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Novel ^{19}F-MRS β-Galactosidase Reporter Molecules Incorporated Nitrogen Mustard Analogues

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Abstract

In this paper, we propose a novel molecular platform integrated fluorinated antitumor nitrogen mustards for ^{19}F-MRS assay of β-galactosidase (β-gal) activity. Following this idea, we have designed, synthesized and characterized 2-fluoro-4-[bis(2'-chloroethyl)amino]phenyl β-*D*-galactopyranoside **5**, 2-fluoro-4-{bis[2'-*O*-(β-*D*-galactopyranosyl)ethyl]amino}phenyl β-*D*-galactopyranoside **8**, 2-fluoro-4-{bis[[1''-(β-*D*-galactopyranosyl)-1'', 2'', 3''-triazol-4''-yl]methyl] amino}phenyl β-*D*-galactopyranoside **14** and 2-fluoro-4-{bis[[1''-(β-*D*-glucopyranosyl)-1'', 2'', 3''-triazol-4''-yl]methyl]amino}phenyl β-*D*-galactopyranoside **15** through glycosylation and click reaction strategies, and their structures were confirmed by NMR and HRMS or elemental analysis data. Among of them, 2-fluoro-4-[bis(2'-chloroethyl)amino]phenyl β-*D*-galacto-pyranoside **5** was found very sensitive to β-gal (E801A) in PBS at 37°C with big $\Delta\delta_F$ response. Here, we demonstrated the feasibility of this platform for assessing β-gal activity in solution, and *in vitro* with *lacZ* transfected human MCF7 breast and PC3 prostate tumor cells, by the characterization of β-gal responsive ^{19}F-chemical shift changes $\Delta\delta_F$ and hydrolytic kinetics.

Keywords

β-Galactosidase detection, ^{19}F-MRS *lacZ* gene reporter, Prodrug, Nitrogen mustard, Synthesis.

INTRODUCTION

^{19}F nucleus has a high gyromagnetic ratio (γ=40.05MHz/T), 100% natural isotopic abundance, large chemical-shift dispersion. Importantly, there are essentially no intrinsic ^{19}F signals detectable in living tissue and thus no interferences from background signals.[1,2] Therefore, ^{19}F-magnetic resonance spectroscopy (MRS) has been widely applied in *in vivo* studies on drug absorption, distribution, metabolism and excretion by reasons of its favorable MR properties, simplicity and high sensitivity.[3] We have developed a series of ^{19}F-MRS β-galactosidase (β-gal) reporters with structures exploiting fluorinated/trifluoromethylated phenols, fluoropyridoxine and fluorinated Fe-chelator aglycones, and demonstrated the success for assessing β-gal activity in solution, *in vitro* with *lacZ* transfected tumor cells, and *in vivo* in human tumor xenografts growing in mice.[4-6] In this paper, we propose a novel molecular platform integrated fluorinated antitumor nitrogen mustards for ^{19}F-MRS detection of β-gal activity.

RESULTS AND DISCUSSION

Design and Synthesis Nitrogen mustards are widely used in the treatment of cancers.[7-9] But, their efficacies for chemotherapy are mainly limited by bone marrow toxicity and genotoxicity,[10] insufficient drug concentrations in tumors, and lack of selectivity for neoplastic cells.[7] The promising solution to these problems is to use targeting strategies such as antibody-directed enzyme prodrug therapy and gene-directed enzyme prodrug therapy. As demonstrated, a number of clinical trials using suicide gene therapy are ongoing.[11,12]

The *lacZ* gene encoding β-gal has been recognized as the standard means of assaying clonal insertion, transcriptional activation, protein expression and interaction in molecular/ cellular biology, small animal investigations, and clinical trials.[13-16] So when using fluorinated nitrogen mustards[17,18] as aglycones, their β-*D*-galactosides would work as prodrugs. Upon delivery and cleavage at the *lacZ*

transfected tumor, the fluorinated antitumor nitrogen mustards will be *lacZ* (or β-gal)-specifically released, in which the companied ^{19}F chemical shift changes and hydrolytic kinetics will recognize the presence and progression of *lacZ* (or β-gal) , offering a potential for simultaneously monitoring of cancer therapy. Moreover, this probe design is based on the *lacZ* gene-directed enzyme prodrug strategy, in which the prodrug proceeds in the β-gal responsive way to selectively convert into a toxic drug at the tumor, such localized activation will then reduce the normal tissue toxicity associated with conventional cytotoxic chemotherapy of nitrogen mustards as reported.[10]

The synthesis started with 2-fluoro-4-nitrophenyl 2', 3', 4', 6'-tetra-*O*-acetyl-β-*D*-galactopyranoside **1**, which was readily obtained in excellent yield (99%) by phase-transfer catalyzed approach using tetrabutylammonium bromide (TBAB) in CH_2Cl_2-H_2O (pH 8-9) at 50°C.[4a] Hydrogenation of **1** in ethanolic solution catalyzed by 5% Pd/C converted to the corresponding 2-fluoro-4-aminephenyl 2', 3', 4', 6'-tetra-*O*-acetyl-β-*D*-galactopyranoside **2** in quantitative yield. Treatment of **2** with ethylene oxide in AcOH/benzene (1:5 V/V') from 0°C to room temperature gave 2-fluoro-4-[bis(2'-hydroxyethyl)amino]phenyl 2'', 3'', 4'', 6''-tetra-*O*-acetyl-β-*D*-galactopyranoside **3** in 85% yield. The direct chlorination of **3** with an excess amount of $SOCl_2$ in CH_2Cl_2 obtained 2-fluoro-4-[bis(2'-chloroethyl)amino]phenyl 2', 3', 4', 6'-tetra-*O*-acetyl-β-*D*-galactopyranoside **4** in 77% yield, which was then deacetylated with NH_3/MeOH from 0°C to room temperature, giving the target molecule 2-fluoro-4-[bis(2'-chloroethyl)amino]phenyl β-*D*-galactopyranoside **5** in excellent yield (**Figure 1**). The structures of **1**-**5** were characterized by NMR and HRMS or elemental analysis data. Their anomeric β-*D*-configuration in the $^4C_1$ chair conformation was confirmed by the ^1H-NMR chemical shifts of the anomeric protons at $\delta_{H\text{-}1'}/\delta_{H\text{-}1''}$=4.60-5.15ppm, and the coupling constants of $J_{1',2'}/J_{1'',2''}$= ~8Hz and $J_{2',3'}/J_{2'',3''}$=~10Hz, as well as the anomeric carbon resonances appeared at $\delta_{C\text{-}1'}/\delta_{C\text{-}1''}$ =100.20-103.00ppm.[4-6]

Carbohydrate-associated prodrugs in clinical applications have showed improved water solubility and permeability, leading to better selectivity and efficacy of chemotherapy.[19] For these reasons, we sought to introduce additional carbohydrate units to the nitrogen mustard. Condensation of **3** directly with 1.3 equivalents of 2, 3, 4, 6-tetra-*O*-acetyl-α-*D*-galactopyranosyl bromide in anhydrous CH_2Cl_2/MeCN catalyzed by $Hg(CN)_2$ as a promoter, furnished the 2-fluoro-4-{bis[2'-*O*-(2'', 3'', 4'', 6''-tetra-*O*-acetyl-β-*D*-galactopyranosyl)ethyl] amino}phenyl 2''', 3''', 4''', 6'''-tetra-*O*-acetyl-β-*D*-galactopyranoside **7** in 89% yield. The HRMS displayed the expected quasimolecular ions $[M+H]^+$ at m/z 1206.3852 (calculated m/z 1206.3889) and $[M+Na]^+$ at m/z 1228.3660 (calculated m/z 1228.3708), corresponding to the fully adorned derivative with additional two 2, 3, 4, 6-tetra-*O*-acetyl-β-*D*-galactopyranosyl units, in which the β-*D*-configuration linkages were established based on the anomeric protons H-1'' of *D*-galactoses at $\delta_{H\text{-}1''}$=4.50ppm with the well resolved doublets $J_{1'',2''}$=8.0Hz and $J_{2'',3''}$=10.6 Hz, whereas the anomeric carbons C-1'' at $\delta_{C\text{-}1''}$=101.13ppm are in accordance. Subsequent deacetylation of **7** with NH_3/MeOH from 0°C to room temperature gave the extra two β-*D*-galactopyranosyl-associated molecule 2-fluoro-4-{bis[2'-*O*-(β-*D*-galactopyranosyl)ethyl]amino} phenyl β-*D*-galactopyranoside **8** in excellent yield (95%). However, similar glycosylation with 2, 3, 4, 6-tetra-*O*-acetyl-α-*D*-glucopyranosyl bromide for the synthesis of 2-fluoro-4-{bis[2'-*O*-(2'', 3'', 4'', 6''-tetra-*O*-acetyl-β-*D*-glucopyranosyl)ethyl]amino}phenyl 2''', 3''', 4''', 6'''-tetra-*O*-acetyl-β-*D*-galactopyranoside **9** then to its corresponding free β-*D*-galactopyranoside **10** (**Figure 2**) was not successful, instead by recovering the starting material 2-fluoro-4-[bis(2'-hydroxyethyl) amino]phenyl 2', 3', 4', 6'-tetra-*O*-acetyl-β-*D*-galactopyranoside **3**.

