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PRINCIPAL INVESTIGATOR: Ralph P. Mason, Ph.D.

CONTRACTING ORGANIZATION: The University of Texas Southwestern Medical Center
Dallas, TX 75390

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Ralph P. Mason, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The University of Texas Southwestern
Medical Center
Dallas, TX 75390

E-Mail: Ralph .Mason@UTSouthwestern.edu

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Gene therapy holds great promise for treatment of breast cancer. In particular, clinical trials are underway to apply therapeutic genes related to pro-drug activation or to modulate the activity of oncogenes by blocking promoter sites. However, there are major problems in terms of assessing the delivery to target tissue, assessing the uniformity (versus heterogeneity) of biodistribution and determining whether the genes are expressed.

We propose to design, evaluate and apply a novel approach to gene activity detection- specifically, using fluorinated substrates of β-galactosidase to reveal gene activity. Our prototype molecule PFONPG (para- fluoro- ortho- nitro- phenyl β-D-galactopyranoside) is a direct analog of the traditional "yellow' biochemical indicator ONPG (ortho- nitro- phenyl β-D-galactopyranoside). This shows useful MR characteristics, sensitivity to enzyme activity and ability to enter cells. We will synthesize analogs of this prototype to optimize MR and biological characteristics and explore the feasibility of tailoring the reporter to specific applications, *e.g.*, exploiting β-gal activity to deliver specific physiological reporter molecules such as pH and potentially specific cytotoxic agents. The agents will be rigorously tested in solution, applied to cultured breast cancer cells and ultimately used to examine β-gal activity *in vivo* in transfected breast tumors in mice and rats.

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Introduction

Gene therapy holds great promise for the treatment of breast cancer (1, 2). In particular, clinical trials are underway to apply therapeutic genes related to pro-drug activation or modulation of the activity of oncogenes by blocking promoter sites. However, there are major problems in terms of assessing the delivery to target tissue, assessing the uniformity (versus heterogeneity) of biodistribution and determining whether the genes are expressed. We propose to design, evaluate and apply a novel approach to gene activity detection – specifically, using fluorinated substrates of β-galactosidase to reveal gene activity. Our prototype molecule PFONPG (para- fluoro-ortho- nitro- phenyl β-D-galactopyranoside) is a direct analog of the traditional "yellow' biochemical indicator ONPG (ortho- nitro- phenyl β-D-galactopyranoside). This shows useful MR characteristics, sensitivity to enzyme activity and ability to enter cells. We will synthesize analogs of this prototype to optimize MR and biological characteristics, and explore the feasibility of tailoring the reporter to specific applications, *e.g.*, exploiting β-gal activity to deliver specific physiological reporter molecules such as pH and potentially specific cytotoxic agents. The agents will be rigorously tested in solution, applied to cultured breast cancer cells and ultimately used to examine β-gal activity *in vivo* in transfected breast tumors in mice and rats.

BODY

Statement of Work

Year 1

Task 1 Synthesize novel molecules to report activity of the β-galactosidase gene-minimum 8 novel agents (Months 1-12)

Task 2 Characterize novel agents (NMR, mass spec, colorimetric analysis) (Months 3-12)

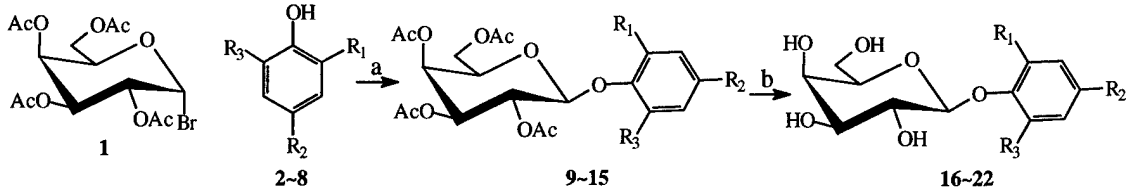
Task 3 Test agents for enzyme activity in solution (Months 4-12)

Task 4 Test initial indicators in cultured breast cancer cells (control + transfected) (Months 6-12)

Task 1 Complete

We have successfully synthesized more than 8 novel fluorine substituted phenylgalactosides, as potential ^{19}F NMR reporter molecules for β-galactosidase activity. The synthesis and evaluation of the prototype molecule 4-fluoro-2-nitrophenyl-β-D-galactopyranoside has been published in "A novel NMR approach to assessing gene transfection: 4-fluoro-2-nitrophenyl-β-D-galactopyranoside as a prototype reporter molecule for β-galactosidase", W. Cui, P. Otten, Y. Li, K. S. Koeneman, J. Yu and R. P. Mason, *Magn. Reson. Med.*, 51, 616-20 (2004). (Appendix 1) A description of synthesis and characterization of properties for the series of analogues is described in a second manuscript, which was submitted to *Bioconjugate Chemistry* in May 2004 and is under review. (Appendix 2) The second manuscript describes a total of 8 novel molecules, including α and β isomers of 2-fluoro-6-nitrophenyl-β-D-galactopyranoside. The molecular structures are described in Figure 1 (below). In addition, we have synthesized trifluoromethylated aryl β-D-galactosides, as detailed in Figure 2.

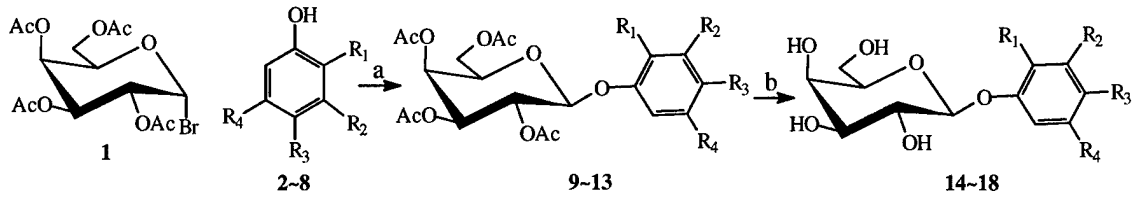
Figure 1. The reactions and structures of **1~22** (fluorophenylgalactosides).



Reaction conditions: (a) CH_2Cl_2-H_2O, pH 8~9, 50 °C, TBAB, ~1 h, near quantitative yield; (b) NH_3-MeOH, 0 °C→r.t., 24 h, quantitative yields.

Compounds	R_1	R_2	R_3
2, 9, 16	**NO_2**	**F**	**H**
3, 10, 17	**F**	**NO_2**	**H**
4, 11, 18	**F**	**H**	**NO_2**
5, 12, 19	**F**	**H**	**H**
6, 13, 20	**H**	**F**	**H**
7, 14, 21	**Cl**	**F**	**H**
8, 15, 22	**Br**	**F**	**H**

Figure 2. The reactions and structures of **1~22** (trifluorophenylgalactosides).



Reaction conditions: (a) CH_2Cl_2-H_2O, pH 8~9, 50 °C, TBAB, ~1 h, near quantitative yield except **13** in only 20% yield; (b) NH_3-MeOH, 0 °C→r.t., 24 h, quantitative yields.

Compounds	R_1	R_2	R_3	R_4
2, 9, 14	NO_2	H	CF_3	H
3, 10, 15	H	CF_3	NO_2	H
4, 11, 16	Cl	CF_3	H	H
5, 12, 17	Cl	H	H	CF_3
6, 13, 18	CF_3	H	H	H
7	H	CF_3	H	H
8	H	H	CF_3	H

Task 2 Complete

Each of the molecules described in Task 1 has been fully characterized and structure validated by mass spectrometry, high resolution NMR, and other techniques, as appropriate. Details for the 8 primary molecules are provided in Appendix 2 and summarized for a representative molecule, as follows:

Fluorinated phenyl β-D-galactopyranoside tetraacetates 9-15.

General procedure (Fig. 1). A solution of **1** (1 mmol; 2, 3, 4, 6-tetra-O-acetyl-α-D-galactopyranosyl bromide, Sigma) in CH_2Cl_2 (5 ml) was added dropwise to a vigorously stirred solution of fluorophenol **2 - 8** (1.2 mmol) and tetrabutylammonium bromide (0.48 g, 1.5 mmol) in H_2O (5 ml; pH 8~9) 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, dried, evaporated under reduced pressure and

recrystallized (EtOH-H_2O) or purified by column chromatography on silica gel to give fluorinated aryl β-D-galactopyranoside tetraacetates **9 - 15**, as white crystals.

2-Nitro-4-fluorophenyl 2, 3, 4, 6-tetra-O-acetyl-β-D-galactopyranoside 9 (0.5 g, 99%), R_f 0.31(3:2 cyclohexane-EtOAc), δ_H: 7.55(1H, dd, *J*=3.0, 8.4 Hz, Ar-H), 7.42(1H, dd, *J*=4.8, 9.0 Hz, Ar-H), 7.27(1H, m, Ar-H), 5.04(1H, d, $J_{1,2}$=7.8 Hz, H-1), 5.52(1H, dd, $J_{2,3}$=8.4 Hz, H-2), 5.11(1H, dd, $J_{3,4}$=3.0 Hz, H-3), 5.47(1H, d, $J_{4,5}$=2.6 Hz, H-4), 4.07(1H, m, H-5), 4.26(1H, dd, $J_{5,6a}$=4.2 Hz, $J_{6a,6b}$=11.4 Hz, H-6a), 4.17(1H, dd, $J_{5,6b}$=5.4 Hz, H-6b), 2.20, 2.14, 2.07, 2.02(12H, 4s, 4×CH_3CO)ppm; δ_C: 170.43, 170.31, 170.24, 169.62(4×CH_3**C**O), 157.70(d, J_{F-C}=164.8 Hz, Ar-C), 145.66(Ar-C), 141.86(d, J_{F-C} =5.7 Hz, Ar-C), 122.68(d, J_{F-C}=4.9 Hz, Ar-C), 120.77(d, J_{F-C}=15.2 Hz, Ar-C), 112.60(d, J_{F-C}=18.3 Hz, Ar-C), 101.40(C-1), 68.01(C-2), 70.63(C-3), 66.86(C-4), 71.58(C-5), 61.45(C-6), 21.25, 21.10, 20.38, 20.25(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.

Fluorinated aryl β-D-galactopyranosides 16 - 22. *General procedure*---- A solution of fluorophenyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside (**9 - 15**) (0.4 g) in anhydrous MeOH (15 ml) containing 0.5M NH_3 was vigorously stirred from 0 °C to room temperature overnight until TLC showed complete reaction. Following solvent removal *in vacuo*, chromatography on silica gel (EtOAc/MeOH) afforded the free galactopyranosides **16 - 22** in near quantitative yield, as white crystalline materials

2-Nitro-4-fluorophenyl β-D-galactopyranoside 16, R_f 0.40(1:9 MeOH-EtOAc), δ_H: 7.84(1H, dd, *J*=2.8, 8.0 Hz, Ar-H), 7.53(1H, ddd, *J*=1.6, 1.0, 2.8 Hz, Ar-H), 7.43(1H, dd, *J*=4.4, 9.2 Hz, Ar-H), 4.96(1H, d, $J_{1,2}$=7.6 Hz, H-1), 3.60(1H, dd, $J_{2,3}$=10.6 Hz, H-2), 3.51(1H, dd, $J_{3,4}$=5.2 Hz, H-3), 3.47(1H, d, $J_{4,5}$=5.6 Hz, H-4), 3.43(1H, m, H-5), 3.67(2H, m, H-6), 5.16(1H, d, $J_{H-2,OH-2}$=5.2 Hz, HO-2), 4.67(1H, d, $J_{H-3,OH-3}$ =4.4 Hz, HO-3), 4.90(1H, d, $J_{H-4,OH-4}$=6.0 Hz, HO-4), 4.67(1H, t, $J_{H-6,OH-6}$=5.2, 5.4 Hz, HO-6)ppm; δ_C: 155.41(d, J_{F-C}=239.6 Hz, Ar-C), 146.19(d, J_{F-C}=3.1 Hz, Ar-C), 140.17(d, J_{F-C} = 9.1 Hz, Ar-C), 120.91(d, J_{F-C}=22.1 Hz, Ar-C), 119.03(d, J_{F-C}=7.7 Hz, Ar-C), 111.89(d, J_{F-C} =27.5 Hz, Ar-C), 101.65(C-1), 70.07(C-2), 73.37(C-3), 68.06(C-4), 75.87 (C-5), 60.33(C-

6)ppm; HRMS: $[M+Na]^+$, $C_{12}H_{14}NO_8FNa$, Calcd: 342.0601, Found: 342.0589; $[M+K]^+$, $C_{12}H_{14}NO_8FK$, Calcd: 358.0341, Found: 358.0328.

The titration curve of each aglycone was analyzed, as presented in Figure 3 and Table 1 and detailed in Appendix 2. Although there were eight product aryl galactosides, one comprised α and β anomers, so that there are 7 aglycones for assessment.

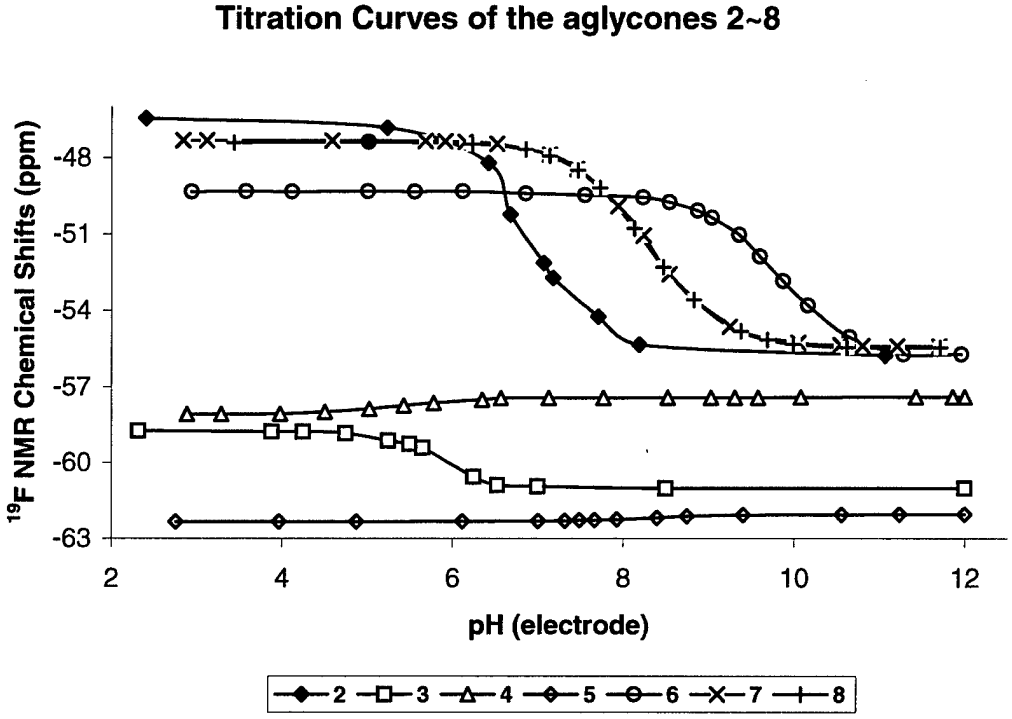


Figure 3 ^{19}F NMR chemical shift pH titration curves of **2-8** in saline at 25 °C

Table 1 ^{19}F NMR pH characterization of aglycones at 25 °C

Compd.	2	3	4	5	6	7	8
pKa	6.87	6.03	5.44	8.33	9.80	8.31	8.28
$\delta_{(acid)}$	-44.44	-58.77	-58.07	-62.33	-49.33	-47.32	-47.40
$\delta_{(base)}$	-55.76	-61.01	-57.39	-62.04	-57.72	-55.40	-55.45
Δδ(ppm)	11.32	2.24	0.68	0.29	6.39	8.08	8.05

Task 3 Complete

Each of the eight primary agents has been evaluated for β-gal enzyme activity, as described in detail in Appendix 2 and summarized in Figure 4 and Table 2 below.

