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Synthesis and evaluation of novel enhanced gene reporter molecules: Detection of β-galactosidase activity using ^{19}F NMR of trifluoromethylated aryl β-D-galactopyranosides

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Abstract—Gene therapy has emerged as a promising strategy for treatment of various diseases, but there is a pressing need for the development of non-invasive reporter techniques based on appropriate molecules and imaging modalities to assay gene expression. We now report the design, synthesis, and evaluation of novel enhanced reporter molecules, which reveal *lacZ* gene expression: trifluoromethylated aryl β-D-galactopyranosides. A series of five molecular structures were screened in solution and with stably transfected *lacZ* expressing human MCF7 breast cancer cells in vitro. *p*-Trifluoromethyl-*o*-nitrophenyl β-D-galactopyranoside (**PCF_3ONPG**) was found to exhibit valuable properties including a single ^{19}F NMR signal, stability in aqueous solution and with wild type cells, but a chemical shift response to enzyme cleavage ($\Delta\delta$ = 1.14 ppm) in breast cancer cells transfected to stably express *lacZ*.

1. Introduction

Strategies for identifying exogenous gene activity have been presented using radionuclide imaging,[1,2] optical imaging,[3,4] and NMR.[5,6] In some cases, natural substrates are used, such as detection of fluorescent molecules or bioluminescence, but in other cases exogenous substrates have been designed to probe enzyme (viz., gene) activity. Recently, attention has turned to β-galactosidase (β-gal), since its introduction has become a standard means of assaying clonal insertion, transcriptional activation, protein expression, and protein interaction. Diverse colorimetric substrates have been developed suitable for histology.[7,8] Tung et al.[9] reported a near infrared active substrate and Louie et al.[10] presented a proton MRI contrast agent. We have demonstrated the feasibility of using ^{19}F NMR to detect chemical shift changes accompanying enzyme-induced cleavage of fluorogalactopyranosides.[11–14] We now report the synthesis of a series of trifluoromethyl (CF_3) aryl β-D-galactopyranosides designed to provide enhanced signal. We report synthesis, relevant characteristics as β-gal substrates, and evaluation of their use to detect *lacZ* gene expression in breast cancer cells. Relative merits are compared with those of previous substrates.

2. Designs and synthesis

β-Galactosidase (β-gal) catalyzes the hydrolysis of galactopyranosides by cleavage of the C–O bonds between D-galactose and the aglycone.[15] However, the enzyme shows remarkably broad substrate specificity. Based on our previous studies using a single fluorine atom as ^{19}F NMR sensitive reporter of β-gal activity,[11–14] it appeared that introduction of a CF_3 group could be advantageous. Inherent signal to noise would be improved, allowing lower concentrations of reporter molecule to be applied, and hence, reducing issues of toxicity or substrate solubility. CF_3– groups are widely used in pharmaceuticals and agrochemicals since they resist enzyme degradation and the typical toxicity of mono- and difluoromethyl groups.[14] Moreover, it has been observed that hydrogen bonding between the active site of the enzyme and the hydroxyl groups of the glycosidic substrate is important in the formation of the enzyme–substrate complex.[16–18] Introduction of strong electron-withdrawing CF_3– group could increase

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the tendency to form the enzyme–substrate complex through the action of the fluorine as an acceptor in hydrogen bonding interactions in the 'glycosylation' step and make the phenolate anion a better leaving group in the 'deglycosylation' step.[19]

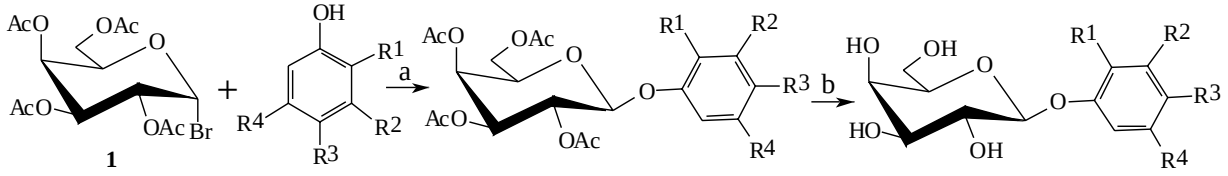
Following the successful high yield phase-transfer catalysis approach to the stereoselective syntheses of fluorinated aryl β-D-galactopyranosides,[12,13] this versatile synthetic method was chosen for preparation of the target compounds **9**–**18** starting with commercially available trifluoromethylphenolic aglycones **2**–**8** (Fig. 1). The aglycones **2**–**8** reacted at 50 °C with 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (**1**) in a dichloromethane-aqueous biphasic system (pH 8–9) using tetrabutylammonium bromide (TBAB) as the phase-transfer catalyst, affording trifluoromethyl aryl β-D-galactopyranoside tetraacetates **9**–**12** in near quantitative yield. However, **13** was obtained in poor yield (20%) and two of the trifluoromethyl phenols (3-trifluoromethylphenol **7** and 4-trifluoromethylphenol **8**) proved to be unreactive. To our knowledge molecules **9**–**18** are new, though we note that an isomer 4-nitro-2-trifluoromethylphenyl β-D-galactopyranoside has been reported previously in a patent related to CEDIA (cloned enzyme donor immunoassay).[20] That work did not appear to exploit the ^{19}F NMR properties, but rather colorimetric changes accompanying β-gal induced cleavage. They reported a very poor yield using an alternate synthetic approach, though it may be characteristic of *o*-CF_3 groups since this was least successful in our hands.

The anomeric β-D-configuration of compounds **9**–**13** in the $^4C_1$ chair conformation was unambiguously established on the basis of the observed ^{1}H NMR chemical shifts (δ_H 4.98–5.25 ppm) of the anomeric protons, and the $J_{1,2}$ (J ~8 Hz) and $J_{2,3}$ (J ~10 Hz) coupling constants. The signals of the ^{13}C NMR spectra of **9**–**13** were assigned by comparison with the chemical shifts of *p*-nitrophenyl β-D-galactopyranosides.[13,21] As expected, the anomeric carbon resonances appeared at 98–101 ppm in accord with the β-D-configuration.

Deacetylation of **9**–**13** with NH_3/MeOH from 0 °C to room temperature gave the free galactopyranosides **14**–**18** in quantitative yield. The signals of the ^{1}H NMR spectra of **14**–**18** were assigned by ^{1}H–^{1}H COSY spectra and D_2O exchange. The ^{1}H NMR chemical shifts (δ_H 5.00–5.15 ppm) of the anomeric protons and the $J_{1,2}$ (J ~8 Hz) and $J_{2,3}$ (J 9–11 Hz) coupling constants showed that the free galactopyranosides **14**–**18** retained the anomeric β-D-configuration with the $^4C_1$ chair conformation.

