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Novel Fe^{3+}-Based ^{1}H MRI β-Galactosidase Reporter Molecules**

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Abstract

There is increasing interest in the development of reporter agents to reveal enzyme activity *in vivo* using small animal imaging. We have previously demonstrated the feasibility of detecting *lacZ* gene activity using the commercially available 3,4-cyclohexenoesculetin-β-*D*-galactopyranoside (S-Gal™) as a ^{1}H MRI reporter. Specifically, β-galactosidase (β-gal) releases the aglycone, which forms an MR contrast-inducing paramagnetic precipitate in the presence of Fe^{3+}. Contrast was primarily T_2-weighted signal loss, but T_1 effects were also observed. Since T_1-contrast generally provides signal enhancement as opposed to loss, it appeared attractive to explore whether analogues could be generated with enhanced characteristics. We now report the design and successful synthesis of novel analogues together with characterization of ^{1}H MRI contrast based on both T_1 and T_2 response to β-gal activity *in vitro* for the lead agent.

Keywords

NMR; enzyme; hydrolases; reporter molecules; relaxivity

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Introduction

Given the importance of reporter genes in various applications ranging from molecular biology to clinical trials, the development of non-invasive techniques to assay gene expression *in vivo* is becoming increasingly significant[1,2]. Traditionally, the *lacZ* gene encoding β-galactosidase (β-gal) was the most popular reporter including assays of clonal insertion, transcriptional activation, protein expression, and protein interaction[3,4]. The broad specificity of β-gal activity allows diverse molecular structures for substrates and successful detection techniques included colorimetric[5,6], fluorescence[7-9], bioluminescence[10], chemiluminescence[11-13], as well as radiotracers for positron emission tomography (PET)[14] or single-photon emission computed tomography (SPECT)[15] and probes for ^{1}H magnetic resonance imaging (MRI)[16-20] and ^{19}F-NMR approaches[21-26]. Many approaches have been demonstrated for *in vitro* detection, but few have been applied *in vivo* to date[8,13,17,18] and these often required direct injection into the tissue of interest[24,25,27]. While we focus on the detection of transgene activity in stably transfected human tumor cells, it is important to note that expression may also arise in normal tissues following exposure to stress such as radiation or doxorubicin induced senescence activated β-galactosidase[28, 29]. Moreover, epithelial exposure of lactase (the human analog of β-galactosidase) has been associated with metaplasia in developing esophageal cancer[12].

The pioneering study of Moats *et al.* demonstrated T_1-weighted MRI contrast based on β-gal activated unmasking of a gadolinium ligand[16] and this was later applied to tracing the developing cell lineages in frog embryos following direct intra cellular injection of substrate[17]. We recently demonstrated the feasibility of detecting β-gal activity *in vitro* in cultured cancer cells and *in vivo* in mice with human breast tumor MCF7 xenografts using 3,4-cyclohexenoesculetin-β-*D*-galactopyranoside (S-Gal™). Specifically, *β*-gal cleaves S-Gal™ to release the cyclohexenoesculetin aglycone, which forms a paramagnetic precipitate in the presence of Fe^{3+} generating T_2*-weighted ^{1}H MRI contrast [20]. This approach was also used to detect genetically engineered β-gal expressing bone marrow cells by MRI *in vivo* following labeling *in vitro* [30]. Both studies exploited T_2*-weighted signal loss to identify β-gal activity. We had noticed that there was additionally T_1 relaxivity, but the high T_2 relaxivity (up to 100 s^{-1} for 15 mM S-Gal™) tended to mask T_1-effects. These results prompted us to examine whether molecular modifications could provide T_1-activity without the high T_2 relaxivity.

β-galactosidase catalyses the hydrolysis of β-*D*-galactopyranosides by cleavage of the C-O bond between *D*-galactose and the aglycone. MRI detection of β-gal based on S-Gal™ depends on contrast produced by the formation of a complex between the 3,4-cyclohexenoesculetin aglycone and Fe^{3+} ions[20,31]. Schwert,[32] Davies,[33] and Raymond *et al.*[34] have described the design and evaluation of series of siderophores that contain catechol binding groups (catecholate ligands) to coordinate Fe^{3+}. Thus, we considered analogous dihydroxy coumarin-based catecholate aglycones. Coumarins are reported to have numerous therapeutic applications including antibacterial, anti-inflammatory and anti-coagulant as well as photochemotherapy and anti-HIV therapy [35]. Therefore, structure-activity relationships and synthetic procedures have been widely examined. Inspired by these studies, we designed 4 analogs based on the structure of 3,4-cyclohexenoesculetin (**1**, aglycone of S-Gal™): 7,8-dihydroxy-3,4-cyclohexenocoumarin (**2**), 6,7-dihydroxy-4-methyl-coumarin (**3**), 7,8-dihydroxy-4-methylcoumarin (**4**), and 7,8-dihydroxy-6-methoxycoumarin (**5**) (Figure 1).

We now report the design, synthesis, and evaluation of these novel analogs of S-Gal™, and *in vitro* assessment of their hydrolytic kinetics. MRI contrast with respect to *lacZ*-transfected

human MCF7 breast and PC3 prostate cancer cells is presented for the most promising agent.

Results and Discussion

Aglycone synthesis

noting the variety of strategies for synthesizing coumarins[32], we chose the Pechmann reaction, coupling the two components (phenol and β-ketoester) with $ZrCl_4$ (10 mol%) as catalyst[36]. We started the synthesis by subjecting pyrogallol or 1,2,4-benzenetriol to the Pechmann reaction with ethyl cyclohexanone-2-carboxylate or ethyl acetoacetate for coumarins **1**~**4**, while 6-methoxy-7,8-dihydroxycoumarin **5**, was purchased commercially. The reactions were performed at 80 °C in toluene, to give **1**~**4** in high yields (92-95%) within 30 minutes. After confirming the structure of coumarins **1**~**4**, we evaluated the T_1- and T_2-weighted MR image contrast of their Fe^{3+}-complexes. Each showed substantial T_1-weighted contrast (Figure 1), suggesting potential as Fe-based ^{1}H MRI *lacZ* gene reporters. Greatest T_1 response was observed for 6,7-dihydroxy-4-methylcoumarin(**3**)/Fe^{3+}, which also showed the greatest T_2-weighted MRI contrast.

Mono β-*D*-galactopyranosides

To generate β-gal reporters a β-*D*-galactopyranosyl group was added to the coumarins forming β-*D*-galactopyranosides (Figure 2). Each coumarin **2**~**5** has two hydroxyl groups located at the 6,7- or 7,8-positions, which were expected to show differences in reactivity and hence an opportunity for regioselective synthesis. Indeed, straightforward regioselective mono-glycopyranosylation [37] and etherification [38] have been reported at 7-hydroxyl group of 3,4-cyclohexenoesculetin **1**. ^{13}C and ^{1}H NMR chemical shifts of coumarin derivatives (δC-7 > δC-5 > δC-6 > δC-8)[39] also suggested that the relative electron deficiency of C-7, C-6, and C-8 would result in relative reactivity: 7-hydroxyl > 6-hydroxyl > 8-hydroxyl. Hydroxyl pK_a values in coumarins **1**~**5** corresponding to their positions (Table 1) suggested that phase-transfer-catalysis at pH = 8~9 could provide regio- and stereoselective synthesis of β-*D*-galactopyranosides, as we also exploited previously for ^{19}F-NMR β-gal reporters [40, 41]. Reaction of 2, 3, 4, 6-tetra-*O*-acetyl-α-*D*-galactopyranosyl bromide with equimolar coumarin **(2**~**5)** at room temperature catalyzed by tetrabutylammonium bromide (TBAB) in a dichloromethane-aqueous biphasic system (pH 8~9) under N_2 afforded 7-*O*-(2′, 3′, 4′, 6′-tetra-*O*-acetyl-β-*D*-galactopyranosyl)-8-hydroxy-3,4-cyclohexenocoumarin (**6**), 7-*O*-(2′, 3′, 4′, 6′-tetra-*O*-acetyl-β-*D*-galactopyranosyl)-6-hydroxy-4-methylcoumarin (**7**), 7-*O*-(2′, 3′, 4′, 6′-tetra-*O*-acetyl-β-*D*-galactopyranosyl)-8-hydroxy-4-methylcoumarin (**8**) and 7-*O*-(2′, 3′, 4′, 6′-tetra-*O*-acetyl-β-*D*-galactopyranosyl)-8-hydroxy-6-methoxycoumarin (**9**) in moderate yields (72~88%) (Figure 2). Nuclear Overhauser enhancements (NOE) showed that the mono β-*D*-galactopyranosylations occurred at the *O*-7 positions, as predicted. Subsequent deacetylation with NH3/MeOH from 0°C to room temperature gave the free mono galactopyranosides **10**~**13** (Figure 3) in nearly quantitative yields. The anomeric β-*D*-configuration of compounds **10**~**13** in the $^4C_1$ chair conformation was confirmed by the ^{1}H-NMR chemical shifts (δ_h 4.75~5.03 ppm) of the anomeric protons and the $J_{1,2}$ (J ~8 Hz), and $J_{2,3}$ (J ~10 Hz) coupling constants. The anomeric carbon resonances appeared at $\delta_{C\text{-}1'}$ 100.85~105.53 ppm in accordance with the β-*D*-configuration[40,42].

The coumarins **1**~**5** are strongly fluorescent (365/440 nm) in PBS (0.1M, pH=7.4), however, their β-*D*-galactopyranosides, S-Gal™ and **10**~**13**, are weakly or non-fluorescent. The measurement of fluorescence intensity increased following reaction of the coumarin with β-gal(E801A) in PBS (0.1M, pH=7.4) at 20~22°C showing that all the β-*D*-galactopyranosides were effective substrates though with varying hydrolytic rates in the

order: $v_1 > v_{11} > v_{13} > v_{10} > v_{12}$ (Figure 4). Given that **11** and **13** were considerably better substrates these were favored for further evaluation. In addition the MRI contrast generated by the aglycones **1**~**5** in the presence of Fe^{3+} (Figure 1) indicated that both **11** and **13** would show considerable T_1 contrast upon hydrolysis by β-gal and since **13** showed much less T_2 sensitivity it was chosen for further evaluation.

MRI in solution

T_1 and T_2 maps were measured for vials containing various combinations of mono β-*D*-galactopyranoside **13** and Fe^{3+} ions, with or without 5 units of β-gal (E801A) (Figure 5**).** Ferric ions alone enhanced R_1 relaxation, but the presence of **13** made no difference. Addition of β-gal to the mixture of **13** + Fe^{3+} generated much more rapid relaxation, which depended on the ratio of the two components: specifically ΔR_1 5.9 s^{-1} (3:1), 5.1 s^{-1} (2:1), and 2.3 s^{-1} (1:1), respectively (Figure 5a). T_2-weighted MR contrast showed a very similar effect though Fe^{3+} alone caused minimal relaxation, as expected: for the complexes [ΔR_2 9.4 s^{-1} (3:1), 7.0 s^{-1} (2:1) and 3.2 s^{-1} (1:1)] (Figure 5b). The relaxation rates R_1 and R_2 varied linearly as a function of the concentration of **13** at a fixed concentration of Fe^{3+} (Figure 5c).

MRI in cells

To demonstrate the potential for detecting β-gal activity *in vivo*, various cells (human MCF7 breast and PC3 prostate cancer), as well as stably transfected clones expressing β-gal (MCF7-*lacZ* and PC3-*lacZ*) were incubated with 15 mM **13** and 5 mM Fe^{3+} in PBS (0.1M, pH=7.4) at 37°C under 5% CO_2 in air with 95% humidity for 30 min. A significant difference in T_1 and T_2 was observed between the *lacZ* transfected and wild type (WT) cells. In MCF7-WT cells T_1 = 1.32 ± 0.12 s and T_2 = 45 ± 6 ms, while for MCF7-*lacZ* T_1= 0.70 ± 0.10 and T_2 = 32 ± 9 ms (Figure 6). Similarly, in PC3-WT cells T_1=1.50 ± 0.07 s, T_2= 39 ± 6 ms while for PC3-*lacZ* T_1=1.16 ± 0.04 s, T_2= 28 ± 4 ms, respectively).