Table 2. ^{19}F chemical shifts (ppm) of **16** - **22** before and after the hydrolysis by β-gal*

Compd.	16	17	18A	18B	19	20	21	22
$\delta_{F(substrate)}$	-42.87	-54.93	-50.67	-49.37	-58.74	-45.87	-43.56	-43.82
$\delta_{F(product)}$	-52.71	-61.04	-58.67	-58.67	-62.30	-49.59	-48.13	-48.24
Observed $\Delta\delta_F$	9.84	6.11	8.00	9.30	3.56	3.72	4.57	4.42
Min $\Delta\delta_F$	1.57	3.84	6.72	8.7	3.3	3.46	3.76	3.58
Max $\Delta\delta_F$	12.89	6.11	8.1	9.4	3.59	11.85	11.84	11.63

* β-gal (E801A, 20 units at 37°C in 0.1M PBS, pH 7.4).

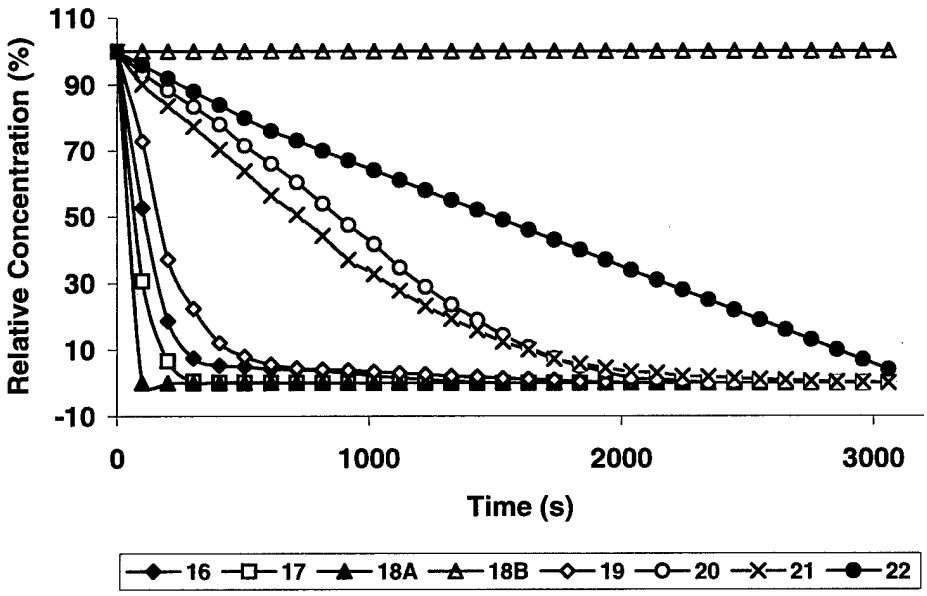


Figure 4 Hydrolysis time courses of the fluorinated phenyl D-galactopyranoside 16-22 (15 mmol) by β-gal (E801A, 20 units) in PBS (0.1M, 0.6 ml) at 37 °C

Task 4 To be completed

We had expected to be able to acquire β-gal expressing breast tumor cells readily. However, these appear to not be readily available from tissue banks and our

colleague's cultures were found to exhibit very low expression. We have now obtained several breast tumor cell lines and we are attempting to generate highly expressing stable transfectants. This work is ongoing and we expect to have several useful cells within the next three months. In the meantime, we felt it was imperative to initiate tests of β-gal expressing cells and we have been fortunate that collaborators in Urology were able to provide highly expressing transiently transfected prostate cells which were used in the published manuscript (Appendix 1) as initial proof of principle. We have now also received stably expressing 9L-glioma cells from our consultant Dr. Brown and have initiated tests. We will undertake further tests for task 4 during the early part of Year 2 to expand application to breast cancer cells and facilitate the subsequent Tasks. We were able to undertake initial tests with a stably transfected rat tumor breast cell line MTLn3-LacZ and initial results are presented in Figure 5 and included in the submitted manuscript (Appendix 2). However, we are seeking to create stable transfectants with higher activity.

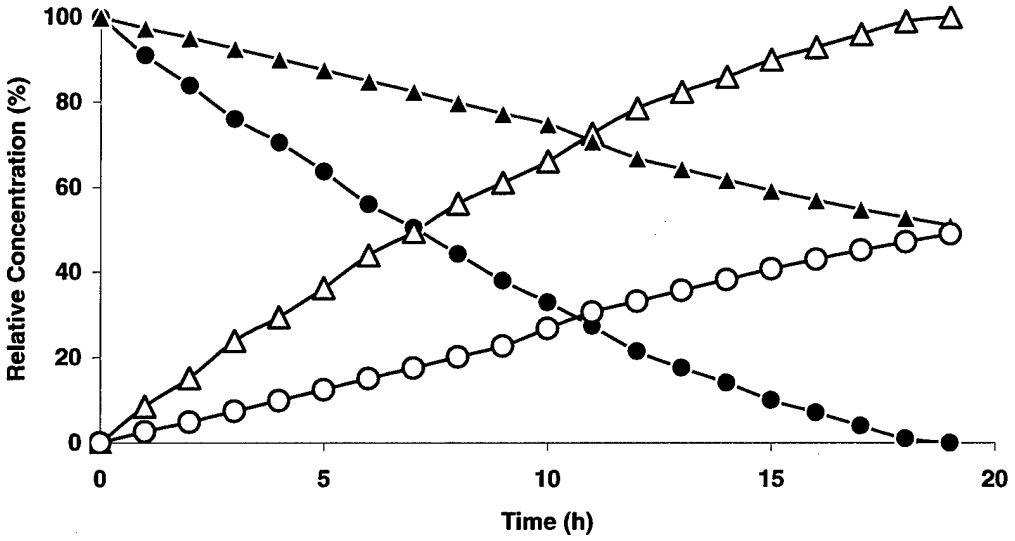
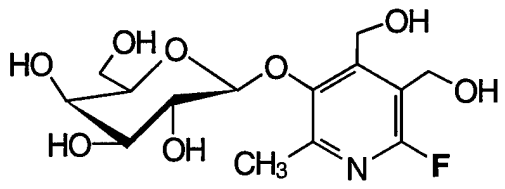


Figure 5 Hydrolysis of 2-fluoro-4-nitrophenyl-β-D-galactopyranoside (open symbols) to 2-fluoro-4-nitrophenol (solid symbols) by stably transfected Dunning prostate R3327 MAT-Lu-*lacZ* cells (Δ 92×10^6) and MTLn3-*lacZ* (O 9.8×10^6) suspended in PBS at 37 °C.

While awaiting the breast tumor cells, we have initiated syntheses related to tasks for Years 2 and 3. Specifically, we have successfully synthesized second generation "smart" pH reporters.

Task 8

Synthesize second generation "smart" β-gal substrates as reporters of physical parameters such as pH or as cytotoxic agents (Months 15-24).



GFPOL (3-*O*-(β-D-galactopyranosyl)-6-fluoropyridoxol)

GFPOL gave a single narrow ^{19}F NMR signal at δ -3.22 ppm essentially invariant ($\Delta\delta \leq 0.06$ppm) with pH in the range 3 to 12 and temperatures from 25 to 37 °C. Addition of β-gal (E801A) in PBS buffer (0.1M, pH=7.4) at 37°C caused hydrolysis releasing the pH indicator aglycone FPOL appearing also the single narrow ^{19}F signal down-field shifting at δ -11.21ppm consistent with our previous titration curve of FPOL (3). This work will be continued during years 2 and 3.

KEY RESEARCH ACCOMPLISHMENTS:

- We have successfully synthesized a series of novel fluorine substituted phenylgalactosides as potential ^{19}F NMR reporter molecules for β-galactosidase activity.
- The fluorophenyl β-D-galactopyranosides are stable in saline, but are rapidly cleaved by the enzyme β-galactosidase.
- The fluorophenyl β-D-galactopyranosides provide a single ^{19}F NMR signal, which is invariant with pH. Enzyme cleavage produces a new signal well removed from the parent compound.

- Preliminary data show that the fluorophenyl β-D-galactopyranosides enter breast tumor cells. In wild type cells, the substrate is stable, but in cells transfected to express β-gal there is cleavage releasing aglycone product as revealed by chemically shifted signal. Thus the proof of principle has been validated.
- A second generation molecule has been generated using trifluoromethyl groups, which should provide enhanced NMR signal to noise.
- Prototype "smart" β-gal substrates have been synthesized using the pH reporter molecule 6-fluoropyridoxol in place of fluorophenol aglycones. The molecule was successfully synthesized and appears to be a good substrate for enzyme activity.

REPORTABLE OUTCOMES:

Published manuscript

"A novel NMR approach to assessing gene transfection: 4-fluoro-2-nitrophenyl-β-D-galactopyranoside as a prototype reporter molecule for β-galactosidase", W. Cui, P. Otten, Y. Li, K. S. Koeneman, J. Yu and R. P. Mason, *Magn. Reson. Med.*, 51, 616-20 (2004)

Conference presentations

1 "Stereoselective synthesis and evaluation of fluorinated vitamin B6 β-d-galactosides as potential novel substrates for *in vivo* and non-invasive detection of lacZ gene expression", J. Yu, R. P. Mason, 38th National Organic Symposium, B3, Bloomington, Indiana, June 2003.

2 "Gene Reporter Molecules: a new platform using ^{19}F NMR substrates for β-galactosidase", J. Yu, W. Cui & R. P. Mason, Proc. 16th International NMR Spectroscopy Conference, C-7, Cambridge, England, July 2003

3 "Novel *in vivo* Gene Reporter Molecule Using Fluorinated Vitamin B6 as ^{19}F NMR indicator", J. Yu and R. P. Mason, Proc. Intl. Soc. Magn. Reson. Med. 11, 674 (2003)

CONCLUSIONS:

Gene therapy holds great promise for tracing breast cancer. A major current obstacle to implementation is assessment of gene expression in terms of heterogeneity and longevity in tissues. Reporter genes and associated molecules should allow assessment of gene expressions. To date successful reporters have been developed for nuclear imaging, but radionuclides can be difficult to handle, and decay, limiting shelf life detectability (4). Optical techniques are favored for gene assessment in small animals, but light penetration can limit utility (5). NMR facilitates assessment of deep tissues without radiation exposure. We have now demonstrated the feasibility of synthesizing an NMR reporter molecule to reveal activity of β-galactosidase, the primary tool of molecular biologists to assess gene transfection. Significantly the molecules enter cells and are effective substrates. Moreover the ^{19}F NMR chemical shift unequivocally reveals enzyme activity. Differences in chemical shift associated with small molecular changes may further allow multiple substrates to be interrogated simultaneously allowing for several genes to be interrogated simultaneously. Proof of principle has been shown in cell culture and the next step is translation to living animals during the next two years.

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APPENDICES:

1) "A novel NMR approach to assessing gene transfection: 4-fluoro-2-nitrophenyl-β-D-galactopyranoside as a prototype reporter molecule for β-galactosidase", W. Cui, P. Otten, Y. Li, K. S. Koeneman, J. Yu and R. P. Mason, *Magn. Reson. Med.*, 51, 616-20 (2004)

2) "A Novel NMR Platform for Detecting Gene Transfection: Synthesis and Evaluation of Fluorinated Phenyl β-D-Galactosides with Potential Application for Assessing LacZ Gene Expression" J. Yu, P. Otten, Z. Ma, W. Cui, R. P. Mason. Submitted *Bioconjugate Chemsitry* (2004)

Magnetic Resonance in Medicine 51:616–620 (2004)

Novel NMR Approach to Assessing Gene Transfection: 4-Fluoro-2-Nitrophenyl-β-D-Galactopyranoside as a Prototype Reporter Molecule for β-Galactosidase

Weina Cui,[1] Pieter Otten,[1] Yingming Li,[2] Kenneth S. Koeneman,[2] Jianxin Yu,[1] and Ralph P. Mason[1*]

Gene therapy holds great promise for the treatment of diverse diseases. However, widespread implementation is hindered by difficulties in assessing the success of transfection in terms of spatial extent, gene expression, and longevity of expression. The development of noninvasive reporter techniques based on appropriate molecules and imaging modalities may help to assay gene expression. 4-Fluoro-2-nitrophenyl-β-D-galactopyranoside (PFONPG) is a novel prototype NMR-sensitive molecule, which is highly responsive to the action of β-galactosidase (β-gal), the product of the *lacZ* gene. The molecule is stable in solution and with respect to wild-type cells, but the enzyme causes very rapid liberation of the aglycone, accompanied by color formation and a ^{19}F NMR chemical shift of 5–10 ppm, depending on pH. Since the product is pH-sensitive, this opens the possibility for direct pH determinations at the site of enzyme activity. Molecular and ^{19}F NMR characteristics of PFONPG in solution, blood, and prostate tumor cells are presented. This prototype molecule facilitates a novel approach for assaying gene activity in vivo. Magn Reson Med 51:616–620, 2004. © 2004 Wiley-Liss, Inc.

Key words: β-galactosidase; gene reporter molecule; lacZ; ^{19}F NMR; nitrophenyl-β-D-galactopyranoside; prostate cancer

Gene therapy holds great promise for the treatment of various diseases, including cancer, cystic fibrosis, and immunodeficiency. However, a major obstacle to widespread successful implementation is the need to verify successful transfection—in particular, the spatial extent of expression in the target tissue—together with assays of the longevity of expression. An image-based assay would greatly facilitate optimal gene therapy vector dosing, in a precise temporal and spatial manner. Numerous preclinical studies have indicated that this therapy shows promise for the treatment of solid tumors. However, useful reporter molecules could accelerate the effective transition to human clinical trials.

[1]Department of Radiology, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas.

[2]Department of Urology, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas.

Presented in part at the 10th Annual Meeting of ISMRM, Honolulu, 2002.

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Weina Cui and Pieter Otten contributed equally to this work.

*Correspondence to: Ralph P. Mason, Ph.D., University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Blvd., Dallas, TX 75390-9058. E-mail: Ralph.Mason@UTSouthwestern.edu

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Many promising methods are being developed to image (i.e., assay noninvasively) tissue gene expression, often by including a reporter gene in tandem with the therapeutic gene (1–4). A critical criterion is that the reporter gene is not normally present or expressed in the cells of interest. One of the earliest examples of gene transfection was the introduction of creatine kinase into the liver of mice, and the subsequent detection of phosphocreatine (PCr) by ^{31}P NMR (5). This particular case involved a benign product and natural substrates, but the technique would be inappropriate in most tissues, where PCr is normally present at high levels. Perhaps the most popular reporter genes today are associated with optical imaging, since this is a cheap modality and highly sensitive results are rapidly obtained. Thus, bioluminescent imaging (BLI) of luciferase (1), and fluorescent imaging of green fluorescent protein (GFP) and longer-wavelength variants (6) are popular. These techniques are very useful in superficial tissues and have extensive applications in mice, but application to larger bodies is limited by the depth of light penetration.