'Click Chemistry' using Cu^I-catalyzed azide-alkyne cycloaddition reaction has been proven as one of the most powerful tools in drug discovery, chemical biology, and proteomic applications.[20] Inspired by these successes, we proposed another strategy to associate additional carbohydrate

units with the nitrogen mustard through the 1, 3, 4-triazole rings, hoping them together to participate in hydrogen-bonding and dipole interactions, thereby favoring molecular recognition processes and enzymatic reaction with β-gal.[20-22] Reaction of 2-fluoro-4-aminephenyl 2', 3', 4', 6'-tetra-*O*-acetyl-β-*D*-galactopyranoside **2** with propargyl bromide in DMF at 80°C for 3h gave 2-fluoro-4-[bis(propargyl)amino]phenyl 2'', 3'', 4'', 6''-tetra-*O*-acetyl-β-*D*-galactopyranoside **11** in 88% yield. The peracetylated β-*D*-galacto- and glucopyranosyl azides were prepared according to the previously described method.[23] Click reaction of alkyne **11** with azides catalyzed by $CuSO_4$/NaAsc in DMF-H_2O at room temperature for 12h afforded 2-fluoro-4-{bis[[1''-(2''', 3''', 4''', 6'''-tetra-*O*-acetyl-β-*D*-galactopyranosyl)-1'', 2'', 3''-triazol-4''-yl]methyl]amino}phenyl 2'''', 3'''', 4'''', 6''''-tetra-*O*-acetyl-β-*D*-galactopyranoside **12** (82%) and 2-fluoro-4-{bis[[1''-(2''', 3''', 4''', 6'''-tetra-*O*-acetyl-β-*D*-glucopyranosyl)-1'', 2'', 3''-triazol-4''-yl]methyl]amino}phenyl 2'''', 3'''', 4'''', 6''''-tetra-*O*-acetyl-β-*D*-galactopyranoside **13** (85%), respectively. After workup and deacetylation, the desired molecules 2-fluoro-4-{bis[[1''-(β-*D*-galactopyranosyl)-1'', 2'', 3''-triazol-4''-yl]methyl]amino}phenyl β-*D*-galactopyranoside **14** and 2-fluoro-4-{bis[[1''-(β-*D*-glucopyranosyl)-1'', 2'', 3''-triazol-4''-yl]methyl]amino}phenyl β-*D*-galactopyranoside **15** were obtained in 91-94% yields (**Figure 3**). The structures of **11**-**15** were characterized by NMR and HRMS data. The expected quasimolecular ions of **12**: $[M+H]^+$ at m/z 1280.4073 (calculated m/z 1280.4018), $[M+Na]^+$ at m/z 1302.3614 (calculated m/z 1302.3838), $[M+K]^+$ at m/z 1318.1919 (calculated m/z 1318.3577); **13**: $[M-H]^+$ at m/z 1278.4196 (calculated m/z 1278.3862), $[M-H+Na]^+$ at m/z 1301.3931 (calculated m/z 1301.3759); **14**/**15**: $[M+H]^+$ at m/z 776.2817/776.2834 (calculated m/z 776.2750), $[M+Na]^+$ at m/z 798.3064/798.3345 (calculated m/z 798.2570), $[M+K]^+$ at m/z 814.2819/814.2741 (calculated m/z 814.2309), are matching to the fully ornamented derivatives with additional two 2, 3, 4, 6-tetra-*O*-acetyl-β-*D*-galactopyranosyl (→**12** and **14**) or 2, 3, 4, 6-tetra-*O*-acetyl-β-*D*-glucopyranosyl (→**13** and **15**) units by the fused 1, 3, 4-triazole

linkers, in which the β-*D*-configuration linkages of *N*-glycosides were established based on the anomeric protons H-1''' of *N*-galacosides (→**12** and **14**) and *N*-glucosides (→**13** and **15**) at $\delta_{H\text{-}1'''}$=5.40-5.90ppm with the well resolved doublets $J_{1''',2'''}$=8.0Hz, whereas the anomeric carbons C-1''' at $\delta_{C\text{-}1'''}$=85.90-88.20ppm are in agreement.[24] In particular, the regioselectivity of the formation 1,4-disubstituted 1, 3, 4-triazoles in **12**-**15** was confirmed by the $\delta_{C\text{-}4''}$, $\delta_{C\text{-}5''}$ and $\Delta(\delta_{C\text{-}4''}\text{-}\delta_{C\text{-}5''})$ values [**12**: $\delta_{C\text{-}4''}$=145.34ppm, $\delta_{C\text{-}5''}$=120.81ppm, $\Delta(\delta_{C\text{-}4''}\text{-}\delta_{C\text{-}5''})$=24.53ppm; **13**: $\delta_{C\text{-}4''}$=145.77ppm, $\delta_{C\text{-}5''}$= 120.82ppm, $\Delta(\delta_{C\text{-}4''}\text{-}\delta_{C\text{-}5''})$=24.95ppm; **14**: $\delta_{C\text{-}4''}$=144.27ppm, $\delta_{C\text{-}5''}$=122.00ppm, $\Delta(\delta_{C\text{-}4''}\text{-}\delta_{C\text{-}5''})$= 22.27ppm; **15**: $\delta_{C\text{-}4''}$=144.14ppm, $\delta_{C\text{-}5''}$=122.45ppm, $\Delta(\delta_{C\text{-}4''}\text{-}\delta_{C\text{-}5''})$=21.69ppm] in the ranges of $\delta_{C\text{-}4''}$=132.00-148.00ppm, $\delta_{C\text{-}5''}$=120.00-128.00ppm, $\Delta(\delta_{C\text{-}4''}\text{-}\delta_{C\text{-}5''})$=21.00-27.00ppm, which are the typical characteristics for the identification of the 1,4-disubstituted 1,3,4-triazole structures.[20,25]

β-Gal Activity The reactivity of **5**, **8**, **14** and **15** to β-gal was evaluated by kinetic ^{19}F-MRS.[4-6] **5**, **8**, **14** and **15** in PBS (0.1M, pH=7.4) at 37°C each gave a narrow ^{19}F-signal at δ_F=-55.65, -56.29, -56.85 and -56.78ppm, respectively. Following the reaction with β-gal (E801A) in PBS (0.1M, pH=7.4) at 37°C, the kinetic ^{19}F-MRS measurements of ^{19}F chemical shift and signal intensity changes at each time point (0-40min) indicated that only **5** converted in the high hydrolytic rate v=738.4μM/min/unit to the fluorinated nitrogen mustard 2-fluoro-4-[bis(2'-chloroethyl)amino]phenol (**2F4ClPh**) (δ_F=-59.44ppm). **8**, **14** and **15** however are not reactive to β-gal as expected at the same condition, keeping their ^{19}F-signals unchanged over the period of 60min. Meanwhile, we found that the liberated fluorinated nitrogen mustard 2-fluoro-4-[bis(2'-chloroethyl)amino]phenol (**2F4ClPh**) was unstable and slowly hydrolyzed with H_2O by displacement of the chlorine atoms in the rate v=378.0μM/min to the dialcohol derivative 2-fluoro-4-[bis(2'-hydroxyethyl)amino]phenol (**2F4OHPh**) (δ_F=-44.33 ppm) (**Figure 4**). This observation was further confirmed by monitoring the reaction of 2-fluoro-4-[bis(2'-hydroxyethyl)amino]phenyl β-*D*-galactopyranoside **6** (δ_F=-55.95ppm) with β-gal at the

same condition, with the direct releasing of 2-fluoro-4-[bis(2'-hydroxyethyl)amino]phenol (**2F4OHPh**) at δ_F=-44.33ppm. **Figure 5** showed the kinetic hydrolysis time courses of 2-fluoro-4-[bis(2'-chloroethyl)amino]phenyl β-*D*-galactopyranoside **5** with β-gal (E801A) in PBS (0.1M, pH=7.4) at 37°C.

***In vitro* Assay** Further *in vitro* evaluation of **5** with the *lacZ* transfected human MCF7 breast and PC3 prostate cancer cells in PBS (0.1M, pH=7.4) at 37°C under 5% CO_2 in air with 95% humidity, using wild type (WT) MCF7 and PC3 cancer cells at the same conditions as the controls, ^{19}F-MRS analysis of the mixtures at different time points (0-180min) exhibited a single narrow ^{19}F-signal of **5** (δ_F=-55.65ppm) at the starting point (0min, or with MCF7-WT and PC3-WT for 3h as controls), and a new single ^{19}F-peak of the fluorinated nitrogen mustard 2-fluoro-4-[bis(2'-chloroethyl)amino] phenol (**2F4ClPh**) at δ_F=-59.44ppm progressively grown with the time going in the rates of v=6.14 (with MCF7-*lacZ* cells) and 2.30 (with PC3-*lacZ* cells) μM/min/per million cells, respectively. However, incubation of **5** with the equal numbers of lysed MCF7-*lacZ* and PC3-*lacZ* cells showed the rates of conversion were 1.5 (with lysed MCF7-*lacZ* cells) and 11.1 (with lysed PC3-*lacZ* cells) times higher than the intact cells, respectively. These differences between intact and lysed cells suggest that membrane permeation is a bigger obstruction for **5** to reaction of β-gal in PC3-*lacZ* cells than in MCF7-*lacZ* cells, due to the cell membranes of human MCF7-*lacZ* breast and PC3-*lacZ* prostate cancer cells have different selective permeabilities.[26]

Cytotoxicity The cytotoxicity of prodrug **5** was examined with both wild-type and *lacZ* expressing MCF7 and PC3 cells in 24h incubation at 37°C under 5% CO_2 in air with 95% humidity. Prodrug **5** showed neither toxicity to MCF7- nor PC3-WT cells, for prodrug **5** viability exceeded 98% for both MCF7- and PC3-WT cells at all concentrations tested. To MCF7- and PC3-*lacZ* cells, prodrug **5** exhibited less cytotoxicity but in different behaviors, for MCF7-*lacZ* cells survival was much more decreased to 65% at 1mM, whereas for PC3-*lacZ* cells survival was >92% at the same concentration

(**Figure 6**). This observation was in good agreement with the differences of selective permeability between MCF7 breast and PC3 prostate cancer cells.