To be useful *in vivo*, reporter molecules must exhibit sufficient water solubility and it appeared that **10~13** were less soluble than S-Gal™. Thus, we sought to enhance solubility by conjugating an additional β-*D*-galactopyranosyl unit to S-Gal™ and **10**~**13**, as applied successfully to ^{19}F NMR β-gal reporters previously[41].

Di-β-*D*-galactopyranosides

condensation of the coumarins **1**~**5** directly with 2.2 equivalents of 2, 3, 4, 6-tetra-*O*-acetyl-α-*D*-galactopyranosyl bromide in anhydrous CH_2Cl_2/MeCN catalyzed by $Hg(CN)_2$ as a promoter, furnished the fully galactopyranosylated coumarins: 6,7-di-*O*-(2″, 3″, 4″, 6″-tetra-*O*-acetyl-β-*D*-galactopyranosyl)-3, 4-cyclohexenocoumarin **14** (90%), 7,8-di-*O*-(2″, 3″, 4″, 6″-tetra-*O*-acetyl-β-*D*-galactopyranosyl)-3, 4-cyclohexenocoumarin **15** (86%), 6,7-di-*O*-(2′, 3′, 4′, 6′-tetra-*O*-acetyl-β-*D*-galactopyranosyl)-4-methylcoumarin **16** (73%), 7,8-di-*O*-(2′, 3′, 4′, 6′-tetra-*O*-acetyl-β-*D*-galactopyranosyl)-4-methylcoumarin **17** (77%) and 7,8-di-*O*-(2′, 3′, 4′, 6′-tetra-*O*-acetyl-β-*D*-galactopyranosyl)-6-methoxycoumarin **18** (87%), respectively (Figure 7). Deacetylation of **14**~**18** in NH_3/MeOH from 0 °C to room temperature accomplished the free di-β-*D*-galactopyranosides **19**~**23** in high yields (Figure 7). The ESI-MS of **19**~**23** showed the expected molecular ions, corresponding to the fully galactopyranosylated derivatives. Again, the identities of **19**~**23** were established from their ^{1}H and ^{13}C NMR spectra. The anomeric protons H-1′, H-1″ or H-1‴ of *D*-galactoses linked to 7 and 6 or 8 positions of coumarins **1**~**5** at 5.26~4.82 ppm with the well resolved doublets ($J_{1,2}$ = 8.0 Hz, $J_{2,3}$ = 10 Hz) confirming both *D*-galactoses in the β-configuration.

As expected, the synthesized di-β-*D*-galactopyranosides **19**~**23** are soluble in PBS (0.1M, pH= 7.4) in high concentrations, unlike **10**~**13**, which required the addition of DMSO. The hydrolysis of di-β-*D*-galactopyranosides **19**~**23** by β-gal (E801A) in PBS (0.1M, pH=7.4) at 20~22°C showed that relative hydrolytic rates were similar though a little slower than for the corresponding mono-galactopyranosides and **23** was considerably slower than expected (Figure 8).

In conclusion we have successfully synthesized 9 novel β-*D*-galactopyranosides and demonstrated the potential to detect β-gal activity based on MRI contrast in the presence of Fe^{3+} ions. The di-β-*D*-galactopyranosides react a little slower, but exhibit much higher water solubility suggesting greater potential for use *in vivo*. MRI clearly revealed WT versus *lacZ* expressing cells in culture upon incubation with **13** based on significant differences in both T_1 and T_2. Signal gain providing contrast in T_1-weighted images is potentially preferable to T_2-weighted signal loss observed previously with S-Gal™ *in vivo*. However the combination of both T_1 and T_2 response may be most promising since the concerted effect will add certainty to observations *in vivo*, where tissue heterogeneity may otherwise be misinterpreted. These mono and di-β-*D*-galactopyranosides show promise as ^{1}H MRI *lacZ* gene reporters and we are currently evaluating them for potential application in human tumor xenografts *in vivo*.

Experimental

General methods -----NMR spectra were recorded on a Varian Unity INOVA 400 spectrometer (400 MHz for ^{1}H, 100 MHz for ^{13}C) with $CDCl_3$, or DMSO-d_6 as solvents at 25°C, and ^{1}H and ^{13}C chemical shifts are referenced to internal TMS. Microanalyses were performed on a Perkin-Elmer 2400CHN microanalyser. Mass spectra were obtained by positive and negative ESI-MS using a Micromass Q-TOF hybrid quadrupole/time-of-flight instrument (Micromass UK Ltd). Solutions in organic solvents were dried with anhydrous sodium sulfate, and concentrated *in vacuo* below 45 °C. 3,4-cyclohexenoesculetin-β-*D*-galactopyranoside (S-Gal™), 2, 3, 4, 6-tetra-*O*-acetyl-α-*D*-galactopyranosyl bromide and 6-methoxy-7, 8-dihydroxycoumarin **5** were purchased from the Sigma Chemical Company. β-Gal (E801A) was purchased from Aldrich Chemical Company and enzyme reactions performed at 20-22 °C in PBS solution (0.1M, pH=7.4). Fluorescence was measured using a Fluorolog 3 spectrometer (Jobin-Yvon Horiba, Edison, NJ) with λ_{ex} at 365 nm and λ_{em} 440 nm. Column chromatography was performed on silica gel (200~300 mesh) by elution with cyclohexane-ethyl acetate and silica gel GF_{254} used for analytical TLC (Aldrich Chemical Company). 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.

Pechmann condensation for synthesis of coumarins 1~4

General procedure -—To an equimolar mixture of the phenol (pyrogallol or 1,2,4-benzenetriol, 10 mmol) and the β-ketoester (ethyl cyclohexanone-2-carboxylate or ethyl acetoacetate, 10 mmol) in toluene (40mL) was added $ZrCl_4$ (377.3 mg, 1.0 mmol, 10mol%) and the mixture was stirred at 80 °C under N_2 until TLC showed complete reaction (<30 minutes). After solvent evaporation under reduced pressure, the mixture was washed with cold water, and recrystallized from hot EtOH/H_2O to give the pure coumarins **1**~**4**.

3,4-cyclohexenoesculetin **1** (2.14 g, 92%), δ_h ([D_6]DMSO, 400 MHz): 6.93 (1 H, s, H-5), 9.25 (1 H, s, OH-6), 9.98 (1 H, s, OH-7), 6.69 (1 H, s, H-8), 2.64 (2 H, t, J = 4.0 Hz, H-1′), 1.69 (4 H, m, H-2′, 3′), 2.34 (2 H, t, J = 4.0 Hz, H-4′) ppm; δ_C ([D_6]DMSO, 100 MHz): 164.02 (-CO), 102.64 (C-3), 148.04 (C-4), 142.74 (C-5), 118.81 (C-6), 146.00 (C-7), 108.40

(C-8), 148.81 (C-9), 111.57 (C-10), 24.78 (CH_2-1′), 21.37, 21.05 (CH_2-2′, 3′), 23.66 (CH_2-4′) ppm.

Anal. Calcd. for $C_{13}H_{12}O_4$ (%): C, 67.23, H, 5.21; Found: C, 67.21, H, 5.19.

7,8-dihydroxy-3,4-cyclohexenocoumarin **2** (2.16 g, 93%), δ_H ([D_6]DMSO, 400 MHz): 7.00 (1 H, d, *J* = 8.0 Hz, H-5), 6.75 (1 H, d, *J* = 8.0 Hz, H-6), 9.84 (1 H, s, OH-7), 9.20 (1 H, s, OH-8), 2.68 (2 H, t, *J* = 4.0 Hz, H-1′), 1.69 (4 H, m, H-2′, 3′), 2.36 (2 H, t, *J* = 4.0 Hz, H-4′) ppm; δ_C ([D_6]DMSO, 100 MHz): 160.08 (-CO), 148.15 (C-9), 141.61 (C-4), 132.00 (C-7), 118.34 (C-5), 113.90 (C-6), 112.82 (C-10), 112.10 (C-8), 107.23 (C-3), 24.79 (CH_2-1′), 21.37, 21.02 (CH_2-2′, 3′), 23.61 (CH_2-4′) ppm.

Anal. Calcd. for $C_{13}H_{12}O_4$ (%): C, 67.23, H, 5.21; Found: C, 67.21, H, 5.20.

6,7-dihydroxy-4-methylcoumarin **3** (1.83 g, 95%), δ_H ([D_6]DMSO, 400 MHz): 6.07 (1 H, s, H-3), 6.98 (1 H, s, H-5), 9.38 (1 H, brs, OH-6), 10.19 (1 H, brs, OH-7), 6.71 (1 H, s, H-8), 2.29 (3 H, s, CH_3-4) ppm; δ_C ([D_6]DMSO, 100 MHz): 160.69 (-CO), 102.74 (C-3), 150.20 (C-4), 142.86 (C-5), 111.58 (C-6), 147.80 (C-7), 110.45 (C-8), 153.31 (C-9), 110.49 (C-10), 18.29 (CH_3-4) ppm.

Anal. Calcd. for $C_{10}H_8O_4$ (%): C, 62.50, H, 4.20; Found: C, 62.47, H, 4.18.

7,8-dihydroxy-4-methylcoumarin **4** (1.81 g, 94%), δ_H ([D_6]DMSO, 400 MHz): 6.10 (1 H, s, H-3), 7.06 (1 H, d, *J* = 8.2 Hz, H-5), 6.80 (1 H, d, *J* = 8.2 Hz, H-6), 10.03 (1 H, s, OH-7), 9.27 (1 H, s, OH-8), 2.33 (3 H, s, CH_3-4) ppm; δ_C ([D_6]DMSO, 100 MHz): 160.29 (-CO), 110.27 (C-3), 149.47 (C-4), 132.24 (C-5), 115.59 (C-6), 143.36 (C-7), 112.19 (C-8), 154.01 (C-9), 112.84 (C-10), 18.33 (CH_3-4) ppm.

Anal. Calcd. for $C_{10}H_8O_4$ (%): C, 62.50, H, 4.20; Found: C, 62.48, H, 4.19.

Regioselective mono β-*D*-galactopyranosylation of coumarins 2∼5 for preparation of acetylated mono β-*D*-galactopyranosides 6∼9

General procedure -----A solution of 2, 3, 4, 6-tetra-*O*-acetyl-α-*D*-galactopyranosyl bromide (1.04 g, 2.52 mmol) in CH_2Cl_2 (30 mL) was added dropwise to a vigorously stirred biphasic mixture (pH 8~9) of coumarins **2**∼**5** (2.52 mmol) and tetrabutylammonium bromide (TBAB) (160 mg, 0.5 mmol) in CH_2Cl_2-H_2O (50 mL, 1:1 V/V′) over a period of 1 hr at room temperature under N_2, and the stirring continued until TLC showed that the reaction complete (~3 hr). Extraction with CH_2Cl_2 (4 × 30 mL), wash, dry (Na_2SO_4), and evaporation under reduced pressure gave a syrup, which was purified by column chromatography on silica gel yielding acetylated mono β-*D*-galactopyranosides **6**∼**9**.