Several nuclear medicine approaches have been demonstrated that employ thymidine kinase with a variety of substrates, including iodo- and fluoronucleosides (such as FIAU and gancyclovir) and various radionuclide labels (e.g., 123-, 124-, 125-I, and ^{18}F) (3,7). An alternative approach uses the sodium iodine symporter (hNIS), which works well with both iodide and pertechnetate substrates (2). For cancer, thymidine kinase is advantageous because the gene serves as a reporter and the gene products themselves may have therapeutic value.

The lac operon was the first gene expression system to be well characterized, some 40 years ago by Jacob and Monod (8). One component, *lacZ*, which produces β-galactosidase, has been the primary choice of reporter gene to verify effective transfection in biochemistry, and many reporter molecules are available for biological and histological analysis. Diverse agents are commercially available with specific characteristics, such as developed color, thermal stability, and cellular retention (e.g., X-gal, o-nitrophenylgalactoside (ONPG), and S-Galacton-star) (9–11). However, β-galactosidase was largely neglected for in vivo work until the elegant studies of Meade et al. (12) were published. The galactose bridged cyclic gadolinium contrast agent ((1-(2-(galactopyranosyloxy)propyl)-4,7,10-tris(carboxymethyl)-1,4,7,10–tetraazacyclo dodecane) gadolinium(III) (EgadMe) shows considerable change in water relaxivity upon exposure to β-galactosidase. While the molecule is a poor substrate for the enzyme (on the order of 500 times less efficient than the colorimetric biochemical agent ONPG) and does not penetrate cells, it can

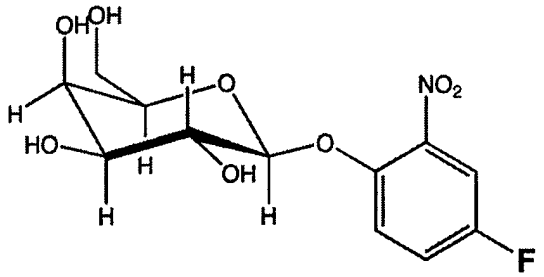

FIG. 1. The structure of PFONPG.

facilitate effective investigations of cell lineages following direct intracellular microinjections (12). These previous findings prompted us to consider other NMR active analogs, and we hypothesized that introducing a fluorine atom into the popular colorimetric biochemical indicator ONPG would produce a strong candidate molecule. We now report the successful synthesis of the reporter molecule, together with NMR characterization and examples of activity in solution and cell culture.

MATERIALS AND METHODS

4-Fluoro-2-nitrophenyl-β-D-galactopyranoside (PFONPG; Fig. 1) was prepared from a reaction of acetobromo-α-D-galactose and the potassium salt of 4-fluoro-2-nitrophenol (PFONP) followed by deprotection with triethylamine using methods similar to those reported by Yoon et al. (13). Details regarding the chemical synthesis will be published in a future work.

^{19}F NMR experiments were performed at 564 MHz using a Varian INOVA Unity spectrometer, and a capillary of sodium trifluoroacetate served as an external chemical shift reference standard (δ 0 ppm). Spectra were obtained from both PFONPG and the aglycone PFONP in saline, buffers, heparinized whole rabbit blood, and prostate cancer cells. The titration curves of PFONP were measured at 25°C and 37°C in saline, and aliquots of HCl (0.2N) and NaOH (0.25N) were added to alter pH, which was independently measured in the NMR tube using a pH electrode.

For single-component kinetic enzyme experiments, PFONPG (5 mg) was dissolved in buffer-I (0.6 ml, pH = 4.5, prepared with 10 mM sodium hydrogen phosphate and 5 mM citric acid). A solution of β-galactosidase (G5160 from *Aspergillus oryzae* (Aldrich); 10 μl of a solution of 19 mg (152 units) in 2 ml buffer-I) was added, and NMR data were acquired immediately at 30°C. Each spectrum was acquired in 36 s and the kinetic curve was assessed over 11 min. A similar experiment was performed using β-gal isolated from *Escherichia coli* (G6008; 250–600 U/mg) in buffer-II at 37°C. Buffer-II was prepared with HEPES (2 mM) and hydrogen phosphate (7 mM), providing the higher pH (7.3) that is optimal for this enzyme.

To test substrate efficacy, a substrate competition experiment was undertaken. NMR experiments were performed with various concentrations of PFONPG (28.8–262 μmol) added to a solution of β-gal (G5160). In a second series, 2-nitrophenyl-β-D-galactopyranoside (ONPG, 65 μmol) was added simultaneously to each sample.

For cell studies, 5×10^6 human prostate tumor cells PC-3 (American Type Culture Collection, Manassas, VA) and LNCAP C4-2 (UroCor, Oklahoma City, OK) were grown on 150-mm culture dishes in T-medium (Invitrogen) with 5% fetal bovine serum at 37°C with 5% CO_2. Control (wild-type) cells and transfected cells (infected by replication defective adenovirus harboring *lacZ* gene under the control of the cytomegalovirus (CMV) or bone sialo protein (BSP) promoter at 10 or 100 multiplicity of infection (MOI), with transfection times of 24 or 48 hr), were harvested. The cells were trypsinized for 2 min and neutralized with medium for harvesting. The cell pellet was obtained by gentle centrifugation, washed twice with phosphate-buffered saline (PBS), and resuspended in 1 ml PBS. PFONPG (2 mg) was added to a suspension of 10^7/ml cells, and ^{19}F NMR spectra were acquired after various incubation times at 30°C or 37°C.

RESULTS

PFONPG is hydrophilic and readily dissolves in saline or whole blood, giving a single narrow ^{19}F NMR signal at δ −42.75 ppm with respect to $\delta_{Na\text{-}TFA}$ 0 ppm (Fig. 2). This signal is essentially invariant in the range of pH 1–11 with a change of <0.05 ppm. The signal appeared stable in solution and whole rabbit blood for a period of 2 days. The addition of β-galactosidase caused rapid cleavage and released the aglycone PFONP, which appeared at δ −46.49 ppm for β-gal G5160 at 30°C and at δ −51.07 ppm for β-gal G6008 at 37°C (Fig. 3), and was accompanied by the development of yellow color. The addition of β-gal (G5160) to PFONPG showed a rapid exponential loss of the substrate accompanied by the appearance of the aglycone over a period of 10 min, as shown by the curves in Fig. 4. Similar activity was seen with G6008, although the change was less rapid under comparable conditions. The substrate competition kinetics showed that ONPG acts as a competitive inhibitor of β-gal with respect to PFONPG: the Michaelis constant increased from 91 μmol to 200 μmol, but Vmax remained unchanged (Fig. 5).

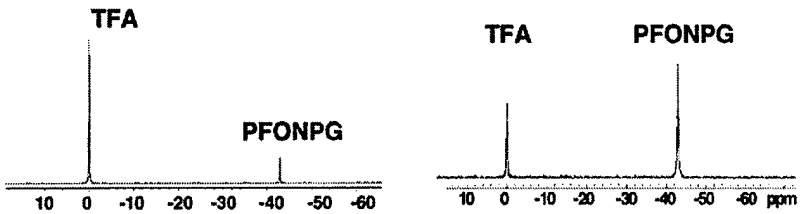

FIG. 2. ^{19}F NMR spectra of PFONPG (2 mg ≡ 8 μmol, 10 mM) in saline (left) and fresh heparinized whole rabbit blood (right). ^{19}F NMR spectra were obtained in 36 s and apodized with a 10-Hz exponential line-broadening prior to Fourier transformation. Sodium trifluoroacetate was used as a chemical shift reference (δ 0 ppm).

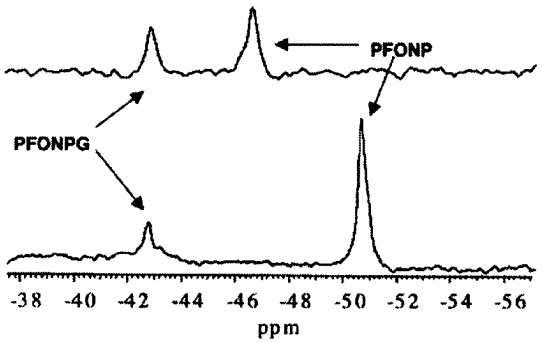

FIG. 3. PFONPG (−42.75 ppm) was rapidly cleaved by β-gal G5160 (pH = 4.5, upper spectrum) and G6008 (initial pH = 7.3, but acidified to 6.8 during reaction, lower spectrum) releasing the aglycone PFONP. Since the product has a pH-sensitive chemical shift, PFONP occurs at different positions for each buffer.

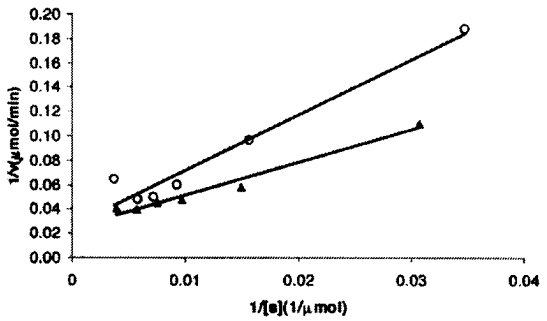

FIG. 5. Lineweaver-Bourke plot showing kinetics of β-gal activity on PFONPG, and competitive inhibition by addition of ONPG (○).

When PFONPG was added to wild-type prostate cancer cells (PC-3 or LNCAP C4-2), there were no spectral changes after incubation for 3 hr at 37°C. When PC-3 cells were transfected with a first-generation adenovirus vector encoding the β-*gal* gene driven by the CMV promoter for 24 hr, 12.7% PFONPG was converted to PFONP after incubation for 3 hr at 37°C (Table 1). When PC-3 cells or LNCAP C4-2 cells were transfected with the CMV system, but for 48 hr, 74% and 100% of PFONPG was converted by PC-3 and LNCAP C4-2 cells, respectively, after incubation for 15 min at room temperature. CMV may be considered a universal promoter, so we also tested the β-*gal* gene under control of the specific BSP promoter. In this case, the substrate cleavage was lower, with only 5% conversion after incubation with PFONPG for 3 hr at 37°C for cells with 10-fold MOI viral transfection, and 14% for 100 MOI.

The ^{19}F NMR signal of the aglycone (PFONP) is very sensitive to pH, exhibiting a range of 9.3 ppm. The titration curves (Fig. 6) were identical at 25°C and 37°C, and gave the Henderson Hasselbalch coefficients pKa = 6.85, δ_{acid} −46.44 ppm, δ_{base} −55.73 ppm.

DISCUSSION

We have demonstrated the potential utility of a novel class of gene reporter molecules—fluoro-phenyl-galactopyranosides (specifically, PFONPG)—as an effective substrate for β-galactosidase. This molecule is an excellent substrate for the enzyme and acts competitively with traditional biochemical indicators. It provides a single ^{19}F NMR signal with a narrow linewidth and good stability in solution. It is apparently stable in normal wild-type cells and whole blood, but exposure to the enzyme or cells transfected to express β-galactosidase causes rapid cleavage, in line with anticipated levels of transfection.

Upon cleavage of the glycosidic bond, a chemical shift difference of >3.6 ppm is observed. However, the chemical shift of the product may have a range of about 9 ppm, since the released aglycone is pH-sensitive and the pKa is in the physiological range. Significantly, there is no overlap between the chemical shift of the substrate and the product. This presents the interesting possibility of selective determination of pH at the site of enzyme activity. Indeed, we have demonstrated that if the aglycone (PFONP) is added to a suspension of red blood cells, two signals are rapidly observed representing the intra- and extracellular pH (14). However, PFONP is somewhat toxic

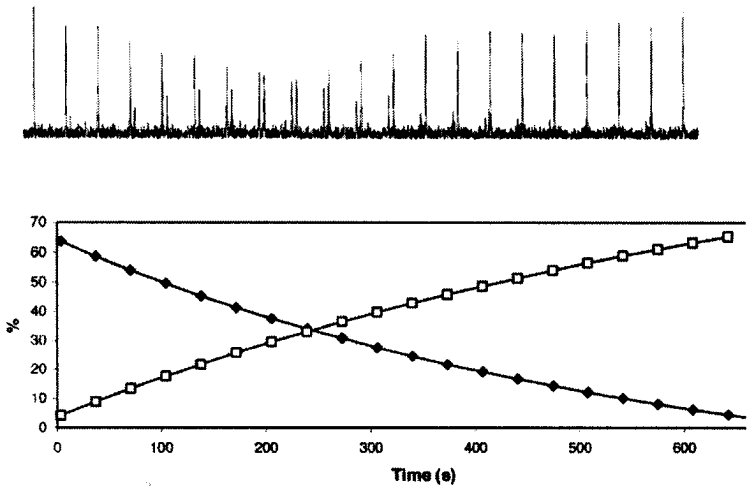

FIG. 4. Kinetic curves showing cleavage of PFONPG to PFONP by β-gal G5160. The upper trace shows the spectral time course, and the amplitudes are plotted below.

and causes lysis of less robust cells, such as cultured tumor cells. Thus, PFONPG may be regarded as an interesting prototype molecule that is primarily representative of a new approach to the use of NMR gene reporter molecules in association with β-galactosidase. Given the well-known promiscuity (lack of specificity) of β-galactosidase (15), many commercial colorimetric molecular substrates are available for this enzyme. We believe that other NMR-sensitive aglycone analogs may readily be introduced in place of the PFONP, and can display preferable characteristics. In particular, analogs may be selected to be less toxic, to be selectively trapped in cells, or to have particular activities as specific reporters or drugs. Indeed, preliminary investigations show the feasibility of conjugating the pH reporter 6-fluoropyridoxol (16) to galactose, and thereby generating an effective substrate for β-gal (17).

While the toxicity of PFONP appears to severely limit the application of PFONPG as a gene reporter molecule, it does present the intriguing possibility of a broad-spectrum, gene-activated chemotherapeutic. Indeed, PFONP is clearly analogous to the classic biochemical uncoupler dinitrophenol. While specific traditional chemotherapeutic drugs may be subject to multidrug resistance and become ineffective, we believe that nitrophenols could exert local cytotoxic effects on many tissues. Gene-activated drug therapy (often termed gene-directed enzyme prodrug therapy (GDEPT)) (18), has been demonstrated by others using the cytosine deaminase (CD) gene. Specifically, CD activates the minimally toxic 5-fluorocytosine (5-FC) to the highly toxic 5-fluorouracil (5-FU). This is being widely exploited in gene therapy trials, in the hope that it may mitigate the toxicity threshold associated with systemic 5-FU delivery (18,19). The conversion of 5-FC to 5-FU causes a chemical shift of ~1.5 ppm, revealing gene activity. This has been demonstrated in a number of systems in vivo (18,20). It is also interesting to note that some of the major metabolic products of 5-FU exhibit chemical shift sensitivity to pH, which may provide an indication of local tissue pH (21).

