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Short communication

Fluorous tag method for the simultaneous synthesis of different kinds of glycolipids

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ABSTRACT

A mixture of saccharide primers with partially fluorinated tails, 2-(perfluorooctyl)ethyl 4′-*O*-(β-D-galactopyranosyl)-β-D-glucopyranoside (Lac H2F8) and 6-(perfluorohexyl)hexyl 2′-acetamido-2′-deoxy-β-D-glucopyranoside (GN H6H6), were introduced to animal cells. The oligosaccharide of Lac H2F8 was elongated by cellular enzymes and gave a GM3-type oligosaccharide. On the other hand, GN H6F6 was galactosylated to afford a lactosamine derivative that was further sialylated. This research confirmed that simultaneous glycosylation processes took place for Lac H2F8 and GN H6F6 primers and that the presence of one did not prevent the glycosylation of the other from proceeding. Each primer was recognized independently and elongated sequentially by cellular enzymes. Significantly, the synthesis of glycolipids from a mixture of these artificial scaffolds did not prevent the synthesis of glycolipids from the natural precursor. The glycosyl transferases recognized both precursors resulting to simultaneous synthesis of glycolipids.

1. Introduction

Biocombinatorial synthesis [1], a strategy that combines chemical synthetic methods with cellular biosynthetic processes, is a promising alternative to conventional methods to generate a library of glycolipid-specific saccharides that play important roles in biological systems. Administration of saccharide primers into cells is a technology for the rapid and convenient synthesis of oligosaccharides with diverse structures. The basic building blocks that are used as substrates by cellular enzymes could be readily available in a few steps. The saccharide primers resemble lactosyl ceramide (LacCer) [2], the natural precursor of glycolipid biosynthesis. Although their glycan headgroups are similar, the saccharide primers have a single alkyl tail as the aglycon moiety. Nevertheless, saccharide primers could pass the cell membrane to the Golgi, the site of glycosylation, and likewise be elongated to give products with structures similar to those biosynthesized from LacCer [3]. Because of the vast number of structural possibilities from a given single scaffold, a library of diverse glycoconjugate structures could be generated by incorporation of different saccharide primers in a wide variety of cells. For example, administration of n-dodecyl β-lactoside (Lac C12) into mouse melanoma B16 cells gave a GM3-type oligosaccharide [4]. On the other hand, introduction of the same primer into African green monkey kidney epithelial cells (Vero) gave GM3-, Gb3- and Gb4-type oligosaccharides [5].

We have reported that glycosides with perfluorinated tails could be incorporated into cells and act as substrates for glycosylation to give products that could be purified by employing the fluorous approach [6] as an alternative to the usual separation by chromatographic methods. A series of fluorous-tagged saccharide primers [7] with different number of fluorine atoms were chemically synthesized and administered individually to cells to determine which among them could be taken in and glycosylated by cells, and the products released to the culture medium. Although the number of fluorine atoms was significant in the primer intake and release of products, results showed that the terminal galactose residue of these primers were glycosylated. Based on the screening results involving various fluorous-tagged primers, we then embarked on the incorporation of a mixture, initially of two kinds of fluorous-tagged primers to establish cellular enzyme response. We envisioned that this preliminary investigation involving a mixture of primers could provide salient information that will eventually lead to the biocombinatorial synthesis of various oligosaccharides by administration of a mixture of several fluorous-tagged primers into cells. We now report the incorporation of a mixture of 2 kinds of fluorous-tagged primers: 2-(perfluorooctyl)ethyl 4′-*O*-(β-D-galactopyranosyl)-β-D-glucopyranoside (Lac H2F8) (each number indicates the number of methylene and fluorinated carbons, respectively) and 6-(perfluorohexyl)hexyl 2′-acetamido-2′-deoxy-β-D-glucopyranoside (GN H6F6). These primers have different types of saccharide