CONCLUSION

We present here a novel molecular platform integrated fluorinated antitumor nitrogen mustards for ^{19}F-MRS detection of β-gal activity. Following this design, we have successfully synthesized carbohydrate-associated prodrugs 2-fluoro-4-[bis(2'-chloroethyl)amino]phenyl β-*D*-galactopyranoside **5**, 2-fluoro-4-{bis[2'-*O*-(β-*D*-galactopyranosyl)ethyl] amino}phenyl β-*D*-galactopyranoside **8**, 2-fluoro-4-{bis[[1''-(β-*D*-galactopyranosyl)-1'', 2'', 3''-triazol-4''-yl]methyl] amino}phenyl β-*D*-galactopyranoside **14** and 2-fluoro-4-{bis[[1''-(β-*D*-glucopyranosyl)-1'', 2'', 3'' -triazol-4''-yl]methyl]amino}phenyl β-*D*-galactopyranoside **15** through glycosylation and click reaction strategies. Among of them, 2-fluoro-4-[bis(2'-chloroethyl)amino]phenyl β-*D*-galactopyranoside **5** was found very sensitive to β-gal (E801A) in PBS at 37°C in the rate v=738.4μM/min/unit and $\Delta\delta$ response ($\Delta\delta$=~4ppm). Herein we demonstrated the feasibility of this platform for assessing β-gal activity in solution, and *in vitro* with *lacZ* transfected human MCF7 breast and PC3 prostate tumor cells, by the characterization of β-gal responsive ^{19}F-chemical shift changes $\Delta\delta_F$ and hydrolytic kinetics.

EXPERIMENTAL

General Methods NMR spectra were recorded on a Varian Unity INOVA 400 spectrometer (400MHz for ^{1}H, 100MHz for ^{13}C, 376 MHz for ^{19}F), ^{1}H and ^{13}C chemical shifts are referenced to TMS as internal standard with $CDCl_3$, or DMSO-d_6 as solvents, ^{19}F to a dilute solution of NaTFA in a capillary as external standard, chemical shifts are given in ppm. All compounds were characterized by NMR at 25°C. Microanalyses were performed on a Perkin-Elmer 2400CHN microanalyser. Mass spectra were obtained by positive and negative ESI-MS using a Micromass Q-TOF hybrid quadrupole/time-of-flight instrument (Micromass UK Ltd).

Solutions in organic solvents were dried with anhydrous Na_2SO_4, and concentrated *in vacuo* below 45°C. $Hg(CN)_2$ was dried before use at 50°C for 1h, CH_2Cl_2 was dried over Drierite, acetonitrile was dried on CaH_2 and kept over molecular sieves under nitrogen. 2, 3, 4, 6-Tetra-*O*-acetyl-α-*D*-galactopyranosyl bromide and 2, 3, 4, 6-tetra-*O*-acetyl-α-*D*-glucopyranosyl bromide were purchased from the Sigma Chemical Company. β-Gal (E801A) was purchased from Promega (Madison, WI, USA) and enzymatic reactions were performed at 37°C in PBS solution (0.1M, pH=7.4). Column chromatography was performed on silica gel (200-300mesh) and silica gel GF_{254} used for analytical TLC was purchased from the Aldrich Chemical Company. Detection was effected by spraying the plates with 5% ethanolic H_2SO_4 (followed by heating at 110°C for 10mins.) or by direct UV illumination of the plate. The purity of the final products was determined by HPLC with ≥ 95%.

2-Fluoro-4-nitrophenyl 2', 3', 4', 6'-tetra-*O*-acetyl-β-*D*-galactopyranoside 1 A solution of 2, 3, 4, 6-tetra-*O*-acetyl-α-*D*-galactopyranosyl bromide (820 mg, 2.0 mmol) in CH_2Cl_2 (15 mL) was added dropwise to a vigorously stirred solution of 2-fluoro-4-nitrophenol (378 mg, 2.4 mmol, 1.2 equiv.) and tetrabutylammonium bromide (480 mg, 1.5 mmol) in H_2O (25 mL, pH 8-9) and CH_2Cl_2 (25 mL) at 50 °C in 3-neck round-bottom flask equipped with condenser and thermometer. After TLC showed complete reaction (~1 h) the organic layer was separated, washed with water, dried (Na_2SO_4), evaporated under reduced pressure and purified by column chromatography on silica gel to give **1** (1.0 g, 99%) as white crystals, R_f 0.35 (3**:**2 cyclohexane-EtOAc), δ_H ($CDCl_3$): 8.03 (2H, m, Ar-H), 7.32 (1H, m, Ar-H), 5.13 (1H, d, $J_{1',2'}$ = 7.8 Hz, H-1'), 5.58 (1H, dd, $J_{2',3'}$ = 10.2 Hz, H-2'), 5.15 (1H, dd, $J_{3',4'}$ = 3.6 Hz, H-3'), 5.40 (1H, d, $J_{4',5'}$ = 3.6 Hz, H-4'), 4.10 (1H, dd, $J_{5',6a'}$ = 4.5 Hz, $J_{5',6b'}$ = 5.0 Hz, H-5'), 4.26 (1H, dd, $J_{6a',6b'}$ = 11.0 Hz, H-6a'), 4.19 (1H, dd, H-6b'), 2.21, 2.11, 2.08, 2.04 (12H, 4s, 4×CH_3CO) ppm; δ_C ($CDCl_3$): 170.45, 170.26, 170.21, 169.44 (4s, 4×CH_3**C**O), 152.23 (d, J_{F-C} = 252.0 Hz, Ar-C), 150.07 (d, J_{F-C} = 10.6 Hz, Ar-C), 120.51 (d, J_{F-C} = 9.2 Hz, Ar-C), 118.53 (d, J_{F-C} = 11.7 Hz,

Ar-C), 113.11 (d, $J_{F\text{-}C}$ = 23.7 Hz, Ar-C), 100.28 (d, $J_{F\text{-}C}$ = 3.1 Hz, C-1'), 68.34 (s, C-2'), 70.57 (s, C-3'), 66.81 (s, C-4'), 71.83 (s, C-5'), 61.45 (s, C-6'), 20.80, 20.77, 20.72, 20.33 (4s, 4×**C**H_3CO) ppm; HRMS: $[M+Na]^+$, $C_{20}H_{22}NO_{12}FNa$, Calcd: 510.1024, Found: 510.1014; $[M+K]^+$, $C_{20}H_{22}NO_{12}FK$, Calcd: 526.0763, Found: 526.0751.

2-Fluoro-4-aminophenyl 2', 3', 4', 6'-tetra-*O*-acetyl-β-*D*-galactopyranoside 2 Hydrogenation (H_2, 30 psi) of **1** (1.12 g, 2.30 mmol) in anhydrous EtOH (80 mL) catalyzed by Pd/C (5%, 200 mg) at room temperature overnight furnished the amine derivative **2** (1.05 g, 100%) as white foam solid, R_f 0.30 (1**:**1 cyclohexane-EtOAc), δ_H ($CDCl_3$): 7.00 (1H, t, *J* = 8.8 Hz, Ar-H), 6.41 (1H, dd, *J* = 2.8, 12.4 Hz, Ar-H), 6.33 (1H, dd, *J* = 2.8, 8.8 Hz, Ar-H), 4.74 (1H, d, $J_{1',2'}$ = 8.0 Hz, H-1'), 5.44 (1H, dd, $J_{2',3'}$ = 10.4 Hz, H-2'), 5.06 (1H, dd, $J_{3',4'}$ = 3.2 Hz, H-3'), 5.42 (1H, d, $J_{4',5'}$ = 3.6 Hz, H-4'), 3.93 (1H, m, H-5'), 4.23 (1H, dd, $J_{5',6a'}$ = 6.4 Hz, $J_{6a',6b'}$ = 11.2 Hz, H-6a'), 4.14 (1H, dd, $J_{5',6b'}$ = 6.8 Hz, H-6b'), 3.60 (2H, br, NH_2), 2.18, 2.11, 2.06, 2.01 (12H, 4s, 4×CH_3CO) ppm; δ_C ($CDCl_3$): 170.55, 170.49, 170.34, 169.86 (4s, 4×CH_3**C**O), 154.57 (d, $J_{F\text{-}C}$ = 244.9 Hz, Ar-C), 144.50 (d, $J_{F\text{-}C}$ = 9.2 Hz, Ar-C), 136.70 (s, Ar-C), 123.47 (d, $J_{F\text{-}C}$ = 1.5 Hz, Ar-C), 110.35 (s, Ar-C), 103.55 (d, $J_{F\text{-}C}$ = 3.8 Hz, Ar-C), 102.99 (s, C-1'), 68.80 (s, C-2'), 70.87 (s, C-3'), 67.02 (s, C-4'), 71.07 (s, C-5'), 61.33 (s, C-6'), 20.83 - 20.76 (4s, 4×**C**H_3CO) ppm.

Anal. Calcd. for $C_{20}H_{24}NO_{10}F$ (%): C, 52.52, H, 5.29, N, 3.06; Found: C, 52.47, H, 5.24, N, 3.02.