NMR reporters have a long shelf life and are easy to handle, both of which are great advantages over radioactive substrates. Functional paramagnetic agents are attractive because they interact with large numbers of water molecules generating amplification, as previously shown for various "smart" contrast agents (22). However, the prototype proton NMR substrate for β-gal (EgadMe) fails to enter cells and is a poor substrate for the enzyme. Introducing a fluorine atom minimally perturbs the structure and reactivity of the traditional efficient biochemical substrate (ONPG), and offers a new approach. Given that there is essentially no ^{19}F NMR background signal in tissues, fluorinated reporter molecules may be assessed by changes in chemical shift. Our ability to detect these molecules is dependent on the signal-to-noise ratio (SNR), rather than the contrast-to-noise ratio (CNR). The chemical shift difference between substrate and aglycone product reveals the unambiguous detection of enzyme activity. This approach was previously demonstrated for the cytosine deaminase reporter gene system (18,20). Spatial resolution will require chemical shift imaging, rather than merely selective excitation, since the PFONP can have a wide pH-dependent chemical shift range. Detectability could be enhanced by the introduction of a trifluoromethyl (CF_3) reporter group, as opposed to the single F-atom. However, a CF_3 group will likely perturb the water solubility to a greater extent, and the chemical shift response is expected to be considerably smaller due to transmission of the electron density redistribution through an additional carbon–carbon bond.

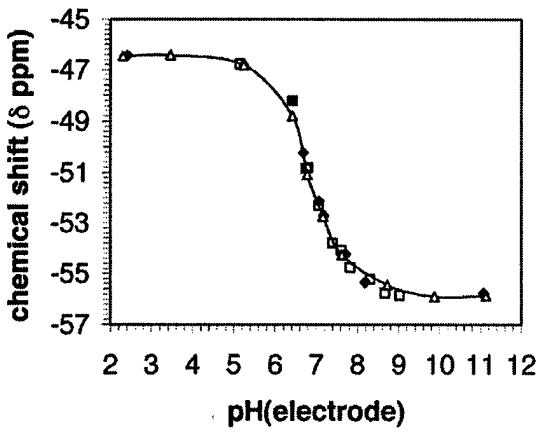

FIG. 6. Titration curves of PFONP in saline (◆), and plasma at 30°C (□), and 37°C (△).

PFONPG is readily synthesized using simple organic chemistry methods. It is water-soluble and appears to enter cells easily. However, the product aglycone is not trapped and in some cases causes cell lysis. Thus, while cleavage provides clear evidence of β-galactosidase activity, it would be difficult to localize this information to specific tissues in vivo. This may limit the ultimate application of this prototype molecule for widespread studies.

Table 1
Relative Gene Expression of Transfected Cells Determined Using PFONPG

Cell line Promoter	PC3 BSP	PC3 CMV	PC3 CMV	C4(2) CMV
Transfection time	24 hr	24 hr	48 hr	48 hr
Incubation temperature	37°C	37°C	Room	Room
Incubation time	3 hr	3 hr	15 min	15 min
Cleaved portion	5.3%	12.7%	74%	100%

ACKNOWLEDGMENTS

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A Novel NMR Platform for Detecting Gene Transfection: Synthesis and Evaluation of Fluorinated Phenyl β-D-Galactosides with Potential Application for Assessing LacZ Gene Expression

Jianxin Yu, Pieter Otten, Zhenyi Ma, Weina Cui, Ralph P. Mason*

Department Radiology, The University of Texas Southwestern Medical Center at Dallas, Texas

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Keywords: gene reporter molecules, lacZ, β-galactosidase, ^{19}F NMR

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* Author for correspondence.

Ralph P. Mason, Ph.D., C.Chem.

Department of Radiology

The University of Texas Southwestern Medical Center at Dallas

5323 Harry Hines Blvd,

Dallas, Texas 75390-9058

USA

Tel: (214)-648-8926, Fax: (214)-648-2991,

E-mail: Ralph.Mason@UTSouthwestern.edu.

Abbreviations

β-gal; β-galactosidase

BLI; bioluminescent imaging

GFP; green fluorescent protein

ONPG; ortho-nitrophenyl-β-galactopyranoside

PFONPG; 4-fluoro-2-nitrophenyl-β-D-galactopyranoside

S-Gal™; 3,4-cyclohexenoesculetin-β-galactopyranoside

X-Gal; 5-bromo-4-chloro-3-indoxyl-β-galactopyranoside

Abstract

Gene therapy holds great promise for the treatment of diverse diseases, but widespread implementation is hindered by difficulties in assessing the success of transfection. The development of non-invasive reporter techniques based on appropriate molecules and imaging modalities may help to assay gene expression. Fluorophenyl-β-D-galactopyranosides provide a novel class of NMR active molecules, which are highly responsive to the action of β-galactosidase (β-gal), the product of the lacZ gene. The reporter molecules are stable in solution and with respect to wild type cells, but the enzyme causes liberation of the aglycone, a fluorophenol, accompanied by distinct color formation and a ^{19}F NMR chemical shift of 5 to 10 ppm, depending on pH. Synthetic strategy, experimental methods and molecular and ^{19}F NMR characteristics are reported for a series of molecules in solution, blood, and tumor cells. This class of molecules presents a new strategy for assaying gene expression with a highly versatile molecular structural platform.

Introduction

Gene therapy holds great promise for treatment of diseases including cancer, cystic fibrosis, and immuno deficiency. However, a major hurdle to widespread successful implementation is the need to verify successful transfection, in particular, the spatial distribution of gene expression in the target tissue, together with assays of the longevity of expression. An image based assay could greatly facilitate optimal gene therapy vector dosing, in a precise temporal and spatial manner.

Two approaches are gaining popularity for reporter genes. One method favors the use of genes producing reporter molecules such as green fluorescent protein, which are directly detectable by physical methods such as fluorescence. The second approach uses genes to produce enzymes, which act upon substrates administered to specifically interrogate gene expression. A critical criterion is that the reporter gene not be normally present or expressed in the cells of interest. The most popular reporter genes today are associated with optical imaging, since this is a cheap modality, is highly sensitive and the results are rapidly available (1, 2). Thus, fluorescent imaging of green fluorescent protein (GFP and longer wavelength variants (3)) and bioluminescent imaging (BLI) of luciferase activity on administered D-luciferin (4) are popular. These techniques are useful only in superficial tissues and have extensive applications in mice, but application to larger bodies is limited by shallow light penetration.

Several nuclear medicine approaches have been demonstrated by exploiting the action of thymidine kinase on a variety of substrates including iodo- and fluoro-nucleosides, such as FIAU and gancyclovir, and various radionuclide labels including 123-, 124-, 125-I, and ^{18}F (5, 6). For cancer, thymidine kinase has the advantage that

the gene serves not only as a reporter, but the gene products can themselves have therapeutic value. An alternative approach uses the sodium iodine symporter (hNIS), which works well with both iodide and pertechnetate substrates (7).

NMR has been applied to cells transfected to express melanin or transferrin resulting in iron accumulation, which produces proton MRI contrast (8, 9). ^{19}F NMR has been used to detect conversion of 5-fluorocytosine to 5-fluorouracil following introduction of cytosine deaminase (10).

Historically, the bacterial *lacZ* gene, encoding the enzyme β-galactosidase (β-gal, EC 3.2.1.23), has been the most popular reporter gene. The lac operon was the first gene expression system to be well characterized, some forty years ago by Jacob and Monod (11), and it is a recognized tool for the study of problems in cell and molecular biology and the recently emerging fields of genomics and proteomics (12). Its induction has become a standard means of assaying clonal insertion and transcriptional activation (13). The long-established tests for β-gal based on colorimetric assay of *ortho*- and *para*-nitrophenyl-β-galactopyranoside hydrolysis to release yellow *ortho*- or *para*-nitrophenols remain popular (14). However, noting the promiscuity of the enzyme, alternate reporter substrates have been proposed (15-19) and many are commercially available. Fluorogenic galactosides based on fluorescein and resorufin, such as p-naphtholbenzein, 1,2-dihydroxyanthraquinone and 4-methylumbeliferone are well established (16, 20, 21). When agar-based methods are used, chromogenic substrates that yield insoluble products are desirable, so that the aglycones released by hydrolysis do not diffuse widely. Indoxylic galactosides, such as indoxyl-β-galactopyranoside, and halogenated derivatives, including 5-bromo-4-chloro-3-indoxyl-β-galactopyranoside (X-

Gal), have the advantage, that upon release the aglycone is oxidized rapidly in air to produce an insoluble colored product, which is restricted to the cells (22). Serious disadvantages of X-gal are its light sensitivity, poor solubility in water and it is often prepared in the presence of toxic organic solvents, such as dimethylformamide. A new substrate, 3,4-cyclohexenoesculetin-β-galactopyranoside (S-Gal^{TM}) provides enhanced contrast and earlier color detection by producing an intense black stain from the formation of an insoluble black chelate with iron (Fe^{3+}) (23). The staining methodologies described above are effective for histological specimens and *in vitro*, but *in vivo* capabilities would promise new applications to study, and clinically evaluate, gene transfection.

Recently, Louie *et al.* (24) demonstrated an elegant MRI assessment of β-gal activity based on 1-[2-(β-D-galactopyranosyloxy) propyl]-4, 7, 10-tris (carboxymethyl)-1, 4, 7, 10-tetraazacyclododecane) gadolinium (III) (EgadMe). Access of water to the first coordination sphere of the paramagnetic Gd^{3+} is blocked by a galactopyranose bridge, but β-gal cleaves the bridge yielding a 20% increase in relaxivity. While EgadMe is a poor substrate for the enzyme (of the order of 500 times less efficient than the colorimetric biochemical agent ONPG) and does not penetrate cells, it facilitated effective investigation of cell lineage following direct intra-cellular microinjections (24). These studies prompted us to consider other NMR active analogs, and it appeared that introduction of a fluorine atom into the popular colorimetric biochemical indicator ONPG could produce a strong candidate molecule. Diverse fluorinated reporter molecules have been successfully applied previously to diverse metabolic and physiological studies (25, 26). ^{19}F-labeled molecules exploit the high NMR visibility of fluorine, the great NMR

sensitivity of ^{19}F to the environmental milieu, and the lack of background signal. We recently reported successful synthesis of a prototype reporter molecule, *para*-fluoro *ortho*-nitrophenyl β-D-galactopyranoside (PFONPG) **16,** and demonstrated initial NMR applications (27). We have now synthesized and evaluated a series of analogs and provide structural characterization, together with evaluation of their activity in solution and in cell culture, and to compare the relative merits of the substrates.

Design and Synthesis

Since ONPG (*ortho*-nitrophenyl β-D-galactopyranoside) and PNPG (*para*nitro-phenyl β-D-galactopyranoside) are widely used and highly effective substrates for β-gal, we chose to generate simple analogs incorporating a ^{19}F atom. The *ortho*- and *para*-fluorinated phenolic derivatives **2 - 8** were chosen as aglycones to seek optimum substrates for β-gal with effective enzyme sensitivity, a large ^{19}F NMR chemical shift response, and minimal toxicity. Although many nitrophenol glycosides have been described (28, 29) including nitrophenyl fluorogalactoside (30), to our knowledge, no published syntheses of the β-D-galactosides **16 – 22** have been reported. For the construction of *O*-glycosidic linkages, many strategies are available as a result of the early work of Fischer (31), Köenigs and Knorr (32), Helferich and Olst (33), Lemieux (34), and Paulsen (35) and the more recent work of Schmidt (36), Kahne (37), Danishefsky (38), and Seeberger (39). Aryl glycopyranosides were generally prepared by coupling of a phenol with the O-acetylated sugar in the presence of anhydrous zinc chloride (for the α anomer) or *p*-toluenesulfonic acid (for the β anomer). Some

improvements of these synthetic techniques have been published (40-42), but the *p*-nitrophenyl glycosides were usually obtained in low yield.

We initially tested the method of Yoon *et al.* (30), which involves the reaction of a potassium salt of an acidic phenol with an α-Dgalactopyranosyl bromide. Aryl β-D galactopyranosides **16, 17** were prepared by a nucleophilic substitution reaction of a phenolate ion on acetobromo-α-D-galactose **1** followed by saponification of the acetyl groups. We applied two different procedures for generating the phenolate ion, depending on the acidity of the phenol. The brightly colored solid potassium salts of fluoro-nitrophenols **2** and **3** could be isolated by lyophilizing an aqueous solution of phenol with a slight excess of aq. KOH. The salts were subsequently used directly in excess for the synthesis of the corresponding fluoronitrophenyl tetra-*O*-acetyl β-D galactopyranosides **9** and **10**. Compounds **9** and **10** were isolated pure in moderate yields (58-77%) after basic extractive work-up and flash chromatography. A different strategy had to be applied for the less acidic 2-fluorophenol **5**. The phenolate was generated *in situ* with K_2CO_3 in refluxing acetone in the presence of a catalytic amount of 18-crown-6 (43).

However, given the reported high yields, mild reaction conditions, and high stereospecificity, we ultimately explored phase-transfer catalysis for the synthesis of our desired glycosides (Fig. 1) (30, 43).

Experimental

General methods

NMR spectra were recorded on a Varian MERCURY 400 (or 600) spectrometer (400

(600) MHz for ^{1}H, 100 (150) MHz for ^{13}C, 376 MHz for ^{19}F) with $CDCl_3$ (for **9 – 15**) or DMSO-d_6 (for **16 – 22**) as solvents and ^{1}H and ^{13}C chemical shifts referenced to TMS, as internal standard. Compounds were characterized by acquisition of ^{1}H, ^{13}C, DEPT, ^{1}H-^{1}H COSY or NOESY experiments at 25 °C. ^{19}F NMR measurements were performed at 25-37 °C in aqueous solution with ^{19}F signals referenced to dilute sodium trifluoroacetate (NaTFA) in a capillary, as an external standard. High-resolution mass spectra were obtained on an ABI Voyager STR MALDI-TOF mass spectrometer in reflector mode (service provided by Dr. Tichy, Department of Chemistry, Texas A&M University).

Reactions requiring anhydrous conditions were performed under nitrogen or argon. Solutions in organic solvents were dried with anhydrous sodium sulfate, and concentrated *in vacuo* below 45 °C. Column chromatography was performed on silica gel (200~300 mesh) with cyclohexane-EtOAc. Analytical TLC (Silica gel GF_{254}; Aldrich Chemical Company) used detection by UV or staining with 5% ethanolic H_2SO_4 at 110 °C for 10 min.

Experimental

Fluorinated phenyl β-D-galactopyranoside tetraacetates 9-15.

General procedure (Fig. 1). A solution of **1** (1 mmol; 2, 3, 4, 6-tetra-O-acetyl-α-D-galactopyranosyl bromide, Sigma) in CH_2Cl_2 (5 ml) was added dropwise to a vigorously stirred solution of fluorophenol **2 - 8** (1.2 mmol) and tetrabutylammonium bromide (0.48 g, 1.5 mmol) in H_2O (5 ml; pH 8~9) 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, dried, evaporated under reduced pressure and

recrystallized (EtOH-H_2O) or purified by column chromatography on silica gel to give fluorinated aryl β-D-galactopyranoside tetraacetates **9 - 15**, as white crystals.