3. ^{19}F NMR

^{19}F NMR spectra of the trifluoromethylphenyl β-D-galactopyranosides **14**–**18** were recorded in aqueous solutions with sodium trifluoroacetate (NaTFA) as an external chemical shift standard. Compounds **14**–**18** each gave a single narrow ^{19}F NMR signal between δ 12–16 ppm essentially invariant ($\Delta\delta \leqslant 0.02$ ppm) with pH in the range 3 to 12 and temperatures from 25 to 37 °C in whole rabbit blood, 0.9% saline, or PBS. Addition of β-gal (G-2513) to **14**–**17** in PBS buffer (0.1 M, pH 7.4) at 37 °C led to rapid hydrolysis releasing the aglycones **2**–**5**, which appeared as single narrow ^{19}F signals shifted downfield (Table 1). Compound **18** was cleaved comparatively slowly. The relative efficacy of **14** (**PCF_3ONPG**) and that of our previously reported *o*-fluoro-*p*-nitrophenyl β-D-galactopyranoside (**OFPNPG**) as β-gal substrate are shown in Figure 2. As expected, **14** provides about 3 times more signal, while cleavage rates are similar. Comparison of β-gal hydrolytic kinetics of **14**–**18** (Fig. 3) showed that each proceeded



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**

Figure 1. The reactions and the structures of **1**–**18**. Reaction and condition: (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 → rt, 24 h, quantitative yields.

Table 1. ^{19}F chemical shifts[a] and hydrolytic rates by β-gal[b]

	14	15	16	17	18
$\delta_{F(substrate)}$	13.40	15.23	13.24	12.70	14.11
$\delta_{F(product)}$	14.54	15.43	13.94	12.95	14.61
$\Delta\delta_F$	1.14	0.20	0.70	0.25	0.50
$v_{(\mu mol/min/U)}$	33.0	52.8	39.6	46.2	2.61

[a] ppm with respect to aq NaTFA.
[b] β-Gal (G-2513, 11 U) at 37 °C in PBS buffer (0.1 M, pH 7.4).

monotonically indicating straightforward first-order kinetics for all substrates and that the liberated aglycones **2**–**6** did not inhibit the β-gal. The substrates **14**–**17** exhibited rates in excess of 33 μmol/min/U exceeding those of *p*-fluoro-*o*-nitrophenyl β-D-galactopyranoside (**PFONPG**; 19 μmol/min/U), **OFPNPG** (32 μmol/min/U), and even *o*-nitrophenyl β-D-galactopyranoside (**ONPG**; 32 μmol/min/U), which we have reported previously.[11,13] The low hydrolysis rate of **18** may be due to the formation of an intramolecular F···H hydrogen bond between the 2-CF_3 and C_1–H or steric effects, which plays an important role in the hydrolytic process rate.[19]

As expected, trifluoromethylphenyl β-D-galactopyranosides showed enhanced ^{19}F signal intensity on a molar basis compared with analogous fluorophenyl β-D-galactopyranosides, but the ^{19}F chemical shift changes were much smaller (Fig. 2, Table 1). The chemical shift ($\Delta\delta$ 1.14 ppm) accompanying cleavage of **14** is sufficient for investigations in vivo, but the other substrates **15**–**18** gave smaller values, which may be insufficient for effective studies. The aglycone *p*-trifluoromethyl-*o*-nitrophenol (**2**, **PCF_3ONP**) also exhibits a ^{19}F NMR chemical shift in response to pH ($\Delta\delta$ = ~1.00 ppm) in the range of pH 4–7 (Fig. 4). Henderson–Hasselbalch coefficients are pK_a = 5.6, δ_{acid} = 13.49 ppm, δ_{base} = 14.52, but importantly there is no overlap with the chemical shift of the substrate **14**.

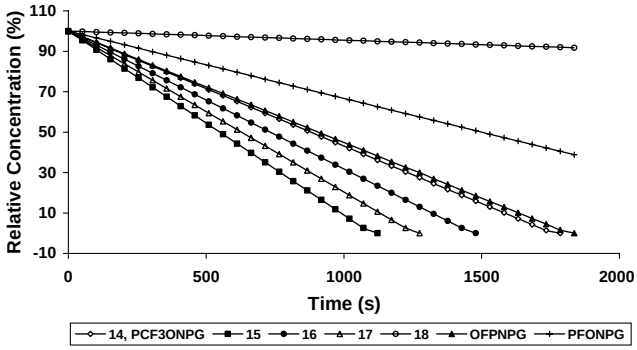


Figure 3. Relative hydrolysis time courses of **14**–**18** (6.0 mmol), **OFPNPG**, and **PFONPG** (5.4 mmol) by β-gal (6 U) in PBS (0.1 M, 600 μL) at 37 °C.

4. In vitro evaluation

CF_3– groups are often associated with increased lipophilicity. As expected, **PCF_3ONPG** has comparatively lower aqueous solubility than either **OFPNPG** or **PFONPG**. However, the higher ^{19}F signal intensity and sensitivity to β-gal allow use of lower concentrations of **PCF_3ONPG**, potentially circumventing issues of toxicity. **PCF_3ONPG** was stable in aqueous solution in the pH range 3–12 at temperatures from 25 to 37 °C over 5 days. Toxicity was evaluated for both aglycone **PCF_3ONP** and conjugate **PCF_3ONPG** using both wild type and *lacZ* expressing human MCF7 breast cancer

