Synthesis and Characterization of a Novel Lysosomotropic Enzyme Substrate That Fluoresces at Intracellular pH

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Fluorogenic substrates that can be used to detect the activity of enzymes within intact cells are needed for biochemical investigations of human lysosomal storage disorders and identification of gene transfer recombinants. The first member of a new series of lysosomotropic fluorescent probes has been synthesized and characterized. This compound, *O*-[4-(1-imidazolyl)butyl]-2,3-dicyano-1,4-hydroquinonyl β-D-galactopyranoside (Im-DCH-β-Gal), and its aglycon, Im-DCH, contain an *N*-alkylimidazole as a lysosomotropic substituent. The concentration of such moderately basic amines within acidic cellular organelles should provide increased specificity toward lysosomal hydrolases. The physiochemical properties of Im-DCH, in particular the intensity of its fluorescence at acidic pH's, make its glycosides well suited for the measurement of enzymatic activity within intact cells.

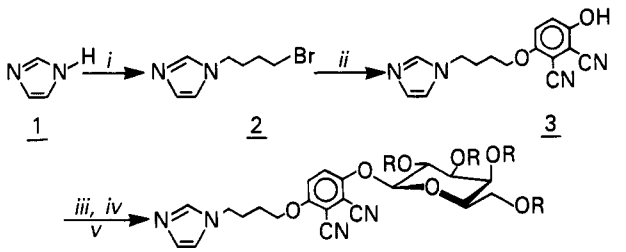
Introduction

Fluorogenic enzyme substrates have become one of the primary methods of quantitating a specific enzymatic activity, largely because of the inherent sensitivity of fluorescent detection. While fluorescent assays using tissue homogenates or cellular extracts have become routine, the detection of enzymatic activity within living cells has proven to be more problematic. Two notable difficulties are the pH dependence of the fluorescence of some probes at physiological pH values[1] and the undesirable tendency of the fluorescent product to diffuse out of the cells.[2] For the measurement of certain enzymes within some cellular systems these problems are minimal.[3] With other cell types, existing fluorescent probes are not optimal, and various techniques have been employed to circumvent their deficiencies. These include cooling of the cells in order to slow efflux of the product,[4] fixing of the fluorescent product as an insoluble precipitate,[5] or performing the incubations in microdroplets containing a single cell.[6] A more general approach is the chemical modification of the fluorophore to obtain the desired properties.[7] A recent example is the synthesis of the 4-nonyl analogue of 4-methylumbelliferone, and the study of its β-glucoside in normal and Gaucher fibroblasts.[8]

With the goal of preparing a new class of fluorogenic substrates for use within intact cells, 2,3-dicyanohydroquinone (DCH) was chosen as a lead compound. This fluorescent compound has been well characterized with regard to the effect of pH on its emission and absorption spectra.[9] DCH and cell-permeant diesters of DCH have been used to measure intracellular pH by flow cytometry[2b,10] and fluorescence microscopy[11] and to test for cell viability.[12] By proper choice of excitation and emission wavelengths, it is possible to discriminate between the neutral and ionized forms of DCH.[9,11] By analogy, it was expected that fluorescent measurement of enzymatic hydrolysis of DCH-glycosides should be possible. An additional advantage of the 2,3-dicyanohydroquinone system is the high acidity of both the ground state and the excited state of the phenols. This should allow the detection of lysosomal enzymes at physiologically relevant pH values (pH 4-7). The relative simplicity of the 2,3-dicyanohydroquinone nucleus also seemed likely to be compatable with steric requirements encountered during enzymatic hydrolysis.

As an approach to the design of in vivo fluorogenic substrates for lysosomal hydrolases, we sought to prepare a fluorogen with lysosomotropic (acidisomotropic)[13] properties. With one of the phenols of DCH glycosylated

Scheme I[a]



[a] (i) $Br(CH_2)_4Br/NaH/THF$; (ii) $DCH/K_2CO_3/DMF/acetone$; (iii) $^nBu_4NOH/NaOH/CHCl_3$; (iv) α-$BrGal(OAc)_4/Ag_2CO_3/CH_3CN$; (v) $NaOCH_3/CH_3OH$.

