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Short communication

Simple and convenient synthesis of a fluorinated GM4 analogue

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Abstract

A series of fluorinated galactosides, dodecyl 2-deoxy-2-fluoro-β-D-galactopyranoside (2F Gal), dodecyl 4-deoxy-4-fluoro-β-D-galactopyranoside (4F Gal) and dodecyl 6-deoxy-6-fluoro-β-D-galactopyranoside (6F Gal), was chemically synthesized and introduced to B16 cells to serve as scaffolds for cellular enzyme glycosylation. Results showed that the presence of fluorine exercised significant effects on cell viability. Among the fluorinated galactosides used, 2F Gal was glycosylated to afford a GM4 analogue.

Keywords: Sialylation; Galactoside primer; Fluorinated saccharide; GM4 analogue

1. Introduction

Structurally, GM4 (NeuAcα2 → 3Galβ1 → Cer) is the simplest ganglioside. Kuhn and Weigandt first revealed GM4 as a minor ganglioside of human brain [1]. The occurrence of this ganglioside has also been detected in chicken cerebellum, mouse erythrocytes, rat kidney and frog liver. GM4 exhibits interesting biological activities—marked immunosuppressive activity in vitro and ability to prevent experimental encephalomyelitis in guinea pigs [2], among others. The biological significance but very low availability of GM4 from natural sources propels the design and synthesis of GM4 and its derivatives. One of the most challenging synthetic considerations involves the regio- and stereoselective sialic acid incorporation.

Facile synthesis of ganglioside analogues could be achieved by biocombinatorial method [3]. Amphiphilic saccharide primers that resemble intermediates in the biosynthetic pathway could be recognized by cellular enzymes and used as substrates for glycosylation [4]. As we described recently, incorporation of dodecyl β-D-galactoside primer to B16 cells resulted to monosialylation of the galactoside primer [5]. This finding initiated experiments to determine the effect of substituting the hydroxyl group of glycoside units and to establish the requirements for efficient cellular glycosylation of saccharide primers. In this research, the replacement of a hydroxyl group by fluorine atom in the galactose residue was pursued to establish the effect in cellular uptake and glycosylation. Fluorinated galactosides with dodecyl aglycon (2F Gal, 4F Gal and 6F Gal) were chemically synthesized and applied to prime oligosaccharide synthesis via cellular enzymatic glycosylation.

2. Results and discussion

The chemical synthesis of the fluorinated galactosides required multi-step sequences. As shown in Scheme 1, modification of the starting material (**1**) gave a protected galactal [6–7] derivative (**2**) that was fluorinated and transformed to a trichloroacetimidate derivative (**3**). Subsequent glycosylation using dodecyl alcohol with *t*-butyldimethylsilyl triflate in dichloromethane as solvent, followed by deacylation afforded dodecyl 2-deoxy-2-fluoro-β-D-galactopyranoside, 2F Gal (**4**). On the other hand, a 4,6-benzylidene derivative [8–10] proved to be a useful intermediate for the synthesis of 4F Gal and 6F Gal primers. Glycosylation [5] of dodecyl alcohol with peracetyl glucose (**5**) in the presence of a Lewis acid followed by benzilidenation and benzylation afforded (**6**) as shown in Scheme 2. Reduction of the benzylidene group of (**6**) followed by fluorination gave compound (**7**) which was debenzylated. However, difficulty of purification prompted further acetylation and finally, deprotected to afford dodecyl 4-deoxy-4-fluoro-β-D-galactopyranoside, 4F Gal (**8**). Similarly, 6F Gal was prepared