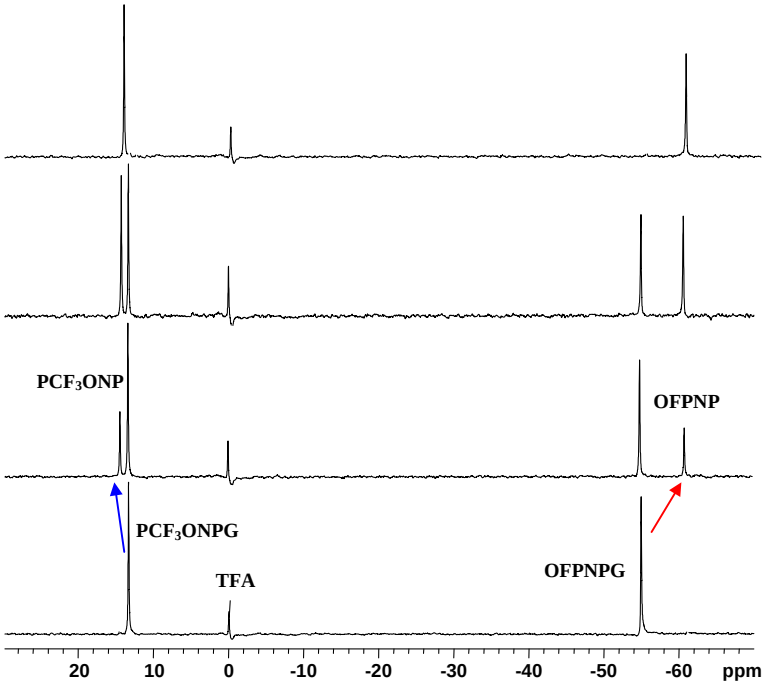


Figure 2. ^{19}F NMR spectra of **PCF_3ONPG** (1.1 mg, 3 mmol) and **OFPNPG** (2.87 mg, 9 mmol) with 1:3 molar ratios in the simultaneous hydrolysis by β-gal (11 U) in PBS (0.1 M, pH 7.4, 600 μL) at 37 °C.

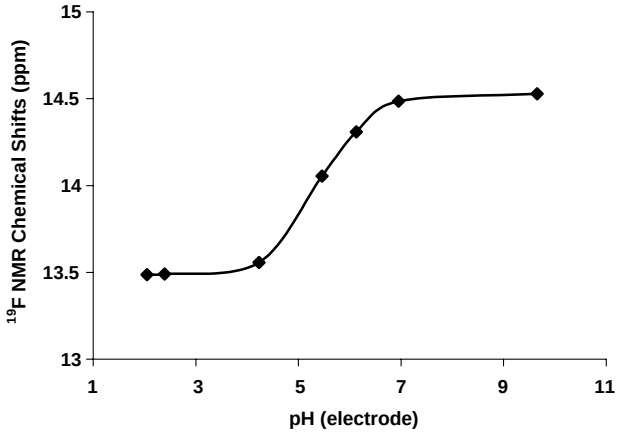


Figure 4. ^{19}F NMR chemical shift pH titration curve of **2** (**PCF_3ONP**) in saline at 37 °C.

cells. Cell viability assays[22] showed that the aglycone **PCF_3ONP** exhibited significant cytotoxicity even at 100 μM with both cell clones (Fig. 5). No toxicity was observed up to 1 mM for **PCF_3ONPG** over 96 h for wild type cells, but some toxicity was found with the *lacZ* expressing cells, presumably due to liberation of the aglycone.

When **PCF_3ONPG** was incubated with MCF7-WT cells for 5 h in PBS buffer at 37 °C under 5% CO_2 in air with 95% humidity, no changes were observed in the ^{19}F NMR spectra. However, addition of **PCF_3ONPG** to cells stably transfected to express β-gal led to cleavage in a smooth monotonic manner releasing the aglycone **PCF_3ONP** (40.0 μmol/min per million MCF7-*lacZ* cells, Figs. 6, 7).

While the chemical shift response of the trifluoromethyl reporters is modest, we demonstrate that it is sufficient for chemical shift selective imaging (CSI) and we have observed the effect of MCF7-*lacZ* cells on **PCF_3ONPG** in vitro (Fig. 8).

5. Conclusion

The phase-transfer approach to synthesizing phenyl galactosides developed previously[13] was also appropriate for several of the trifluoromethyl galactosides. The substrates are stable in aqueous solution and with wild type cancer cells, but the CF_3 agents are responsive to β-gal activity with rates exceeding those of the fluorophenyl analogs. Signal to noise is enhanced and although the ^{19}F NMR chemical shift response to enzyme cleavage is smaller, it is adequate for detecting hydrolysis with **PCF_3ONPG**. Overall, the trifluoromethyl galactosides show promise as reporter molecules for β-gal activity and we are initiating investigations of *lacZ* expressing tumors in animals.

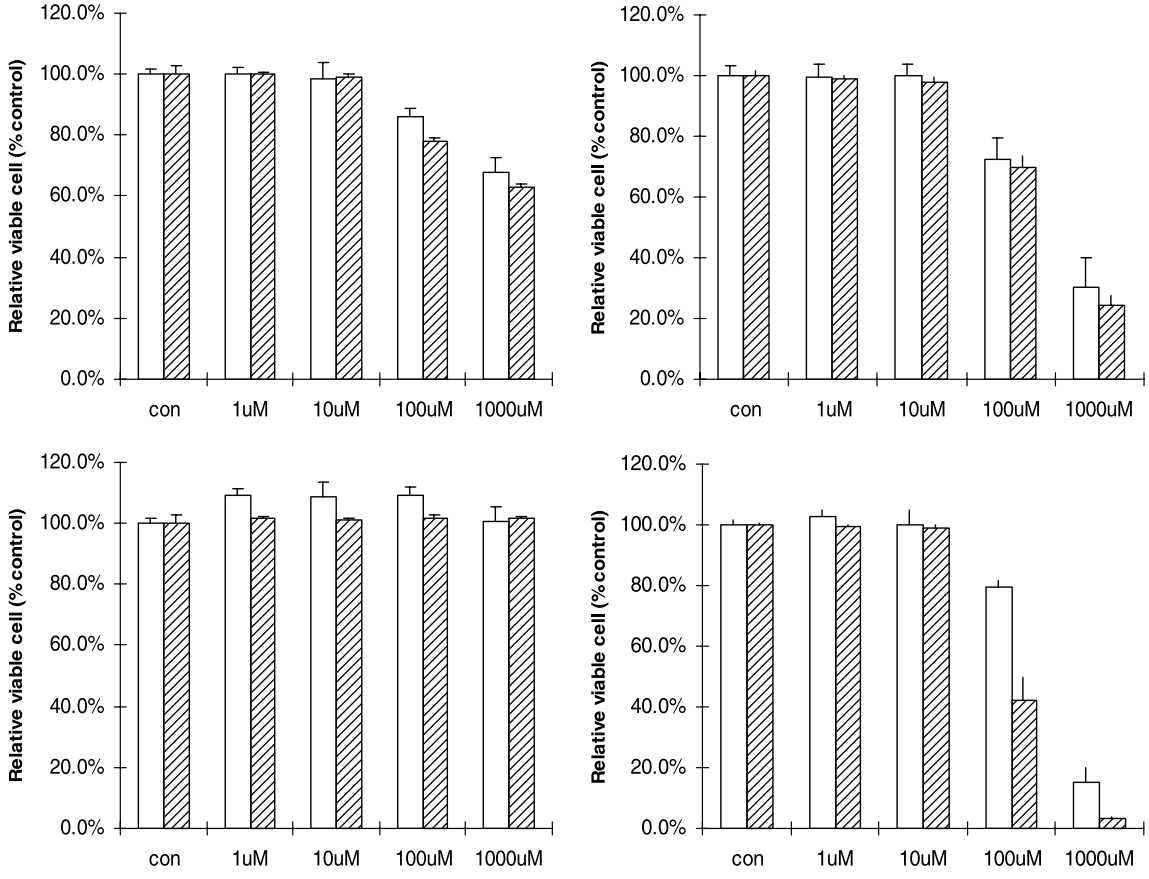


Figure 5. Cell viability of human MCF7 breast cancer cells in PBS (pH 7.4) with respect to exposure to substrate **14** (left panels) or aglycone **2** (right panels). Upper panels MCF7-*lacZ* cells; lower panels MCF7-WT. Open bars 48 h exposure; hatched bars 96 h exposure.