2-Fluoro-4-[bis(2'-hydroxyethyl)amino]phenyl 2'', 3'', 4'', 6''-tetra-*O*-acetyl-β-*D*-galactopyranoside 3 To a solution of the amine **2** (1.0 g, 2.2 mmol) in benzene (50 mL) and acetic acid (10 mL) at 0°C was added ethylene oxide (2.2 mL, 44.0 mmol, 10 equiv.), and the mixture was stirred for 48 h at room temperature at the end of time TLC showed reaction complete, then evaporated under reduced pressure and purified by column chromatography on silica gel to give **3**

(1.0 g, 85%) as syrup, R_f 0.45 (EtOAc), δ_H ($CDCl_3$): 7.07 (1H, t, J = 9.2 Hz, Ar-H), 6.42 (1H, dd, J = 2.8, 13.6 Hz, Ar-H), 6.33 (1H, dd, J = 2.8, 9.2 Hz, Ar-H), 4.74 (1H, d, $J_{1'',2''}$ = 8.0 Hz, H-1''), 5.44 (1H, dd, $J_{2'',3''}$ = 10.4 Hz, H-2''), 5.06 (1H, dd, $J_{3'',4''}$ = 3.2 Hz, H-3''), 5.41 (1H, d, $J_{4'',5''}$ = 2.4 Hz, H-4''), 3.92 (1H, m, H-5''), 4.23 (1H, dd, $J_{5'',6a''}$ = 6.4 Hz, $J_{6a'',6b''}$ = 11.2 Hz, H-6a''), 4.15 (1H, dd, $J_{5'',6b''}$ = 7.2 Hz, H-6b''), 3.53 (4H, t, $J_{1',2'}$ = 4.8 Hz, -NC**H**$_2$CH$_2$OH), 3.83 (4H, t, $J_{1',2'}$ = 4.8 Hz, -NCH$_2$C**H**$_2$OH), 3.35 (2H, br, -N(CH$_2$)$_2$O**H**), 2.19, 2.11, 2.04, 2.01 (12H, 4s, 4×CH_3CO) ppm; δ_C ($CDCl_3$): 170.58, 170.49, 170.37, 169.91 (4s, 4×CH_3**C**O), 154.74 (d, J_{F-C} = 243.4 Hz, Ar-C), 146.09 (d, J_{F-C} = 10.0 Hz, Ar-C), 135.62 (d, J_{F-C} = 12.0 Hz, Ar-C), 123.43 (s, Ar-C), 107.91 (s, Ar-C), 103.10 (s, Ar-C), 101.03 (s, C-1''), 68.82 (s, C-2''), 70.88 (s, C-3''), 66.98 (s, C-4''), 71.06 (s, C-5''), 61.27 (s, C-6''), 55.57 (s, -N**C**H$_2$CH$_2$OH), 60.69 (s, -NCH$_2$**C**H$_2$OH), 21.85 - 20.78 (4s, 4×**C**H$_3$CO) ppm.

Anal. Calcd. for $C_{24}H_{32}NO_{12}F$ (%): C, 52.84, H, 5.91, N, 2.57; Found: C, 52.78, H, 5.88, N, 2.53.

2-Fluoro-4-[bis(2'-chloroethyl)amino]phenyl 2'', 3'', 4'', 6''-tetra-*O*-acetyl-β-*D*-galactopyranoside 4 To a well stirred solution of **3** (0.5 g, 1.0 mmol) in anhydrous CH_2Cl_2 (30 mL) at 0°C was added 2M $SOCl_2$ in CH_2Cl_2 (3.0 mL, 6.0 mmol, 3 equiv.), and the mixture was continually stirred for 2 h at 0°C and 1 h at room temperature until TLC showed reaction complete, then evaporated under reduced pressure and purified by flash silica gel column chromatography to afford **4** (411 mg, 77%) as syrup, R_f 0.67 (2**:**3 cyclohexane-EtOAc), δ_H ($CDCl_3$): 7.10 (1H, t, J = 8.2 Hz, Ar-H), 6.31 (1H, dd, J = 3.8, 12.2 Hz, Ar-H), 6.23 (1H, dd, J = 3.8, 8.2 Hz, Ar-H), 4.76 (1H, d, $J_{1'',2''}$ = 8.0 Hz, H-1''), 5.45 (1H, dd, $J_{2'',3''}$ = 10.2 Hz, H-2''), 5.07 (1H, dd, $J_{3'',4''}$ = 3.8 Hz, H-3''), 5.41 (1H, d, $J_{4'',5''}$ = 3.4 Hz, H-4''), 3.91 (1H, m, H-5''), 4.22 (1H, dd, $J_{5'',6a''}$ = 6.6 Hz, $J_{6a'',6b''}$ = 11.6 Hz, H-6a''), 4.14 (1H, dd, $J_{5'',6b''}$ = 7.4 Hz, H-6b''), 3.58 (4H, t, $J_{1',2'}$ = 4.8 Hz, -NC**H**$_2$CH$_2$Cl), 3.77 (4H, t, $J_{1',2'}$ = 4.8 Hz, -NCH$_2$C**H**$_2$Cl), 2.18, 2.11, 2.03, 2.00 (12H, 4s, 4×CH_3CO) ppm; δ_C ($CDCl_3$): 170.52, 170.47, 170.34,

169.83 (4s, 4×CH_3**C**O), 155.08 (d, $J_{F\text{-}C}$ = 244.0 Hz, Ar-C), 144.04 (d, $J_{F\text{-}C}$ = 5.0 Hz, Ar-C), 135.78 (d, $J_{F\text{-}C}$ = 12.0 Hz, Ar-C), 123.97 (s, Ar-C), 106.90 (s, Ar-C), 103.01 (s, Ar-C), 100.43 (s, C-1''), 68.86 (s, C-2''), 70.93 (s, C-3''), 68.34 (s, C-4''), 71.14 (s, C-5''), 67.03 (s, C-6''), 59.08 (s, -N**C**H_2CH_2Cl), 61.28 (s, -NCH_2**C**H_2Cl), 21.24 - 20.80 (4s, 4×**C**H_3CO) ppm.

Anal. Calcd. for $C_{24}H_{30}NO_{10}FCl_2$ (%): C, 49.50, H, 5.19, N, 2.41; Found: C, 49.44, H, 5.13, N, 2.37.

2-Fluoro-4-[bis(2'-chloroethyl)amino]phenyl β-*D*-galactopyranoside 5 A solution of **4** (400 mg) in anhydrous MeOH (30 mL) containing 0.5M NH_3 was vigorously stirred from 0°C to room temperature overnight until TLC showed that the reaction was completed, and evaporated to dryness *in vacuo*. Chromatography of the crude syrup on silica gel with ethyl acetate-methanol afforded the corresponding β-*D*-galactopyranoside **5** (270 mg, 95%) as white foam solid, R_f 0.37 (5**:**1 MeOH-EtOAc), δ_H (DMSO-d_6): 7.11 (1H, t, J = 12.0 Hz, Ar-H), 6.63 (1H, dd, J = 4.8, 12.4 Hz, Ar-H), 6.47 (1H, dd, J = 4.8, 12.0 Hz, Ar-H), 4.66 (1H, d, $J_{1'',2''}$ = 8.0 Hz, H-1''), 3.80 - 3.24 (14H, m, H-2'', 3'', 4'', 5'', 6'', -NC**H**$_2$C**H**$_2$Cl), 5.13 (1H, d, $J_{H\text{-}2'',OH\text{-}2''}$ = 4.0 Hz, HO-2''), 4.49 (1H, d, $J_{H\text{-}3'',OH\text{-}3''}$ = 4.2 Hz, HO-3''), 4.82 (1H, d, $J_{H\text{-}4'',OH\text{-}4''}$ = 3.2 Hz, HO-4''), 4.60 (1H, t, $J_{H\text{-}6'',OH\text{-}6''}$ = 4.2 Hz, HO-6'') ppm; δ_C (DMSO-d_6): 153.14 (d, $J_{F\text{-}C}$ = 241.0 Hz, Ar-C), 142.56 (d, $J_{F\text{-}C}$ = 9.0 Hz, Ar-C), 136.04 (d, $J_{F\text{-}C}$ = 11.0 Hz, Ar-C), 120.01 (s, Ar-C), 107.65 (s, Ar-C), 102.80 (s, Ar-C), 100.81 (s, C-1''), 70.30 (s, C-2''), 73.35 (s, C-3''), 68.05 (s, C-4''), 75.47 (s, C-5''), 60.31 (s, C-6''), 41.23 (s, -N**C**H_2CH_2Cl), 52.32 (s, -NCH_2**C**H_2Cl) ppm.

Anal. Calcd. for $C_{16}H_{22}NO_6FCl_2$ (%): C, 46.39, H, 5.35, N, 3.38; Found: C, 46.34, H, 5.31, N, 3.34.