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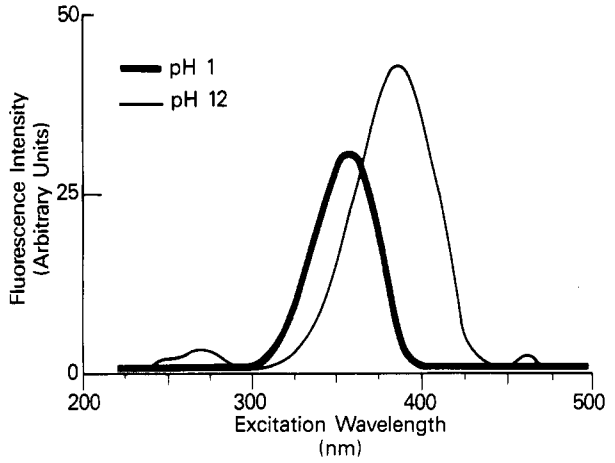

Figure 1. Comparison of the excitation spectra of Im-DCH, **3**, at pH 1 (—) and pH 12 (—). The excitation maxima are 358 nm at pH 1 and 385 nm at pH 12. Fluorescence was measured at 460 nm using 1 μM solutions in 0.2 M potassium chloride/hydrochloric acid buffer (pH 1) and 0.1 M sodium phosphate buffer (pH 12), with maximum absorbances of 0.0054 and 0.0057, respectively.

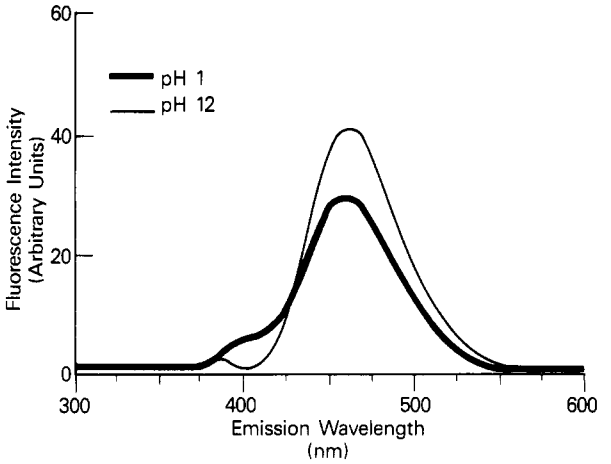

Figure 2. Comparison of the emission spectra of Im-DCH, **3**, at pH 1 (—) and pH 12 (—). Conditions are as described for Figure 1 with excitation at 355 and 386 nm for the pH 1 and pH 12 solutions, respectively. The emission maxima occur at 460 nm for both solutions.

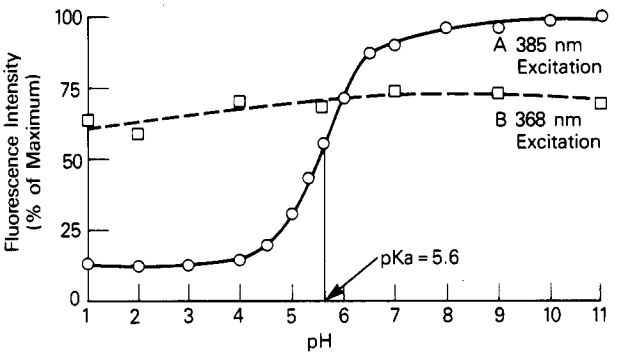

Figure 3. (A) Fluorescence of Im-DCH as a function of pH, measured with excitation at 385 nm and emission at 460 nm. Buffers were 0.2 M potassium chloride/hydrochloric acid (pH 1) and 0.1 M sodium phosphate (pH 2–11). The pK_a corresponds to the midpoint of the intensity change. (B) Fluorescence of Im-DCH as measured at an excitation wavelength (368 nm) that was selected to give high fluorescence across the physiological pH range (pH 4–7). The intensity scale, emission wavelength (460 nm), and buffers are the same as in Figure 3A.

to serve as an enzyme substrate, the other phenol may be modified to alter the cellular transport properties of the probe. To this end, a dicyanophenol containing an *N*-alkylimidazole moiety (Im-DCH, **3**) was chosen as the synthetic target. There is ample precedent in the literature for the concentration of such amines with pK_a's near 7 within acidic subcellular structures such as the lysosomes.[14]

For this initial study, the β-galactoside was chosen as the enzymatic substrate. This particular glycoside was selected because of both the ready availability of a variety of purified β-galactosidases and the existence of the human disorder, G_{M1} gangliosidosis, which involves the lack of a major lysosomal β-galactosidase. This disease has been well characterized,[15] and fibroblast lines derived from a number of patients are available. We now describe the chemical synthesis and the fluorescent properties of the fluorophor Im-DCH, **3**, and its β-galactoside Im-DCH-β-Gal, **5**.