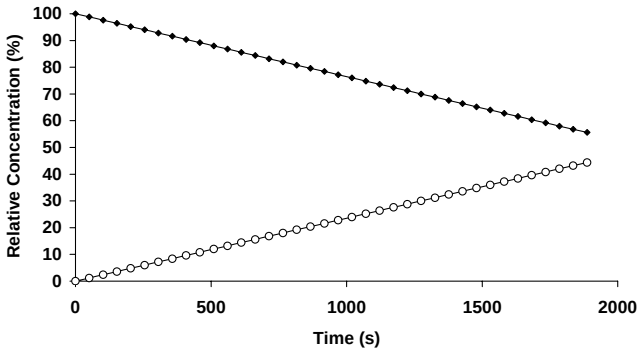


Figure 6. Hydrolysis of **14** (♦, 5.0 mmol) to **2** (○) by stably transfected MCF7-*lacZ* breast cancer cells (1.75×10^6) in PBS buffer at 37 °C.

6. Experimental

6.1. General methods

NMR spectra were recorded on a Varian Inova 400 spectrometer (400 MHz for ^{1}H, 100 MHz for ^{13}C, 376 MHz for ^{19}F) with $CDCl_3$ or DMSO-d_6 as solvents. ^{1}H and ^{13}C chemical shifts are referenced to TMS as internal standard and ^{19}F to dil sodium trifluoroacetate (NaTFA) in a capillary as external standard. All compounds were characterized by acquisition of ^{1}H, ^{13}C, DEPT, ^{1}H–^{1}H, and COSY experiments at 25 °C and ^{19}F spectra at 37 °C. Imaging experiments used a Varian INOVA Unity scanner 4.7 T (188.2 MHz) with a 2D spin echo CSI sequence (FOV = 30 × 30 mm, spectral window = 30 ppm, slice thickness: 10 mm, matrix = 16 × 16, and TR/TE = 1000/12 ms in 4.5 min per image). Microanalyses were performed on a Perkin-Elmer 2400CHN microanalyzer. 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) by elution with cyclohexane–EtOAc and silica gel GF_{254} (Aldrich Chemical Company, St. Louis, MO) was used for analytical TLC. Detection was effected by spraying the plates with 5% ethanolic H_2SO_4 (followed by heating at 110 °C for 10 min) or by direct UV illumination of the plate.

For enzyme kinetic experiments, **PCF_3ONPG** (2.2 mg, 6 mmol) was dissolved in PBS (0.1 M, pH 7.4, 573 μL) and a PBS solution of β-gal (27 μL, G-2513 from *Escherichia coli*, 0.22 U/μL, Aldrich) was added and NMR data were acquired immediately at 37 °C.

Human MCF7 breast cancer cells were stably transfected with recombinant vector phCMV/*lacZ*, which inserted the *E. coli lacZ* gene (from pSV-β-gal vector, Promega) to high expression human cytomegalovirus (CMV) immediate-early enhancer/promoter vector phCMV (Gene Therapy Systems, Inc) using GenePORTER2 (Gene Therapy Systems, Inc). For MCF7 cells, clonal selection was applied to identify those cells with highest β-gal expression. Control (wild type) and transfected (*lacZ*) cells were grown in culture dishes under standard conditions and harvested. **PCF_3ONPG** (2.2 mg) in PBS (70 μL) was added to a suspension of 10^6 cells in PBS (530 μL) and ^{19}F NMR spectra were acquired immediately and again after incubation for various times up to 5 h at 37 °C.

The sensitivities of MCF7-WT and -*lacZ* cells to **PCF_3ONPG** and **PCF_3ONP** were quantified using the Crystal Violet Mitogenic Assay[23] performed in triplicate using 24-well plates seeded with 2×10^4 cells per well in 1 mL DMEM supplemented with 10% FBS and 2 mM

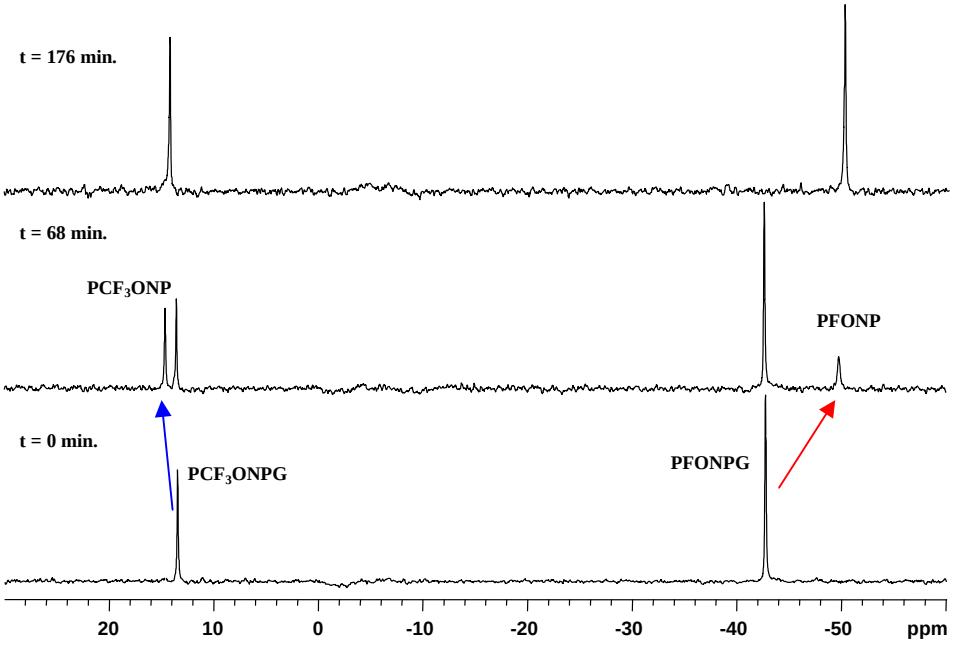


Figure 7. ^{19}F NMR spectra of **PCF_3ONPG** (1.7 mg, 4.5 mmol) and **PFONPG** (6.0 mg, 18.8 mmol) showing simultaneous hydrolysis by stably transfected MCF7-*lacZ* cells (1.75×10^6) in PBS (0.1 M, pH 7.4, 600 μL) at 37 °C. Each spectrum acquired in 51 s.

