to 2:3 diethyl ether; hexanes) afforded the desired product as a crystalline white solid (**7**, 310 mg, 0.93 mmol, 56 % over two steps). ^{1}H NMR (400 MHz, CD_3OD) δ: 7.43 (2 H, AB, J = 8.8), 7.23 (2 H, AB, J = 8.8), 4.70 (1 H, dd, J = 9.6, 2.4), 4.23 (1 H, dt, J = 50.4, 9.2), 3.77-3.64 (3 H, m), 3.50 (1 H, dd, J = 13.2, 8.8), 3.22-3.18 (1 H, m). ^{13}C NMR (100.6 MHz, CD_3OD) δ: 133.72, 133.67, 131.26, 128.68, 89.07 (J = 183.3), 84.65 (J = 24.6), 77.78, 72.64 (J = 18.0), 69.95 (J = 9.1), 52.48. +FAB (LiCl): *m/z* 340

(M + Li)$^+$; Anal. Calcd for $C_{12}H_{13}ClFN_3O_3S$; C, 43.18; H, 3.93; N, 12.59; Exp. C, 43.06; H, 3.90; N, 12.55.

para-Chlorothiophenyl 3,4-*O*-acetyl-6-azido-2,6-dideoxy-2-fluoro-β-D-galactopyranoside

To a solution of *para*-chlorothiophenyl 6-azido-2,6-dideoxy-2-fluoro-β-D-galactopyranoside (**7**, 260 mg, 0.78 mmol) in pyridine (10 mL) was added acetic anhydride (1 mL). The reaction mixture was stirred overnight after which time the reaction was judged complete by TLC analysis. The solvent was removed in vacuo and the residue was dissolved in ethyl acetate (50 mL) and washed successively with saturated $NaHCO_3$, 1 M HCl, water, sat. $NaHCO_3$, and brine. The organic was dried over $MgSO_4$, filtered, and the solvent removed *in vacuo* to yield an amorphous solid. The residue was dissolved in diethyl ether and crystallized from cold diethyl ether and hexanes to yield, after three crystallizations, the desired product (**8**, 270 mg, 0.64 mmol, 82 %). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.55 (2 H, AB, *J* = 8.3), 7.33 (2 H, AB, *J* = 8.3), 5.38 (1 H, t, *J* = 2.5), 5.11 (1 H, ddd, *J* = 13.0, 9.4, 4.4), 4.72 (1 H, dd, *J* = 9.7, 2.7), 4.44 (1 H, dt, *J* = 49.9, 9.6), 3.82 (1 H, dd, *J* = 7.9, 4.6), 3.48 (1 H, dd, *J* = 13.0, 7.9), 3.18 (1 H, dd, *J* = 13.0, 4.5), 2.10 (3 H, s), 2.04 (3 H, s). ^{13}C NMR (125.77 MHz, $CDCl_3$) δ: 169.82 (*J* = 6.0), 135.31, 129.19, 86.12, 85.18 (*J* = 25.2), 84.62, 76.05, 71.91 (*J* = 20.1), 68.57 (*J* = 8.8), 50.63, 20.55, 20.47 +FAB (LiCl): *m/z* 315 (M + Li)$^+$; Anal. Calcd for $C_{16}H_{17}ClFN_3O_5S$; C, 45.99; H, 4.10; N, 10.06; Exp. C, 45.89; H, 4.12; N, 9.94.