7-*O*-(2″, 3″, 4″, 6″-tetra-*O*-acetyl-β-*D*-galactopyranosyl)-8-hydroxy-3, 4-cyclohexeno-coumarin **6** (1.25 g, 88%), R_f 0.40 (1:1 cyclohexane-EtOAc), δ_h ($CDCl_3$, 400 MHz): 7.23 (1 H, d, *J* = 8.0 Hz, H-5), 6.83 (1 H, d, *J* = 8.0 Hz, H-6), 4.84 (1 H, d, $J_{1'',2''}$ = 8.0 Hz, H-1″), 5.53 (1 H, dd, $J_{2'',3''}$ = 12.0 Hz, H-2″), 5.07 (1 H, dd, $J_{3'',4''}$ = 4.0 Hz, H-3″), 5.40 (1 H, d, $J_{4'',5''}$ = 3.2 Hz, H-4″), 3.95 (1 H, m, H-5″), 4.19 (1 H, dd, $J_{5'',6a''}$ = 7.2 Hz, $J_{6a'',6b''}$ = 11.8 Hz, H-6a″), 4.16 (1 H, dd, $J_{5'',6b''}$ = 5.6 Hz, H-6b″), 2.68 (2 H, t, *J* = 4.0 Hz, H-1′), 1.76 (4 H, m, H-2′, 3′), 2.47 (2 H, t, *J* = 4.0 Hz, H-4′), 2.00, 1.99, 1.98, 1.96 (12 H, 4 s, 4 × CH_3CO), ppm; δ_C ($CDCl_3$, 100 MHz): 170.92, 170.56, 170.30, 170.14 (4 × CH3CO), 161.24 (-CO), 112.83 (C-3), 147.64 (C-4), 131.06 (C-5), 120.88 (C-6), 145.87 (C-7), 114.35 (C-8), 151.96 (C-9), 120.69 (C-10), 104.52 (C-1″), 68.11 (C-2″), 70.68 (C-3″), 66.83 (C-4″), 71.87 (C-5″), 61.12 (C-6″), 25.49 (CH_2-1′), 21.80, 21.49 (CH_2-2′, 3′), 24.00 (CH_2-4′), 20.91, 20.82, 20.75, 20.70 (4 × CH_3CO) ppm.

Anal. Calcd. for $C_{27}H_{30}O_{13}$ (%): C, 57.65, H, 5.38; Found: C, 57.63, H, 5.35.

7-*O*-(2′, 3′, 4′, 6′-tetra-*O*-acetyl-β-*D*-galactopyranosyl)-6-hydroxy-4-methylcoumarin **7** (0.95 g, 72%), R_f 0.33 (2:3 cyclohexane-EtOAc), δ_h ($CDCl_3$, 400 MHz): 6.16 (1 H, s, H-3), 7.07 (1 H, s, H-5), 6.95 (1 H, s, H-8), 5.03 (1 H, d, $J_{1',2'}$ = 8.0 Hz, H-1′), 5.45 (1 H, dd, $J_{2',3'}$ = 114 Hz, H-2′), 5.15 (1 H, dd, $J_{3',4'}$ = 3.4 Hz, H-3′), 5.42 (1 H, d, $J_{4',5'}$ = 7.2 Hz, H-4′), 4.10 (1 H, m, H-5′), 4.16 (1 H, dd, $J_{5',6a'}$ = 4.0 Hz, $J_{6a',6b'}$ = 7.2 Hz, H-6a′), 4.13 (1 H, dd, $J_{5',6b'}$ = 5.0 Hz, H-6b′), 2.32 (3 H, s, CH_3-4), 2.15, 2.08, 2.07, 1.99 (12 H, 4 s, 4 × CH_3CO) ppm; δ_C ($CDCl_3$, 100 MHz): 170.95, 170.64, 170.24, 170.04 (4 × CH_3CO), 161.15 (-CO), 104.15 (C-3), 147.88 (C-4), 143.45 (C-5), 116.17 (C-6), 146.77 (C-7), 110.03 (C-8), 152.30 (C-9), 114.03 (C-10), 100.85 (C-1′), 69.18 (C-2′), 70.23 (C-3′), 66.82 (C-4′), 71.91 (C-5′), 61.46 (C-6′), 21.06, 20.84, 20.77, 20.69 (4 × CH_3CO), 18.98 (CH_3-4) ppm.

Anal. Calcd. for $C_{24}H_{26}O_{13}$ (%): C, 55.17, H, 5.02; Found: C, 55.15, H, 5.00.

7-*O*-(2′, 3′, 4′, 6′-tetra-*O*-acetyl-β-*D*-galactopyranosyl)-8-hydroxy-4-methylcoumarin **8** (1.07 g, 81%), R_f 0.47 (1:1 cyclohexane-EtOAc), δ_h ($CDCl_3$, 400 MHz): 6.17 (1 H, s, H-3), 7.01 (1 H, d, *J* = 8.0 Hz, H-5), 6.93 (1 H, d, *J* = 8.0 Hz, H-6), 4.96 (1 H, d, $J_{1',2'}$ = 8.0 Hz, H-1′), 5.44 (1 H, dd, $J_{2',3'}$ = 10.7 Hz, H-2′), 5.09 (1 H, dd, $J_{3',4'}$ = 4.0 Hz, H-3′), 5.41 (1 H, d, $J_{4',5'}$ = 3.0 Hz, H-4′), 4.01 (1 H, m, H-5′), 4.18 (1 H, dd, $J_{5',6a'}$ = 5.8 Hz, $J_{6a',6b'}$ = 11.6 Hz, H-6a′), 4.16 (1 H, dd, $J_{5',6b'}$ = 7.8 Hz, H-6b′), 2.34 (3 H, s, CH_3-4), 2.14, 2.06, 2.01, 1.96 (12 H, 4 s, 4 × CH_3CO) ppm; δ_C ($CDCl_3$, 100 MHz): 170.58, 170.46, 170.33, 170.24 (4 × CH_3CO), 160.06 (-CO), 113.77 (C-3), 146.35 (C-4), 135.34 (C-5), 117.28 (C-6), 143.65 (C-7), 113.93 (C-8), 152.63 (C-9), 115.25 (C-10), 101.64 (C-1′), 69.01 (C-2′), 70.42 (C-3′), 66.83 (C-4′), 71.54 (C-5′), 61.37 (C-6′), 20.91, 20.83, 20.81, 20.75 (4 × CH_3CO), 18.99 (CH_3-4) ppm.

Anal. Calcd. for $C_{24}H_{26}O_{13}$ (%): C, 55.17, H, 5.02; Found: C, 55.16, H, 5.01.

7-*O*-(2′, 3′, 4′, 6′-tetra-*O*-acetyl-β-*D*-galactopyranosyl)-8-hydroxy-6-methoxycoumarin **9** (1.06 g, 78%), R_f 0.52 (1:4 cyclohexane-EtOAc), δ_h ($CDCl_3$, 400 MHz): 6.29 (1 H, d, *J* = 9.8 Hz, H-3), 7.51 (1 H, d, *J* = 9.8 Hz, H-4), 6.90 (1 H, s, H-5), 4.80 (1 H, d, $J_{1',2'}$ = 8.0 Hz, H-1′), 5.44 (1 H, dd, $J_{2',3'}$ = 10.2 Hz, H-2′), 5.04 (1 H, dd, $J_{3',4'}$ = 3.5 Hz, H-3′), 5.34 (1 H, d, $J_{4',5'}$ = 6.8 Hz, H-4′), 3.93 (1 H, m, H-5′), 4.12 (1 H, dd, $J_{5',6a'}$ = 4.4 Hz, $J_{6a',6b'}$ = 7.8 Hz, H-6a′), 4.04 (1 H, dd, $J_{5',6b'}$ = 5.2 Hz, H-6b′), 3.79 (3 H, s, CH3O-6), 2.14, 2.08, 1.97, 1.94 (12 H, 4 s, 4 × CH_3CO) ppm; δ_C ($CDCl_3$, 100 MHz): 170.64, 170.34, 170.20, 169.75 (4 × CH_3CO), 160.24 (-CO), 115.49 (C-3), 143.36 (C-4), 138.34 (C-5), 136.07 (C-6), 139.23 (C-7), 116.27 (C-8), 149.31 (C-9), 116.77 (C-10), 103.57 (C-1′), 68.60 (C-2′), 70.51 (C-3′), 66.74 (C-4′), 71.76 (C-5′), 61.03 (C-6′), 56.41 (CH3O-6), 20.06, 20.88, 20.79, 20.76 (4 × CH_3CO) ppm.

Anal. Calcd. for $C_{24}H_{26}O_{14}$ (%): C, 53.53, H, 4.87; Found: C, 53.52, H, 4.85.

Mono β-*D*-galactopyranosides 10~13

General procedure ----—A solution of 7-*O*-(acetylated β-*D*-galactopyranosyl) coumarins **6**~**9** (900 mg) in anhydrous ammoniacal MeOH (0.5M 100 mL) was vigorously stirred from 0 °C to room temperature overnight until TLC showed that the reaction was complete, evaporated to dryness *in vacuo*. Chromatography of the crude syrup on silica gel with EtOAc/MeOH afforded the corresponding free mono β-D-galactopyranosides **10**~**13** in nearly quantitative yields.

7-*O*-(β-*D*-galactopyranosyl)-8-hydroxy-3, 4-cyclohexenocoumarin **10** (599.43 mg, 95%), R_f 0.41 (1:3 MeOH-EtOAc), δ_h ([D_6]DMSO, 400 MHz): 7.30 (1 H, d, *J* = 8.4 Hz, H-5), 6.82 (1

H, d, J = 8.4 Hz, H-6), 4.75 (1 H, d, J1″,2″ = 8.0 Hz, H-1″), 3.67 (1 H, dd, J2″,3″ = 10.2 Hz, H-2″), 3.56 (1 H, dd, $J_{3'',4''}$ = 4.0 Hz, H-3″), 3.45 (1 H, d, $J_{4'',5''}$ = 6.3 Hz, H-4″), 3.40 (1 H, m, H-5″), 3.38 (2 H, m, H-6″), 2.74 (2 H, t, *J* = 4.0 Hz, H-1′), 1.72 (4 H, m, H-2′, 3′), 2.51 (2 H, t, *J* = 4.0 Hz, H-4′) ppm; δ_C ([D_6]DMSO, 100 MHz): 160.81 (-CO), 112.12 (C-3), 147.99 (C-4), 131.60 (C-5), 119.62 (C-6), 145.89 (C-7), 113.45 (C-8), 153.46 (C-9), 118.15 (C-10), 105.53 (C-1″), 71.40 (C-2″), 73.27 (C-3″), 67.86 (C-4″), 75.77 (C-5″), 59.95 (C-6″), 24.83 (CH_2-1′), 21.36, 21.02 (CH_2-2′, 3′), 23.60 (CH_2-4′) ppm.

Anal. Calcd. for $C_{19}H_{22}O_9$ (%): C, 57.87, H, 5.62; Found: C, 57.84, H, 5.58.

7-*O*-(β-*D*-galactopyranosyl)-6-hydroxy-4-methylcoumarin **11** (567.62 mg, 93%), R_f 0.40 (1:3 MeOH-EtOAc), δ_H ([D_6]DMSO, 400 MHz): 6.16 (1 H, s, H-3), 7.07 (1 H, s, H-5), 6.95 (1 H, s, H-8), 5.03 (1 H, d, $J_{1',2'}$ = 8.0 Hz, H-1′), 5.45 (1 H, dd, $J_{2',3'}$ = 11.4 Hz, H-2′), 5.15 (1 H, dd, $J_{3',4'}$ = 3.4 Hz, H-3′), 5.42 (1 H, d, $J_{4',5'}$ = 7.2 Hz, H-4′), 4.10 (1 H, m, H-5′), 4.16 (1 H, dd, $J_{5',6a'}$ = 4.0 Hz, $J_{6a',6b'}$ = 7.2 Hz, H-6a′), 4.13 (1 H, dd, $J_{5',6b'}$ = 5.0 Hz, H-6b′), 2.32 (3 H, s, CH_3-4) ppm; δ_C ([D_6]DMSO, 100 MHz): 161.15 (-CO), 104.15 (C-3), 147.88 (C-4), 143.45 (C-5), 116.17 (C-6), 146.77 (C-7), 110.03 (C-8), 152.30 (C-9), 114.03 (C-10), 100.85 (C-1′), 69.18 (C-2′), 70.23 (C-3′), 66.82 (C-4′), 71.91 (C-5′), 61.46 (C-6′), 18.98 (CH_3-4) ppm.