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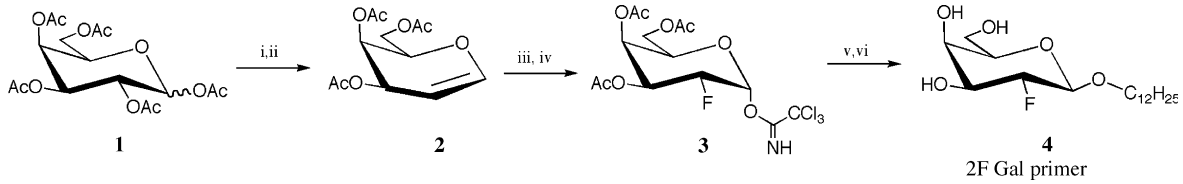
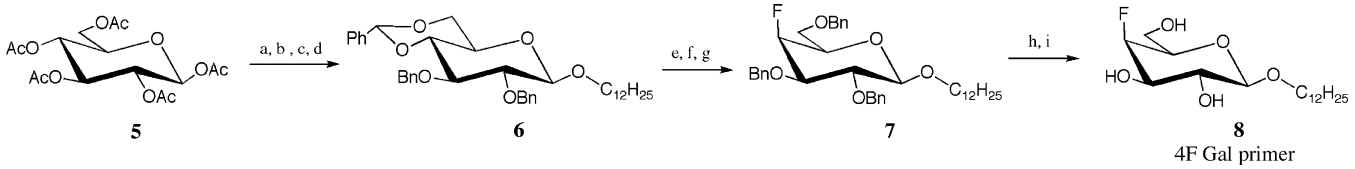


Scheme 1. Chemical synthesis of 2F Gal primer. (i) HBr–AcOH, CH_2Cl_2; (ii) $CuSO_4$ Zn dust, AcOH (71%, 2 steps); (iii) Selectfluor™, MeCN, H_2O (32%); (iv) CCl_3CN, K_2CO_3, CH_2Cl_2; (v) $C_{12}H_{25}OH$, TBSOTf, CH_2Cl_2 (36%, 2 steps); (vi) MeONa, MeOH (89%).



Scheme 2. Chemical synthesis of 4F and 6F Gal primers. (a) $C_{12}H_{25}OH$, $ClCH_2CH_2Cl$, $BF_3{\cdot}Et_2O$ (51%); (b) NaOMe, MeOH (100%); (c) $PhCH(OMe)_2$, DMF, CSA; (d) BnBr, NaH, DMF (76%, 2 steps); (e) TFA, Et_3SiH, CH_2Cl_2 (87%); (f) Tf_2O, CH_2Cl_2, pyridine; (g) TBAF, THF (35% 2 steps); (h) Pd/C, H_2 then Ac_2O, pyridine (47%, 2 steps); (i) NaOMe, MeOH (93%); (j) 80% AcOH (aq) (87%); (k) DAST, CH_2Cl_2 (30%); (l) Pd/C, H_2 (16%).

from peracetyl galactose (**9**) as shown in Scheme 2. Treatment of the benzylidene derivative (**10**) with acetic acid followed by fluorination gave compound **11** which was debenzylated to afford dodecyl 6-deoxy-6-fluoro-β-D-galactopyranoside, 6F Gal (**12**). Fluorine was introduced at the 2, 4 and 6 positions of the galactose unit by treatment of corresponding galactosyl derivative with Selectfluor™, tetrabutylammonium fluoride (TBAF) and *N*,*N*-diethylamino-sulfur trifluoride (DAST), respectively [11–15]. The chemical synthetic methods for the synthesis of all the primers were carried out according to the references cited therein. The structures of the synthesized compounds were confirmed from the ^1H and ^{13}C NMR results. The ^{13}C NMR spectra of the fluorinated galactoside primers in comparison with the dodecyl β-D-galactoside are shown in Fig. 1.

The primers were administered to mouse melanoma B16 cells to verify their potential as scaffolds for oligosaccharide synthesis using cells. Cell culture, incubation of cells with primer and lipid extraction from cell and medium fractions were carried out as described previously [3,5]. After the incubation of B16 cells with 50 μM of the primers for 48 h, lipids from the culture media and from the cell homogenate were collected and were extracted. Among the three kinds of primers, 2F Gal did not affect cell morphology and viability (Fig. 2). In contrast, 4F Gal primer exhibited slight toxicity to cells and 6F Gal primer was cytotoxic. The HPTLC results of the lipids confirmed that the primers were taken-up by the cells as shown in Fig. 3. However, only the 2F Gal was glycosylated and the elongated product released to the culture medium. The structure of the glycosylated product was determined. The MALDI TOF mass spectral results obtained suggested a sialylated galactoside primer. Treatment of the elongated 2F Gal with 2,3-sialidase (cloned from *S. typhimurium* LT2 and expressed in *E. coli*) confirmed the structure of the glycosylation product, a GM4 analogue. From the results, we could infer that 2F Gal diffused through the cell membrane to the Golgi where the primer functioned as acceptor substrate for the sialyl transferase.