Results and Discussion

The synthesis of the fluorescent phenol **3**, the first analogue of DCH that contains a lysosomotropic amine substituent, followed the route outlined in Scheme I. Imidazole was converted into *N*-(4-bromobutyl)imidazole, **2**,[16] using a procedure which has been described for *N*-(6-bromohexyl)imidazole.[17] Purification by chromatography was precluded by the instability of the free amine, which was therefore stored as the acetate salt. Conditions have been reported for monoalkylation of DCH with primary *n*-alkyl tosylates.[18] When these conditions were employed with simple alkyl tosylates and bromides, mixtures of mono- and dialkylated product and unreacted DCH were formed (typical mole ratios 25:45:30, respectively, data not shown). In order to maximize the yield of **3** from the unstable bromide **2**, a 3-fold excess of DCH was employed. The monoalkylated product, **3**, was purified most conviently by recrystallization of the hydrochloride salt from water, although chromatography of the sparingly soluble free amine was also possible. Phenol **3** was glycosylated by conversion to the highly soluble tetrabutylammonium salt, which was then alkylated with α-bromogalactose tetraacetate in the presence of silver carbonate. This technique has previously been used for glycosylation of other aromatic alcohols.[19] The unprotected galactoside, **5**, was readily prepared by deacetylation of **4** in basic methanol. The galactosides, **4** and **5**, were shown to have the β-glycosidic linkage by NMR spectroscopy. In particular, the C-13 chemical shifts of the galactose C1 carbons (101.4 ppm in **4**, 101.3 ppm in **5**) and the magnitude of the galactose H1–H2 coupling (7.9 Hz in **4**, 7.6 Hz in **5**) match those of model aromatic β galactosides.[20] No α-galactoside byproduct could be detected by NMR spectroscopy in either the tetraacetate **4** or the free galactoside **5**.

The fluorescent behavior of the dicyanophenol, **3**, in aqueous buffers is shown in Figure 1 and 2. As anticipated

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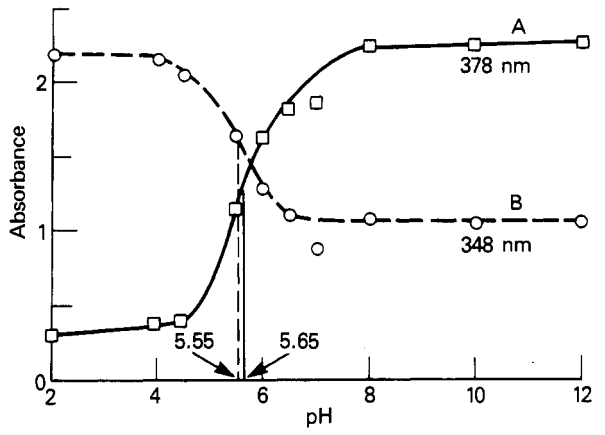

Figure 4. Measurement of the pK_a of the phenol/phenolate transition of Im-DCH using UV absorption. The samples were 0.4 mM in 0.1 M sodium phosphate buffers and were measured with a path length of 1 cm. (A) Absorbance at the phenolate maximum of 378 nm. (B) Absorbance at the phenol maximum of 348 nm.

by the behavior of the parent compoud, 2,3-dicyanohydroquinone,[9] a shift of the excitation maxima from 358 to 385 nm occurs upon deprotonation.

The pK_a of the phenol/phenolate equilibrium of **3** was determined to be 5.6 by fluorescence measurement (Figure 3, curve A). That this pK_a reflects the acidity of the ground state of **3** was verified by measurements of the pH dependence of the UV absorbance spectrum (Figure 4).

The maximum in the excitation spectra of **3** shifts from 358 to 385 nm between pH 1 and 12, while there is no corresponding shift in the emission spectra. This suggests that the emission is from the same molecular species at both pH's. In common with many aromatic alcohols, **3** is much more acidic in the excited state than in the ground state. Thus, even at pH values below 3, where the ground-state molecules are essentially all in the phenol form, following absorption of a 358 nm photon the excited state molecules rapidly deprotonate with a much more acidic equilibrium constant ($pK_a^* \ll pK_a$). At pH values much above the pK_a^*, essentially all the fluorescence will therefore be from the phenolate form of the excited state. At pH's near the pK_a^*, fluorescence may also be observed from the excited state of the phenol. A small shoulder in the emission spectra at approximately 400 nm is seen at pH 1 (Figure 2) but not at pH 2 or above. This is assigned to emission from a small amount of excited state phenol that is present at pH 1. When the concentration of acid is increased further, this shoulder grows, until in 6 M hydrochloric acid essentially all the emission occurs from the undissociated phenol (Figure 5). The phenol form of **3** therefore displays emission and excitation maxima at 405 and 355 nm, respectively (Figure 5, spectrum in 6 M HCl).