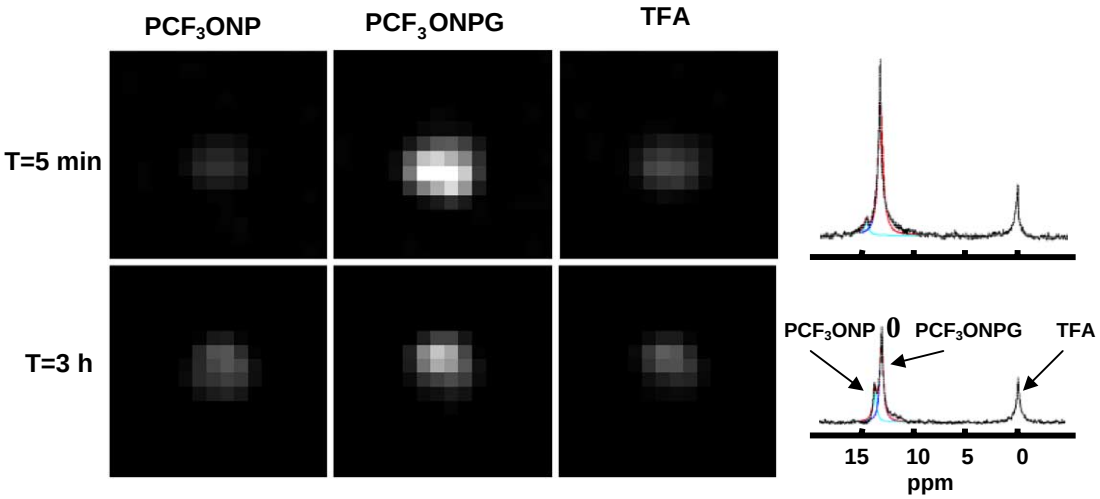


Figure 8. ^{19}F CSI of **PCF$_3$ONPG** (13.2 mg) and **TFA** (2.0 mg) during hydrolysis by stably transfected MCF7-*lacZ* cells (10^7) in PBS (0.1 M, pH 7.4, 700 μL) at 20 °C. Upper images show data acquired in 4.5 min, ten minutes after addition of substrate to cells. The lower images were acquired 3 h later. At right are corresponding ^{19}F NMR spectra obtained from a single voxel in the image. The curve fits are also presented to demonstrate the deconvolution to allow CSI.

glutamine. After 24 h incubation, the medium was replaced with fresh DMEM containing 0.1% DMSO and various concentrations of **PCF$_3$ONPG** or **PCF$_3$ONP** (0–1 mM) and incubated for 48 or 96 h, followed by the Crystal Violet Mitogenic Assay.

6.2. Trifluoromethylphenyl β-D-galactopyranoside tetraacetates 9–13

6.2.1. General procedure. A solution of 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (Sigma) (**1**) (1 mmol) and tetrabutyl-ammonium bromide (0.48 g, 1.5 mmol) in CH_2Cl_2 (5 mL) was stirred vigorously at 50 °C with the solution of fluorophenols (**2**–**8**) (1.2 mmol) in H_2O (5 mL; pH 8–9) until TLC showed complete reaction (~1 h). The organic layer was separated, washed, dried (Na_2SO_4), and evaporated under reduced pressure to give a syrup, which was purified by column chromatography on silica gel to give trifluoromethylphenyl β-D-galactopyranoside tetraacetates **9**–**13**.

2-Nitro-4-trifluoromethylphenyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside **9** (0.54 g, 99%) as white crystals, R_f 0.38 (3:2 cyclohexane/EtOAc), δ_H: 8.07 (1H, d, J = 2.0 Hz, Ar-H), 7.79 (1H, dd, J = 1.6, 7.2 Hz, Ar-H), 7.48 (1H, d, J = 8.8 Hz, Ar-H), 5.17 (1H, d, $J_{1,2}$ = 7.6 Hz, H-1), 5.57 (1H, dd, $J_{2,3}$ = 10.4 Hz, H-2), 5.12 (1H, dd, $J_{3,4}$ = 3.2 Hz, H-3), 5.48 (1H, d, $J_{4,5}$ = 3.2 Hz, H-4), 4.13 (1H, m, H-5), 4.24 (1H, dd, $J_{5,6a}$ = 4.4 Hz, $J_{6a,6b}$ = 11.2 Hz, H-6a), 4.18 (1H, dd, $J_{5,6b}$ = 5.6 Hz, H-6b), 2.19, 2.15, 2.10, 2.01 (12H, 4s, 4× CH_3CO) ppm; δ_C: 170.49, 170.31, 169.45 (4× $CH_3\mathbf{C}O$), 140.93 (Ar-$C_{1'}$), 151.87 (Ar-$C_{2'}$), 130.74 (q, $^3J_{F-C}$ = 3.8 Hz, Ar-$C_{3'}$), 126.14 (q, $^2J_{F-C}$ = 34.0 Hz, Ar-$C_{4'}$), 123.10 (q, $^3J_{F-C}$ = 3.9 Hz, Ar-$C_{5'}$), 119.59 (Ar-$C_{6'}$), 122.82 (q, $^1J_{F-C}$ = 284.0 Hz, CF_3), 100.46 (C-1), 67.80 (C-2), 70.55 (C-3), 66.79 (C-4), 71.89 (C-5), 61.55 (C-6), 20.84, 20.80, 20.78, 20.73 (4× $\mathbf{C}H_3CO$) ppm. Anal. Calcd for $C_{21}H_{22}NO_{12}F_3$ (%): C, 46.92; H, 4.13; N, 2.61. Found: C, 46.90; H, 4.10; N, 2.58.