In a related work, we reported that a fluorous-tagged lactoside primer (6-(perfluorohexyl)hexyl 4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranoside) could be taken in by B16 melanoma cells, the saccharide chain elongated by cellular enzymes to afford a GM3-type oligosaccharide, and the

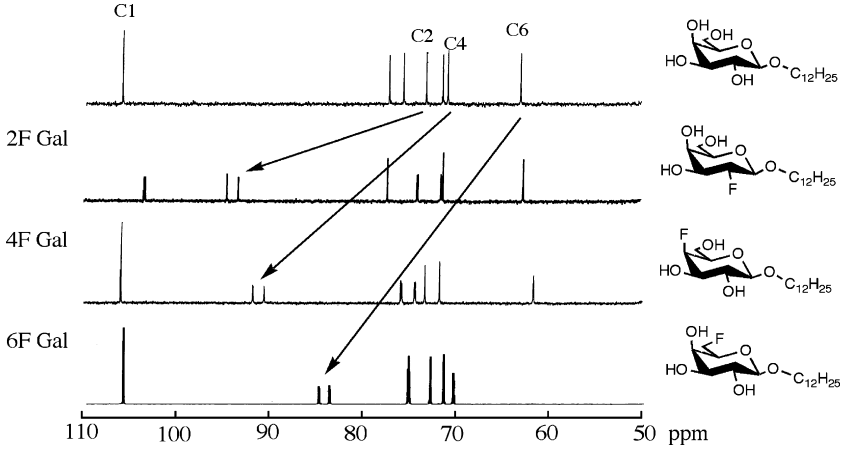


Fig. 1. ^{13}C NMR spectra of dodecyl β-D-galactopyranoside and fluorinated galactoside primers in MeOH-d_4.

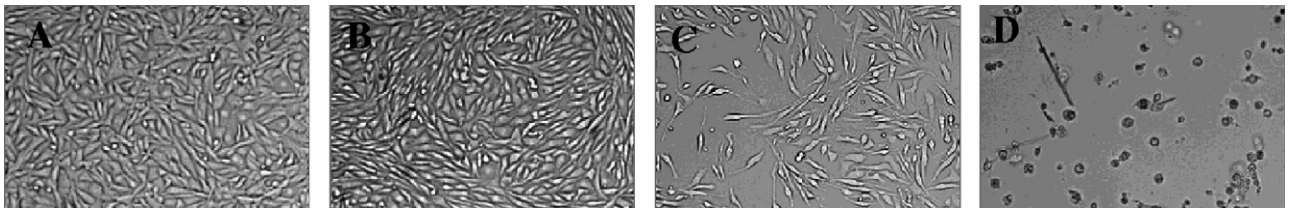


Fig. 2. Effect on B16 cells after 48-h incubation in the (A) absence, or in the presence of primer (B) 2F Gal; (C) 4F Gal; (D) 6F Gal.

elongated product released by the cells to the culture medium [16]. The fluorous tag did not exert a steric influence to cellular uptake neither did the numerous fluorine at the aglycon exhibit cytotoxicity. To take advantage of the positive effect of the fluorine substituent to the cellular enzyme glycosylation of primers, the hydroxyl group of the galactose residue was replaced by fluorine. Relative to the hydroxyl group, the size of the fluorine substituent does not dramatically influence to create a steric hindrance to sialylation by the enzyme. Hence, replacement of one hydroxyl unit by a fluorine atom is expected to have only the hydrophobic effect on the conformation of the galactose residue. Moreover, it was assumed that the presence of only one fluorine atom in the saccharide unit would have no adverse effects on the cells. However, results showed otherwise. Interestingly, replacement of one hydroxyl unit by a fluorine atom in different positions of the galactose residue elicited different cellular responses. Modification at 2 position of the galactose residue did not have adverse effects to the cell and 2F Gal was glycosylated. Surprisingly, modification of 4 and 6 positions slightly, or greatly, affected cell viability. Consequently, saccharide elongation of 4F and 6F Gal primers could not possibly take place.