2-Fluoro-4-[bis(2'-hydroxyethyl)amino]phenyl β-*D*-galactopyranoside 6 Deacetylation of **3** (500 mg) in anhydrous MeOH (50 mL) containing 0.5M NH_3, according to the procedures as described for the preparation of free β-*D*-galactopyranoside **5**, yielded **6** (336 mg, 97%) as white foam

solid, R_f 0.30 (6**:**1 MeOH-EtOAc), δ_H (DMSO-d_6): 7.06 (1H, t, J = 9.6 Hz, Ar-H), 6.52 (1H, dd, J = 3.8, 14.4 Hz, Ar-H), 6.40 (1H, dd, J = 3.8, 9.6 Hz, Ar-H), 4.61 (1H, d, $J_{1'',2''}$ = 8.0 Hz, H-1''), 3.60 - 3.30 (14H, m, H-2'', 3'', 4'', 5'', 6'', -NC**H**$_2$C**H**$_2$OH), 5.13 (1H, d, $J_{H\text{-}2'',OH\text{-}2''}$ = 5.2 Hz, HO-2''), 4.50 (1H, d, $J_{H\text{-}3'',OH\text{-}3''}$ = 4.8 Hz, HO-3''), 4.83 (1H, d, $J_{H\text{-}4'',OH\text{-}4''}$ = 5.6 Hz, HO-4''), 4.73 (1H, t, $J_{H\text{-}6'',OH\text{-}6''}$ = 5.2 Hz, HO-6''), 3.68 (2H, t, $J_{H\text{-}2',OH\text{-}2'}$ = 4.0 Hz, -NCH$_2$CH$_2$O**H**) ppm; δ_C (DMSO-d_6): 153.34 (d, $J_{F\text{-}C}$ = 239.0 Hz, Ar-C), 144.53 (d, $J_{F\text{-}C}$ = 9.0 Hz, Ar-C), 134.65 (d, $J_{F\text{-}C}$ = 11.0 Hz, Ar-C), 120.23 (s, Ar-C), 106.77 (s, Ar-C), 103.18 (s, Ar-C), 99.64 (s, C-1''), 70.36 (s, C-2''), 73.37 (s, C-3''), 68.07 (s, C-4''), 75.46 (s, C-5''), 60.32 (s, C-6''), 53.46 (s, -N**C**H$_2$CH$_2$OH), 58.17 (s, -NCH$_2$**C**H$_2$OH) ppm.

Anal. Calcd. for $C_{16}H_{24}NO_8F$ (%): C, 50.93, H, 6.41, N, 3.71; Found: C, 50.89, H, 6.38, N, 3.67.

2-Fluoro-4-{bis[2'-*O*-(2'', 3'', 4'', 6''-tetra-*O*-acetyl-β-*D*-galactopyranosyl)ethyl] amino}phenyl 2''', 3''', 4''', 6'''-tetra-*O*-acetyl-β-*D*-galactopyranoside 7 A solution of 2, 3, 4, 6-tetra-O-acetyl-α-*D*-galactopyranosyl bromide (784 mg, 1.9 mmol, 1.3 equiv.) in anhydrous CH_2Cl_2 (10 mL) was added dropwise into the solution of **3** (400 mg, 0.7 mmol) and $Hg(CN)_2$ (556 mg, 2.2 mmol, 1.5 equiv.) as a promoter in dry CH_2Cl_2 (20 mL) containing powdered molecular sieves (4 Å, 1.5 g) with vigorous stirring at room temperature under an argon atmosphere in the dark for 12 h. The mixture was diluted with CH_2Cl_2 (70 mL), filtered through Celite, washed with water, dried (Na_2SO_4) and concentrated *in vacuo*. The residue was purified on a silica gel column to yield **7** (787 mg, 89%) as syrup, R_f 0.56 (EtOAc), δ_H ($CDCl_3$): 7.07 (1H, t, J = 8.2 Hz, Ar-H), 6.48 (1H, dd, J = 3.8, 15.6 Hz, Ar-H), 6.39 (1H, dd, J = 3.8, 8.2 Hz, Ar-H), 4.50 (2H, d, $J_{1'',2''}$ = 8.0 Hz, H-1''), 4.77 (1H, d, $J_{1''',2'''}$ = 8.0 Hz, H-1'''), 5.23 (2H, dd, $J_{2'',3''}$ = 10.6 Hz, H-2''), 5.45 (1H, dd, $J_{2''',3'''}$ = 10.2 Hz, H-2'''), 5.00 (2H, dd, $J_{3'',4''}$ = 3.4 Hz, H-3''), 5.08 (1H, dd, $J_{3''',4'''}$ = 3.2 Hz, H-3'''), 5.39 (2H, d, $J_{4'',5''}$ = 2.8 Hz, H-4''), 5.43 (1H, d, $J_{4''',5'''}$ = 2.6 Hz, H-4'''), 4.03 - 3.88 (3H, m, H-5'', 5'''), 4.30 - 4.05 (6H, m, H-6'', 6'''), 3.55 (4H, t, $J_{1',2'}$ = 4.0 Hz, -NC**H**$_2$CH$_2$O-), 3.76 (4H, t, $J_{1',2'}$ = 4.0 Hz, -NCH$_2$C**H**$_2$O-), 2.20, 2.19, 2.18, 2.13, 2.12, 2.07,

2.06, 2.05, 2.04, 2.02, 1.99, 1.98 (36H, 12s, 12×CH_3CO) ppm; δ_C ($CDCl_3$): 170.56, 170.54, 170.53, 170.51, 170.49, 170.46, 170.40, 170.38, 170.33, 170.27, 169.82, 169.70 (12s, 12×CH_3**C**O), 154.62 (d, $J_{F\text{-}C}$ = 244.0 Hz, Ar-C), 146.34 (d, $J_{F\text{-}C}$ = 9.0 Hz, Ar-C), 135.52 (d, $J_{F\text{-}C}$ = 11.0 Hz, Ar-C), 123.28 (s, Ar-C), 108.04 (s, Ar-C), 103.07 (s, Ar-C), 101.13 (s, C-1''), 101.26 (s, C-1'''), 66.93 (s, C-2''), 67.04 (s, C-2'''), 68.76 (s, C-3'', 3'''), 61.19 (s, C-4''), 61.23 (s, C-4'''), 70.85 (s, C-5''), 70.94 (s, C-5'''), 60.09 (s, C-6''), 60.55 (s, C-6'''), 51.70 (s, -N**C**H_2CH_2O-), 54.76 (s, -NCH_2**C**H_2O-), 21.21 - 20.64 (12s, 12×**C**H_3CO) ppm; HRMS: $[M+H]^+$, $C_{52}H_{69}NO_{30}F$, Calcd: 1206.3889, Found: 1206.3852; $[M+Na]^+$, $C_{52}H_{68}NO_{30}FNa$, Calcd: 1228.3708, Found: 1228.3660.

2-Fluoro-4-{bis[2'-*O*-(β-*D*-galactopyranosyl)ethyl]amino}phenyl β-*D*-galacto-pyranoside 8

Deacetylation of **7** (700 mg) in anhydrous MeOH (60 mL) containing 0.5M NH_3 using the procedures as described for the preparation of free β-*D*-galactopyranosides **5** and **6** afforded **8** (387 mg, 95%) as white foam solid, R_f 0.33 (10**:**1 MeOH-EtOAc), δ_H (DMSO-d_6): 7.08 (1H, t, J = 8.0 Hz, Ar-H), 6.54 (1H, dd, J = 3.8, 12.2 Hz, Ar-H), 6.42 (1H, dd, J = 3.8, 8.0 Hz, Ar-H), 4.13 (2H, d, $J_{1'',2''}$ = 8.0 Hz, H-1''), 4.63 (1H, d, $J_{1''',2'''}$ = 8.0 Hz, H-1'''), 3.85 - 3.15 (26H, m, H-2'', 2''', 3'', 3''', 4'', 4''', 5'', 5''', 6'', 6''', -NC**H**$_2$C**H**$_2$O-), 4.85 (2H, d, $J_{H\text{-}2'',OH\text{-}2''}$ = 4.2 Hz, HO-2''), 5.14 (1H, d, $J_{H\text{-}2''',OH\text{-}2'''}$ = 6.2 Hz, HO-2'''), 4.38 (2H, d, $J_{H\text{-}3'',OH\text{-}3''}$ = 4.0 Hz, HO-3''), 4.49 (1H, d, $J_{H\text{-}3''',OH\text{-}3'''}$ = 4.2 Hz, HO-3'''), 4.71 (2H, d, $J_{H\text{-}4'',OH\text{-}4''}$ = 4.8 Hz, HO-4''), 4.83 (1H, d, $J_{H\text{-}4''',OH\text{-}4'''}$ = 5.8 Hz, HO-4'''), 4.59 (2H, t, $J_{H\text{-}6'',OH\text{-}6''}$ = 5.4 Hz, HO-6''), 4.68 (1H, t, $J_{H\text{-}6''',OH\text{-}6'''}$ = 4.4 Hz, HO-6''') ppm; δ_C (DMSO-d_6): 153.40 (d, $J_{F\text{-}C}$ = 245.0 Hz, Ar-C), 144.47 (d, $J_{F\text{-}C}$ = 9.0 Hz, Ar-C), 134.79 (d, $J_{F\text{-}C}$ = 12.0 Hz, Ar-C), 124.28 (s, Ar-C), 106.93 (s, Ar-C), 106.47 (s, Ar-C), 103.24 (s, C-1''), 103.95 (s, C-1'''), 70.44 (s, C-2''), 70.66 (s, C-2'''), 73.44 (s, C-3''), 73.49 (s, C-3'''), 68.15 (s, C-4''), 68.20 (s, C-4'''), 75.30 (s, C-5''), 75.52 (s, C-5'''), 60.39 (s, C-6''), 60.47 (s, C-6'''), 56.13 (s, -N**C**H_2CH_2O-), 58.25 (s, -NCH_2**C**H_2O-) ppm; HRMS: $[M+H]^+$, $C_{28}H_{45}NO_{18}F$, Calcd: 702.2621, Found: 702.2667; $[M+Na]^+$, $C_{28}H_{44}NO_{18}FNa$, Calcd: 724.2440, Found: 724.2505.