4-Nitro-3-trifluoromethylphenyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside **10** (0.54 g, 99%) as syrup, R_f 0.36 (3:2 cyclohexane/EtOAc), δ_H: 7.98 (1H, d, J = 8.8 Hz, Ar-H), 7.44 (1H, s, Ar-H), 7.28 (1H, d, J = 9.2 Hz, Ar-H), 5.25 (1H, d, $J_{1,2}$ = 8.0 Hz, H-1), 5.57 (1H, dd, $J_{2,3}$ = 10 Hz, H-2), 5.17 (1H, dd, $J_{3,4}$ = 2.8 Hz, H-3), 5.50 (1H, d, $J_{4,5}$ = 3.2 Hz, H-4), 4.15 (1H, m, H-5), 4.18 (2H, m, H-6), 2.20, 2.09, 2.07, 2.03 (12H, 4s, 4× CH_3CO) ppm; δ_C: 170.61, 170.26, 170.16, 169.45 (4× $CH_3\mathbf{C}O$), 142.91 (Ar-$C_{1'}$), 116.15 (q, $^3J_{F-C}$ = 6.1 Hz, Ar-$C_{2'}$), 126.18 (q, $^2J_{F-C}$ = 34.4 Hz, Ar-$C_{3'}$), 120.07 (Ar-$C_{4'}$), 127.91 (Ar-$C_{5'}$), 159.31 (Ar-$C_{6'}$), 121.76 (q, $^1J_{F-C}$ = 271.6 Hz, CF_3), 98.70 (C-1), 68.30 (C-2), 70.65 (C-3), 67.01 (C-4), 71.99 (C-5), 61.95 (C-6), 20.82, 20.79, 20.71, 20.67 (4× $\mathbf{C}H_3CO$) ppm. Anal. Calcd for $C_{21}H_{22}NO_{12}F_3$ (%): C, 46.92; H, 4.13; N, 2.61. Found: C, 46.89; H, 4.11; N, 2.60.

2-Chloro-3-trifluoromethylphenyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside **11** (0.50 g, 95%) as syrup, R_f 0.61 (3:2 cyclohexane/EtOAc), δ_H: 7.46 (1H, dd, J = 1.2, 6.6 Hz, Ar-H), 7.40 (1H, d, J = 8.4 Hz, Ar-H), 7.32 (1H, dd, J = 7.8, 8.4 Hz, Ar-H), 4.98 (1H, d, $J_{1,2}$ = 8.4 Hz, H-1), 5.59 (1H, dd, $J_{2,3}$ = 10.5 Hz, H-2), 5.11 (1H, dd, $J_{3,4}$ = 3.6 Hz, H-3), 5.48 (1H, d, $J_{4,5}$ = 3.6 Hz, H-4), 4.06 (1H, m, H-5), 4.25 (1H, dd, $J_{5,6a}$ = 6.6 Hz, $J_{6a,6b}$ = 11.7 Hz, H-6a), 4.16 (1H, dd, $J_{5,6b}$ = 6.0 Hz, H-6b), 2.20, 2.10, 2.06, 2.02 (12H, 4s, 4× CH_3CO) ppm; δ_C: 170.45, 170.42, 170.34, 169.42 (4× $CH_3\mathbf{C}O$), 153.82 (Ar-$C_{1'}$), 122.39 (q, $^3J_{F-C}$ = 3.6 Hz, Ar-$C_{2'}$), 130.11 (q, $^2J_{F-C}$ = 20.6 Hz, Ar-$C_{3'}$), 122.39 (q, $^3J_{F-C}$ = 3.4 Hz, Ar-$C_{4'}$), 127.42 (Ar-$C_{5'}$), 132.58 (Ar-$C_{6'}$), 124.01 (q, $^1J_{F-C}$ = 282.5 Hz, CF_3), 100.83 (C-1), 68.28 (C-2), 70.69 (C-3), 66.87 (C-4),

71.47 (C-5), 61.42 (C-6), 20.92, 20.77, 20.70 (4× **C**H_3CO) ppm. Anal. Calcd for $C_{21}H_{22}O_{10}ClF_3$ (%): C, 47.90; H, 4.22. Found: C, 47.89; H, 4.20.

2-Chloro-5-trifluoromethylphenyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside **12** (0.51 g, 96%) as white crystals, R_f 0.56 (3:2 cyclohexane/EtOAc), δ_H: 7.51 (1H, s, Ar-H), 7.49 (1H, dd, J = 1.8, 3.6 Hz, Ar-H), 7.30 (1H, dd, J = 1.2, 7.8 Hz, Ar-H), 5.02 (1H, d, $J_{1,2}$ = 8.4 Hz, H-1), 5.60 (1H, dd, $J_{2,3}$ = 10.2 Hz, H-2), 5.12 (1H, dd, $J_{3,4}$ = 3.6 Hz, H-3), 5.48 (1H, d, $J_{4,5}$ = 3.6 Hz, H-4), 4.12 (1H, m, H-5), 4.22 (1H, dd, $J_{5,6a}$ = 4.2 Hz, $J_{6a,6b}$ = 11.4 Hz, H-6a), 4.17 (1H, dd, $J_{5,6b}$ = 7.8 Hz, H-6b), 2.20, 2.10, 2.07, 2.02 (12H, 4s, 4× CH_3CO) ppm; δ_C: 170.70, 170.27, 170.17, 169.40 (4× CH_3**C**O), 152.97 (Ar-$C_{1'}$), 109.92 (Ar-$C_{2'}$), 131.03 (Ar-$C_{3'}$), 115.13 (q, $^3J_{F-C}$ = 3.0 Hz, Ar-$C_{4'}$), 130.34 (q, $^2J_{F-C}$ = 22.2 Hz, Ar-$C_{5'}$), 121.06 (q, $^3J_{F-C}$ = 2.9 Hz, Ar-$C_{6'}$), 122.59 (q, $^1J_{F-C}$ = 282.0 Hz, CF_3), 100.65 (C-1), 68.13 (C-2), 70.62 (C-3), 67.21 (C-4), 71.95 (C-5), 62.31 (C-6), 20.89, 20.73, 20.50 (4× **C**H_3CO) ppm. Anal. Calcd for $C_{21}H_{22}O_{10}ClF_3$ (%): C, 47.90; H, 4.22. Found: C, 47.88; H, 4.21.