The difference in the ground- and excited-state pK_a's can be estimated by eq 1.[21] In this formula, $\Delta\nu$ is the

$$pK_a^* = pK_a - 0.625\Delta\nu/T(K) \quad (1)$$

wavenumber difference (in cm^{-1}) between the neutral and deprotonated forms. In order to take into account differences in solvation between the ground and excited states, $\Delta\nu$ should be the average of the spectral shifts which are measured by absorbance and fluorescence.[22] Using the observed shifts in absorption (386–355 nm) and emission (460–405 nm), eq 1 predicts a value of 0.13 for the pK_a^* of Im-DCH. Analysis of the data in Figure 5 by

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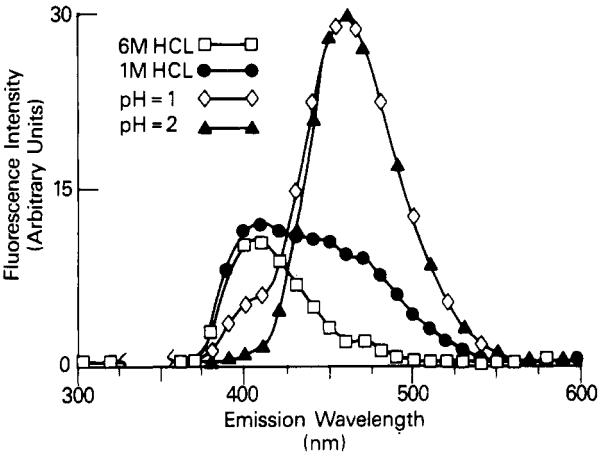

Figure 5. Fluorescence emission of Im-DCH, **3**, in strongly acidic solutions. Peaks at 405 and 460 nm are assigned to emission from the excited states of the phenol and phenolate, respectively. Excitation was at 358 nm for pH 2, 355 nm for pH 1, and 340 nm for the hydrochloric acid solutions. The concentration of Im-DCH was 1 μM for all samples.

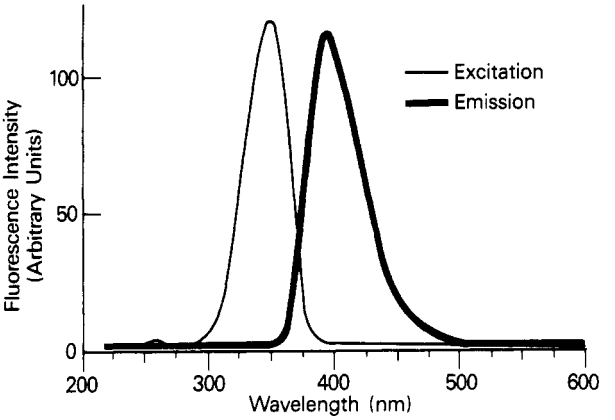

Figure 6. Fluorescence of Im-DCH-β-Gal, **5**, showing the excitation spectrum (—, emission at 394 nm) and emission spectrum (—, excitation at 349 nm). Measurements were made at 10 μM with a maximum absorbance of 0.065 in 0.1 M sodium phosphate pH 7 buffer.

the method of Brown and Porter[9] gives a range of 0.2–0.8 for the pK_a^* of Im-DCH. This is reasonable when compared with the pK_a^* of 0.04, which was measured for the first deprotonation of the parent compound, DCH.[9]

The importance practical consequence of the high acidity of the excited state of **3** is that for any pH above 2, the emission maximum remains fixed at 460 nm. If the excitation wavelength is set to the isosbestic point at 368 nm in the absorption spectra (Figure 1), the intensity of the fluorescence becomes essentially independent of pH (Figure 3, curve B). Using this approach, a fluorescence signal of approximately 60–75% of the maximum fluorescence of **3** is obtainable across the entire pH range of 1–12.

The fluorescence data for the β-galactoside **5** are shown in Figure 6. Several aspects of the fluorescence behavior of galactoside **5** mirror the properties of the undissociated phenol **3**. The excitation maximum of 350 nm coincides with that of the undissociated phenol seen at low pH. The emission maximum of 395 nm is also quite similar to that observed for **3** under highly acidic conditions where emission from the undissociated excited state occurs (compare Figures 5 and 6).

In order to be useful as a fluorogenic substrate, conditions must exist under which the measured fluorescence of the hydrolytic product is much higher than that of the

glycosylated substrate. A comparison of the spectroscopic properties of the galactoside **5** (Figure 6) with those of the phenol **3** (Figures 1 and 2) shows that both the excitation and emission maxima are shifted to shorter wavelength in the galactoside spectra. These shifts in absorption and emission maxima are augmented by a reduction in the intrinsic fluorescence of the galactoside, which is estimated to be only approximately one-fourth that of the phenolate. As a result, with excitation at 385 nm and emission at 460 nm, the measured intensity of fluorescence of the phenol is higher than that of the unhydrolyzed galactoside by a factor of 620:1. This favorable ratio in combination with the narrow bandwidth interference filters currently available for fluorescence microscopy and multiwell plate readers should be sufficient for detection of the enzymatic hydrolysis of **5** within intact cells.