2-Nitro-4-fluorophenyl 2, 3, 4, 6-tetra-O-acetyl-β-D-galactopyranoside 9 (0.5 g, 99%), R_f 0.31(3:2 cyclohexane-EtOAc), δ_H: 7.55(1H, dd, *J*=3.0, 8.4 Hz, Ar-H), 7.42(1H, dd, *J*=4.8, 9.0 Hz, Ar-H), 7.27(1H, m, Ar-H), 5.04(1H, d, $J_{1,2}$=7.8 Hz, H-1), 5.52(1H, dd, $J_{2,3}$=8.4 Hz, H-2), 5.11(1H, dd, $J_{3,4}$=3.0 Hz, H-3), 5.47(1H, d, $J_{4,5}$=2.6 Hz, H-4), 4.07(1H, m, H-5), 4.26(1H, dd, $J_{5,6a}$=4.2 Hz, $J_{6a,6b}$=11.4 Hz, H-6a), 4.17(1H, dd, $J_{5,6b}$=5.4 Hz, H-6b), 2.20, 2.14, 2.07, 2.02(12H, 4s, 4×CH_3CO)ppm; δ_C: 170.43, 170.31, 170.24, 169.62(4×CH_3**C**O), 157.70(d, J_{F-C}=164.8 Hz, Ar-C), 145.66(Ar-C), 141.86(d, J_{F-C} =5.7 Hz, Ar-C), 122.68(d, J_{F-C}=4.9 Hz, Ar-C), 120.77(d, J_{F-C}=15.2 Hz, Ar-C), 112.60(d, J_{F-C}=18.3 Hz, Ar-C), 101.40(C-1), 68.01(C-2), 70.63(C-3), 66.86(C-4), 71.58(C-5), 61.45(C-6), 21.25, 21.10, 20.38, 20.25(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-nitrophenyl 2, 3, 4, 6-tetra-O-acetyl-β-D-galactopyranoside 10 (0.5 g, 99%), R_f 0.35(3:2 cyclohexane-EtOAc), δ_H: 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, dd, $J_{4,5}$=3.6 Hz, H-4), 4.10(1H, ddd, $J_{5,6a}$=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: 170.45, 170.26, 170.21, 169.44(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-C}=23.7 Hz, Ar-C), 100.28(d, J_{F-C}=3.1 Hz, C-1), 68.34(C-2), 70.57(C-3), 66.81(C-4), 71.83(C-5), 61.45(C-6), 20.80, 20.77, 20.72,

20.33(4×**C**H_3CO)ppm; HRMS: $[M+Na]^+$, $C_{20}H_{22}NO_{12}FNa$, Calcd: 510.1010, Found: 510.1014; $[M+K]^+$, $C_{20}H_{22}NO_{12}FK$, Calcd: 526.0763, Found: 526.0751.

2-Nitro-6-fluorophenyl 2, 3, 4, 6-tetra-O-acetyl-β-D-galactopyranoside 11 (0.49 g, 98%), R_f 0.30(3:2 cyclohexane-EtOAc), δ_H: 7.58(1H, ddd, *J*=1.2, 1.4, 8.8 Hz, Ar-H), 7.39(1H, ddd, *J*=1.8, 8.4, 9.8 Hz, Ar-H), 7.29(1H, m, Ar-H), 5.02(1H, d, $J_{1,2}$=8.0 Hz, H-1), 5.48(1H, dd, $J_{2,3}$=10.4 Hz, H-2), 5.08(1H, dd, $J_{3,4}$=5.4 Hz, H-3), 5.41(1H, dd, $J_{4,5}$=3.6 Hz, H-4), 3.90(1H, m, H-5), 4.11(1H, dd, $J_{5,6a}$=7.8 Hz, $J_{6a,6b}$=10.2 Hz, H-6a), 4.08(1H, dd, $J_{5,6b}$=7.2 Hz, H-6b), 2.20, 2.14, 2.01, 2.00(12H, 4s, 4×CH_3CO)ppm; δ_C: 170.45, 170.19, 169.61(4×CH_3**C**O), 155.84(d, J_{F-C}=251.8 Hz, Ar-C), 146.01(Ar-C), 137.17(d, J_{F-C}=15.3 Hz, Ar-C), 125.66(d, J_{F-C}=7.6 Hz, Ar-C), 121.11(d, J_{F-C}=20.6 Hz, Ar-C), 120.22(d, J_{F-C}= 3.8 Hz, Ar-C), 102.84(d, J_{F-C}=2.3 Hz, C-1), 68.75(C-2), 70.76(C-3), 66.74(C-4), 71.41(C-5), 61.02(C-6), 20.82, 20.79, 20.70, 20.66(4×**C**H_3CO)ppm; HRMS: $[M+Na]^+$, $C_{20}H_{22}NO_{12}FNa$, Calcd: 510.1024, Found: 510.1011; $[M+K]^+$, $C_{20}H_{22}NO_{12}FK$, Calcd: 526.0763, Found: 526.0750.

2-Fluorophenyl 2, 3, 4, 6-tetra-O-acetyl-β-D-galactopyranoside 12 (0.45g, 98%), R_f 0.40(3:2 cyclohexane-EtOAc), δ_H: 7.48(1H, dd, *J*=2.0, 10 Hz, Ar-H), 7.36(1H, m, Ar-H), 7.30(1H, m, Ar-H), 7.22(1H, m, Ar-H), 5.54(1H, d, $J_{1,2}$=8.4 Hz, H-1), 5.48(1H, dd, $J_{2,3}$=10.0 Hz, H-2), 5.09(1H, dd, $J_{3,4}$= 4.4 Hz, H-3), 5.40(1H, d, $J_{4,5}$=3.0 Hz, H-4), 4.24(1H, m, H-5), 4.18(1H, dd, $J_{5,6a}$=7.5 Hz, $J_{6a,6b}$=10.4 Hz, H-6a), 4.08(1H, dd, $J_{5,6b}$=7.0 Hz, H-6b), 2.19, 2.10, 2.04, 2.02(12H, 4s, 4×CH_3CO)ppm; δ_C: 170.66, 170.23, 170.10, 169.58(4×CH_3**C**O), 156.14(Ar-C), 146.01(d, J_{F-C}=245.9 Hz, Ar-C), 136.58(d, J_{F-C} =14.8 Hz, Ar-C), 126.08(d, J_{F-C}=7.9 Hz, Ar-C), 122.33(d, J_{F-C}=26.3 Hz, Ar-C), 121.33(d, J_{F-C}=6.8 Hz, Ar-C), 101.89(d, J_{F-C}=2.6 Hz, C-1), 68.85(C-2), 70.58(C-3), 66.79(C-4),

71.56(C-5), 61.33(C-6), 20.77, 20.70, 20.60, 20.58(4×**C**H_3CO)ppm; HRMS: $[M+Na]^+$, $C_{20}H_{23}NO_{10}FNa$, Calcd: 465.1173, Found: 465.1169; $[M+K]^+$, $C_{20}H_{23}NO_{10}FK$, Calcd: 481.0912, Found: 481.0907.

4-Fluorophenyl 2, 3, 4, 6-tetra-O-acetyl-β-D-galactopyranoside 13 (0.42 g, 95%), R_f 0.45(3:2 cyclohexane-EtOAc), δ_H: 6.99(4H, m, Ar-H), 4.98(1H, d, $J_{1,2}$=8.0 Hz, H-1), 5.48(1H, dd, $J_{2,3}$=10.0 Hz, H-2), 5.11(1H, dd, $J_{3,4}$=4.2 Hz, H-3), 5.45(1H, d, $J_{4,5}$=2.4 Hz, H-4), 4.06(1H, m, H-5), 4.24(1H, dd, $J_{5,6a}$=7.2 Hz, $J_{6a,6b}$=10.4 Hz, H-6a), 4.16(1H, dd, $J_{5,6b}$=6.4 Hz, H-6b), 2.19, 2.09, 2.06, 2.02(12H, 4s, 4×CH_3CO)ppm; δ_C: 170.45, 170.36, 170.28, 169.48(4×CH_3**C**O), 160.08(Ar-C), 157.69(Ar-C), 153.10(d, J_{F-C}= 20.0 Hz, Ar-C), 118.82~115.97(m, Ar-C), 100.62(C-1), 68.68(C-2), 70.71(C-3), 66.90(C-4), 71.06(C-5), 61.44(C-6), 20.79, 20.72, 20.68, 20.64(4×**C**H_3CO)ppm; HRMS: $[M+Na]^+$, $C_{20}H_{23}NO_{10}FNa$, Calcd: 465.1173, Found: 465.1170; $[M+K]^+$, $C_{20}H_{23}NO_{10}FK$, Calcd: 481.0912, Found: 481.1014.

2-Bromo-4-fluorophenyl 2, 3, 4, 6-tetra-O-acetyl-β-D-galactopyranoside 14 (0.50 g, 97%), R_f 0.48(3:2 cyclohexane-EtOAc), δ_H: 7.28(1H, ddd, *J*=3.2, 2.8, 3.2 Hz, Ar-H), 7.16(1H, dd, *J*=4.8, 9.2 Hz, Ar-H), 6.97(1H, m, Ar-H), 4.91(1H, d, $J_{1,2}$= 8.0 Hz, H-1), 5.56(1H, dd, $J_{2,3}$=10.8 Hz, H-2), 5.10(1H, dd, $J_{3,4}$=3.6 Hz, H-3), 5.46(1H, d, $J_{4,5}$=4.0 Hz, H-4), 4.24(1H, m, H-5), 4.25(1H, dd, $J_{5,6a}$=7.2 Hz, $J_{6a,6b}$=11.4 Hz, H-6a), 4.08(1H, dd, $J_{5,6b}$=6.0 Hz, H-6b), 2.19, 2.11, 2.06, 2.02(12H, 4s, 4×CH_3CO)ppm; δ_C: 170.52, 170.43, 170.34, 169.57(4×CH_3**C**O), 159.80(Ar-C), 157.35(Ar-C), 150.22(Ar-C), 120.91~113.93(m, Ar-C), 101.36(C-1), 68.33(C-2), 70.66(C-3), 67.03(C-4), 71.49(C-5), 61.51(C-6), 21.28, 21.15, 21.10, 20.91(4×**C**H_3CO)ppm; HRMS: $[M+Na]^+$, $C_{20}H_{22}O_{10}F^{79}BrNa$, Calcd: 543.0279, Found: 543.0266; $C_{20}H_{22}O_{10}F^{81}BrNa$, Calcd:

545.0259, Found: 545.0205; $[M+K]^+$, $C_{20}H_{22}O_{10}F^{79}BrK$, Calcd: 559.0043, Found: 559.0043; $C_{20}H_{22}O_{10}F^{81}BrNa$, Calcd: 560.9997, Found: 560.9892.

2-Chloro-4-fluorophenyl 2, 3, 4, 6-tetra-O-acetyl-β-D-galactopyranoside 15 (0.48 g, 99%), R_f 0.46(3:2 cyclohexane-EtOAc), δ_H: 7.21(1H, dd, *J*=4.8, 8.8 Hz, Ar-H), 7.13(1H, dd, *J*=3.2, 8.0 Hz, Ar-H), 6.92(1H, m, Ar-H), 4.90(1H, d, $J_{1,2}$=8.0 Hz, H-1), 5.54(1H, dd, $J_{2,3}$=10.8 Hz, H-2), 5.12(1H, dd, $J_{3,4}$=3.2 Hz, H-3), 5.46(1H, d, $J_{4,5}$=3.6 Hz, H-4), 4.04(1H, m, H-5), 4.26(1H, dd, $J_{5,6a}$= 6.8 Hz, $J_{6a,6b}$=11.4 Hz, H-6a), 4.16(1H, dd, $J_{5,6b}$=6.4 Hz, H-6b), 2.19, 2.11, 2.06, 2.02(12H, 4s, 4×CH_3CO)ppm; δ_C: 170.40, 170.32, 170.21, 169.49(4×CH_3**C**O), 159.72(Ar-C), 157.25(Ar-C), 149.14(d, J_{F-C}= 3.1 Hz, Ar-C), 125.55(d, J_{F-C}=9.9 Hz, Ar-C), 120.62~114.34(m, Ar-C), 101.46(C-1), 68.29(C-2), 70.51(C-3), 66.97(C-4), 71.38(C-5), 61.40(C-6), 20.99, 20.87, 20.77, 20.66(4×**C**H_3CO)ppm; HRMS: $[M+Na]^+$, $C_{20}H_{22}O_{10}F^{35}ClNa$, Calcd: 499.0783, Found: 499.0761; $C_{20}H_{22}O_{10}F^{37}ClNa$, Calcd: 501.0754, Found: 501.0701; $[M+K]^+$, $C_{20}H_{22}O_{10}F^{35}ClK$, Calcd: 515.0523, Found: 515.0495; $C_{20}H_{22}O_{10}F^{37}ClK$, Calcd: 517.0494, Found: 517.0488.

Fluorinated aryl β-D-galactopyranosides 16 - 22. *General procedure*---- A solution of fluorophenyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside (**9 - 15**) (0.4 g) in anhydrous MeOH (15 ml) containing 0.5M NH_3 was vigorously stirred from 0 °C to room temperature overnight until TLC showed complete reaction. Following solvent removal *in vacuo*, chromatography on silica gel (EtOAc/MeOH) afforded the free galactopyranosides **16 - 22** in near quantitative yield, as white crystalline materials

2-Nitro-4-fluorophenyl β-D-galactopyranoside 16, R_f 0.40(1:9 MeOH-EtOAc), δ_H: 7.84(1H, dd, *J*=2.8, 8.0 Hz, Ar-H), 7.53(1H, ddd, *J*=1.6, 1.0, 2.8 Hz, Ar-H), 7.43(1H, dd,

J=4.4, 9.2 Hz, Ar-H), 4.96(1H, d, $J_{1,2}$=7.6 Hz, H-1), 3.60(1H, dd, $J_{2,3}$=10.6 Hz, H-2), 3.51(1H, dd, $J_{3,4}$=5.2 Hz, H-3), 3.47(1H, d, $J_{4,5}$=5.6 Hz, H-4), 3.43(1H, m, H-5), 3.67(2H, m, H-6), 5.16(1H, d, $J_{H\text{-}2,OH\text{-}2}$=5.2 Hz, HO-2), 4.67(1H, d, $J_{H\text{-}3,OH\text{-}3}$ =4.4 Hz, HO-3), 4.90(1H, d, $J_{H\text{-}4,OH\text{-}4}$=6.0 Hz, HO-4), 4.67(1H, t, $J_{H\text{-}6,OH\text{-}6}$=5.2, 5.4 Hz, HO-6)ppm; δ_C: 155.41(d, $J_{F\text{-}C}$=239.6 Hz, Ar-C), 146.19(d, $J_{F\text{-}C}$=3.1 Hz, Ar-C), 140.17(d, $J_{F\text{-}C}$ = 9.1 Hz, Ar-C), 120.91(d, $J_{F\text{-}C}$=22.1 Hz, Ar-C), 119.03(d, $J_{F\text{-}C}$=7.7 Hz, Ar-C), 111.89(d, $J_{F\text{-}C}$ =27.5 Hz, Ar-C), 101.65(C-1), 70.07(C-2), 73.37(C-3), 68.06(C-4), 75.87 (C-5), 60.33(C-6)ppm; HRMS: $[M+Na]^+$, $C_{12}H_{14}NO_8FNa$, Calcd: 342.0601, Found: 342.0589; $[M+K]^+$, $C_{12}H_{14}NO_8FK$, Calcd: 358.0341, Found: 358.0328.