2-Trifluoromethylphenyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside **13** (0.30 g, 20%) as white crystals, R_f 0.54 (3:2 cyclohexane/EtOAc), δ_H: 7.60 (1H, dd, J = 1.2, 7.8 Hz, Ar-H), 7.50 (1H, m, Ar-H), 7.27 (1H, dd, J = 1.8, 6.6 Hz, Ar-H), 7.16 (1H, dd, J = 7.8 Hz, Ar-H), 5.06 (1H, d, $J_{1,2}$ = 8.4 Hz, H-1), 5.58 (1H, dd, $J_{2,3}$ = 10.5 Hz, H-2), 5.12 (1H, dd, $J_{3,4}$ = 3.6 Hz, H-3), 5.47 (1H, dd, $J_{4,5}$ = 0.6 Hz, H-4), 4.11 (1H, m, H-5), 4.27 (1H, dd, $J_{5,6a}$ = 6.0 Hz, $J_{6a,6b}$ = 11.4 Hz, H-6a), 4.17 (1H, dd, $J_{5,6b}$ = 6.0 Hz, H-6b), 2.20, 2.08, 2.05, 2.01 (12H, 4s, 4× CH_3CO) ppm; δ_C: 170.26, 170.20, 170.05, 169.10 (4× CH_3**C**O), 154.59 (Ar-$C_{1'}$), 120.10 (q, $^2J_{F-C}$ = 21.0 Hz, Ar-$C_{2'}$), 127.03 (q, $^3J_{F-C}$ = 3.4 Hz, Ar-$C_{3'}$), 122.80 (Ar-$C_{4'}$), 116.69 (Ar-$C_{5'}$), 133.30 (Ar-$C_{6'}$), 129.78 (q, $^1J_{F-C}$ = 208.4 Hz, CF_3), 99.79 (C-1), 67.85 (C-2), 70.73 (C-3), 66.76 (C-4), 71.20 (C-5), 61.40 (C-6), 20.59, 20.36 (4× **C**H_3CO) ppm. Anal. Calcd for $C_{21}H_{23}O_{10}F_3$ (%): C, 51.21; H, 4.71. Found: C, 51.19; H, 4.69.

6.3. Trifluoromethylphenyl β-D-galactopyranosides 14–18

6.3.1. General procedure. A solution of trifluoromethylphenyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside (**9**–**13**) (0.4 g) in anhydrous MeOH (15 mL) containing 0.5 M NH_3 was vigorously stirred from 0 °C to room temperature overnight until TLC showed complete reaction and evaporated to dryness in vacuo. Chromatography of the crude syrup on silica gel with EtOAc–MeOH afforded the free β-D-galactopyranosides **14**–**18** in quantitative yield.

2-Nitro-4-trifluoromethylphenyl β-D-galactopyranoside **14** as white crystals, R_f 0.35 (1:9 MeOH/EtOAc), δ_H: 8.28 (1H, d, J = 2.0 Hz, Ar-H), 7.99 (1H, dd, J = 2.0, 9.2 Hz, Ar-H), 7.59 (1H, d, J = 8.8 Hz, Ar-H), 5.15 (1H, d, $J_{1,2}$ = 7.6 Hz, H-1), 3.55 (1H, dd, $J_{2,3}$ = 8.9 Hz, H-2), 3.50 (1H, dd, $J_{3,4}$ = 6.0 Hz, H-3), 3.47 (1H, d, $J_{4,5}$ = 6.0 Hz, H-4), 3.42 (1H, m, H-5), 3.67 (1H, m, H-6), 5.24 (1H, d, $J_{H\text{-}2,OH\text{-}2}$ = 4.8 Hz, HO-2), 4.63 (1H, d, $J_{H\text{-}3,OH\text{-}3}$ = 4.4 Hz, HO-3), 4.92 (1H, d, $J_{H\text{-}4,OH\text{-}4}$ = 6.0 Hz, HO-4), 4.69 (1H, t, $J_{H\text{-}6,OH\text{-}6}$ = 5.4, 5.6 Hz, HO-6) ppm; δ_C: 152.18 (Ar-$C_{1'}$), 139.97 (Ar-$C_{2'}$), 130.82 (q, $^3J_{F-C}$ = 3.9 Hz, Ar-$C_{3'}$), 122.06 (q, $^2J_{F-C}$ = 33.8 Hz, Ar-$C_{4'}$), 122.42 (q, $^3J_{F-C}$ = 3.8 Hz, Ar-$C_{5'}$), 117.91 (Ar-$C_{6'}$), 123.33 (q, $^1J_{F-C}$ = 270.9 Hz, CF_3), 100.95 (C-1), 69.97 (C-2), 73.29 (C-3), 68.03 (C-4), 76.04 (C-5), 60.30 (C-6) ppm. Anal. Calcd for $C_{13}H_{14}NO_8F_3$ (%): C, 42.27; H, 3.82; N, 3.79. Found: C, 42.25; H, 3.80; N, 3.77.

4-Nitro-3-trifluoromethylphenyl β-D-galactopyranoside **15** as white crystals, R_f 0.40 (1:9 MeOH/EtOAc), δ_H: 8.16 (1H, d, J = 8.8 Hz, Ar-H), 7.53 (1H, d, J = 2.8 Hz, Ar-H), 7.49 (1H, dd, J = 2.8, 8.8 Hz, Ar-H), 5.08 (1H, d, $J_{1,2}$ = 7.6 Hz, H-1), 3.53 (1H, dd, $J_{2,3}$ = 10.0 Hz, H-2), 3.49 (1H, dd, $J_{3,4}$ = 5.2 Hz, H-3), 3.44 (1H, d, $J_{4,5}$ = 2.4 Hz, H-4), 3.61 (1H, m, H-5), 3.68 (2H, m, H-6), 5.33 (1H, d, $J_{H\text{-}2,OH\text{-}2}$ = 4.8 Hz, HO-2), 4.60 (1H, d, $J_{H\text{-}3,OH\text{-}3}$ = 4.8 Hz, HO-3), 4.96 (1H, d, $J_{H\text{-}4,OH\text{-}4}$ = 5.6 Hz, HO-4), 4.70 (1H, dd, $J_{H\text{-}6,OH\text{-}6}$ = 5.2, 5.4 Hz, HO-6) ppm; δ_C: 160.45 (Ar-$C_{1'}$), 115.95 (q, $^3J_{F-C}$ = 6.1 Hz, Ar-$C_{2'}$), 123.67 (q, $^2J_{F-C}$ = 32.8 Hz, Ar-$C_{3'}$), 141.10 (Ar-$C_{4'}$), 120.32 (Ar-$C_{5'}$), 128.48 (Ar-$C_{6'}$), 121.94 (q, $^1J_{F-C}$ = 271.7 Hz, CF_3), 100.95 (C-1), 70.09 (C-2), 73.11 (C-3), 68.12 (C-4), 75.99 (C-5), 60.36 (C-6) ppm. Anal. Calcd for $C_{13}H_{14}NO_8F_3$ (%): C, 42.27; H, 3.82; N, 3.79. Found: C, 42.26; H, 3.79; N, 3.78.