2-Fluoro-4-[bis(propargyl)amino]phenyl 2'', 3'', 4'', 6''-tetra-*O*-acetyl-β-*D*-galactopyranoside 11 To a well stirred dry DMF (50 mL) solution of the amine **2** (1.0 g, 2.2 mmol) and K_2CO_3 (1.2 g, 8.8 mmol) at 80°C was added propargyl bromide solution (1.0 g, 6.6 mmol, 80% wt. in toluene) dropwise, and the stirring continued for 3 h until completion of the reaction (TLC). The mixture was diluted with CH_2Cl_2 (100 mL), filtered, washed (H_2O), dried (Na_2SO_4) and concentrated *in vacuo*. The residue was purified on a silica gel column to yield **11** (1.0 g, 88%) as syrup, R_f 0.51 (1**:**1 cyclohexane-EtOAc), δ_H ($CDCl_3$): 7.13 (1H, t, J = 8.8 Hz, Ar-H), 6.70 (1H, dd, J = 3.8, 12.1 Hz, Ar-H), 6.60 (1H, dd, J = 3.8, 8.8 Hz, Ar-H), 4.80 (1H, d, $J_{1'',2''}$ = 8.0 Hz, H-1''), 5.46 (1H, dd, $J_{2'',3''}$ = 10.0 Hz, H-2''), 5.07 (1H, dd, $J_{3'',4''}$ = 3.8 Hz, H-3''), 5.41 (1H, d, $J_{4'',5''}$ = 2.8 Hz, H-4''), 3.94 (1H, m, H-5''), 4.23 (1H, dd, $J_{5'',6a''}$ = 6.6 Hz, $J_{6a'',6b''}$ = 11.8 Hz, H-6a''), 4.15 (1H, dd, $J_{5'',6b''}$ = 7.0 Hz, H-6b''), 4.05 (4H, d, J = 4.0 Hz, -NC**H**$_2$C≡CH), 2.27 (2H, t, J = 4.0 Hz, -NCH$_2$C≡C**H**), 2.18, 2.11, 2.04, 2.01 (12H, 4s, 4×CH_3CO) ppm; δ_C ($CDCl_3$): 170.47, 170.41, 170.27, 169.74 (4s, 4×CH_3**C**O), 154.29 (d, J_{F-C} = 245.0 Hz, Ar-C), 145.54 (d, J_{F-C} = 8.0 Hz, Ar-C), 137.70 (d, J_{F-C} = 12.0 Hz, Ar-C), 122.75 (s, Ar-C), 111.17 (d, J_{F-C} = 3.0 Hz, Ar-C), 104.50 (d, J_{F-C} = 22.0 Hz, Ar-C), 102.63 (s, C-1''), 68.77 (s, C-2''), 70.86 (s, C-3''), 67.00 (s, C-4''), 71.14 (s, C-5''), 61.33 (s, C-6''), 40.95 (s, -N**C**H$_2$C≡CH), 78.77 (s, -NCH$_2$**C**≡CH), 73.24 (s, -NCH$_2$C≡**C**H), 21.81 - 20.74 (4s, 4×**C**H$_3$CO) ppm.

Anal. Calcd. for $C_{26}H_{28}NO_{10}F$ (%): C, 58.53, H, 5.29, N, 2.63; Found: C, 58.50, H, 5.25, N, 2.60.

2-Fluoro-4-{bis[[1''-(2''', 3''', 4''', 6'''-tetra-*O*-acetyl-β-*D*-galacto- or glucopyranosyl) -1'', 2'', 3''-triazol-4''-yl]methyl]amino}phenyl 2'''', 3'''', 4'''', 6''''-tetra-*O*-acetyl-β-*D*-galactopyranosides 12, 13 A freshly prepared solution of (+)-sodium L-ascorbate (40 mg, 0.2 mmol) in H_2O (5 mL) was dropwise added into the solution of **11** (530 mg, 1.0 mmol), 2, 3, 4, 6-tetra-*O*-acetyl-β-*D*-galactopyranosyl azide or 2, 3, 4, 6-tetra-*O*-acetyl-β-*D*-glucopyranosyl azide (900 mg,

2.4 mmol), and $CuSO_4 \cdot 5H_2O$ (25 mg, 0.1 mmol) in DMF (30 mL)-H_2O (5 mL) with vigorous stirring at room temperature under an argon atmosphere, and the reaction mixture was continually stirred for 12 h until completion as monitored by TLC (cyclohexane-EtOAc 1**:**5). EtOAc (200 mL) was added to dilute the mixture. The organic phase was washed by saturated brine, dried (Na_2SO_4), concentrated and purified by column chromatography.

2-Fluoro-4-{bis[[1''-(2''', 3''', 4''', 6'''-tetra-*O*-acetyl-β-*D*-galactopyranosyl)-1'', 2'', 3''-triazol-4''-yl]methyl]amino}phenyl 2'''', 3'''', 4'''', 6''''-tetra-*O*-acetyl-β-*D*-galactopyranoside **12** (1.05 g, 82%) as syrup, R_f 0.47 (1**:**5 cyclohexane-EtOAc), δ_H ($CDCl_3$): 7.73 (1H, s, H-5''), 7.64 (1H, dd, J = 4.0, 8.0 Hz, Ar-H), 7.48 (1H, dd, J = 4.0, 12.0 Hz, Ar-H), 6.56 (1H, t, J = 12.0 Hz, Ar-H), 5.80 (2H, d, $J_{1''',2'''}$ = 8.0 Hz, H-1'''), 4.73 (1H, d, $J_{1'''',2''''}$ = 8.0 Hz, H-1''''), 5.46 (2H, dd, $J_{2''',3'''}$ = 10.2 Hz, H-2'''), 5.39 (1H, dd, $J_{2'''',3''''}$ = 10.0 Hz, H-2''''), 5.21 (2H, dd, $J_{3''',4'''}$ = 3.8 Hz, H-3'''), 5.02 (1H, dd, $J_{3'''',4''''}$ = 4.0 Hz, H-3''''), 5.50 (2H, d, $J_{4''',5'''}$ = 3.0 Hz, H-4'''), 5.36 (1H, d, $J_{4'''',5''''}$ = 2.8 Hz, H-4''''), 3.92 - 3.85 (3H, m, H-5''', 5''''), 4.25 - 4.04 (6H, m, H-6''', 6''''), 4.56 (4H, s, -NCH_2-), 2.19 - 1.80 (36H, 7s, 12×CH_3CO) ppm; δ_C ($CDCl_3$): 170.40 - 169.70 (7s, 12×CH_3**C**O), 154.33 (d, $J_{F\text{-}C}$ = 244.0 Hz, Ar-C), 146.12 (d, $J_{F\text{-}C}$ = 9.0 Hz, Ar-C), 145.34 (s, C-4''), 132.49 (s, Ar-C), 130.95 (s, Ar-C), 128.84 (s, Ar-C), 120.81 (s, C-5''), 109.73 (s, Ar-C), 86.30 (s, C-1'''), 103.00 (s, C-1''''), 68.18 (s, C-2'''), 67.94 (s, C-2''''), 70.76 (s, C-3'''), 68.70 (s, C-3''''), 66.95 (s, C-4'''), 66.90 (s, C-4''''), 74.12 (s, C-5'''), 71.00 (s, C-5''''), 61.22 (s, C-6'''), 61.16 (s, C-6''''), 46.59 (s, -NCH_2-), 20.75 - 20.19 (7s, 12×**C**H_3CO) ppm; HRMS: $[M+H]^+$, $C_{54}H_{67}N_7O_{28}F$, Calcd: 1280.4018, Found: 1280.4073; $[M+Na]^+$, $C_{54}H_{66}N_7O_{28}FNa$, Calcd: 1302.3838, Found: 1302.3614; $[M+K]^+$, $C_{54}H_{66}N_7O_{28}FK$, Calcd: 1318.3577, Found: 1318.1919.

2-Fluoro-4-{bis[[1''-(2''', 3''', 4''', 6'''-tetra-*O*-acetyl-β-*D*-glucopyranosyl)-1'', 2'', 3''-triazol-4''-yl]methyl]amino}phenyl 2'''', 3'''', 4'''', 6''''-tetra-*O*-acetyl-β-*D*-galactopyranoside **13** (1.10 g, 85%) as syrup, R_f 0.41 (1**:**5 cyclohexane-EtOAc), δ_H ($CDCl_3$): 7.73 (1H, s, H-5''), 7.70 (1H, dd, J = 3.0, 8.0 Hz,

Ar-H), 7.52 (1H, dd, J = 3.0, 12.0 Hz, Ar-H), 6.54 (1H, t, J = 12.0 Hz, Ar-H), 5.85 (2H, d, $J_{1''',2'''}$ = 8.0 Hz, H-1'''), 4.78 (1H, d, $J_{1'''',2''''}$ = 8.0 Hz, H-1''''), 5.45 (2H, dd, $J_{2''',3'''}$ = 10.6 Hz, H-2'''), 4.61 (1H, dd, $J_{2'''',3''''}$ = 10.8 Hz, H-2''''), 5.20 (2H, dd, $J_{3''',4'''}$ = 4.0 Hz, H-3'''), 5.05 (1H, dd, $J_{3'''',4''''}$ = 4.0 Hz, H-3''''), 5.40 (3H, d, $J_{4''',5'''} = J_{4'''',5''''}$ = 3.4 Hz, H-4''', 4''''), 4.03 - 3.91 (3H, m, H-5''', 5''''), 4.29 - 4.08 (6H, m, H-6''', 6''''), 4.58 (4H, s, -NCH_2-), 2.20 - 2.03 (36H, 8s, 12×CH_3CO) ppm; δ_C ($CDCl_3$): 170.59 - 169.18 (8s, 12×CH_3**C**O), 154.44 (d, $J_{F\text{-}C}$ = 244.0 Hz, Ar-C), 146.05 (d, $J_{F\text{-}C}$ = 9.0 Hz, Ar-C), 145.77 (s, C-4''), 132.62 (s, Ar-C), 131.04 (s, Ar-C), 128.96 (s, Ar-C), 120.82 (s, C-5''), 109.71 (s, Ar-C), 85.94 (s, C-1'''), 102.81 (s, C-1''''), 70.36 (s, C-2'''), 68.80 (s, C-2''''), 72.69 (s, C-3'''), 70.89 (s, C-3''''), 68.31 (s, C-4'''), 67.89 (s, C-4''''), 75.37 (s, C-5'''), 71.11 (s, C-5''''), 61.76 (s, C-6'''), 61.26 (s, C-6''''), 47.52 (s, -NCH_2-), 20.83 - 20.24 (8s, 12×**C**H_3CO) ppm; HRMS: $[M]^+$, $C_{54}H_{66}N_7O_{28}F$, Calcd: 1279.3940, Found: 1279.4218; $[M\text{-}H]^+$, $C_{54}H_{65}N_7O_{28}F$, Calcd: 1278.3862, Found: 1278.4196; $[M\text{-}H+Na]^+$, $C_{54}H_{65}N_7O_{28}FNa$, Calcd: 1301.3759, Found: 1301.3931.