The differences in cellular response could be addressed considering not only the conformation of primers but also cellular enzyme specificity [13]. Even if it has been reported that sialyl transferases are amenable to substrate modifications, primers should be able to cross the plasma membrane, reach the Golgi where the enzymes reside that is the site of glycosylation. All three primers satisfy the requirement for cellular enzyme

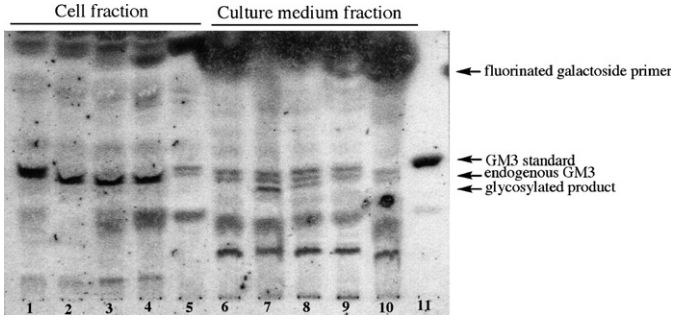


Fig. 3. B16 melanoma cells (1×10^6, 100 mm dish) were incubated for 48 h in serum free 1:1 DMEM-F12 supplemented with transferrin and insulin (TI/DF) in the absence or presence of 50 μM galactoside primer. Lipids were extracted from the cell and culture medium fractions and separated by HPTLC. Lanes 1 and 6: *Control* (Me_2SO only in TI-DF) for the cell and culture medium fractions, respectively; lanes 2–5 and 7–10: *Treated* (50 μM galactoside primer) for the cell and culture medium fractions, respectively; lanes 2 and 7, dodecyl galactoside primer; lanes 3 and 8: 2F Gal primer; lanes 4 and 9: 4F Gal primer; lanes 5 and 10: 6F Gal primer; lane 11: GM3 standard.

glycosylation, a terminal galactose residue [5]. However, the type and position of substituent at the vicinity of the glycosylation site (C3 position) and their positive effect on cell viability are prerequisites for cellular enzyme glycosylation and substrate recognition by 2,3-sialyl transferase. Although it may be too hasty to draw a conclusion and explain the phenomenon based on present results, the position of the substituent should also be a significant consideration for oligosaccharide synthesis using primers and cells. Whether or not other types of cells or substituents will give the same results remain to be seen.

3. Concluding remarks

Fluoro-glycosyl compounds are of increasing importance in biochemical studies [14]. For example, substitution of 2-OH by fluorine in glycosides has been reported to alter biological activities and improve antifungal, antiviral and anticancer properties relative to parent compounds. In the light of increased demand for fluorinated saccharides and gangliosides, the simple and convenient method of the regio- and stereoselective synthesis of the fluorine-containing GM4 analogue described in this study is a viable alternative to the conventional yet tedious chemical synthetic method.

4. Experimental

4.1. Chemical synthesis of primers

4.1.1. Chemical synthesis of 2F Gal primer

4.1.1.1. Preparation of 2,3,4,6-tetra-O-acetyl-α-bromo-D-galactopyranoside. 1,2,3,4,6-Penta-*O*-acetyl-D-galactopyranoside (**1**) (15.6 g, 40.0 mmol) was dissolved in dichloromethane (280 ml) and to the solution was added 30% HBr in acetic acid (26.0 ml). The solution was stirred for 1 h at 0 °C and for another 6 h at room temperature. The solution was quenched with ice water (600 ml), and the organic layer was extracted with chloroform, neutralized with $NaHCO_3$, washed with water and with brine. The organic layer was dried over anhydrous sodium sulfate, filtered and evaporated to dryness to afford the desired product, 2,3,4,6-tetra-*O*-acetyl-α-bromo-D-galactopyranoside, that was used for the next reaction without further purification.