Anal. Calcd. for $C_{16}H_{18}O_9$ (%): C, 54.24, H, 5.12; Found: C, 54.20, H, 5.09.

7-*O*-(β-*D*-galactopyranosyl)-8-hydroxy-4-methylcoumarin **12** (585.93 mg, 96%), R_f 0.38 (1:3 MeOH-EtOAc), δ_H ([D_6]DMSO, 400 MHz): 6.17 (1 H, s, H-3), 7.01 (1 H, d, *J* = 8.0 Hz, H-5), 6.93 (1 H, d, *J* = 8.0 Hz, H-6), 4.96 (1 H, d, $J_{1',2'}$ = 8.0 Hz, H-1′), 5.44 (1 H, dd, $J_{2',3'}$ = 10.7 Hz, H-2′), 5.09 (1 H, dd, $J_{3',4'}$ = 4.0 Hz, H-3′), 5.41 (1 H, d, $J_{4',5'}$ = 3.0 Hz, H-4′), 4.01 (1 H, m, H-5′), 4.18 (1 H, dd, $J_{5',6a'}$ = 5.8 Hz, $J_{6a',6b'}$ = 11.6 Hz, H-6a′), 4.16 (1 H, dd, $J_{5',6b'}$ = 7.8 Hz, H-6b′), 2.34 (3 H, s, CH_3-4) ppm; δ_C ([D_6]DMSO, 100 MHz): 160.06 (-CO), 113.77 (C-3), 146.35 (C-4), 135.34 (C-5), 117.28 (C-6), 143.65 (C-7), 113.93 (C-8), 152.63 (C-9), 115.25 (C-10), 101.64 (C-1′), 69.01 (C-2′), 70.42 (C-3′), 66.83 (C-4′), 71.54 (C-5′), 61.37 (C-6′), 18.99 (CH_3-4) ppm.

Anal. Calcd. for $C_{16}H_{18}O_9$ (%): C, 54.24, H, 5.12; Found: C, 54.19, H, 5.08.

7-*O*-(β-*D*-galactopyranosyl)-8-hydroxy-6-methoxycoumarin **13** (575.63 mg, 93%), R_f 0.45 (1:3 MeOH-EtOAc), δ_H ([D_6]DMSO, 400 MHz): 6.29 (1 H, d, *J* = 9.8 Hz, H-3), 7.51 (1 H, d, *J* = 9.8 Hz, H-4), 6.90 (1 H, s, H-5), 4.80 (1 H, d, $J_{1',2'}$ = 8.0 Hz, H-1′), 5.44 (1 H, dd, $J_{2',3'}$ = 10.2 Hz, H-2′), 5.04 (1 H, dd, $J_{3',4'}$ = 3.5 Hz, H-3′), 5.34 (1 H, d, $J_{4',5'}$ = 6.8 Hz, H-4′), 3.93 (1 H, m, H-5′), 4.12 (1 H, dd, $J_{5',6a'}$ = 4.4 Hz, $J_{6a',6b'}$ = 7.8 Hz, H-6a′), 4.04 (1 H, dd, $J_{5',6b'}$ = 5.2 Hz, H-6b′), 3.79 (3 H, s, CH3O-6) ppm; δ_C ([D_6]DMSO, 100 MHz): 160.24 (-CO), 115.49 (C-3), 143.36 (C-4), 138.34 (C-5), 136.07 (C-6), 139.23 (C-7), 116.27 (C-8), 149.31 (C-9), 116.77 (C-10), 103.57 (C-1′), 68.60 (C-2′), 70.51 (C-3′), 66.74 (C-4′), 71.76 (C-5′), 61.03 (C-6′), 56.41 (CH3O-6) ppm.

Anal. Calcd. for $C_{16}H_{18}O_{10}$ (%): C, 51.90, H, 4.90; Found: C, 51.85, H, 4.86.

Full β-*D*-galactopyranosylation of coumarins 1~5 for preparation of acetylated di-β-*D*-galactopyranosides 14~18

General procedure -----To a vigorously stirred solution of coumarins **1**~**5** (2.52 mmol) and $Hg(CN)_2$ (2.10 g, 8.31 mmol) in anhydrous MeCN (100 mL) containing freshly activated 4Å molecular sieves (5.00 g) was added dropwise 2, 3, 4, 6-tetra-*O*-acetyl-α-*D*-galactopyranosyl bromide (2.29 g, 5.54 mmol, 2.2 equiv.) in CH_2Cl_2 (50 mL). The mixture was stirred in the dark at room temperature under N_2 atmosphere until TLC indicated

complete reaction, then diluted with CH_2Cl_2 (150 mL), filtered through Celite, washed, dried (Na_2SO_4), evaporated under reduced pressure to give a syrup, which was purified by column chromatography on silica gel to give the acetylated di-β-*D*-galactopyranosides **14**~**18**.

6,7-di-*O*-(2″, 3″, 4″, 6″-tetra-*O*-acetyl-β-*D*-galactopyranosyl)-3, 4-cyclohexenoesculetin **14** (2.03 g, 90%), R_f 0.41 (2:3 cyclohexane-EtOAc), δ_h ($CDCl_3$, 400 MHz): 7.32 (1 H, s, H-5), 7.08 (1 H, s, H-8), 5.14 (1 H, d, $J_{1″,2″}$ = 8.0 Hz, H-1″), 5.20 (1 H, d, $J_{1‴,2‴}$ = 8.0 Hz, H-1‴), 5.48 (2 H, dd, $J_{2″,3″}$ = $J_{2‴,3‴}$ = 10.0 Hz, H-2″, 2‴), 5.16 (2 H, dd, $J_{3″,4″}$ = $J_{3‴,4‴}$ = 4.0 Hz, H-3″, 3‴), 5.43 (2 H, d, $J_{4″,5″}$ = $J_{4‴,5‴}$ = 3.2 Hz, H-4″, 4‴), 4.06 (2 H, m, H-5″, 5‴), 4.20 (2 H, dd, $J_{5″,6a″}$ = $J_{5‴,6a‴}$ = 5.0 Hz, $J_{6a″,6b″}$ = $J_{6a‴,6b‴}$ = 11.2 Hz, H-6a″, 6a‴), 4.13 (2 H, dd, $J_{5″,6b″}$ = $J_{5‴,6b‴}$ = 4.0 Hz, H-6b″, 6b‴), 2.72 (2 H, t, *J* = 4.0 Hz, H-1′), 1.81 (4 H, m, H-2′, 3′), 2.57 (2 H, t, *J* = 4.0 Hz, H-4′), 2.21, 2.14, 2.10, 2.09, 2.03, 2.02, 2.01, 2.00 (24 H, 8 s, 8 × CH_3CO) ppm; δ_C ($CDCl_3$, 100 MHz): 170.63, 170.43, 170.35, 170.27, 170.24, 170.22, 169.36, 169.20 (8 × CH_3CO), 161.71 (-CO), 105.34 (C-3), 148.84 (C-4), 143.17 (C-5), 122.95 (C-6), 146.53 (C-7), 114.10 (C-8), 149.30 (C-9), 115.68 (C-10), 100.88 (C-1″), 101.02 (C-1‴), 68.66 (C-2″), 69.00 (C-2‴), 70.87 (C-3″), 70.96 (C-3‴), 67.17 (C-4″), 67.22 (C-4‴), 71.48 (C-5″), 71.82 (C-5‴), 61.57 (C-6″), 61.64 (C-6‴), 25.38 (CH_2-1′), 21.72, 21.46 (CH_2-2′, 3′), 24.13 (CH_2-4′), 20.95, 20.90, 20.86, 20.83, 20.80, 20.78, 20.76, 20.74 (8 × CH_3CO) ppm; ESIMS: *m/z* 893 [M^+] (27%), 894 [M+1] (11%).

Anal. Calcd. for $C_{41}H_{48}O_{22}$ (%): C, 55.16, H, 5.42; Found: C, 55.14, H, 5.40.

7,8-di-*O*-(2″, 3″, 4″, 6″-tetra-*O*-acetyl-β-*D*-galactopyranosyl)-3, 4-cyclohexenocoumarin **15** (1.94 g, 86%), R_f 0.36 (2:3 cyclohexane-EtOAc), δ_h ($CDCl_3$, 400 MHz): 7.27 (1 H, d, *J* = 8.0 Hz, H-5), 7.06 (1 H, d, *J* = 8.0 Hz, H-6), 5.37 (1 H, d, $J_{1″,2″}$ = 8.0 Hz, H-1″), 5.25 (1 H, d, $J_{1‴,2‴}$ = 8.0 Hz, H-1‴), 5.48 (1 H, dd, $J_{2″,3″}$ = 10.4 Hz, H-2″), 5.45 (1 H, dd, $J_{2‴,3‴}$ = 10.2 Hz, H-2‴), 5.13 (1 H, dd, $J_{3″,4″}$ = 4.0 Hz, H-3″), 5.10 (1 H, dd, $J_{3‴,4‴}$ = 4.0 Hz, H-3‴), 5.44 (1 H, d, $J_{4″,5″}$ = 3.6 Hz, H-4″), 5.42 (1 H, d, $J_{4‴,5‴}$ = 3.8 Hz, H-4‴), 3.99 (1 H, m, H-5″), 3.93 (1 H, m, H-5‴), 4.21 (2 H, dd, $J_{5″,6a″}$ = $J_{5‴,6a‴}$ = 7.6 Hz, $J_{6a″,6b″}$ = $J_{6a‴,6b‴}$ = 6.4 Hz, H-6a″, 6a‴), 4.16 (2 H, dd, $J_{5″,6b″}$ = $J_{5‴,6b‴}$ = 5.8 Hz, H-6b″, 6b‴), 2.73 (2 H, t, *J* = 4.0 Hz, H-1′), 1.84 (4 H, m, H-2′, 3′), 2.54 (2 H, t, *J* = 4.0 Hz, H-4′), 2.19, 2.18, 2.17, 2.16, 2.12, 2.00, 1.99, 1.94 (24 H, 8 s, 8 × CH_3CO) ppm; δ_C ($CDCl_3$, 100 MHz): 170.36, 170.33, 170.31, 170.27, 170.13, 170.10, 169.87, 169.65 (8 × CH_3CO), 160.44 (-CO), 116.79 (C-3), 148.91 (C-4), 134.35 (C-5), 122.81 (C-6), 145.51 (C-7), 117.87 (C-8), 150.07 (C-9), 118.62 (C-10), 101.45 (C-1″), 100.72 (C-1‴), 69.29 (C-2″), 68.80 (C-2‴), 71.07 (C-3″), 70.62 (C-3‴), 67.01 (C-4″, 4‴), 71.31 (C-5″), 71.24 (C-5‴), 61.25 (C-6″), 61.20 (C-6‴), 25.47 (CH_2-1′), 21.58, 21.36 (CH_2-2′, 3′), 24.06 (CH_2-4′), 20.91, 20.90, 20.76, 20.74, 20.71, 20.69, 20.67, 20.66 (8 × CH_3CO) ppm; ESIMS: *m/z* 893 [M^+] (29%), 894 [M+1] (15%).

Anal. Calcd. for $C_{41}H_{48}O_{22}$ (%): C, 55.16, H, 5.42; Found: C, 55.13, H, 5.38.