2-Fluoro-4-{bis[[1''-(β-*D*-galacto- or glucopyranosyl)-1'', 2'', 3''-triazol-4''-yl] methyl]amino}phenyl β-*D*-galactopyranosides 14, 15 Deacetylation of **12** and **13** (900 mg) in anhydrous MeOH (80 mL) containing 0.5M NH_3, as described for the preparation of free β-*D*-galactopyranosides **5**, **6** and **8**, afforded **14** and **15**, respectively.

2-Fluoro-4-{bis[[1''-(β-*D*-galactopyranosyl)-1'', 2'', 3''-triazol-4''-yl]methyl]amino}phenyl β-*D*-galactopyranoside **14** (513 mg, 94%) as syrup, R_f 0.36 (10**:**1 MeOH-EtOAc), δ_H (DMSO-d_6): 8.20 (1H, s, H-5''), 7.08 (1H, t, J = 8.0 Hz, Ar-H), 6.81 (1H, dd, J = 4.0, 12.0 Hz, Ar-H), 6.66 (1H, dd, J = 4.0, 8.0 Hz, Ar-H), 5.48 (2H, d, $J_{1''',2'''}$ = 8.0 Hz, H-1'''), 4.13 (1H, d, $J_{1'''',2''''}$ = 8.0 Hz, H-1''''), 4.05 - 3.33 (18H, m, H-2''', 2'''', 3''', 3'''', 4''', 4'''', 5''', 5'''', 6''', 6''''), 5.24 (2H, d, $J_{H\text{-}2''',OH\text{-}2'''}$ = 4.4 Hz, HO-2'''), 5.13 (1H, d, $J_{H\text{-}2'''',OH\text{-}2''''}$ = 4.0 Hz, HO-2''''), 4.49 (2H, d, $J_{H\text{-}3''',OH\text{-}3'''}$ = 4.0 Hz, HO-3''), 4.28 (1H, d, $J_{H\text{-}3'''',OH\text{-}3''''}$ = 4.0 Hz, HO-3''''), 5.03 (2H, d, $J_{H\text{-}4''',OH\text{-}4'''}$ = 4.0 Hz, HO-4'''), 4.82 (1H, d, $J_{H\text{-}4'''',OH\text{-}4''''}$ = 5.2 Hz, HO-4''''), 4.73

(2H, t, $J_{H\text{-}6''',OH\text{-}6'''}$ = 5.6 Hz, HO-6'''), 4.68 (1H, t, $J_{H\text{-}6'''',OH\text{-}6''''}$ = 4.6 Hz, HO-6''''), 4.63 (4H, s, -NCH_2-) ppm; δ_C (DMSO-d_6): 152.91 (d, $J_{F\text{-}C}$ = 241.0 Hz, Ar-C), 144.27 (s, C-4''), 144.08 (d, $J_{F\text{-}C}$ = 9.0 Hz, Ar-C), 135.86 (d, $J_{F\text{-}C}$ = 12.0 Hz, Ar-C), 131.65 (d, $J_{F\text{-}C}$ = 20.0 Hz, Ar-C), 128.65 (s, Ar-C), 122.00 (s, C-5''), 108.29 (s, Ar-C), 88.12 (s, C-1'''), 102.87 (s, C-1''''), 69.39 (s, C-2'''), 70.35 (s, C-2''''), 73.73 (s, C-3'''), 73.39 (s, C-3''''), 67.40 (s, C-4'''), 68.52 (s, C-4''''), 74.48 (s, C-5'''), 75.47 (s, C-5''''), 62.82 (s, C-6'''), 60.50 (s, C-6''''), 48.64 (s, -NCH_2-) ppm; HRMS: $[M+H]^+$, $C_{30}H_{43}N_7O_{16}F$, Calcd: 776.2750, Found: 776.2817; $[M+Na]^+$, $C_{30}H_{42}N_7O_{16}FNa$, Calcd: 798.2570, Found: 798.3064; $[M+K]^+$, $C_{30}H_{42}N_7O_{16}FK$, Calcd: 814.2309, Found: 814.2819.

2-Fluoro-4-{bis[[1''-(β-*D*-glucopyranosyl)-1'', 2'', 3''-triazol-4''-yl]methyl]amino}phenyl β-*D*-galactopyranoside **15** (496 mg, 91%) as syrup, R_f 0.32 (10**:**1 MeOH-EtOAc), δ_H (DMSO-d_6): 8.26 (1H, s, H-5''), 7.71 (1H, dd, *J* = 4.0, 8.0 Hz, Ar-H), 7.68 (1H, dd, *J* = 4.0, 12.0 Hz, Ar-H), 7.09 (1H, t, *J* = 12.0 Hz, Ar-H), 5.53 (2H, d, $J_{1''',2'''}$ = 8.0 Hz, H-1'''), 4.13 (1H, d, $J_{1'''',2''''}$ = 8.0 Hz, H-1''''), 3.82 - 3.22 (18H, m, H-2''', 2'''', 3''', 3'''', 4''', 4'''', 5''', 5'''', 6''', 6''''), 5.39 (2H, d, $J_{H\text{-}2''',OH\text{-}2'''}$ = 4.0 Hz, HO-2'''), 5.28 (1H, d, $J_{H\text{-}2'''',OH\text{-}2''''}$ = 4.0 Hz, HO-2''''), 4.50 (2H, d, $J_{H\text{-}3''',OH\text{-}3'''}$ = 3.8 Hz, HO-3''), 4.83 (1H, d, $J_{H\text{-}3'''',OH\text{-}3''''}$ = 4.0 Hz, HO-3''''), 5.17 (2H, d, $J_{H\text{-}4''',OH\text{-}4'''}$ = 4.0 Hz, HO-4'''), 5.13 (1H, d, $J_{H\text{-}4'''',OH\text{-}4''''}$ = 5.0 Hz, HO-4''''), 4.66 (2H, t, $J_{H\text{-}6''',OH\text{-}6'''}$ = 5.8 Hz, HO-6'''), 4.60 (1H, t, $J_{H\text{-}6'''',OH\text{-}6''''}$ = 5.2 Hz, HO-6''''), 4.62 (4H, s, -NCH_2-) ppm; δ_C (DMSO-d_6): 152.90 (d, $J_{F\text{-}C}$ = 241.0 Hz, Ar-C), 144.14 (s, C-4''), 144.03 (d, $J_{F\text{-}C}$ = 9.0 Hz, Ar-C), 135.89 (d, $J_{F\text{-}C}$ = 12.0 Hz, Ar-C), 131.66 (d, $J_{F\text{-}C}$ = 21.0 Hz, Ar-C), 128.66 (s, Ar-C), 122.45 (s, C-5''), 108.33 (s, Ar-C), 87.51 (s, C-1'''), 102.88 (s, C-1''''), 72.11 (s, C-2'''), 70.34 (s, C-2''''), 77.02 (s, C-3'''), 73.40 (s, C-3''''), 69.61 (s, C-4'''), 68.11 (s, C-4''''), 79.98 (s, C-5'''), 75.49 (s, C-5''''), 62.83 (s, C-6'''), 60.73 (s, C-6''''), 48.63 (s, -NCH_2-) ppm; HRMS: $[M+H]^+$, $C_{30}H_{43}N_7O_{16}F$, Calcd: 776.2750, Found: 776.2834; $[M+Na]^+$, $C_{30}H_{42}N_7O_{16}FNa$, Calcd: 798.2570, Found: 798.3345; $[M+K]^+$, $C_{30}H_{42}N_7O_{16}FK$, Calcd: 814.2309, Found: 814.2741.

Stable *lacZ* Transfected Cancer Cells. *E.coli lacZ* gene (from pSV-β-gal vector, Promega, Madison, WI, USA) was inserted into high expression human cytomegalovirus (CMV) immediate-early enhancer/promoter vector phCMV (Gene Therapy Systems, San Diego, CA, USA) giving a recombinant vector phCMV/*lacZ*. This was used to transfect wild type MCF7 (human breast cancer) and PC3 (human prostate cancer) cells (ATCC, Manassas, VA, USA) using GenePORTER2 (Gene Therapy Systems, Genlantis, Inc., San Diego, CA, USA), as described in detail previously.[4-6] The highest β-gal expressing colony was selected using the antibiotic G418 disulfate (800μg/mL, Research Products International Corp, Mt Prospect, IL, USA) and G418 (200μg/mL) was also included for routine culture. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Mediatech Inc., Herndon, VA, USA) containing 10% fetal bovine serum (FBS, 0.1M, pH=7.4, Atlanta Biologicals, Inc., Lawrenceville, GA, USA) with 100units/mL of penicillin, 100units/mL streptomycin, and cultured in a humidified 5% CO_2 incubator at 37°C. The β-gal activity of *lacZ*-transfected tumor cells was measured using a β-gal assay kit with *o*-nitrophenyl-β-*D*-galactopyranoside (Promega, Madison, WI, USA), and confirmed by X-gal staining.[4-6] Cell lysis was achieved by a freeze/thaw method: equal numbers of MCF7-*lacZ* or PC3-*lacZ* cells were suspended in PBS (0.1M, pH=7.4) and then frozen at -80°C for 10 mins before thawing at room temperature over 3 cycles.