The ability of Im-DCH-β-Gal to serve as an in vitro substrate for lysosomal β-galactosidase was tested using cultured human skin fibroblasts as the enzyme source. The assay conditions were based upon those described by Ho and O'Brien[23] and Suzuki,[24] which are optimized for lysosomal β-galactosidase by virtue of a high chloride ion concentration in a pH 4.0 citrate/phosphate buffer. When Im-DCH-β-Gal, **5**, at a concentration of 0.12 mM was incubated with cell homogenate from normal skin fibroblasts, rapid hydrolysis to Im-DCH was observed. With four normal cell lines the rate of hydrolysis of **5** was 140 (±26 SD) nmol/h per mg cell protein. In comparison, the rate of hydrolysis of **5** by cell homogenates of fibroblasts from four patients with G_{M1} gangliosidosis was reduced to 3.4 (±1.5 SD) nmol/h per mg cell protein. This demonstrates that Im-DCH-β-Gal is an active substrate for human lysosomal β-galactosidase and can be efficiently used to identify patients who are deficient in this enzyme. Experiments to study its use as a fluroescent substrate within intact cells are in progress.

Experimental Section

Materials. Tetrabutylammonium hydroxide and 2,3-dicyanohydroquinone were from Aldrich Chemical Co., Milwaukee, WI; (acetobromo)-α-D-galactose and bovine serum albumin (Fraction V) were from Sigma Chemical Co., St. Louis, MO; 1,4-dibromobutane and sodium hydride were from Fluka USA, Ronkonkoma, NY; deuterated solvents were from MSD Isotopes, St. Louis, MO; acetonitrile containing less than 0.003% water was used directly as supplied by Burdick & Jackson (Baxter), Muskegon, MI; tetrahydrofuran was freshly distilled from sodium ketyl; TLC analysis was performed on silica gel plates (Polygram SilG/UV_{254}; Macherey-Nagel) Brinkmann Instruments, Westbury, NY. G_{M1} gangliosidoses cell lines (GM-3589, GM-2439, GM-5652A, and GM-5335) were obtained from the NIGMS Human Genetic Mutant Cell Repository, Camden, NJ. Control fibroblasts were established from skin biopsies obtained from healthy volunteers. McCoy's 5A medium and *l*-glutamine were from Biofluids, Rockville, MD. Fetal calf serum was from Hyclone Labs, Logan, UT.

Instrumentation. Fluorescence was measured on a Perkin-Elmer 204-A fluorescence spectrophotometer, using 10-nm slit widths, and 1 cm × 1 cm quartz cuvettes. Solvent blank values were subtracted from the sample readings, and the results were reported as uncorrected fluorescence. All fluorescence intensities in this report were measured at a consistent set of instrument parameters (photomultiplier gain of 1, sensitivity ratio of 0.3). Intensities were normalized to the fluorescence of quinine (5 mg/L in 0.1 N sulfuric acid) at excitation and emission wavelengths of 360 and 430 nm, respectively. Melting points are uncorrected.

***N*-(4-Bromobutyl)imidazolium Acetate Salt (2).** An 80% suspension of NaH (2.36 g, 1.05 equiv) was washed twice with THF under Ar. A solution of 5.1 g (75 mmol) of imidazole in 160 mL of THF was added, refluxed for 15 min, and then cooled to 20 °C. After the rapid addition of 48.4 g (3 equiv) of 1,4-dibromobutane, the thick mixture was stirred at 20 °C for 18 h. Acetic acid (8.6 mL, 2 equiv) was added, and the solvent was removed under vacuum. Unreacted dibromide was removed by addition of 100 mL of 10% HCl, and extraction with $CHCl_3$ (5 × 30 mL). The solution was then basified with solid Na_2CO_3 to pH 8, and the product was rapidly extracted with diethyl ether (5 × 75 mL). Acetic acid (4.3 mL, 1 equiv) was added to the first ether extract, and subsequent extracts were pooled with it. The solvent was removed under vacuum, affording 10.6 g of product as a colorless oil. NMR analysis revealed the presence of 1.8 equiv of acetate per mole of product, and confirmed a 45% yield of (bromobutyl)imidazole product. The remainder of the starting imidazole had been converted into byproducts of higher polarity which were not extractable into diethyl ether. Storage of *N*-(4-bromobutyl)imidazole as the free base resulted in the rapid formation of further quantities of these byproducts. In contrast, no decomposition of the acetate salt was detected after several months at −20 °C. TLC: $CHCl_3/MeOH/H_2O$, 80:10:1, R_f = 0.40, acetate salt; R_f = 0.52, free amine. ^1H NMR (DMSO-d_6 = 2.49 ppm): 7.67 (1 H, br s, Ar), 7.17 (1 H, d, J = 1.0 Hz, Ar), 6.91 (1 H, br s, Ar), 4.00 (2 H, t, J = 6.4 Hz, CH_2N), 3.51 (2 H, t, J = 6.4 Hz, CH_2Br), 1.90 (3 H, s, CH_3CO_2), 1.8 (4 H, mult, CH_2CH_2). ^{13}C NMR (DMSO-d_6 = 39.5 ppm): 171.99, 137.13, 128.01, 119.30, 45.13, 34.18, 29.26, 29.16, 21.04. IR: (neat, NaCl plate) 3130, 2940, 1715, 1550, 1370, 1250, 1080, 1005 cm^{-1}. UV (0.3 mM in EtOH): 211 nm, $\epsilon = 4.6 \times 10^3$ M^{-1} cm^{-1}.