6-Azido-2,6-dideoxy-2-fluoro-β-D-galactopyranosyl fluoride

To a solution of *para*-chlorothiophenyl 3,4-di-*O*-acetyl-6-azido-2,6-dideoxy-2-fluoro-β-D-galactopyranoside (**8**, 78 mg, 0.19 mmol) in a mixture of acetone (3 mL) and water (0.5 mL) at RT was added *N*-bromosuccinimide (10 eq., 330 mg, 2.8 mmol). The reaction mixture was stirred for 3 h after which aqueous $NaHCO_3$ (2 mL) was added followed by dichloromethane was added (20 mL). The organic layer was then washed successively with saturated $NaHCO_3$, water, and brine. The organic phase was dried over $MgSO_4$, filtered, and the solvent removed *in vacuo* to yield a pale yellow residue which was used in the subsequent reaction without further purification. This residue was dissolved in a mixture of THF (2 mL) and dichloromethane (2 mL) and the resulting mixture was cooled to -40 °C after which was added DAST (250 μL, 1.9 mmol). The reaction mixture was allowed to warm to RT overnight and was stirred for a further day. The reaction mixture was cooled to 0 °C and aqueous $NaHCO_3$ was added to the reaction mixture after which dichloromethane was added (15 mL). The organic layer was then washed successively with saturated $NaHCO_3$, water, and brine. The organic phase was dried over $MgSO_4$, filtered, and the solvent removed *in vacuo* to yield a pale yellow residue. Gradient flash column silica chromatography of this residue (1: 2 diethyl ether : hexanes; 2: 3 diethyl ether : hexanes) yielded 3,4-*O*-acetyl-6-azido-2,6-dideoxy-2-fluoro-β-D-galactopyranosyl fluoride. This intermediate was used without further purification. To a solution of 3,4-*O*-acetyl-6-azido-2,6-dideoxy-2-fluoro-β-D-galactopyranosyl fluoride (**9**, 64 mg, 0.22 mmol) in dry methanol (40 mL) was added a catalytic quantity of 1 M NaOMe in methanol. The reaction was allowed to proceed at RT for fifty min at

which time the reaction was judged complete by TLC analysis. A sufficient quantity of Amberlyst IR-20 (H^+ form) resin was added to the mixture to render the pH of the solution neutral. The resin was removed by filtration and the solvent removed *in vacuo* to yield an amorphous white solid. Gradient flash column silica chromatography of this material (3: 1 diethyl ether : hexanes; 5: 1 diethyl ether : hexanes) yielded 3,4-*O*-acetyl-6-azido-2,6-dideoxy-2-fluoro-β-D-galactopyranosyl fluoride (**10**, 42 mg, 0.20 mmol, 58 % over two steps). ^{1}H NMR (500 MHz, CD_3OD) δ: 4.42 (1 H, dddd, *J* = 52.4, 14.1, 9.3, 7.0), 3.84-3.77 (3 H, m), 3.64 (1 H, dd, *J* = 12.9, 8.2), 3.40 (1 H, dd, *J* = 12.9, 4.6). ^{13}C NMR (125.77 MHz, CD_3OD) δ: 108.54 (*J* = 213.8, 26.4), 92.68 (*J* = 182.4, 22.64), 75.64 (*J* = 3.8), 72.18 (*J* = 17.6, 10.1) 70.62 (*J* = 8.8, 1.3), 51.96. High resolution +CIMS $(M + H)^+$: Calculated, 210.06903; Found, 210.06891. mp 109-111 ˚C.

Cell and protein labeling:

Agrobacterium sp. β-glucosidase and *Xanthomonas manihotis* β-galactosidase were generously provided by Stephen G. Withers. Other glycosidases were obtained from Sigma. Cultures (6 x 5 mL) of *Escherichia coli* K-12 were grown to an OD_{600} value of 0.6 and were either induced with 0.1 mM IPTG or untreated. After growing the cells at RT overnight to stationary phase the cells were harvested by centrifugation (5000 x g) and frozen at -80 °C in a solution (500 μL) of 100 mM tris buffer, pH 7.4, containing 10 % glycerol, 1 mM PMSF, and hen egg white lysozyme (0.1 mg/mL). The suspension of frozen cells was sonicated at 4 °C (4 x 30 sec, power level 2-3, microtip, Misonix, Ultrasonic Processor XL). The crude lysate was clarified by centrifugation at 14 000 rpm in an Eppendorf 5415C microcentrifuge for 20 min at 4 °C. The supernatant was