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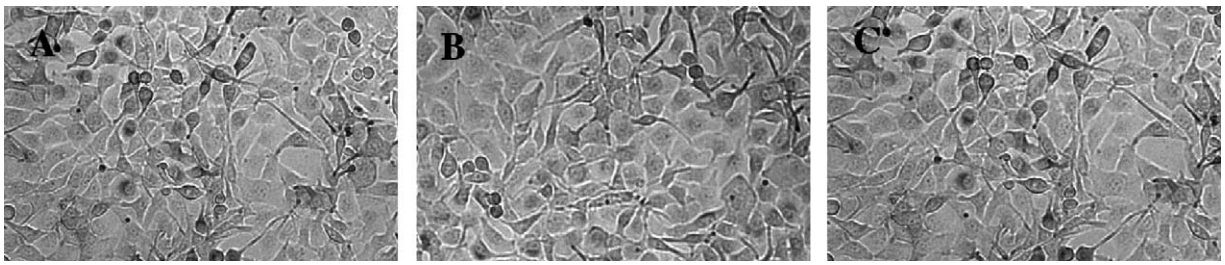


Fig. 1. Effect on B16 cells after incubation in the presence of (A) Control: Lac H2F8 only (B) Control: GN H6F6 only, and (C) mixture of Lac H2F8 and GN H6F6 primers. 5×10^6 B16 cells were seeded into 15 cm dishes containing 20 ml of 10% FBS DMEM/F12 and incubated for 48 h. 50 μM fluorous-tagged saccharide primer (or mixture of 50 μM of each primer) in 20 ml DMEM/F12 supplemented with insulin, transferrin and selenium (ITS-X) were administered into cells.

moeity and different numbers of methylene and difluoromethylene in the aglycon unit. They were chosen among the previously reported ones on the basis of efficiency in assimilation into cells, elongation and release of products into the culture medium. This work also aims to possibly separate the perceived mixture of products that can be obtained. Hence, the number of fluorine atoms in the aglycon unit has been varied for this purpose. The mixture of these primers was introduced into mouse melanoma B16 cells and the effect on the glycosyl transferases and the glycosylation sequence was determined.

2. Results and discussion

The primers were prepared in two synthetic steps [7,8], glycosylation and deacylation, as reported in literature. A mixture of 50 μM each of the fluorous-tagged saccharide primers Lac H2F8 and GN H6F6 was non-cytotoxic when introduced into mouse melanoma B16 cells (seeded cell number: 5×10^6 cells/15 cm dish) as shown in Fig. 1. Although the total concentration of administered mixture was twice as compared to control (50 μM Lac H2F8 only or 50 μM GN H6F6 only), results did not show any significant effects on morphology and viability.

After incubation for 48 h, the culture medium fraction was collected and the lipids were extracted. The HPTLC profile of lipids showed bands corresponding to endogenous GM3 and the product of glycosylation as shown in Fig. 2. Interestingly, the product of glycosylation from the mixture of Lac H2F8 and GN H6F6 gave a single strong band with the same Rf value as those obtained when the primers were introduced separately suggesting that the cells elongated the primers to give the same kind of oligosaccharide whether the primers were introduced separately or as a mixture. Moreover, the single band indicates that the major products of glycosylation may be structurally related. To elucidate the structure of the respective products, separation of the lipids was carried out by gradient elution using fluorous solid phase extraction cartridges [9]. The elongated products were efficiently separated from the rest of the components of the culture medium by using 40% and 50% MeOH in water as shown in Fig. 3. Electrospray ionization mass spectral result (negative mode) obtained from the mixture of Lac H2F8 and GN H6F6 gave 2 major peaks at *m/z* 1078.1 ($C_{33}H_{42}F_{17}NO_{19}$) and at *m/z* 1075.2 ($C_{37}H_{53}F_{13}N_2O_{19}$). As shown in Fig. 4, a comparison of this result with authentic samples confirmed the products to be a sialylated Lac H2F8, and a sialylated galactosyl GN H6F6.