6,7-di-*O*-(2′, 3′, 4′, 6′-tetra-*O*-acetyl-β-*D*-galactopyranosyl)-4-methylcoumarin **16** (1.56 g, 73%), R_f 0.35 (1:2 cyclohexane-EtOAc), δ_h ($CDCl_3$, 400 MHz): 6.17 (1 H, s, H-3), 7.30 (1 H, s, H-5), 7.04 (1 H, s, H-8), 5.15 (2 H, d, $J_{1′,2′}$ = $J_{1″,2″}$ = 8.0 Hz, H-1′,1″), 5.41 (2 H, dd, $J_{2′,3′}$ = $J_{2″,3″}$ = 10.4 Hz, H-2′, 2″), 5.10 (2 H, dd, $J_{3′,4′}$ = $J_{3″,4″}$ = 4.0 Hz, H-3′, 3″), 5.37 (2 H, d, $J_{4′,5′}$ = $J_{4′,5′}$ = 3.2 Hz, H-4′, 4″), 4.07 (1 H, m, H-5′), 4.00 (1 H, m, H-5″), 4.15 (2 H, dd, $J_{5′,6a′}$ = $J_{5″,6a″}$ = 5.4 Hz, $J_{6a′,6b′}$ = $J_{6a″,6b″}$ = 110 Hz, H-6a′, 6a″), 4.13 (2 H, dd, $J_{5′,6b′}$ = $J_{5″,6b″}$ = 4.2 Hz, H-6b′, 6b″), 2.34 (3 H, s, CH3-4), 2.14, 2.12, 2.07, 2.04, 2.03, 1.96, 1.95, 1.94 (24 H, 8 s, 8 × CH_3CO) ppm; δ_C ($CDCl_3$, 100 MHz): 170.52, 170.33, 170.25, 170.16, 170.13, 170.10, 169.27, 169.11 (8 × CH_3CO), 160.69 (-CO), 105.43 (C-3),

150.41 (C-4), 132.52 (C-5), 115.27 (C-6), 143.05 (C-7), 114.00 (C-8), 151.74 (C-9), 114.38 (C-10), 100.68 (C-1′), 100.74 (C-1″), 68.53 (C-2′), 68.90 (C-2″), 70.79 (C-3′), 70.85 (C-3″), 67.05 (C-4′, 4″), 71.49 (C-5′), 71.87 (C-5″), 61.41 (C-6′), 61.51 (C-6″), 21.12, 20.86, 20.75, 20.73, 20.70, 20.68, 20.66, 20.64 (8 × CH_3CO), 18.69 (CH_3-4) ppm; ESIMS: *m/z* 853 [M^+] (25%), 854 [M+1] (9%).

Anal. Calcd. for $C_{38}H_{44}O_{22}$ (%): C, 53.52, H, 5.20; Found: C, 53.50, H, 5.18.

7,8-di-*O*-(2′, 3′, 4′, 6′-tetra-*O*-acetyl-β-*D*-galactopyranosyl)-4-methylcoumarin **17** (1.66 g, 77%), Rf 0.30 (1:2 cyclohexane-EtOAc), δ_h ($CDCl_3$, 400 MHz): 6.16 (1 H, s, H-3), 7.25 (1 H, d, *J* = 8.0 Hz, H-5), 7.04 (1 H, d, *J* = 8.0 Hz, H-6), 5.33 (1 H, d, $J_{1',2'}$ = 8.0 Hz, H-1′), 5.21 (1 H, d, $J_{1'',2''}$ = 8.0 Hz, H-1″), 5.42 (1 H, dd, $J_{2',3'}$ = 10.8 Hz, H-2′), 5.38 (1 H, dd, $J_{2'',3''}$ = 10.4 Hz, H-2″), 5.08 (1 H, dd, $J_{3',4'}$ = 4.0 Hz, H-3′), 5.05 (1 H, dd, $J_{3'',4''}$ = 4.0 Hz, H-3″), 5.34 (1 H, d, $J_{4',5'}$ = 2.8 Hz, H-4′), 5.32 (1 H, d, $J_{4'',5''}$ = 3.0 Hz, H-4″), 3.95 (1 H, m, H-5′), 3.90 (1 H, m, H-5″), 4.16 (2 H, dd, $J_{5',6a'} = J_{5'',6a''}$ = 5.6 Hz, $J_{6a',6b'} = J_{6a'',6b''}$ = 11.2 Hz, H-6a′, 6a″), 4.09 (2 H, dd, $J_{5',6b'} = J_{5'',6b''}$ = 4.8 Hz, H-6b′, 6b″), 2.35 (3 H, s, CH_3-4), 2.15, 2.14, 2.12, 2.06, 1.97, 1.96, 1.95, 1.94 (24 H, 8 s, 8 × CH_3CO) ppm; δ_C ($CDCl_3$, 100 MHz): 170.35, 170.31, 170.29, 170.27, 170.26, 170.10, 169.78, 169.56 (8 × CH_3CO), 159.39 (-CO), 113.93 (C-3), 151.33 (C-4), 134.49 (C-5), 120.02 (C-6), 147.17 (C-7), 116.53 (C-8), 152.25 (C-9), 117.53 (C-10), 101.37 (C-1′), 100.55 (C-1″), 69.32 (C-2′), 68.75 (C-2″), 71.04 (C-3′), 70.58 (C-3″), 67.01 (C-4′), 66.92 (C-4″), 71.39 (C-5′), 71.30 (C-5″), 61.23 (C-6′), 61.16 (C-6″), 21.13, 20.88, 20.77, 20.75, 20.71, 20.69, 20.67, 20.65 (8 × CH_3CO), 18.97 (CH_3-4) ppm; ESIMS: *m/z* 853 [M^+] (29%), 854 [M+1] (17%).

Anal. Calcd. for C38H44O22 (%): C, 53.52, H, 5.20; Found: C, 53.49, H, 5.17.

7,8-di-*O*-(2′, 3′, 4′, 6′-tetra-*O*-acetyl-β-*D*-galactopyranosyl)-6-methoxycoumarin **18** (1.91 g, 87%), R_f 0.38 (1:3 cyclohexane-EtOAc), δ_h ($CDCl_3$, 400 MHz): 6.27 (1 H, d, *J* = 8.2 Hz, H-3), 7.54 (1 H, d, *J* = 8.2 Hz, H-4), 6.69 (1 H, s, H-5), 4.08 (1 H, d, $J_{1',2'}$ = 8.0 Hz, H-1′), 4.01 (1 H, d, $J_{1'',2''}$ = 8.0 Hz, H-1″), 5.42 (1 H, dd, $J_{2',3'}$ = 10.1 Hz, H-2′), 5.40 (1 H, dd, $J_{2'',3''}$ = 10.0 Hz, H-2″), 5.04 (1 H, dd, $J_{3',4'}$ = 3.0 Hz, H-3′), 5.01 (1 H, dd, $J_{3'',4''}$ = 3.2 Hz, H-3″), 5.36 (1 H, d, $J_{4',5'}$ = 6.4 Hz, H-4′), 5.32 (1 H, d, $J_{4'',5''}$ = 6.2 Hz, H-4″), 3.89 (1 H, m, H-5′), 3.83 (1 H, m, H-5″), 4.04 (4 H, m, H-6′, 6″), 3.78 (3 H, s, CH_3O-6), 2.11, 2.10, 2.05, 1.96, 1.91, 1.90, 1.88, 1.85 (24 H, 8 s, 8 × CH_3CO) ppm; δ_C (CDCl3, 100 MHz): 170.44, 170.36, 170.26, 170.20, 170.15, 170.08, 169.83, 169.42 (8 × CH_3CO), 159.58 (-CO), 105.61 (C-3), 143.15 (C-4), 141.77 (C-5), 136.64 (C-6), 142.44 (C-7), 115.30 (C-8), 149.75 (C-9), 116.11 (C-10), 105.58 (C-1′), 101.45 (C-1″), 69.48 (C-2′), 69.17 (C-2″), 70.96 (C-3′, 3″), 66.93 (C-4′), 66.88 (C-4″), 71.20 (C-5′), 71.10 (C-5″), 60.93 (C-6′), 60.88 (C-6″), 56.74 (CH_3O-6), 21.07, 20.86, 20.84, 20.80, 20.65, 20.62, 20.60, 20.58 (8 × CH_3CO) ppm; ESIMS: *m/z* 869 [M^+] (27%), 870 [M+1] (18%).

Anal. Calcd. for $C_{38}H_{44}O_{23}$ (%): C, 52.54, H, 5.11; Found: C, 52.50, H, 5.08.

Free di-β-*D*-galactopyranosides 19~23

Di-*O*-(2′, 3′, 4′, 6-tetra-*O*-acetyl-β-*D*-galactopyranosyl) coumarins **14**~**18** (1.00 g) were deacetylated as described above for free mono β-*D*-galactopyranosides **10**~**13** to afford the corresponding free di-β-*D*-galactopyranosides **19**~**23** in high yields.

6,7-di-*O*-(β-*D*-galactopyranosyl)-3, 4-cyclohexenoesculetin **19** (585.93 mg, 94%), R_f 0.29 (1:3 MeOH-EtOAc), δ_H ([D_6]DMSO, 400 MHz): 7.40 (1 H, s, H-5), 7.14 (1 H, s, H-8), 5.29 (1 H, br, HO-2″, 2‴, exchangeable with D_2O), 4.67 (2 H, br, HO-3″, 3‴, exchangeable with D_2O), 5.00 (2 H, br, HO-4″, 4‴, exchangeable with D_2O), 4.70 (2 H, br, HO-6″, 6‴, exchangeable with D_2O), 4.82 (1 H, d, $J_{1'',2''}$ = 8.0 Hz, H-1″), 4.91 (1 H, d, $J_{1''',2'''}$ = 8.0 Hz,

H-1‴), 3.68 (2 H, dd, $J_{2″,3″} = J_{2‴,3‴}$ = 10.0 Hz, H-2″, 2‴), 3.65 (2 H, dd, $J_{3″,4″} = J_{3‴,4‴}$ = 4.0 Hz, H-3″, 3‴), 3.47 (2 H, d, $J_{4″,5″} = J_{4‴,5‴}$ = 3.4 Hz, H-4″, 4‴), 3.52 (2 H, m, H-5″, 5‴), 3.72 (2 H, m, H-6″, 6‴), 2.74 (2 H, t, J = 4.0 Hz, H-1′), 1.75 (4 H, m, H-2′, 3′), 2.39 (2 H, t, J = 4.0 Hz, H-4′) ppm; δ_C ([D$_6$]DMSO, 100 MHz): 161.30 (-CO), 103.82 (C-3), 147.74 (C-4), 143.49 (C-5), 120.55 (C-6), 147.23 (C-7), 103.92 (C-8), 149.28 (C-9), 113.66 (C-10), 101.23 (C-1″), 102.05 (C-1‴), 70.05 (C-2″), 70.26 (C-2‴), 72.76 (C-3″), 72.94 (C-3‴), 67.99 (C-4″), 68.17 (C-4‴), 75.61 (C-5″), 75.70 (C-5‴), 60.34 (C-6″), 60.54 (C-6‴), 24.60 (CH$_2$-1′), 21.14, 20.84 (CH$_2$-2′, 3′), 23.58 (CH$_2$-4′) ppm; ESIMS: *m/z* 557 [M$^+$] (7%), 558 [M+1] (10%).

Anal. Calcd. for $C_{25}H_{32}O_{14}$ (%): C, 53.96, H, 5.80; Found: C, 53.94, H, 5.76.