2,3-Dicyano-4-[4-(1-imidazoyl)butoxy]phenol Hydrochloride Salt (3). A solution of 4.5 g of the acetate salt of *N*-(4-bromobutyl)imidazole (14.5 mmol, containing 15% wt/wt excess of acetic acid) in 12 mL of dimethylformamide (DMF) was added over 40 min to a degassed suspension of anhydrous K_2CO_3 (19.9 g) in 200 mL of 10% acetone in DMF containing 6.92 g (43 mmol) of 2,3-dicyanohydroquinone. The resulting thick mixture was stirred under N_2 at 20 °C for 18 h, after which the solvents were removed under vacuum (40 °C, 1 mmHg). Water (50 mL) was added, followed by sufficient acetic acid (ca. 35 mL) to solubilize the product. After filtration through Celite, the filtrate was brought to pH 7.5 with concentrated NH_4OH to precipitate the product. Filtration and vacuum drying provided 10.3 g of yellow solids which contained the desired product. A 2.0-g portion of this crude product was heated with 20 mL of 1 M HCl and extracted while warm with EtOAc (3 × 25 mL). Upon cooling, the aqueous phase deposited crystals which were collected and washed with two 2-mL portions of cold 1.5 M HCl. Vacuum drying provided 0.47 g of product as white needles, mp 250–54 °C (calculated yield, 35%). Recrystallization from 1 M HCl sharpened the mp to 256–257.5 °C. TLC: R_f = 0.14 in $CHCl_3/MeOH/H_2O$, 80:10:1; R_f = 0.49 in $CHCl_3/MeOH/H_2O$, 65:25:4. ^1H NMR (HCl salt in 0.1 M DCl/D_2O, DSS = 0 ppm): 8.76 (1 H, br s, Imid), 7.55 (1 H, t, J = 1.7 Hz, Imid), 7.47 (1 H, t, J = 1.7 Hz, Imid), 7.35 (1 H, d, J = 9.6 Hz, Ar), 7.26 (1 H, d, J = 9.6 Hz, Ar), 4.36 (2 H, t, J = 7.0 Hz, CH_2O), 4.18 (2 H, t, J = 5.8 Hz, CH_2N), 2.13 (2 H, p, J = 7.3 Hz, CH_2CH_2O), 1.8 (2 H, mult, CH_2CH_2N). IR (4% in KBr): 3050, 2240, 1577, 1545, 1500, 1450, 1290, 1075, 988 cm^{-1}. UV (0.4 mM in 0.1 M sodium phosphate buffer, pH = 2): 347, $\epsilon = 5.4 \times 10^3$; 275 nm, $\epsilon = 1.0 \times 10^3$. (pH = 12): 380, $\epsilon = 5.7 \times 10^3$; 270 nm, $\epsilon = 8.6 \times 10^2$. Anal. $C_{15}H_{15}ClN_4O_2$ Calcd (Found): C, 56.52 (56.27); H, 4.74 (4.73); N, 17.58 (17.41).

Tetrabutylammonium Salt of 2,3-Dicyano-4-[4-(1-imidazoyl)butoxy]phenol. A suspension of 250 mg (0.78 mmol) of the hydrochloride salt, **3**, in 4.3 mL of 0.2 M sodium hydroxide (1.1 equiv) was added to 2 mL of $CHCl_3$ and 0.56 g (1.1 equiv) of 40% (wt/wt) aqueous tetrabutylammonium hydroxide. After extraction of the fluorescent tetrabutylammonium salt into the $CHCl_3$, the aqueous phase was re-extracted with five 2-mL portions of fresh $CHCl_3$. Drying (Na_2SO_4) and evaporation of $CHCl_3$ provided 489 mg of the strongly fluorescent salt as a yellow oil. This material was used within 24 h in the subsequent alkylation reaction. TLC: R_f = 0.14 in $CHCl_3/MeOH/H_2O$, 80:10:1. ^1H NMR ($N(Bu)_4$ salt in $CDCl_3$, TMS = 0 ppm): 7.50 (1 H, br s), 7.03 (1 H, br s), 6.97 (1 H, br s), 6.87 (1 H, d, J = 9.6 Hz), 6.80 (1 H, d, J = 9.6 Hz), 4.06 (2 H, t, J = 7.1 Hz), 3.93 (2 H, t, J =