With reference to control (treatment of cells with GN H6F6 primer only), it is noteworthy that almost all of the lactosamine was sialylated as evidenced by the absence of a band that would most likely have an Rf value that is very close to that of Lac H2F8 primer as shown in Fig. 2. The absence of a band corresponding to a lactosamine in the HPTLC result of the culture medium indicates that after elongation of GN H6F6 by galactosyl transferases, the resulting lactosamine remained inside the cell and was readily available for further elongation by sialyl transferases. Hence, it implied that sialylation was almost instantaneous.

Although a mixture of primers was introduced in cells, the cellular enzymes recognized the primers and glycosylated each one independently as shown in Scheme 1. Sialyl transferases acted immediately on Lac H2F8 to give a sialylated lactoside derivative but did not act on GN H6F6. On the other hand, galactosyl transferases elongated the saccharide chain of GN H6F6 to afford a lactosamine derivative. Hence, the action of enzymes on primers is predetermined sequentially and in this case, it is most likely cell specific since GM3 is the major ganglioside produced by B16 cells.

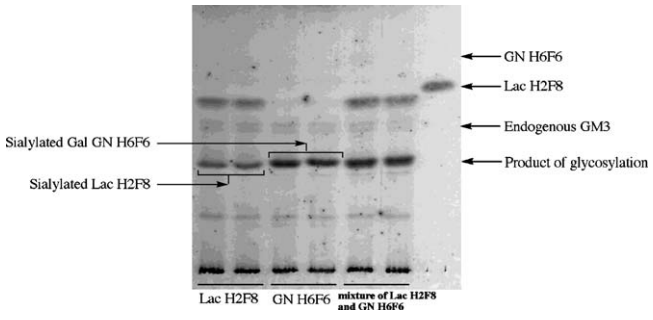


Fig. 2. HPTLC profile of lipids from the culture medium fraction obtained after incubation of B16 cells in the presence of Lac H2F8 only, GN H6F6 only and a mixture of Lac H2F8 and GN H6F6 primers. 5×10^6 B16 cells were seeded into 15 cm dishes containing 20 ml of 10% FBS DMEM/F12 and incubated for 48 h. 50 μM fluorous-tagged saccharide primer (or mixture of 50 μM of each primer) in 20 ml ITS-X DMEM/F12 were administered into cells. After incubation for 48 h, culture medium was collected and lipids were extracted and separated.

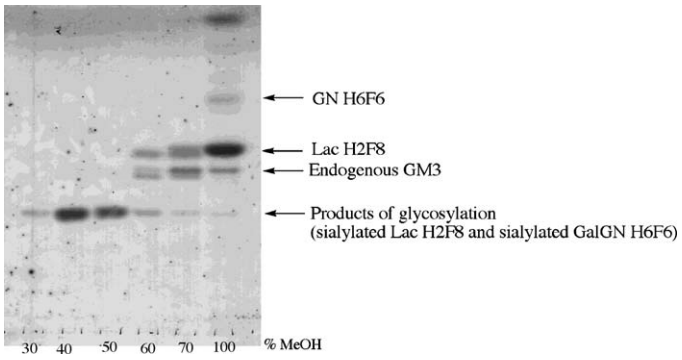


Fig. 3. HPTLC profile after separation of lipids obtained from a mixture of Lac H2F8 and GN H6F6 using fluorous solid phase extraction cartridge (FSPE). 5×10^6 B16 cells were seeded into 15 cm dish containing 20 ml of 10% FBS DMEM/F12. Then, 50 μM each of Lac H2F8 and GN H6F6 primers in 20 ml ITS-X DMEM/F12 were administered into cells and incubated for 48 h. The culture medium was collected and the lipids were extracted. Separation was carried out as follows: pre-treatment of the FSPE cartridges (2 g) with THF (4 ml) followed by water (4 ml), then sample loading and washing with water (5 ml), and finally gradient elution with 5 ml MeOH/water.