2-Fluorine-4-nitrophenyl β-D-galactopyranoside 17, R_f 0.45(1:9 MeOH-EtOAc), δ_H: 7.58(1H, dd, J=3.3, 8.0 Hz, Ar-H), 7.42(1H, dd, J=4.2, 8.7 Hz, Ar-H), 7.28(1H, m, Ar-H), 5.16(1H, d, $J_{1,2}$=7.8 Hz, H-1), 4.60(1H, dd, $J_{2,3}$=8.8 Hz, H-2), 3.70(1H, dd, $J_{3,4}$=3.2 Hz, H-3), 3.63(1H, d, $J_{4,5}$=3.1 Hz, H-4), 3.60(1H, m, H-5), 3.50(2H, m, H-6), 4.98(1H, d, $J_{H\text{-}2,OH\text{-}2}$=7.2 Hz, HO-2), 4.66(1H, d, $J_{H\text{-}3,OH\text{-}3}$=4.0 Hz, HO-3), 4.80(1H, d, $J_{H\text{-}4,OH\text{-}4}$=5.0 Hz, HO-4), 4.90(1H, t, $J_{H\text{-}6,OH\text{-}6}$=5.5, 5.8 Hz, HO-6)ppm; δ_C: 156.80(d, J=160.4 Hz, Ar-C), 146.55(Ar-C), 140.68(d, J=5.5 Hz, Ar-C), 123.44(d, J=4.2 Hz, Ar-C), 121.33(d, J=18.2 Hz, Ar-C), 114.80(d, J=17.9 Hz, Ar-C), 101.73(C-1), 68.33(C-2), 70.86(C-3), 66.90(C-4), 71.47(C-5), 61.77(C-6)ppm; HRMS: $[M+Na]^+$, $C_{12}H_{14}NO_8FNa$, Calcd: 342.0601, Found: 342.0585; $[M+K]^+$, $C_{12}H_{14}NO_8FK$, Calcd: 358.0341, Found: 358.0320.

2-Nitro-6-fluorophenyl β-D-galactopyranoside 18A, Rf 0.40(1:9 MeOH-EtOAc), δ_H: 7.73(1H, ddd, J=1.6, 3.2, 8.4 Hz, Ar-H), 7.63(1H, ddd, J_{HH}=1.2, 4.0, 8.4 Hz, J_{HF}=19.2 Hz, Ar-H), 7.46(1H, ddd, J_{HH}=5.2, 8.4 Hz, J_{HF}=16.8 Hz, Ar-H), 5.00(1H, d, $J_{1,2}$=7.6 Hz, H-1), 3.88(1H, dd, $J_{2,3}$=9.2 Hz, H-2), 3.82(1H, dd, $J_{3,4}$=4.0 Hz, H-3), 3.70(1H, dd,

$J_{4,5}$=3.2 Hz, H-4), 3.66(1H, m, H-5), 4.00(2H, m, H-6), 3.80(1H, d, $J_{H\text{-}2,OH\text{-}2}$=4.2 Hz, HO-2), 3.69(1H, d, $J_{H\text{-}3,OH\text{-}3}$=3.0 Hz, HO-3), 3.86(1H, d, $J_{H\text{-}4,OH\text{-}4}$=1.6 Hz, HO-4), 3.62(1H, dd, $J_{H\text{-}6,OH\text{-}6}$=4.6, 5.0 Hz, HO-6)ppm; δ_C: 156.66(d, $J_{F\text{-}C}$=246.2 Hz, Ar-C), 145.78(Ar-C), 138.54(d, $J_{F\text{-}C}$ =12.2 Hz, Ar-C), 125.83(d, $J_{F\text{-}C}$=8.4 Hz, Ar-C), 121.65(d, $J_{F\text{-}C}$=19.9 Hz, Ar-C), 120.80(d, $J_{F\text{-}C}$=3.8 Hz, Ar-C), 106.06(d, $J_{F\text{-}C}$=3.8 Hz, C-1), 70.59(C-2), 74.48(C-3), 69.30(C-4), 77.00(C-5), 61.73(C-6)ppm; HRMS: $[M+Na]^+$, $C_{12}H_{14}NO_8FNa$, Calcd: 342.0601, Found: 342.0599; $[M+K]^+$, $C_{12}H_{14}NO_8FK$, Calcd: 358.0341, Found: 358.0337.

2-Nitro-6-fluorophenyl α-D-galactopyranoside 18B, R_f 0.47(1:9 MeOH-EtOAc), δ_H: 7.76(1H, ddd, *J*=1.6, 2.8, 8.4 Hz, Ar-H), 7.62(1H, ddd, J_{HH}=0.4, 4.8, 8.4 Hz, J_{HF}=12.0 Hz, Ar-H), 7.40(1H, ddd, J_{HH}=4.8, 8.4 Hz, J_{HF}=13.2 Hz, Ar-H), 5.85(1H, d, $J_{1,2}$=3.2 Hz, H-1), 4.01(1H, dd, $J_{2,3}$=8.9 Hz, H-2), 4.03(1H, dd, $J_{3,4}$=3.6 Hz, H-3), 4.11(1H, dd, $J_{4,5}$=3.6 Hz, H-4), 3.74(1H, m, H-5), 3.63(2H, m, H-6), 3.92(1H, d, $J_{H\text{-}2,OH\text{-}2}$=3.2 Hz, HO-2), 3.74(1H, d, $J_{H\text{-}3,OH\text{-}3}$=3.6 Hz, HO-3), 3.83(1H, d, $J_{H\text{-}4,OH\text{-}4}$=2.0 Hz, HO-4), 3.80(1H, t, $J_{H\text{-}6,OH\text{-}6}$=5.6, 6.0 Hz, HO-6)ppm; δ_C: 157.06(d, $J_{F\text{-}C}$=248.7 Hz, Ar-C), 146.05(Ar-C), 139.20(d, $J_{F\text{-}C}$= 13.8 Hz, Ar-C), 124.59(d, $J_{F\text{-}C}$=8.4 Hz, Ar-C), 122.05(d, $J_{F\text{-}C}$=19.9 Hz, Ar-C), 121.37(d, $J_{F\text{-}C}$=3.0 Hz, Ar-C), 104.14(d, $J_{F\text{-}C}$=7.6 Hz, C-1), 70.23(C-2), 70.59(C-3), 69.94(C-4), 74.21(C-5), 62.03(C-6)ppm; HRMS: $[M+Na]^+$, $C_{12}H_{14}NO_8FNa$, Calcd: 342.0601, Found: 342.0590; $[M+K]^+$, $C_{12}H_{14}NO_8FK$, Calcd: 358.0341, Found: 358.0333.

2-Fluorophenyl β-D-galactopyranoside 19, R_f 0.48(1:9 MeOH-EtOAc), δ_H: 7.26(1H, m, Ar-H), 7.21(1H, ddd, *J*=1.2, 3.6, 8.0 Hz, Ar-H), 7.11(1H, ddd, J_{HH}=1.2, 7.2, J_{HF}=14.4 Hz, Ar-H), 6.98(1H, m, Ar-H), 4.91(1H, d, $J_{1,2}$=7.6 Hz, H-1), 3.41(1H, dd, $J_{2,3}$=9.6 Hz, H-2), 3.47(1H, dd, $J_{3,4}$=4.0 Hz, H-3), 3.71(1H, d, $J_{4,5}$=3.2 Hz, H-4), 3.54(1H, m, H-5), 3.60(2H, m, H-6), 3.58(1H, d, $J_{H\text{-}2,OH\text{-}2}$=4.0 Hz, HO-2), 3.48(1H, d, $J_{H\text{-}3,OH\text{-}3}$ =3.1

Hz, HO-3), 3.57(1H, d, $J_{H\text{-}4,OH\text{-}4}$=2.2 Hz, HO-4), 3.54(1H, t, $J_{H\text{-}6,OH\text{-}6}$=4.0, 5.2 Hz, HO-6)ppm; δ_C: 152.28(d, $J_{F\text{-}C}$=242.7 Hz, Ar-C), 145.50(d, $J_{F\text{-}C}$=9.9 Hz, Ar-C), 125.10(d, $J_{F\text{-}C}$ =3.8 Hz, Ar-C), 122.60(d, $J_{F\text{-}C}$=6.9 Hz, Ar-C), 117.73(Ar-C), 116.66(d, $J_{F\text{-}C}$=18.3 Hz, Ar-C), 101.53(C-1), 70.59(C-2), 73.81(C-3), 68.52(C-4), 76.04(C-5), 60.74(C-6)ppm; HRMS: $[M+Na]^+$, $C_{12}H_{15}O_6FNa$, Calcd: 297.0750, Found: 297.0739; $[M+K]^+$, $C_{12}H_{15}O_6FK$, Calcd: 313.0490, Found: 313.0486.

4-Fluorophenyl β-D-galactopyranoside 20, R_f 0.40(1:9 MeOH-EtOAc), δ_H: 7.14~7.03(4H, m, Ar-H), 4.74(1H, d, $J_{1,2}$=7.6 Hz, H-1), 3.54(1H, dd, $J_{2,3}$=10.6 Hz, H-2), 3.50(1H, dd, $J_{3,4}$=3.6 Hz, H-3), 3.72(1H, d, $J_{4,5}$=3.2 Hz, H-4), 3.43(1H, m, H-5), 3.58(2H, m, H-6), 5.30~4.4(4H, br, HO-2, 3, 4, 6)ppm; δ_C: 157.25(d, $J_{F\text{-}C}$= 235 Hz, Ar-C), 153.98(Ar-C), 117.93~115.69(Ar-C), 101.88(C-1), 70.40(C-2), 73.32(C-3), 68.30(C-4), 75.60(C-5), 60.46(C-6)ppm; HRMS: $[M+Na]^+$, $C_{12}H_{15}O_6FNa$, Calcd: 297.0750, Found: 297.0731.

2-Bromo-4-fluorophenyl β-D-galactopyranoside 21, R_f 0.52(1:9 MeOH-EtOAc), δ_H: 7.54(1H, dd, J=2.4, 8.0 Hz, Ar-H), 7.22(2H, m, Ar-H), 4.90(1H, d, $J_{1,2}$=7.6 Hz, H-1), 3.41(1H, dd, $J_{2,3}$=9.6 Hz, H-2), 3.47(1H, dd, $J_{3,4}$=4.0 Hz, H-3), 3.71(1H, d, $J_{4,5}$=3.2 Hz, H-4), 3.54(1H, m, H-5), 3.60(2H, m, H-6), 5.11(1H, d, $J_{H\text{-}2,OH\text{-}2}$=5.6 Hz, HO-2), 4.58(1H, d, $J_{H\text{-}3,OH\text{-}3}$=4.8 Hz, HO-3), 4.89(1H, d, $J_{H\text{-}4,OH\text{-}4}$=5.2 Hz, HO-4), 4.67(1H, t, $J_{H\text{-}6,OH\text{-}6}$=5.2, 5.2 Hz, HO-6)ppm; δ_C: 157.38(d, $J_{F\text{-}C}$=240.4 Hz, Ar-C), 151.09(Ar-C), 120.79~121.32(m, Ar-C), 101.96(C-1), 70.87(C-2), 73.78(C-3), 68.84(C-4), 76.13(C-5), 61.13(C-6)ppm; HRMS: $[M+Na]^+$, $C_{12}H_{14}O_6F^{79}BrNa$, Calcd: 374.9855, Found: 374.9851; $C_{12}H_{14}O_6F^{81}BrNa$, Calcd: 376.9835, Found: 376.9665; $[M+K]^+$, $C_{12}H_{14}O_6F^{79}BrK$, Calcd: 390.9595, Found: 390.9803; $C_{12}H_{14}O_6F^{81}BrK$, Calcd: 392.9575, Found: 392.9687.

2-Chloro-4-fluorophenyl β-D-galactopyranoside 22, R_f 0.45(1:9 MeOH-EtOAc), δ_H: 7.42(1H, dd, J=2.8, 8.4 Hz, Ar-H), 7.28(1H, dd, J=5.2, 9.6 Hz, Ar-H), 7.17(1H, m, Ar-H), 4.90(1H, d, $J_{1,2}$=7.6 Hz, H-1), 3.57(1H, dd, $J_{2,3}$=11.6 Hz, H-2), 3.52(1H, dd, $J_{3,4}$=5.2 Hz, H-3), 3.72(1H, m, H-4), 3.17(1H, m, H-5), 3.60(1H, dd, $J_{5,6a}$=8.8 Hz, $J_{6a,6b}$=12.4 Hz, H-6a), 3.45(1H, dd, $J_{5,6b}$=6.0 Hz, H-6b), 5.17(1H, d, $J_{H\text{-}2,OH\text{-}2}$ =4.0 Hz, HO-2), 4.59(1H, d, $J_{H\text{-}3,OH\text{-}3}$=3.2 Hz, HO-3), 4.89(1H, d, $J_{H\text{-}4,OH\text{-}4}$=7.0 Hz, HO-4), 4.69(1H, br, HO-6) ppm; δ_C: 156.38(d, $J_{F\text{-}C}$=238.80 Hz, Ar-C), 149.62(Ar-C), 122.55(d, $J_{F\text{-}C}$=10.7 Hz, Ar-C), 117.45~114.54(m, Ar-C), 101.45(C-1), 70.25(C-2), 73.47(C-3), 68.23(C-4), 75.75(C-5), 60.39(C-6)ppm; HRMS: $[M+Na]^+$, $C_{12}H_{14}O_6F^{35}ClNa$, Calcd: 331.0361, Found: 331.0349; $C_{12}H_{14}O_6F^{37}ClNa$, Calcd: 333.0331, Found: 333.0249.

Kinetic experiments

Relative substrate efficacy of **16 - 22** was compared using ^{19}F NMR. Enzyme reactions were conducted at 37 °C in PBS (0.1M, pH 7.4) using β-gal (E801A, Promega, Madison, WI, USA). Fluorophenyl β-D-galactopyranosides **16 - 22** (15 mmol) were dissolved in PBS (600 μL, pH=7.4) and β-gal (20 μL, 1 unit/μL E801A in PBS) added, followed by immediate ^{19}F NMR data acquisition at 37 °C with subsequent spectra every 101 s providing a kinetic curve over 51 min.

Substrate efficacy relative to traditional indicators was assessed by spectrophotometry. PFONPG **16** and ONPG (2.5 μmol/ml), respectively, were dissolved in buffer (pH=4.5, 10 mM sodium hydrogen phosphate, 5 mM citric acid). A solution of β-gal (G5160 from Aspergillus oryzae (Aldrich), 95 μg in 10 μl buffer) was added and the absorption (λ= 420 nm) measured every 30 s for 10 mins at r.t.