7,8-di-*O*-(β-*D*-galactopyranosyl)-3, 4-cyclohexenocoumarin **20** (592.16 mg, 95%), R_f 0.31 (1:3 MeOH-EtOAc), δ_H ([D$_6$]DMSO, 400 MHz): 7.43 (1 H, d, J = 8.0 Hz, H-5), 7.26 (1 H, d, J = 8.0 Hz, H-6), 5.43 (2 H, br, HO-2″, 2‴, exchangeable with D$_2$O), 4.47 (2 H, br, HO-3″, 3‴, exchangeable with D$_2$O), 4.95 (2 H, br, HO-4″, 4‴, exchangeable with D$_2$O), 4.63 (2 H, br, HO-6″, 6‴, exchangeable with D$_2$O), 5.08 (1 H, d, $J_{1″,2″}$ = 8.0 Hz, H-1″), 4.82 (1 H, d, $J_{1‴,2‴}$ = 8.0 Hz, H-1‴), 3.72 (2 H, dd, $J_{2″,3″} = J_{2‴,3‴}$ = 10.2 Hz, H-2″, 2‴), 3.45 (2 H, dd, $J_{3″,4″} = J_{3‴,4‴}$ = 4.0 Hz, H-3″, 3‴), 3.39 (2 H, d, $J_{4″,5″} = J_{4‴,5‴}$ = 3.6 Hz, H-4″, 4‴), 3.35 (2 H, m, H-5″, 5‴), 3.53 (4 H, m, H-6″, 6‴), 2.77 (2 H, t, J = 4.0 Hz, H-1′), 1.78 (4 H, m, H-2′, 3′), 2.43 (2 H, t, J = 4.0 Hz, H-4′) ppm; δ_C ([D$_6$]DMSO, 100 MHz): 160.51 (-CO), 112.98 (C-3), 147.40 (C-4), 132.95 (C-5), 120.66 (C-6), 145.51 (C-7), 115.42 (C-8), 151.56 (C-9), 118.94 (C-10), 103.85 (C-1″), 102.95 (C-1‴), 71.37 (C-2″), 70.66 (C-2‴), 73.28 (C-3″), 72.60 (C-3‴), 68.15 (C-4″), 68.05 (C-4‴), 75.94 (C-5″), 75.77 (C-5‴), 60.46 (C-6″), 60.20 (C-6‴), 24.76 (CH$_2$-1′), 21.13, 20.83 (CH$_2$-2′, 3′), 23.61 (CH$_2$-4′) ppm; ESIMS: *m/z* 557 [M$^+$] (9%), 558 [M+1] (13%).

Anal. Calcd. for $C_{25}H_{32}O_{14}$ (%): C, 53.96, H, 5.80; Found: C, 53.92, H, 5.77.

6,7-di-*O*-(β-*D*-galactopyranosyl)-4-methylcoumarin **21** (545.07 mg, 90%), R_f 0.31 (1:3 MeOH-EtOAc), δ_H ([D$_6$]DMSO, 400 MHz): 6.24 (1 H, s, H-3), 7.45 (1 H, s, H-5), 7.16 (1 H, s, H-8), 5.10 (1 H, d, $J_{H\text{-}2′,OH\text{-}2′}$ = 4.0 Hz, HO-2′, exchangeable with D$_2$O), 5.12 (1 H, d, $J_{H\text{-}2″,OH\text{-}2″}$ = 4.0 Hz, HO-2″, exchangeable with D$_2$O), 4.54 (1 H, d, $J_{H\text{-}3′,OH\text{-}3′}$ = 4.0 Hz, HO-3′, exchangeable with D$_2$O), 4.58 (1 H, d, $J_{H\text{-}3″,OH\text{-}3″}$ = 4.0 Hz, HO-3″, exchangeable with D$_2$O), 4.88 (1 H, d, $J_{H\text{-}4′,OH\text{-}4′}$ = 4.0 Hz, HO-4′, exchangeable with D$_2$O), 4.90 (1 H, d, $J_{H\text{-}4″,OH\text{-}4″}$ = 4.0 Hz, HO-4″, exchangeable with D$_2$O), 4.69 (1 H, d, $J_{H\text{-}6a′,OH\text{-}6′}$ = 4.0 Hz, $J_{H\text{-}6b′,OH\text{-}6′}$ = 6.1 Hz, HO-6′, exchangeable with D$_2$O), 4.71 (1 H, d, $J_{H\text{-}6a″,OH\text{-}6″}$ = 4.0 Hz, $J_{H\text{-}6b″,OH\text{-}6″}$ = 6.0 Hz, HO-6″, exchangeable with D$_2$O), 4.85 (1 H, d, $J_{1′,2′}$ = 8.0 Hz, H-1′), 4.97 (1 H, d, $J_{1″,2″}$ = 8.0 Hz, H-1″), 3.68 (2 H, dd, $J_{2′,3′} = J_{2″,3″}$ = 10.1 Hz, H-2′, 2″), 3.71 (2 H, dd, $J_{3′,4′} = J_{3″,4″}$ = 4.0 Hz, H-3′, 3″), 3.53 (2 H, d, $J_{4′,5′} = J_{4″,5″}$ = 3.6 Hz, H-4′, 4″), 3.46 (2 H, m, H-5′, 5″), 3.58 (4 H, m, H-6′, 6″), 2.37 (3 H, s, CH$_3$-4) ppm; δ_C ([D$_6$]DMSO, 100 MHz): 160.24 (-CO), 104.25 (C-3), 150.70 (C-4), 143.65 (C-5), 113.51 (C-6), 149.09 (C-7), 112.04 (C-8), 153.25 (C-9), 113.04 (C-10), 101.30 (C-1′), 102.24 (C-1″), 70.24 (C-2′), 70.40 (C-2″), 73.11 (C-3′), 73.33 (C-3″), 68.18 (C-4′), 68.35 (C-4″), 75.78 (C-5′), 75.92 (C-5″), 60.49 (C-6′), 60.65 (C-6″), 18.13 (CH$_3$-4) ppm; ESIMS: *m/z* 517 [M$^+$] (5%), 518 [M+1] (9%).

Anal. Calcd. for $C_{22}H_{28}O_{14}$ (%): C, 51.16, H, 5.47; Found: C, 51.12, H, 5.44.

7,8-di-*O*-(β-*D*-galactopyranosyl)-4-methylcoumarin **22** (563.24 mg, 93%), R_f 0.26 (1:3 MeOH-EtOAc), δ_H ([D$_6$]DMSO, 400 MHz): 6.27 (1 H, s, H-3), 7.48 (1 H, d, J = 8.0 Hz, H-5), 7.26 (1 H, d, J = 8.0 Hz, H-6), 5.36 (1 H, d, $J_{H\text{-}2′,OH\text{-}2′}$ = 4.0 Hz, HO-2′, exchangeable with D$_2$O), 4.94 (1 H, d, $J_{H\text{-}2″,OH\text{-}2″}$ = 4.0 Hz, HO-2″, exchangeable with D$_2$O), 4.60 (1 H,

d, $J_{H\text{-}3',OH\text{-}3'}$ = 4.0 Hz, HO-3′, exchangeable with D_2O), 4.58 (1 H, d, $J_{H\text{-}3'',OH\text{-}3''}$ = 4.0 Hz, HO-3″, exchangeable with D_2O), 4.91 (1 H, d, $J_{H\text{-}4',OH\text{-}4'}$ = 4.0 Hz, HO-4′, exchangeable with D_2O), 4.89 (1 H, d, $J_{H\text{-}4'',OH\text{-}4''}$ = 4.0 Hz, HO-4″, exchangeable with D_2O), 4.73 (1 H, d, $J_{H\text{-}6a',OH\text{-}6'}$ = 4.0 Hz, $J_{H\text{-}6b',OH\text{-}6'}$ = 6.2 Hz, HO-6′, exchangeable with D_2O), 4.43 (1 H, d, $J_{H\text{-}6a'',OH\text{-}6''}$ = 4.0 Hz, $J_{H\text{-}6b'',OH\text{-}6''}$ = 6.6 Hz, HO-6″, exchangeable with D_2O), 5.07 (1 H, d, $J_{1',2'}$ = 8.0 Hz, H-1′), 4.83 (1 H, d, $J_{1'',2''}$ = 8.0 Hz, H-1″), 3.70 (2 H, dd, $J_{2',3'}$ = $J_{2'',3''}$ = 10.0 Hz, H-2′, 2″), 3.44 (2 H, dd, $J_{3',4'}$ = $J_{3'',4''}$ = 4.2 Hz, H-3′, 3″), 3.76 (2 H, d, $J_{4',5'}$ = $J_{4'',5''}$ = 3.8 Hz, H-4′, 4″), 3.36 (2 H, m, H-5′, 5″), 3.51 (4 H, m, H-6′, 6″), 2.40 (3 H, s, CH_3-4) ppm; δ_C ([D_6]DMSO, 100 MHz): 159.72 (-CO), 112.29 (C-3), 152.63 (C-4), 133.08 (C-5), 120.48 (C-6), 147.12 (C-7), 112.98 (C-8), 153.32 (C-9), 115.39 (C-10), 103.82 (C-1′), 102.72 (C-1″), 71.38 (C-2′), 70.64 (C-2″), 73.26 (C-3′), 72.61 (C-3″), 68.16 (C-4′), 68.02 (C-4″), 75.97 (C-5′), 75.76 (C-5″), 60.45 (C-6′), 60.19 (C-6″), 18.23 (CH_3-4) ppm; ESIMS: *m/z* 517 [M^+] (7%), 518 [M+1] (15%).

Anal. Calcd. for $C_{22}H_{28}O_{14}$ (%): C, 51.16, H, 5.47; Found: C, 51.13, H, 5.43.

7,8-di-*O*-(β-*D*-galactopyranosyl)-6-methoxycoumarin **23** (706.17 mg, 97%), R_f 0.35 (1:3 MeOH-EtOAc), δ_H ([D_6]DMSO, 400 MHz): 6.41 (1 H, d, *J* = 8.0 Hz, H-3), 7.96 (1 H, d, *J* = 8.0 Hz, H-4), 7.15 (1 H, s, H-5), 5.36 (1 H, br, HO-2′, exchangeable with D_2O), 5.32 (1 H, br, HO-2″, exchangeable with D_2O), 4.46 (2 H, br, HO-3′, 3″, exchangeable with D_2O), 4.88 (2 H, br, HO-4′, 4″, exchangeable with D_2O), 4.57 (2 H, br, HO-6′, 6″, exchangeable with D_2O), 5.22 (1H, d, $J_{1',2}$ = 8.0 Hz, H-1′), 5.26 (1 H, d, $J_{1'',2''}$ = 8.0 Hz, H-1″), 3.72 (2 H, dd, $J_{2',3'}$ = $J_{2'',3''}$ = 10.0 Hz, H-2′, 2″), 3.57 (2 H, dd, $J_{3',4'}$ = $J_{3'',4''}$ = 4.0 Hz, H-3′, 3″), 3.40 (2 H, d, $J_{4',5'}$ = $J_{4'',5''}$ = 3.8 Hz, H-4′, 4″), 3.46 (2 H, m, H-5′, 5″), 3.75 (4 H, m, H-6′, 6″), 3.83 (3 H, s, CH_3O-6) ppm; δ_C ([D_6]DMSO, 100 MHz): 159.94 (-CO), 105.99 (C-3), 144.23 (C-4), 141.58 (C-5), 136.52 (C-6), 142.56 (C-7), 114.38 (C-8), 149.89 (C-9), 114.83 (C-10), 103.33 (C-1′), 103.23 (C-1″), 71.31 (C-2′), 71.26 (C-2″), 73.26 (C-3′), 73.18 (C-3″), 68.06 (C-4′, 4″), 75.88 (C-5′), 75.82 (C-5″), 60.09 (C-6′, 6″), 56.65 (CH3O-6) ppm; ESIMS: *m/z* 533 [M^+] (8%), 534 [M+1] (16%).

Anal. Calcd. for $C_{22}H_{28}O_{15}$ (%): C, 49.63, H, 5.30; Found: C, 49.59, H, 5.26.