collected and these samples were used as the source of LacZ in cell lysates. The relative activities of induced and uninduced cultures were measured using an aliquot (100 μL) of a 50-fold dilution of culture lysate in a solution of 100 mM tris, pH 7.4, containing 6.6 mM pNPGal and 10 % glycerol in a final volume of 850 μL at RT and it was determined that uninduced cultures contained only 0.6 % of β-galactosidase activity (0.0003 A_{400} min^{-1} μL^{-1} of cell lysate) as compared to induced cultures (0.055 A_{400} min^{-1} μL^{-1} of cell lysate). An aliquot (5 μL) of each sample was subject to SDS-PAGE analysis (precast 10 or 12 % Tris-HCl polyacrylamide gels, Biorad) using coomassie stain to verify differences in LacZ protein expression levels between induced and uninduced cultures. An aliquot of each of the cell lysate samples (90 μL) was treated with a solution (10 μL) of 6Az2FGalF (526 mM, final [6Az2FGalF] = 52.6 mM) in PBS containing 50% DMF and incubated overnight at 37 °C. Glycosidase samples used in this study were inactivated overnight at 25 ˚C but otherwise were analyzed in the same way as the cell lysate samples. The inactivated samples were then dialysed overnight (Pierce Slide-A-Lyzer Mini Dialysis Unit, 10 000 MWCO) at 4 °C. After dialysis solutions of 1 M pH 2.0 sodium phosphate (20 μL), β-mercaptoethanol in water (100 mM), PMSF in ethanol (100 mM), and saturated urea (one third of the total volume) were added to yield a solution of pH ≈ 3.5 containing 4 mM β-mercaptoethanol and 2 mM PMSF. One volume of a solution of FLAG-phosphine in water (500 μM) was added (250 μM final concentration) and the mixture was allowed to incubate overnight at RT. The sample was concentrated (Amicon, microconcentrator 10000 MWCO) to a final volume of ≈ 20 μL and an aliquot of the sample was mixed with SDS-PAGE loading buffer. Without heating the sample was loaded onto precast 10 or 12 % Tris-HCl polyacrylamide gels

(Biorad). After electrophoresis, the samples were electroblotted to nitrocellulose membrane membrane (0.45 μm, Biorad). Transfer was verified by visual inspection of the transfer of prestained markers (Benchmark, Invitrogen). The membrane was blocked using PBS conatining 5 % low fat dry powdered milk, containing 0.1 % Tween-20 (blocking buffer) for 1 hour at room temperature or overnight at 4 °C. The blocking solution was decanted and a solution of blocking buffer A containing either anti-β-galactosidase mouse IgG mAb (1:5000, Promega) or anti-FLAG-HRP mAb conjugate (1:3500, Sigma). Membranes were incubated at room temperature for 1 hour or overnight at 4 °C after which the blocking solution was decanted and the membrane was rinsed with PBS, containing 0.1 % Tween-20 (wash buffer). Membranes were then rinsed for 2 x 5 min and 2 x 20 min with wash buffer. For detection of LacZ, the membrane was incubated in blocking solution for 1 hour at RT and, after washing, the membrane was incubated with a secondary goat anti-mouse-HRP conjugate (1:10000, Zymed Laboratories) for one hour at RT or 4 °C overnight in blocking solution. The membrane was washed and detection of membrane bound goat anti-mouse-HRP conjugate was accomplished as for anti-FLAG-HRP. Membranes were stripped using 2 % SDS, 100 mM β-mercaptoethanol, 50 mM TRIS, pH 6.8 at 50°C for 30 min. Control samples were treated in the same manner except that specific reagents were replaced by buffer where appropriate. Detection of membrane-bound goat anti-mouse-HRP or anti-FLAG-HRP conjugates was accomplished with chemiluminescent detection using the SuperSignal West Pico Chemiluminescent Detection Kit (Pierce) and film (Kodak Biomax MR or X-OMAT).

References:

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