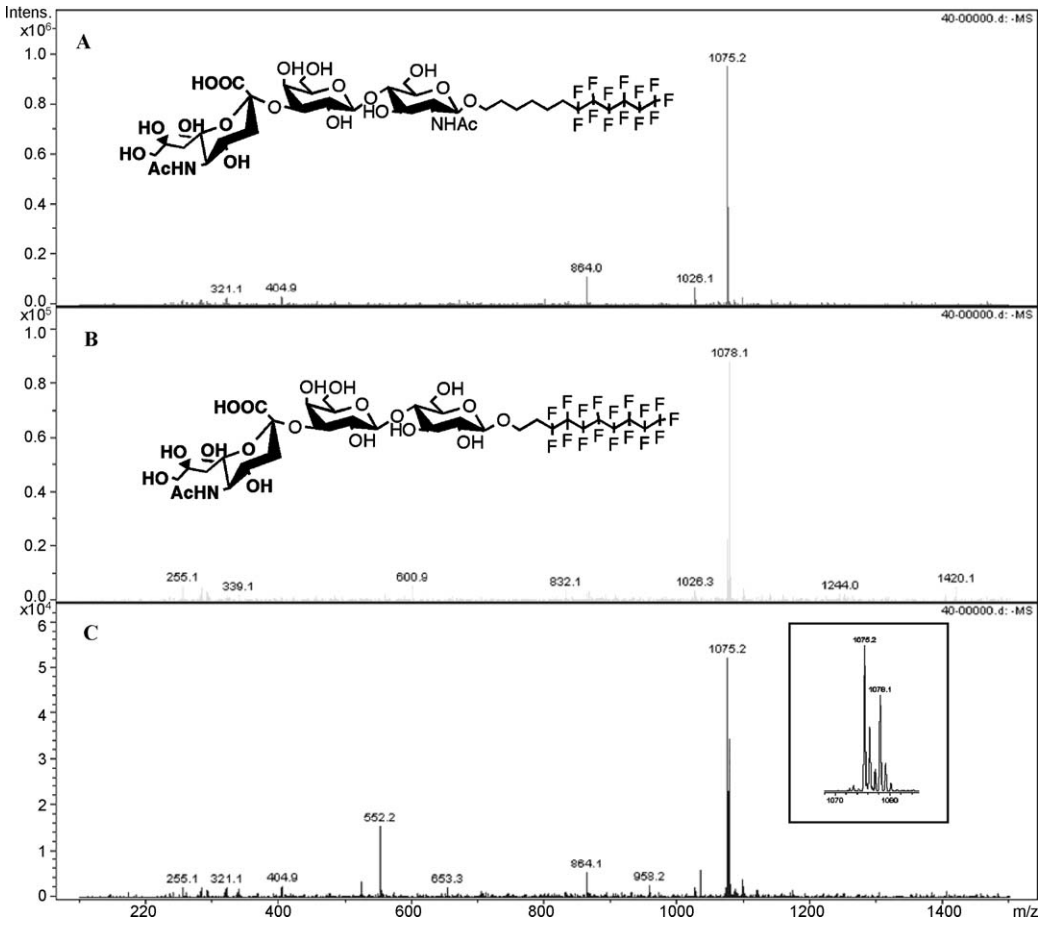
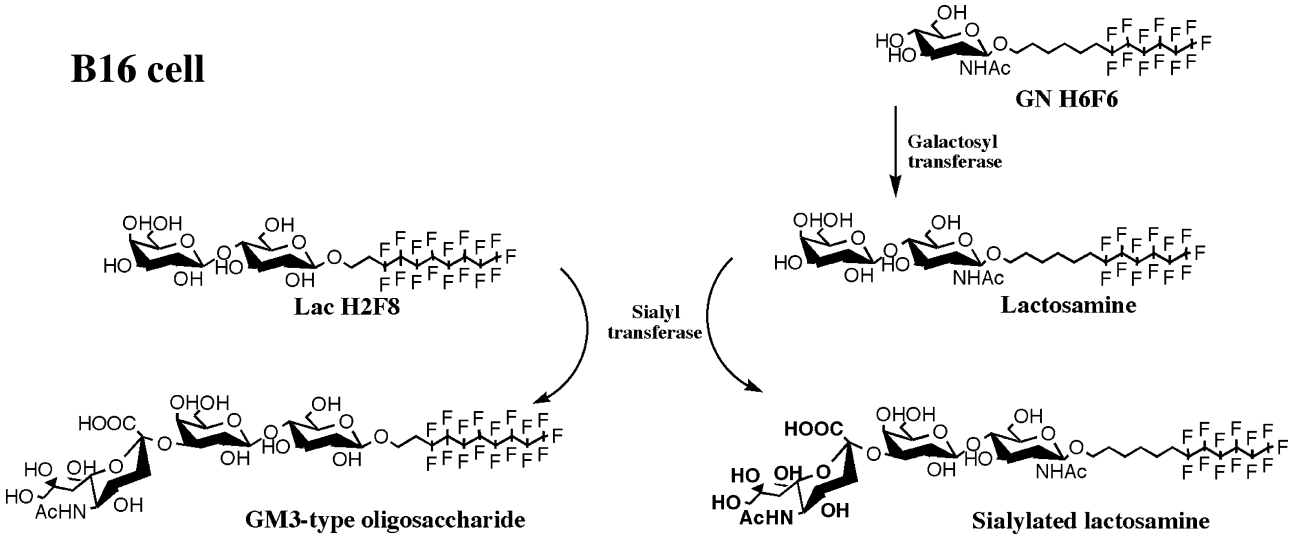