*4.1.1.2. Preparation of 3,4,6-tri-O-acetyl-D-galactal (**2**).* 2,3,4,6-Tetra-*O*-acetyl-α-bromo-D-galactopyranoside was dissolved in acetic acid (200 ml) and to the cooled solution was slowly added zinc dust (30.0 g, 459 mmol) and $CuSO_4$

(3.00 g, 18.8 mmol) dissolved in water. The solution was stirred for 30 min at 0 °C and for another 18 h at room temperature. The solution was filtered through a bed of Celite. Chloroform was added and the organic layer was neutralized with $NaHCO_3$ and washed successively with water and with saturated sodium chloride. The organic layer was dried over anhydrous sodium sulfate, filtered and concentrated in vacuo. Separation by silica gel column chromatography (ethyl acetate:hexane, 1:2) afforded the desired product (**2**) (2 steps: 7.76 g, 71%).

4.1.1.3. Preparation of 3,4,6-tri-O-acetyl-2-deoxy-2-fluoro-D-galactopyranoside. To a mixture of 3,4,6-tri-*O*-acetyl-D-galactal (**2**) (949 mg, 3.49 mmol), Selectfluor (1.48 g, 4.19 mmol) was added acetonitrile (15 ml) and water (3 ml) and the resulting solution was stirred for 16 h at room temperature and refluxed for an additional 30 min. The solution was evaporated to dryness and subsequent separation by silica gel column chromatography (ethyl acetate:hexane, 1:2) afforded the desired product (344 mg, 32%).

*4.1.1.4. Preparation of O-(3,4,6-tri-O-acetyl-2-deoxy-2-fluoro-D-galactopyranosyl)trichloroacetimidate (**3**).* 3,4,6-Tri-*O*-acetyl-2-deoxy-2-fluoro-D-galactopyranoside (1.09 g, 3.55 mmol) and K_2CO_3 (2.45 g, 17.8 mmol) were dissolved in dichloromethane (50 ml) and the solution stirred for 1 h. To the solution was added acetonitrile (1.08 ml, 10.7 mmol). The solution was stirred for 16 h, then filtered through a bed of Celite and evaporated in vacuo to afford the desired product (**3**) that was used in the next reaction without further purification.

4.1.1.5. Preparation of n-dodecyl 3,4,6-tri-O-acetyl-2-deoxy-2-fluoro-β-D-galactopyranoside. The *O*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-D-galactopyranosyl) trichloroacetimidate that was obtained in the previous reaction was dissolved in dichloromethane (50 ml) and the solution was stirred for 1 h. Then 1-dodecanol (823 μl, 3.67 mmol) was added and the solution was stirred for 3 h at −78 °C. Then, TBSOTf (168 μl, 0.73 mmol) was slowly added and the solution was stirred at room temperature for 12 h. The reaction was stopped by the addition of aqueous sodium bicarbonate. Chloroform was added and the organic layer was washed successively with saturated sodium bicarbonate, water and saturated sodium chloride, dried with anhydrous sodium sulfate, filtered and evaporated to dryness. Separation by silica gel column chromatography (ethyl acetate:hexane, 1:7) afforded 0.632 mg (36%, 2 steps) of the β-glycosylation product.

4.1.1.6. Preparation of n-dodecyl 2-deoxy-2-fluoro-β-D-galactopyranoside (2F Gal primer). Deacylation of *n*-dodecyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-β-D-galactopyranoside (268 mg, 0.56 mmol) was carried out by dissolving in 3 ml methanol and to the solution was added sodium methoxide (18 mg, 0.33 mmol). The mixture was stirred for 30 min at room temperature and then neutralized with cation-exchange resin (DIAION SK1B (H^+ form), filtered and evaporated to afford *n*-dodecyl 2-deoxy-2-fluoro-β-D-galactopyranoside (174 mg, 89%).

Chemical synthesis of 4F and 6F Gal primers were carried out according to literature cited

4.2. Cellular uptake of glycoside primers

Cell culture, incubation of cells with primer and identification of glycosylated product were carried out according to the literature cited in the text.

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