Cell culture and cell Proliferation Assay

Dunning R3327-MAT-Lu rat prostate cancer cells (isolated for growth in culture by us from solid tumor tissues originally provided by Dr. Peter Peschke of the DKFZ, Heidelberg, Germany) and MTLn3 rat breast cancer cells (ATCC, Manassas, VA, USA) were maintained in RPMI1640 medium supplemented with 100 U/ml penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum (FBS) at 37 °C, with 5% CO_2 and 95% humidified air. Using TransFast™ Transfection Reagent (Promega, Madison, WI, USA), MAT-Lu and MTLn3 cells were co-transfected with pCMVβ (Clontech, Palo Alto, CA, USA) comprising the *E.coli lacZ* gene located under the human cytomegalovirus (CMV) immediate-early enhancer/ promoter region and pCI-neo (Promega, Madison, WI, USA) carrying the neomycin phosphotransferase gene and were selected in growth medium containing 400 μg/ml G418 (Cellgro, Merndon, VA, USA). Cells were harvested, trypsinized and resuspended in PBS, pH 7.4. **17** (1.9 mg, 100 μL) was added to cell suspension in PBS (10^8 cells in 600 μL) and ^{19}F NMR spectra were acquired immediately at 37 °C, and again at various times up to 72 h. MTLn3 cells (10^7) were tested, as above. The β-gal activity of MAT-Lu-LacZ cells was 196 milliunits/10^7 cells and 67 milliunits/10^7 cells for the MTLn3-LacZ cells.

Results

The methodology presented by Yoon *et al.* (30) to synthesize phenyl fluorogalactoside tetraacetates using potassium salts of the phenol, produced mediocre yields for the fluorophenyl analogs **9, and 10.** By contrast the phase-transfer approach using TBAB produced fluorophenyl β-D-galactopyranoside tetraacetates **9 - 15** in near

quantitative yields. The anomeric β-D-configuration of compounds **9 - 15** in the $^4C_1$ chair conformation was unambiguously established on the basis of the observed ^{1}H-NMR chemical shifts (δ_H 5.00~5.25 ppm) of the anomeric protons and the $J_{1,2}$(J ~8 Hz) and $J_{2,3}$(J ~10 Hz) coupling constants (34). The signals of the ^{13}C-NMR spectra of **9 - 15** were assigned by comparison with the chemical shifts of *p*-nitrophenyl β-D-galactopyranoside (30). As expected, the anomeric carbon resonances appeared at ~100 ppm in accord with the β-D-configuration.

Deacetylation of **9-15** gave the free galactopyranosides **16-22** in near quantitative yields. ^{1}H-NMR spectra of **16-22** were assigned by ^{1}H-^{1}H COSY spectra and D_2O exchange. The ^{1}H-NMR chemical shifts (δ_H 4.91- 5.85ppm) of the anomeric protons and the coupling constants ($J_{1,2}$ ~8 Hz; $J_{2,3}$ ~10 Hz) showed that the free galactopyranosides **16-22** (except **18**) retained the anomeric β-D-configuration with the $^4C_1$ chair conformation. The synthesis of **18** was more complex with the isolation of two distinct and separable isomers **18A, and B** in a 1:1 ratio, which also gave distinct ^{19}F NMR spectra. The downfield shifts of **18B** δ_{H1} at 5.85 ppm with $J_{1,2}$ 3.2 Hz and δ_C at 104.14 ppm compared to those of **18A** at 5.00 ppm with $J_{1,2}$ 7.6 Hz and δ_C at 106.06 ppm suggest α and β isomers respectively (44-47). Further evidence supporting the structural assignment of **18B** is that the α anomer resists activity of β-gal (Fig. 4). Epimerization likely occurs during removal of the acetyl groups, possibly via a carbocation pathway due to the excellent leaving group ability of , the 2-fluoro-6-nitrophenol anion group.

All except **18A** were stable in saline (0.9%), PBS (0.1M), and fresh whole rabbit blood, at 25 and 37 °C for extended periods showing no breakdown by ^{19}F NMR even

after 1 week. **18A** hydrolysed completely within 2 h in 0.1M PBS buffer solution (pH=7.4) at 25 °C. By contrast, **18B** was stable even at 60 or 100 °C. As expected, the more polar substituted phenyl β-D-galactopyranosides **16-18** were most soluble and **19-21** quite soluble in water, buffer, and whole blood, but 2-bromo-4-fluorophenyl β-D-galactopyranoside **22** was poorly soluble.

^{19}F NMR

Compounds **16** - **22** each gave a single, narrow ^{19}F NMR signal essentially invariant ($\Delta\delta \leq 0.03$ppm) in rabbit whole blood, 0.9% saline and PBS in the pH range 3 to 12 and at various temperatures ranging from 25 to 37 °C. The liberated aglycones display a chemical shift range of about 16 ppm (Table 1, Fig. 2). Addition of β-galactosidase (E801A) in PBS (0.1M, pH=7.4) at 37 °C caused each substrate to hydrolyze releasing the pH sensitive aglycone **2** - **8** appearing also as a single narrow ^{19}F NMR signal (Fig. 2, Table 1), consistent with the titration curves of **2** - **8** (Fig. 3, Table 2). The minimum ^{19}F NMR chemical shift response observed was 3.56 ppm for **19** and maximum was 9.84 ppm for **16** at pH 7.4 (Table 1).

The various substrates **16-22** exhibited differential sensitivity to β-galactosidase in PBS (pH=7.4) and kinetics were monitored by changes in the integration of the ^{19}F NMR signals (Figs. 2 & 4). The shapes of the kinetic curves suggest straightforward first order kinetics for all substrates. The best substrate was **18A** with an initial rate of 0.74 mM/min/unit, although this molecule hydrolyzes spontaneously within 2 h. **16**, **17** and **19** also showed rapid cleavage, while **20-22** were somewhat slower and **18B** resisted enzyme action (as expected for an α-anomer). By colorimetric assay **16** was essentially equivalent to ONPG as a substrate (Fig. 5).

Several of the aglycones (**2-4**, **6**, **7**) exhibit a large ^{19}F NMR chemical shift in response to pH with $\Delta\delta$ reaching 9.3 ppm. However, **5** and **8** showed little response (Fig. 3). The observed pKa's were found to be in the range of 5.7 to 9.9 (Table 2). There was a strong linear correlation between initial rate of enzymatic hydrolysis of the substrates and pKa of the aglycone (Fig. 6).

When **17** was incubated with wild type MAT-Lu rat prostate cancer cells (4 h, PBS, 37 °C under air-5%CO_2 with 95% humidity), ^{19}F NMR spectra showed no changes. When **17** was incubated with Mat-Lu-LacZ cells (92×10^6, 1803 milliunits β-gal) in PBS, cleavage proceeded in a smooth monotonic manner with hydrolysis complete after 19 h (Fig. 7). MTLn3-*LacZ* breast cancer cells (9.8×10^6, 67 milliunits β-gal) hydrolyzed about 50% of **17** in 19 h.

Discussion

We have presented a method to efficiently and stereoselectively synthesize a series of fluorophenyl β-D-galactopyranosides **16-22** as potential ^{19}F NMR-based gene reporter molecules. We previously demonstrated that **16** could be used to assess β-gal activity in transiently transfected human prostate cancer cells (27). We have now assessed the relative substrate efficacy of a series of analogs and demonstrated the utility of **17** to assess β-gal activity in stably transfected rat breast and prostate tumor cells.

^{19}F NMR provides a large chemical shift response to small changes in molecular structure or microenvironment (25, 26). Here, hydrolysis provided a minimum $\Delta\delta$ 3.56 ppm between the substrate and the aglycone at pH 7.4, though depending on the pH, it

could range from 2.3 to 12.9 ppm. Significantly, because of the chemical shift range of the ^{19}F signals of the aglycones **2 - 8** (up to 9.3 ppm) several substrates and aglycones can be detected simultaneously, allowing direct comparison of substrate efficacy (Fig. 2). It could also allow simultaneous detection of other fluorinated molecules and could be applied in combination with drugs (*e.g.*, 5-FU) (48), physiological reporters (such as 6-FPOL and hexafluorobenzene for measuring pH, pO_2) (25, 49) or additional enzyme activated molecules (10). Preliminary data suggest that the approach used here, could also be applied to glucosidases and glucoronides. Indeed, Schmidt and Monneret (50) presented ^{19}F NMR analysis of a fluorine tagged nitrogen mustard pro-drug activated by glucuronidase. ^{19}F NMR spectroscopy lacks the spatial resolution of ^{1}H MRI, but the chemical shift accompanying substrate cleavage by β-gal unequivocally reveals enzyme activity. Interestingly, **16-22** provide both an NMR chemical shift upon release of the aglycones **2-8** and a pH dependant intense yellow to orange color This could provide opportunities for dual modality analysis, though colorimetric assays would be problematic *in vivo* (19). Comparing the substrates shows a 10 fold range in rate of substrate reaction at pH 7.4, the optimal pH for activity of β-gal derived from E.coli. The observed initial rates were found to have a strong linear correlation correlated with the pKa of the aglycone (Fig. 6) consistent with previous work by Richard *et al. (51)*, who examined a series of alkyl β-galactosides. At pH 4.5 - the optimal pH for β-gal derived from *A. oryzae*- **16** showed very similar activity in comparison with the traditional colorimetric indicator ONPG.

Substrates **16** and **17** share many attributes with similar sensitivity to β -gal at pH 7.4 (Fig. 4), a large chemical shift range, and effective response to cells expressing β-gal

(Fig. 7). All the aglycones may be considered to be electronically similar to the classic biochemical uncoupler dinitrophenol. In a preliminary study (unpublished), we assessed cytotoxicity. **16** had allowed us to assess β-gal expression (27), but we found extensive cell lysis upon direct exposure to the product aglycone **2** (IC_{50}= 0.35 mM). Other aglycones **3, 4, 6, 8** were less toxic IC_{50}> 1 mM making them more suitable for *in vivo* NMR investigations. **5** and **7** were severely cytotoxic.

The severe cytotoxicity of **5** and **7** and considerable cytolytic activity of **2** suggest that these are less suitable for detection of gene activity. As a corollary, they may however be useful for gene-activated broad spectrum chemotherapy. There is increasing application of cytosine deaminase in cancer gene therapy, including Phase 1 clinical trials (52). A specific drug such as 5-FU may encounter drug resistance, whereas this is less likely for a cytolytic agent such as the toxic aglycones. Thus, one can envisage tumor transfection with LacZ to express β-gal stimulating local release of aglycones such as **2, 5, 7** following administration of the less toxic conjugates. A close analogy has been examined by ^{19}F NMR previously, where fluorinated mustard drugs were released by activity of glucuronidase (50).

Release of the less toxic, pH sensitive aglycones suggests a novel approach to measuring pH at the site of enzyme activity. Moreover, noting the promiscuity of β-gal, we have synthesized an alternative galactoside with the ^{19}F NMR pH indicator fluoropyridoxol (25), as the aglycone, and preliminary data show that it is also sensitive to enzyme activity (53).

While synthesis appeared facile using the method of Yoon *et al.* (30), the method ultimately chosen involves only two steps based on commercial reagents and provides

essentially quantitative yields. No α-anomers of **9-15** were detected, which indicates that the fluorophenyl β-D galactopyranosides are formed exclusively in an Sn2 fashion and no carbocation formation at C1 of the carbohydrate is involved. During the hydrolysis of the acetyl protecting groups, a small amount of the corresponding **2, 3, 4**, 6-tetra-O-acetyl-D-galactopyranoses was observed. However, **18** occurred as two separate isolatable isomers, which showed very different stability and tendency for enzyme susceptibility. **18A** showed some hydrolysis in aqueous solution and was cleaved very rapidly in the presence of β-gal (Fig. 4) due to the excellent leaving group. The α-anomeric status of **18B** is confirmed by the NMR spectra and lack of response to β-gal, since the enzyme is known to have strict specificity for the β configuration (11).

We believe that non-invasive detection of gene reporter molecules will become increasingly important in biomedicine. It will be important to have diverse agents, genes, and modalities for specific applications. Fluorophenyl β-D-Galactosides offer a novel approach for addressing β-gal activity. NMR has the great advantage over optical approaches based on chromogenic substrates that the signal can be detected in colored solutions, *e.g.*, blood (19, 54) and in deep tissue. A key advantage of NMR reporters over radiolabeled substrates is a long shelf life and the absence of radioactivity. Of course NMR does require mM reporter molecule concentrations, as opposed to μM (or lower) needed for optical and radionuclide approaches.

Currently, **17** appears the strongest candidate molecule for assessing β-gal activity and we are initiating investigations with transfected tumors implanted in animals. Moreover, we believe the concept of using ^{19}F NMR to monitor gene transfection,

together with the molecular approach presented here, can serve as a platform technology with widespread application to many diverse genes and enzymes.

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Figure Legends

Figure 1 The reactions and structures of **1** - **22**.

Figure 2 ^{19}F NMR spectra of β-D-galactopyranosides **16** (**PFONPG**), **17** (**OFPNPG**) and **19** (**OFPG**) in PBS at 37°C (a) and following addition of β-gal (2 mins) (b) 34 mins (c)

Figure 3 ^{19}F NMR chemical shift pH titration curves of **2-8** in saline at 25 °C

Figure 4 Hydrolysis time courses of **16-22** (15 mmol) by β-gal (E801A, 20 units) in PBS (0.1M, 0.6 ml) at 37 °C

Figure 5 Colorimetric comparison of enzyme (G5160, pH 4.5) sensitivity for **16** and ONPG.

Figure 6 Brønsted plot of substrate susceptibility to β-gal (E801A, pH 7.4) versus of pKa of liberated aglycone (r^2>0.85).

Figure 7 Hydrolysis of **17** (open symbols) to **3** (solid symbols) by stably transfected Dunning prostate R3327 MAT-Lu-*lacZ* cells (Δ 92×10^6) and MTLn3-*lacZ* (O 9.8×10^6) suspended in PBS at 37 °C.

Tables

Table 1. ^{19}F chemical shifts (ppm) of **16** - **22** before and after the hydrolysis by β-gal*

Compd.	16	17	18A	18B	19	20	21	22
$\delta_{F(substrate)}$	-42.87	-54.93	-50.67	-49.37	-58.74	-45.87	-43.56	-43.82
$\delta_{F(product)}$	-52.71	-61.04	-58.67	-58.67	-62.30	-49.59	-48.13	-48.24
Observed $\Delta\delta_F$	9.84	6.11	8.00	9.30	3.56	3.72	4.57	4.42
Min $\Delta\delta_F$	1.57	3.84	6.72	8.7	3.3	3.46	3.76	3.58
Max $\Delta\delta_F$	12.89	6.11	8.1	9.4	3.59	11.85	11.84	11.63

* β-gal (E801A, 20 units at 37°C in 0.1M PBS, pH 7.4).