Preparation of stable *lacZ* transfected MCF7 and PC3 cell lines

E.coli lacZ—gene (from pSV-β-gal vector, Promega, Madison, WI) was inserted into high expression human cytomegalovirus (CMV) immediate-early enhancer/promoter vector phCMV (Gene Therapy Systems, San Diego, CA) giving a recombinant vector phCMV/lacZ. This was used to transfect wild type MCF7 (human breast cancer) and PC3 (human prostate cancer) cells (ATCC, Manassas, VA) using GenePORTER2 (Gene Therapy Systems, Genlantis, Inc., San Diego, CA), as described in detail previously [24, 25]. The highest β-gal expressing colony was selected using the antibiotic G418 disulfate (Research Products International Corp, Mt Prospect, IL, USA); 800 μg/ml) and G418 (200 μg/ml) was also included for routine culture. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Mediatech Inc., Herndon, VA, USA) containing 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA) with 100 units/ml of penicillin, 100 units/ml streptomycin, and cultured in a humidified 5% CO_2 incubator at 37°C. The β-gal activity of tumor cells was measured using a β-gal assay kit with *o*-nitrophenyl-β-*D*-galactopyranoside (Promega, Madison, WI).

MRI

MRI studies were performed using a 4.7 T horizontal bore magnet equipped with a Varian INOVA Unity system (Palo Alto, CA, USA). T_1 and T_2 maps were acquired using a spin-echo sequence with varying repetition times (TR) and echo times (TE), respectively.

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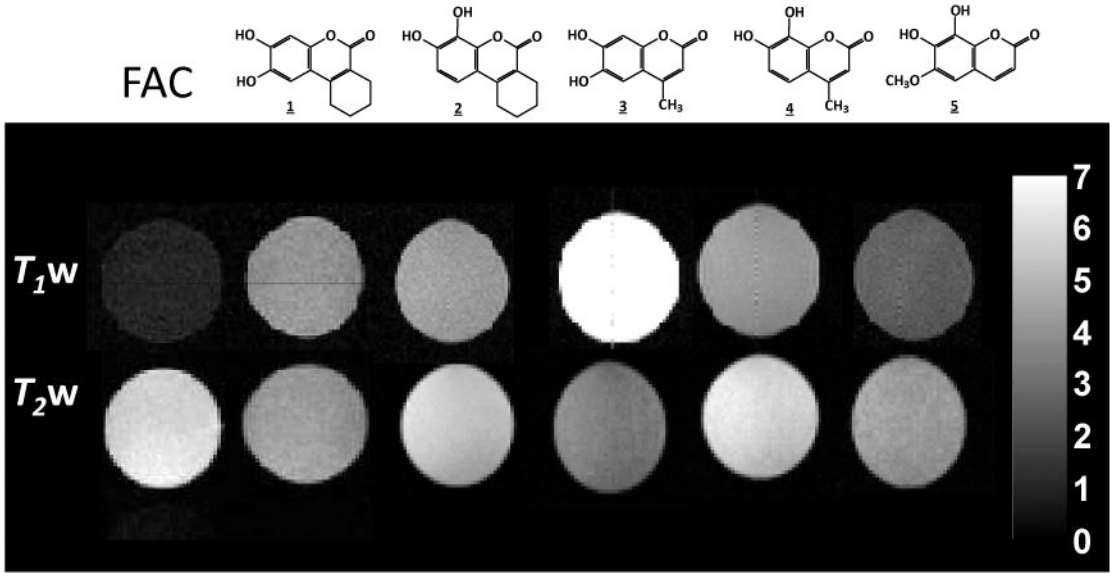

Figure 1. Comparison of MRI Contrast for aglycone ligands 1-5 with Fe^{3+}

200 MHz ^{1}H MRI of vials of ferric ammonium citrate (FAC) (3.0 mM) in PBS/DMSO (V/V ′ 1:1) alone (leftmost) or mixed with ligands shown above (**1**-**5**; 9.0 mM). Upper row of images: T_1-weighted ^{1}H MRI with TR = 300 ms, TE = 20 ms, 1.5 mm slice with, 128 × 128 resolution over 50 × 50 mm^2. Lower row Corresponding T_2-weighted ^{1}H MRI with TR = 2000 ms, TE= 80 ms.

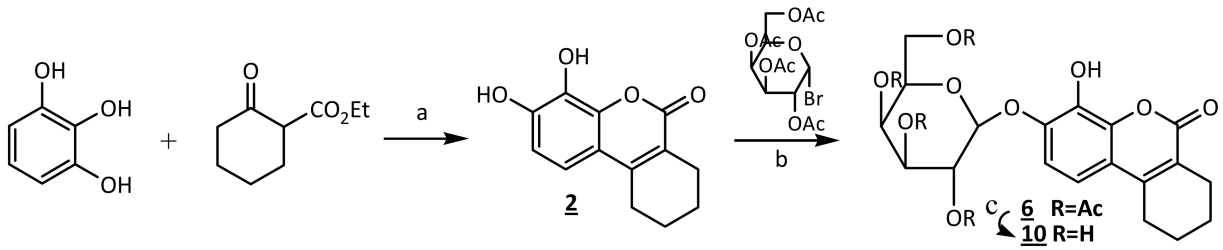

Figure 2. General reaction scheme

(a) pyrogallol (5 mmol), ethyl cyclohexanone-2-carboxylate (5 mmol), $ZrCl_4$ (0.5 mmol), toluene (20 mL), 80 °C, N_2, 20 min, 93%(→**2**); (b) 2, 3, 4, 6-tetra-*O*-acetyl-α-*D*-galactopyranosyl bromide (2.5 mmol), **2** (2.5 mmol), TBAB (0.5 mmol), CH_2Cl_2-H_2O (60 mL), pH 8~9, rt °C, N2, 3~4 hr, 88%(→**6**); (c) 0.5M NH3-MeOH, 0°C→r.t., 24 hr, quantitative yields.