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5.9 Hz), 2.03 (2 H, p, J = 7.3 Hz), 1.7 (2 H, m, J = 6 Hz). Counterion resonances: 3.26 (8 H, br t, J = 8.0 Hz), 1.66 (8 H, mult), 1.43 (8 H, 6 lines, J = 7.1 Hz), 0.98 (12 H, t, J = 7.1 Hz). ^{13}C NMR ($N(Bu)_4$ salt, $CDCl_3$ = 77 ppm): 168.20, 148.69, 137.02, 129.27, 128.30, 121.97, 118.97, 118.77, 115.68, 102.94, 97.93, 70.50, 46.63, 27.84, 26.19. Counterion resonances: 58.82, 23.86, 19.60, 13.51.

2,3-Dicyano-1-hydroxy-4-[4-(1-imidazoyl)butoxy]phenyl β-D-Galactopyranoside 2',3',4',6'-Tetra-*O*-acetate (4). The tetrabutylammonium salt of the starting material 3 (0.49 g, 0.8 mmol) was dissolved in 50 mL of CH_3CN containing 4 g of 3A molecular sieves under an atmosphere of dry N_2. Freshly prepared[25] Ag_2CO_3 (0.43 g, 2 equiv) was added, followed by 0.64 g of α-bromogalactose tetraacetate (2 equiv). After 18 h at 20 °C the suspension was filtered through Celite. The filter cake was washed with four 6-mL portions of $CHCl_3$, which were combined with the CH_3CN and evaporated to dryness. The resulting 1.2 g of dark oil was purified by flash chromatography (silica gel, 3 × 23 cm, $CHCl_3$/MeOH, 80:5), and the fractions between 130 and 270 mL were evaporated to give 0.42 g of the galactoside (88% yield) as a slowly crystallizing oil. Recrystallization from ethanol provided 255 mg (53% yield) of white crystals. This material undergoes a phase change with partial melting at 115 °C, recrystallizes at higher temperatures, and displays a sharp mp at 169.5–170 °C. This behavior was not changed upon subsequent recrystallizations from ethanol. TLC: R_f = 0.36 in $CHCl_3$/MeOH/H_2O, 80:10:1; R_f = 0.73 in $CHCl_3$/MeOH/H_2O, 65:25:4. ^{1}H NMR ($CDCl_3$, TMS = 0 ppm): 7.52 (2 H, d, J = 9.4 Hz, Ar), 7.50 (1 H, br s, Im), 7.10 (2 H, d, J = 9.4 Hz, Ar), 7.06 (1 H, t, J = 1 Hz, Im), 6.96 (1 H, t, J = 1 Hz, Im), 5.54 (1 H, dd, J = 10.4, 7.9 Hz, G2), 5.47 (1 H, dd, J = 3.4, 0.9 Hz, G4), 5.10 (1 H, dd, J = 10.4, 3.4 Hz, G3), 4.97 (1 H, d, J = 7.9 Hz, G1), 4.3–4.0 (3 H, mult, G5 and G6), 4.08 (2 H, t, J = 7 Hz, OCH_2), 4.05 (2 H, t, J = 5 Hz, NCH_2), 2.20 (3 H, s, Me), 2.16 (3 H, s, Me), 2.06 (3 H, s, Me), 2.05 (2 H, mult, CH_2), 2.02 (3 H, s, Me), 1.84 (2 H, mult, CH_2). ^{13}C NMR ($CDCl_3$ = 77 ppm): 170.21, 170.03, 170.00, 169.41, CH_3CO_2; 157.28, 152.16, Ar1 and 4; 137.08, Im2; 129.75, 118.69, Im4 and 5; 125.50, 117.88, Ar5 and 6; 112.50, 111.91, ArCN; 108.78, 105.30, Ar2 and 3; 101.43, G1; 71.54, 70.40, G5 and 3; 69.50, $ArOCH_2$; 67.76, 66.62, G2 and 4; 61.11, G6; 46.57, NCH_2; 27.57, 25.98, CH_2CH_2; 20.85, 20.70, 20.67, 20.58, CH_3CO_2. IR (4% in KBr): 3440, 2940, 2230, 1755, 1487, 1370, 1240, 1065, 950, 905, 823, 740 cm^{-1}. UV (30 μM in EtOH): 334, ε = 6.9 × 10^3; 235, ε = 9.9 × 10^3; 220 nm, ε = 3.8 × 10^4. Fluorescence: 1 μM in EtOH; maxima, Exc = 345 nm, Em = 385 nm. Anal. $C_{29}H_{32}N_4O_{11}$ Calcd (Found): C, 56.86 (56.60); H, 5.26 (5.26); N, 9.15 (9.05).