Table 2 ^{19}F NMR pH characterization of aglycones at 25 °C

Compd.	2	3	4	5	6	7	8
pKa	6.87	6.03	5.44	8.33	9.80	8.31	8.28
$\delta_{(acid)}$	-44.44	-58.77	-58.07	-62.33	-49.33	-47.32	-47.40
$\delta_{(base)}$	-55.76	-61.01	-57.39	-62.04	-57.72	-55.40	-55.45
Δδ(ppm)	11.32	2.24	0.68	0.29	6.39	8.08	8.05

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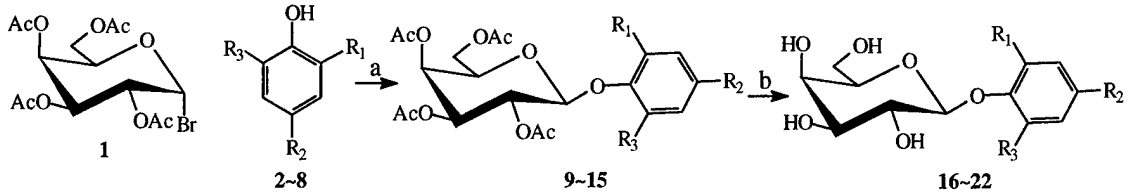
Figure 1. The reactions and structures of **1~22**.



Reaction conditions: (a) CH_2Cl_2-H_2O, pH 8~9, 50 °C, TBAB, ~1 hr, near quantitative yield; (b) NH_3-MeOH, 0 °C→r.t., 24 hr, quantitative yields.

Compounds	R_1	R_2	R_3
2, 9, 16	**NO_2**	**F**	**H**
3, 10, 17	**F**	**NO_2**	**H**
4, 11, 18	**F**	**H**	**NO_2**
5, 12, 19	**F**	**H**	**H**
6, 13, 20	**H**	**F**	**H**
7, 14, 21	**Cl**	**F**	**H**
8, 15, 22	**Br**	**F**	**H**

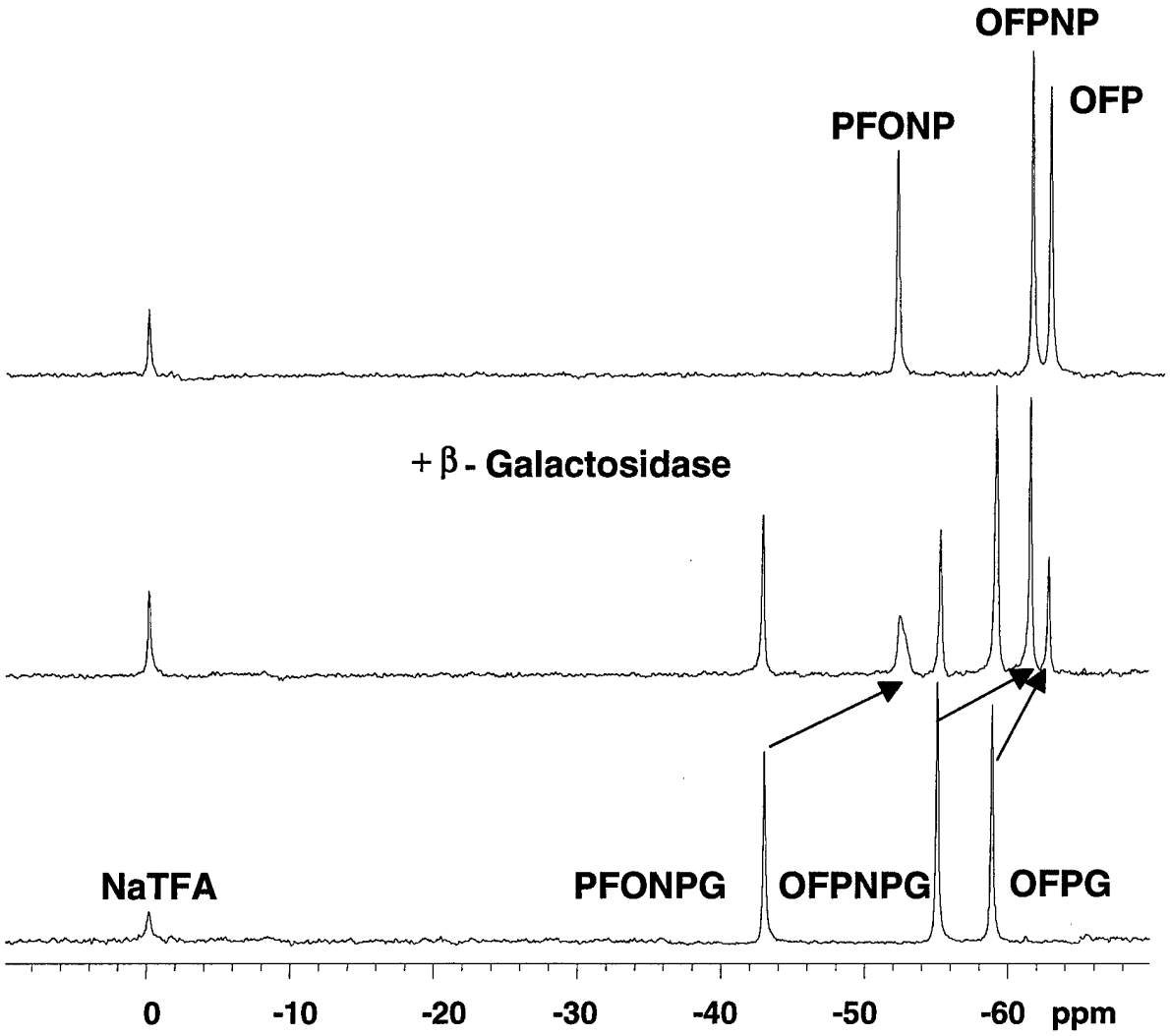

OFPNP
OFP
PFONP
+ β - Galactosidase
NaTFA
PFONPG
OFPNPG
OFPG
0
-10
-20
-30
-40
-50
-60
ppm

Figure 2

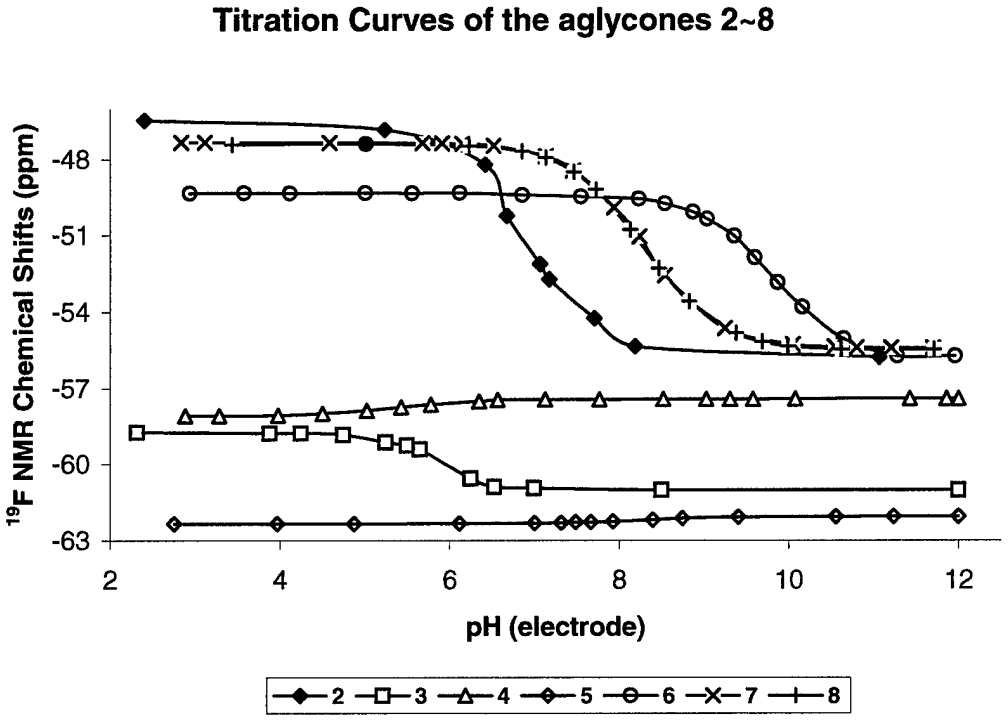


Figure 3

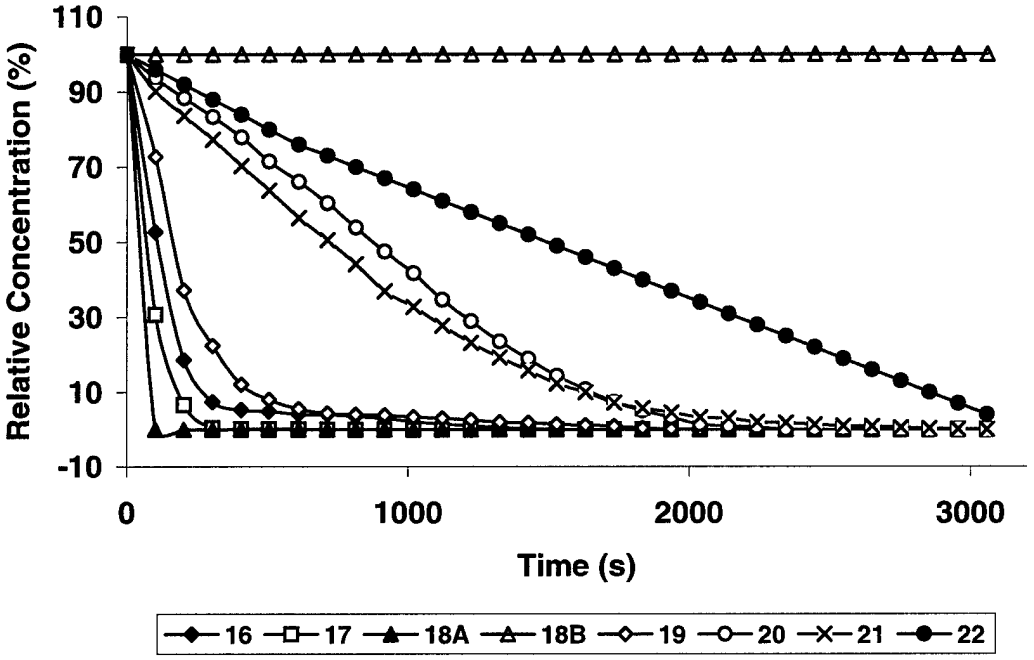


Figure 4

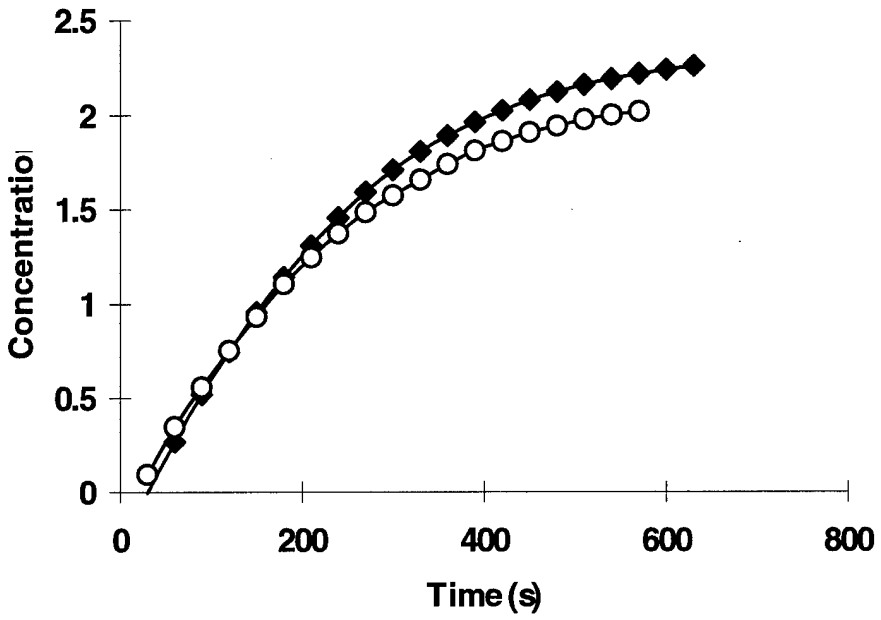


Figure 5

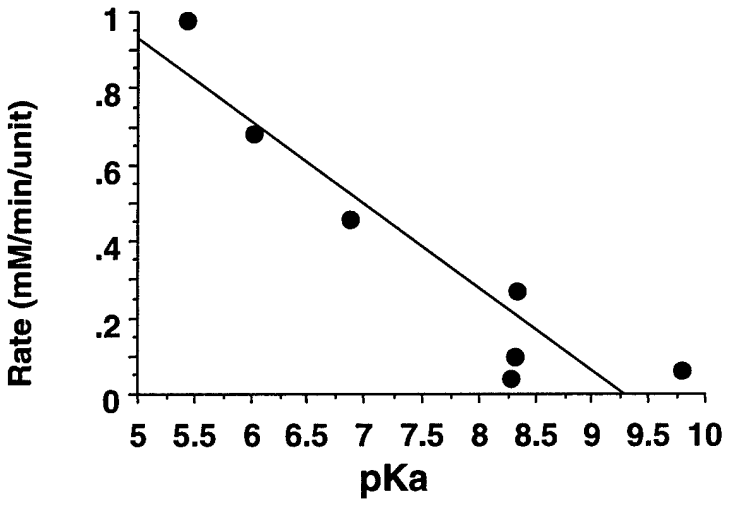


Figure 6

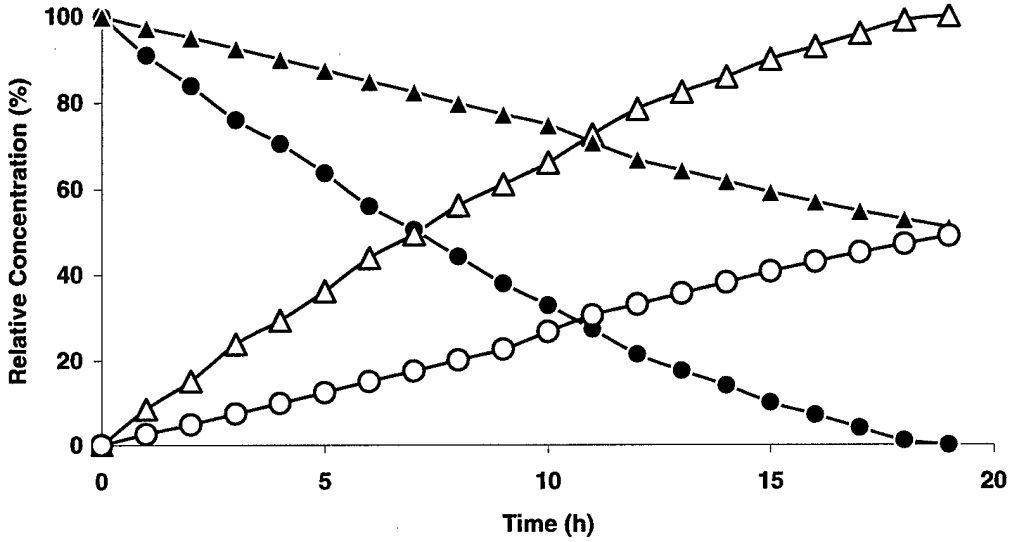


Figure 7