Kinetic ^{19}F-MRS Experiments. Relative substrate efficacy of 2-fluoro-4-[bis(2'-chloroethyl)amino]phenyl β-*D*-galactopyranoside **5** and 2-fluoro-4-[bis(2'-hydroxyethyl)amino] phenyl β-*D*-galactopyranoside **6** was evaluated using ^{19}F-MRS. Enzyme reactions were conducted at 37°C in PBS (0.1M, pH=7.4) using β-gal (E801A, Promega, Madison, WI, USA). The prodrugs **5** and **6** (13.8μmol) were dissolved in PBS (595μL, pH=7.4) and β-gal (E801A, 5 μL, 1unit/μL) added, followed by immediate ^{19}F-MRS data acquisition at 37°C with subsequent spectra every 102s providing a

kinetic curve over 40min. The β-gal hydrolysis rates were calculated according to the formula: ν = [concentration change of **5** or **6** (C_{t1} - C_{t2}) × 1000] / [reaction time (min) × unites of β-gal], and expressed in μM/min/unit.

In vitro evaluation of **5** with the wild type (WT), *lacZ* transfected human MCF7 breast and PC3 prostate cancer cells (5×10^6) in PBS (0.1M, pH=7.4) at 37°C under 5% CO_2 in air with 95% humidity by using ^{19}F-MRS analysis was conducted at different time points (0-180min) at 37°C with subsequent spectra every 261s. The hydrolysis rates by MCF7- or PC3-*lacZ* cells were measured according to the formula: ν = [concentration change of **5** (C_{t1} - C_{t2}) × 1000] / [reaction time (min) × numbers of million MCF7- or PC3-*lacZ* cells], and expressed in μM/min/per million cells.

Cytotoxicity. The cytotoxicity was assessed for both prodrug **5** and the released fluorinated nitrogen mustard 2-fluoro-4-[bis(2'-chloroethyl)amino]phenol (**2F4ClPh**) in both wild-type and *lacZ* expressing MCF7 and PC3 cells using a colorimetric CellTiter 96 Aqueous Nonradioactive MTS Cell Proliferation Assay (Promega). Assays were performed in triplicate using 24-well plates seeded with 10^3 cells per well in 500 μL of RPMI-1640 without phenol red and supplemented with 10% FCS and 2 mM glutamine.[5,6]

ASSOCIATED CONTENT

Supporting Information

Figure S1. The numbering of structures **3**-**6**; **Figure S2**. The numbering of structures **7**-**8**; **Figure S3**. The numbering of structures **12**-**15**.

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ABBREVIATIONS

MRS: Magnetic resonance spectroscopy; **NMR:** Nuclear magnetic resonance; **HRMS:** High resolution mass spectrometry; **β-gal:** β-Galactosidase; **TBAB:** Tetrabutylammonium bromide; **CH_2Cl_2:** Dichloromethane; **AcOH:** Acetic acid; **$SOCl_2$:** Thionyl chloride; **PBS:** Phosphate buffered saline; **DMF:** Dimethylformamide; **NaASc:** (+)-Sodium L-ascorbate; **DMSO:** Dimethyl sulfoxide; **TLC:** Thin layer chromatography.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest in this work.

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FIGURE LEGENDS

Graphic Abstract

Figure 1. The synthesis and the structures of **1-6**. **Reaction conditions**: (a) H_2 (30psi), EtOH, Pd/C (5% W/W'), r.t., overnight, 100%; (b) ethylene oxide (10 equiv.), AcOH-benzene (1:5 V/V'), 0°C→r.t., 48h, 85% yield; (c) $SOCl_2$ (3.0 equiv.), CH_2Cl_2, stirring at 0°C for 2h then r.t. for 1h, 77% yield; (d) 0.5M NH_3-MeOH, 0°C→r.t., 24h, 95%(→**5**) and 97%(→**6**), respectively**.**

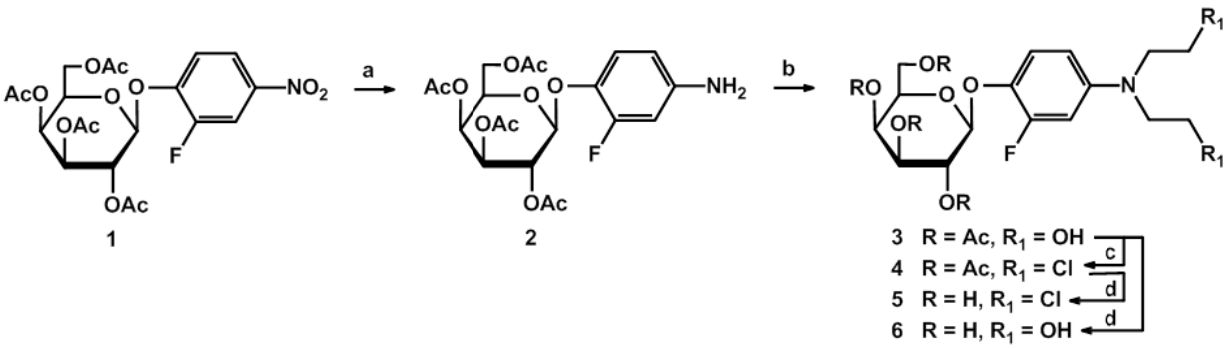
Figure 2. The synthetic route and the structures of **7-10**. **Reaction conditions**: (a) 2, 3, 4, 6-tetra-*O*-acetyl-α-*D*-galactopyranosyl bromide or 2, 3, 4, 6-tetra-*O*-acetyl-α-*D*-glucopyranosyl bromide (1.3 equiv.), $Hg(CN)_2$ (1.5 equiv.), 4Å M.S., CH_2Cl_2, r.t., 12h, 89%(→**7**); (b) 0.5M NH_3-MeOH, 0°C→r.t., 24h, 95%(→**8**)**.**

Figure 3. The synthetic route and the structures of **11-15**. **Reaction conditions**: (a) $CH{\equiv}CCH_2Br$ (1.5 equiv.), K_2CO_3 (2.0 equiv.), DMF, 80°C, 3h, 88%; (b) 2, 3, 4, 6-tetra-*O*-acetyl-β-*D*-galactopyranosyl azide or 2, 3, 4, 6-tetra-*O*-acetyl-β-*D*-glucopyranosyl azide (1.2 equiv.), $CuSO_4{\cdot}5H_2O$ (10% mol), (+)-sodium L-ascorbate (20% mol), DMF-H_2O (3:1 V/V'), r.t., 12h, Ar, 82%(→**12**) and 85%(→**13**), respectively; (c) 0.5M NH_3-MeOH, 0°C→r.t., 24h, 91-94% yields.

Figure 4. ^{19}F-MRS detection of β-gal (E801A) in PBS at 37°C. (**1**) Control: 2-Fluoro-4-[bis(2'-chloroethyl)amino]phenyl β-*D*-galactopyranoside **5** (13.8 μmol), without β-gal (5 units, E801A) in PBS (0.1 M, pH=7.4, 600 μL) at 37°C; (**2**) 2-Fluoro-4-[bis(2'-chloroethyl)amino]phenyl β-*D*-galactopyranoside **5** (13.8 μmol), with β-gal (5 units, E801A) in PBS (0.1 M, pH=7.4, 600 μL) at 37°C at the time point, t = 2 minutes. **^{19}F-MRS:** parameter sets: relaxation delay: 1 s, pulse width (90°): 16.6 μs, number of points acquired: 59966, filter bandwidth: 28000 Hz, each spectrum acquisition time: 0.6 s, number of acquisitions: 64, and enhanced with an exponential line broadening of 40 Hz. 2-Fluoro-4-[bis(2'-chloroethyl)amino] phenyl β-*D*-galactopyranoside **5** at δ_F=-55.65 ppm, 2-fluoro-4-[bis(2'-chloroethyl) amino]phenol (**2F4ClPh**) at δ_F=-59.44 ppm, and 2-fluoro-4-[bis(2'-hydroxyethyl)amino]phenol (**2F4OHPh**) at δ_F=-44.33 ppm.

Figure 5. The kinetic hydrolysis time courses of 2-fluoro-4-[bis(2'-chloroethyl)amino]phenyl β-*D*-galactopyranoside **5** (16.8 mM) (•) → 2-fluoro-4-[bis(2'-chloroethyl)amino]phenol (**2F4ClPh**) (■) → 2-fluoro-4-[bis(2'-hydroxyethyl)amino]phenol (**2F4OHPh**) (▲) with β-gal (5 units, E801A) in PBS (0.1 M, pH=7.4, 600 μL) at 37°C.

Figure 6. The cytotoxicity of prodrug **5** to wild-type (WT) and *lacZ* expressing MCF7 and PC3 cells, incubation at 37°C under 5% CO_2 in air with 95% humidity for 24h; **Histology:** β-gal activity validation in MCF7- and PC3-*lacZ* cells with deep blue (bottom) and no activity in MCF7- and PC3-WT cells (top), X-gal staining (×200).



1

2

3 R = Ac, R_1 = OH

4 R = Ac, R_1 = Cl

5 R = H, R_1 = Cl

6 R = H, R_1 = OH