2-Chloro-3-trifluoromethylphenyl β-D-galactopyranoside **16** as white crystals, R_f 0.48 (1:9 MeOH/EtOAc), δ_H: 8.28 (1H, d, J = 2.0 Hz, Ar-H), 7.99 (1H, dd, J = 2.0, 9.2 Hz, Ar-H), 7.59 (1H, d, J = 8.8 Hz, Ar-H), 5.15 (1H, d, $J_{1,2}$ = 7.6 Hz, H-1), 3.55 (1H, dd, $J_{2,3}$ = 8.9 Hz, H-2), 3.50 (1H, dd, $J_{3,4}$ = 6.0 Hz, H-3), 3.47 (1H, d, $J_{4,5}$ = 6.0 Hz, H-4), 3.42 (1H, m, H-5), 3.67 (2H, m, H-6), 5.24 (1H, d, $J_{H\text{-}2,OH\text{-}2}$ = 4.8 Hz, HO-2), 4.63 (1H, d, $J_{H\text{-}3,OH\text{-}3}$ = 4.4 Hz, HO-3), 4.92 (1H, d, $J_{H\text{-}4,OH\text{-}4}$ = 6.0 Hz, HO-4), 4.69 (1H, dd, $J_{H\text{-}6,OH\text{-}6}$ = 5.4, 5.6 Hz, HO-6) ppm; δ_C: 153.98 (Ar-$C_{1'}$), 120.13 (q, $^3J_{F-C}$ = 3.4 Hz, Ar-$C_{2'}$), 128.48 (q, $^2J_{F-C}$ = 30.1 Hz, Ar-$C_{3'}$), 131.62 (q, $^3J_{F-C}$ = 12.2 Hz, Ar-$C_{4'}$), 128.32 (Ar-$C_{5'}$), 131.56 (Ar-$C_{6'}$), 124.70 (q, $^1J_{F-C}$ = 260.0 Hz, CF_3), 100.95 (C-1), 69.97 (C-2), 73.29 (C-3), 68.03 (C-4), 76.04 (C-5), 60.30 (C-6) ppm. Anal. Calcd for $C_{13}H_{14}ClO_6F_3$ (%): C, 43.57; H, 3.94. Found: C, 43.55; H, 3.91.

2-Chloro-5-trifluoromethylphenyl β-D-galactopyranoside **17** as white crystals, R_f 0.50 (1:9 MeOH/EtOAc), δ_H: 7.69 (1H, d, J = 8.0 Hz, Ar-H), 7.55 (1H, d, J = 0.6 Hz, Ar-H), 7.59 (1H, dd, J = 2,1, 8.8 Hz, Ar-H), 5.11 (1H, d, $J_{1,2}$ = 8.4 Hz, H-1), 3.53 (1H, dd, $J_{2,3}$ = 10.2 Hz, H-2), 3.48 (1H, dd, $J_{3,4}$ = 5.2 Hz, H-3), 3.48 (1H, d, $J_{4,5}$ = 6.4 Hz, H-4), 3.44 (1H, m, H-5), 3.69 (2H, m, H-6), 5.20 (1H, d, $J_{H\text{-}2,OH\text{-}2}$ = 3.6 Hz, HO-2), 4.58 (1H, d, $J_{H\text{-}3,OH\text{-}3}$ = 2.8 Hz, HO-3), 4.91 (1H, d, $J_{H\text{-}4,OH\text{-}4}$ = 3.6 Hz, HO-4), 4.65 (1H, dd, $J_{H\text{-}6,OH\text{-}6}$ = 5.0, 6.0 Hz, HO-6) ppm; δ_C: 153.01 (Ar-$C_{1'}$), 109.31 (Ar-$C_{2'}$), 130.97 (Ar-$C_{3'}$), 112.90 (q, $^3J_{F-C}$ = 2.8 Hz, Ar-$C_{4'}$), 122.32 (q, $^2J_{F-C}$ = 32.0 Hz,

Ar-$C_{5'}$), 121.42 (q, $^3J_{F-C}$ = 2.7 Hz, Ar-$C_{6'}$), 122.22 (q, $^1J_{F-C}$ = 262.0 Hz, CF_3), 100.81 (C-1), 70.02 (C-2), 73.31 (C-3), 68.02 (C-4), 75.73 (C-5), 60.18(C-6) ppm. Anal. Calcd for $C_{13}H_{14}ClO_6F_3$ (%): C, 43.57; H, 3.94. Found: C, 43.56; H, 3.93.

2-Trifluoromethylphenyl β-D-galactopyranoside **18** as white crystals, R_f 0.52 (1:9 MeOH/EtOAc), δ_H: 7.67 (1H, m, Ar-H), 7.60 (1H, dd, J = 5.2, 5.6 Hz, Ar-H), 7.33 (1H, d, J = 5.6 Hz, Ar-H), 7.12 (1H, dd, J = 4.8, 5.2 Hz, Ar-H), 5.00 (1H, d, $J_{1,2}$ = 7.8 Hz, H-1), 3.62 (1H, dd, $J_{2,3}$ = 10.8 Hz, H-2), 3.54 (1H, dd, $J_{3,4}$ = 4.8 Hz, H-3), 3.49 (1H, dd, $J_{4,5}$ = 5.4, 6.0 Hz, H-4), 3.40 (1H, m, H-5), 3.70 (2H, m, H-6), 4.97 (1H, d, $J_{H\text{-}2,OH\text{-}2}$ = 6.0 Hz, HO-2), 4.57 (1H, d, $J_{H\text{-}3,OH\text{-}3}$ = 4.2 Hz, HO-3), 4.91 (1H, d, $J_{H\text{-}4,OH\text{-}4}$ = 6.0 Hz, HO-4), 4.65 (1H, dd, $J_{H\text{-}6,OH\text{-}6}$ = 5.4, 6.5 Hz, HO-6) ppm; δ_C: 155.60 (Ar-$C_{1'}$), 131.65 (q, $^2J_{F-C}$ = 36.0 Hz, Ar-$C_{2'}$), 126.49 (q, $^3J_{F-C}$ = 3.6 Hz, Ar-$C_{3'}$), 121.14 (Ar-$C_{4'}$), 116.69 (Ar-$C_{5'}$), 133.95 (Ar-$C_{6'}$), 127.53 (q, $^1J_{F-C}$ = 208.4 Hz, CF_3), 100.46 (C-1), 70.13 (C-2), 73.58 (C-3), 68.04 (C-4), 75.62 (C-5), 60.30 (C-6) ppm. Anal. Calcd for $C_{13}H_{15}O_6F_3$ (%): C, 48.14; H, 4.67. Found: C, 48.12; H, 4.66.

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