2,3-Dicyano-1-hydroxy-4-[4-(1-imidazoyl)butoxy]phenyl β-D-Galactopyranoside (5). To a solution of 0.19 g (0.31 mmol) of Im-DCH-β-Gal(OAc)$_4$, 4, in 5 mL of warm MeOH was added 62 μL of 2.5 M NaOMe in MeOH (0.16 mmol). Product began to precipitate within 5 min, and the reaction was quenched with 10 μL of glacial acetic acid (0.17 mmol) after 2 h. Removal of solvent under vacuum afforded the crude product as a yellow solid, mp 171–2 °C. Recrystallization from MeOH provided 84 mg (60% yield) of Im-DCH-β-Gal, mp 190–192 °C. Further recrystallization from ethanol sharpened the mp to 193–194.5 °C. TLC: R_f = 0.03 in $CHCl_3$/MeOH/H_2O, 80:10:1. R_f = 0.20 in $CHCl_3$/MeOH/H_2O, 65:25:4. ^{1}H NMR ($CDCl_3$/CD_3OD, 1:1, TMS = 0 ppm): 7.62 (1 H, s, Im), 7.60 (1 H, d, J = 9.5 Hz, Ar), 7.34 (1 H, d, J = 9.5 Hz, Ar), 7.09 (1 H, s, Im), 6.99 (1 H, s, Im), 4.96 (1 H, d, J = 7.6 Hz, G1), 4.15 (2 H, t, J = 5.5 Hz, NCH_2), 4.12 (2 H, t, J = 7.1 Hz, OCH_2), 3.94 (1 H, br d, J = 2.4 Hz, G4), 3.9–3.8 (3 H, mult, G3 and G6), 3.68 (1 H, mult, G5), 3.59 (1 H, dd, J = 9.6, 3.4 Hz, G2), 2.07 (2 H, mult, CH_2), 1.86 (2 H, mult, CH_2). ^{13}C NMR (0.04 M DCl in D_2O, CH_3OD = 49 ppm): 156.69, 152.82, Ar1 and 4; 134.46, Im2; 123.37, 120.46, Ar5 and 6; 121.84, 119.86, Im4 and 5; 114.11, 113.78, ArCN; 104.58, 102.98, Ar2 and 3; 101.29, G1; 75.79, 72.56, G5 and 3; 70.26, 68.43, G2 and 4; 69.76, OCH_2; 60.89, G6; 49.12, NCH_2; 26.34, 25.00, CH_2CH_2. IR (4% in KBr): 3390, 2235, 1653, 1578, 1493, 1398, 1282, 1080 cm^{-1}. UV (30 μM in pH 7 sodium phosphate buffer): 339, ε = 6.5 × 10^3; 241, ε = 9.9 × 10^3; 225 nm, ε = 3.6 × 10^4. Anal. $C_{21}H_{24}N_4O_7$ Calcd (Found): C, 56.75 (56.65); H, 5.44 (5.50); N, 12.61 (12.35).

Enzyme Assays. G_{M1} gangliosidoses cell lines (GM-3589, GM-2439, GM-5652A, and GM-5335) and control fibroblasts were cultured at 37 °C in 5% CO_2 in McCoy's 5A medium supplemented with 10% fetal calf serum and 2 mM *l*-glutamine. For enzyme assays, postconfluent cultures were harvested by trypsinization. The cells were resuspended in media in order to neutralize the trypsin, centrifuged at 500*g* and then washed twice in cold phosphate buffered saline. Cell pellets were either used immediately or frozen at −70 °C for up to 30 days. The cells were lysed by suspension in distilled H_2O (~300 μL for 400 μg of protein) followed by disruption (two 5-s pulses) with a Sonifier-W140 sonicator. Cell homogenates were kept at 0 °C and used within 4 h. A 0.12 mM solution of Im-DCH-β-Gal, 5, was prepared in pH 4 citrate/phosphate buffer (0.1 M citric acid titrated with 0.2 M Na_2HPO_4). Assays were started by the addition of 20 μL of cell homogenate to a 37 °C mixture of 200 μL of substrate solution, 100 μL of 0.2 M NaCl, and 80 μL of H_2O. Controls were performed by substituting 20 μL of H_2O for the cell homogenate. After 30 or 60 min at 37 °C, the incubations were terminated by the addition of 2.6 mL of a 1:1 mixture of 0.1 M glycine and 0.1 M NaOH. The fluorescence of 3 was measured at 460 nm with excitation at 385 nm. Proteins were determined by the method of Lowry,[26] using bovine serum albumin standards.

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