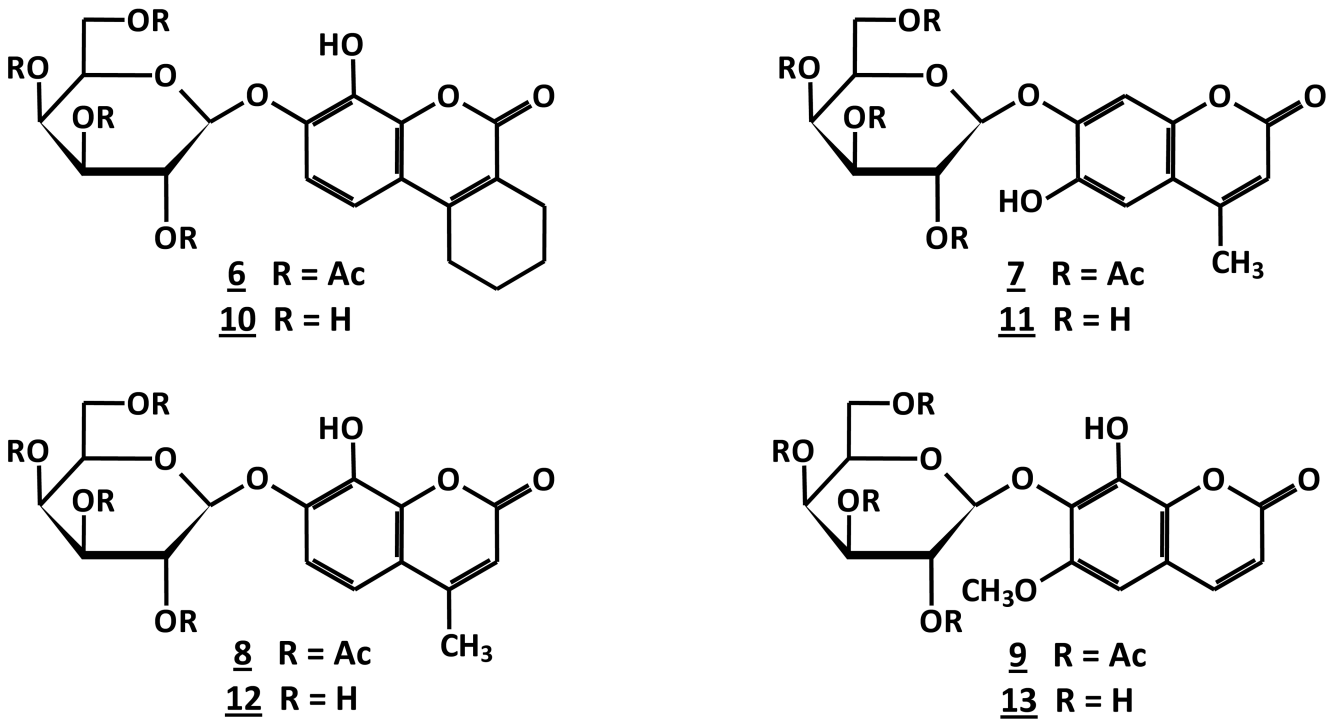

Figure 3. Mono β-*D*-galactopyranosides 6-13

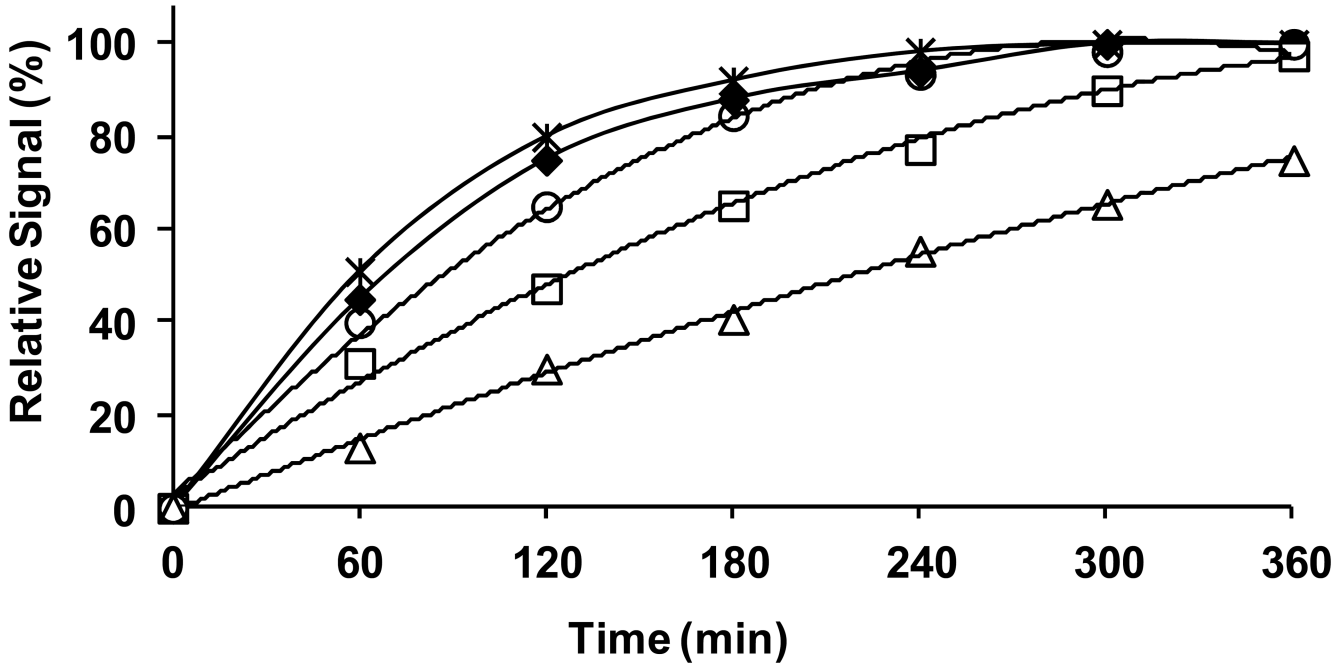

Figure 4. The kinetic hydrolysis time courses of mono β-*D*-galactopyranosides

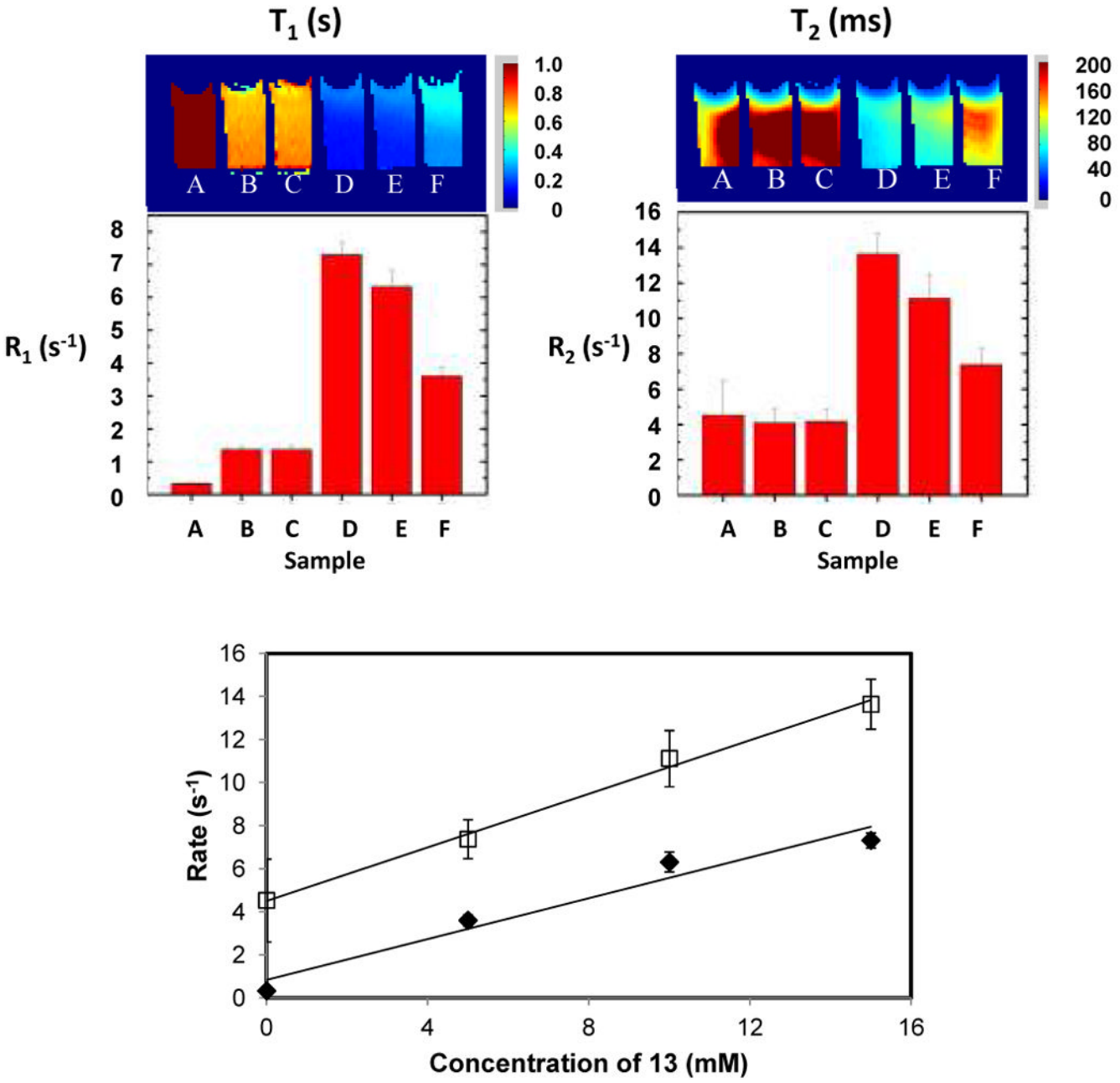

Figure 5. T_1 and T_2 response to β-gal

The T_1 (**a**) and T_2 (**b**) maps of solutions of various concentrations of mono β-*D*-galactopyranoside **13** in PBS (0.1M, pH=7.4) in the presence of ferric ammonium citrate (FAC; 5mM) together with bar charts for corresponding R_1 and R_2 values. β-gal (E801A, 5 units) was added to D-F. (A) **13** (15mM) alone in PBS; (B) FAC (5mM) alone in H_2O; (C) **13** (15mM) plus FAC (5mM) in PBS; (D) **13** (15mM), FAC (5mM) and β-gal in PBS; (E) **13** (10mM), FAC (5mM) and β-gal in PBS; (F) **13** (5mM), FAC (5mM) and β-gal in PBS. ^{1}H MRI at 200 MHz. C) Dependence of relaxation rates R_1 (♦) and R_2 (□) on concentration of **13** for constant β-gal (E801A, 5 units) and FAC (5mM) in PBS (0.1M, pH=7.4).

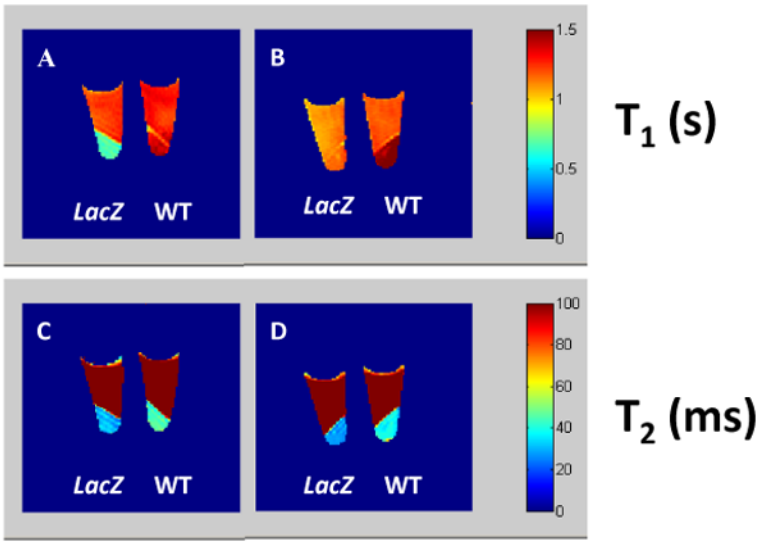

Figure 6. T_1 and T_2 effects due to *lacZ* transfected cells

The T_1 and T_2 maps of mono β-*D*-galactopyranoside **13** (15mM) and FAC (5mM) in PBS (0.1M, pH=7.4, 200 μ;L) incubated at 37 °C under 5% CO_2 in air with 95% humidity for 30 min. T_1 maps: (**A**) MCF7-*lacZ* and MCF7-WT: 4×10^6 cells each; (**B**) PC3-*lacZ* and PC3-WT: 4×10^6 cells each; corresponding T_2 maps (**C,D**). MRI parameters: 200 MHz, matrix=128 × 128, FOV=40 × 40, 2 mm slice

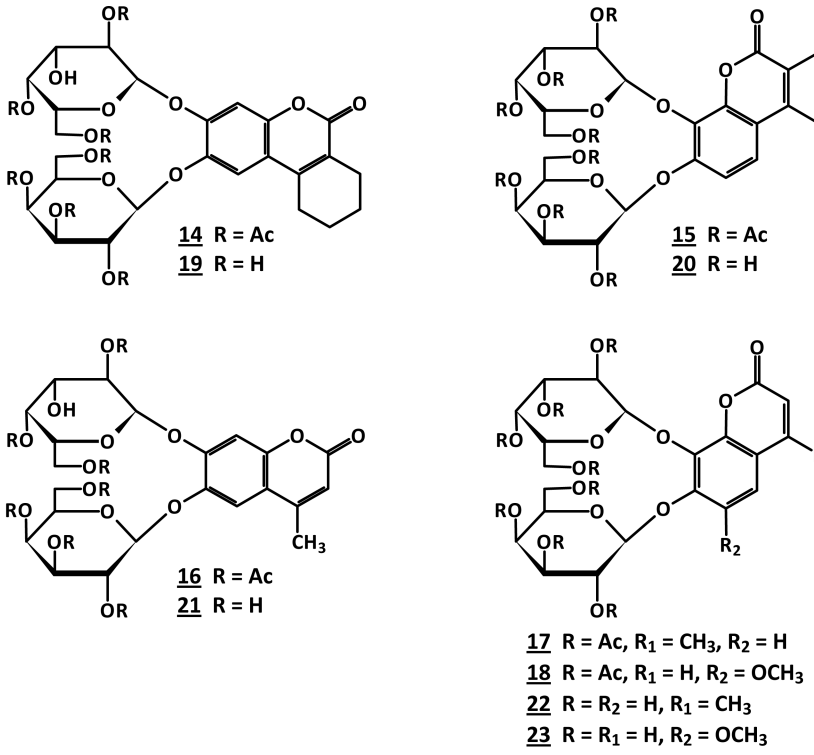

Figure 7. The structures of di β-*D*-galactopyranosides 14-23

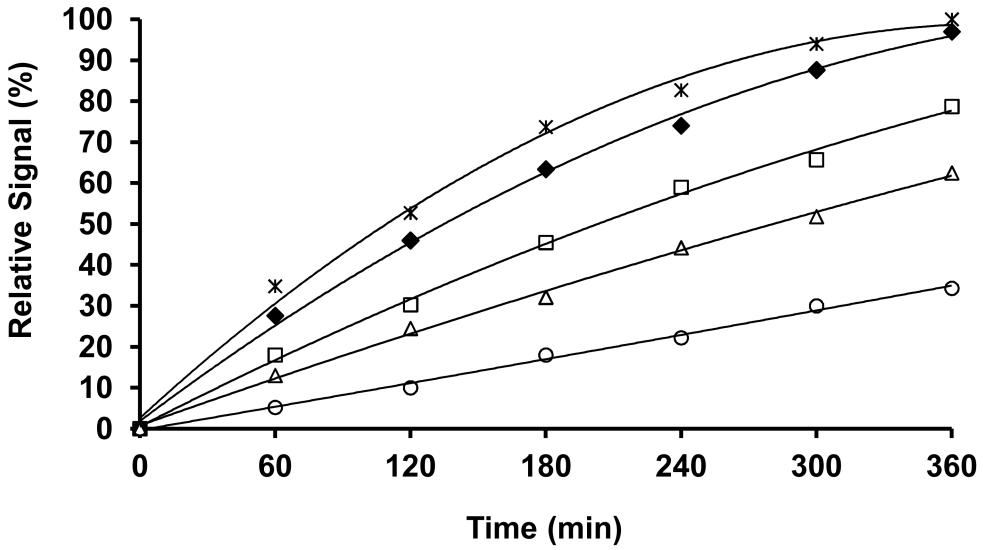

Figure 8. The kinetichydrolysis time courses of di-β-D-galactopyranosides

Evolution of fluorescence (365/440 nm) following addition of β-gal (10 units, E801A) to solutions of **19-23** (10mM) in PBS (1.0mL, 0.1M, pH=7.4) at 20-22°C showing release of the corresponding aglycones **19→1(*), 20→2(□), 21→3(♦), 22→4(Δ), 23→5(○)**.

Table 1

The hydroxyl *pKa* values of coumarins 1-5

Coumarins	$pKa_{(OH\text{-}7)}$	$pKa_{(OH\text{-}6)}$	$pKa_{(OH\text{-}8)}$
3,4-cyclohexenoesculetin **1**	11.84	8.74	---
7,8-dihydroxy-3,4-cyclohexenocoumarin **2**	11.48	---	7.95
6,7-dihydroxy-4-methylcoumarin **3** *	10.28	8.52	---
7,8-dihydroxy-4-methylcoumarin **4** *	10.35	---	8.00
7,8-dihydroxy-6-methoxycoumarin **5**	10.49	---	7.22

*Values from [36], while others were calculated using Advanced Chemistry Development Software (www.acdlabs.com).