Fig. 4. Electrospray ionization mass spectrum (negative ion mode) of (A) sialylated La H2F8, (B) sialylated Gal GN H6F6 and (C) products obtained after 48 h incubation of B16 cell in the presence of a mixture of Lac H2F8 and GN H6F6 primers.

According to the natural biosynthetic sequence in the synthesis of glycolipids, galactosyl transferases act on glucosyl ceramide to produce lactosyl ceramide that is further sialylated to give ganglioside GM3. Based on HPTLC results, B16 cells also produced GM3 endogenously from lactosyl ceramide. Hence, simultaneous syntheses occurred and the sialyl transferases elongated both the natural and artificial scaffolds according to the natural glycosylation sequence in the biosynthesis of glycolipids.

The glycosyl transferases, although specific, did not exhibit preferences. As long as the primers fulfilled the basic requirement (terminal galactose or N-acetyl glucosamine residue for sialyl or galactosyl transferase, respectively) and reached the Golgi,



Scheme 1. Simultaneous synthesis of glycolipids in B16 cells using fluorous-tagged primers GN H6F6 and Lac H2F8.

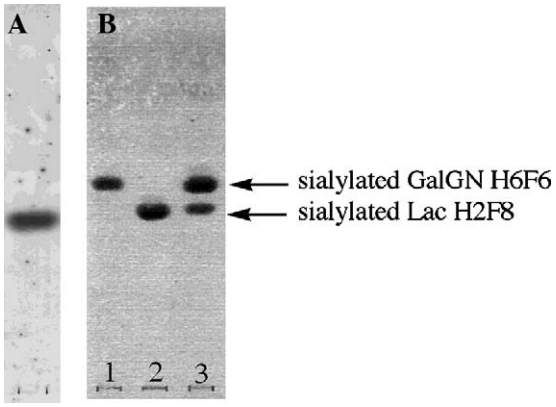


Fig. 5. Separation of products (sialylated Lac H2F8 and sialylated GalGN H6F6) using (A) Normal silica gel HPTLC and $CHCl_3$: MeOH: 0.25% KCl (5:4:1) as developing solvent, and (B) FluoroFlash TLC plates with F254 indicator and 80%MeOH as developing solvent. Lane 1, sialylated GalGN H6F6; Lane 2, sialylated Lac H2F8; Lane 3, a mixture of sialylated Lac H2F8 and sialylated GalGN H6F6.

elongation took place. Although two simultaneous glycosylation processes were taking place for Lac H2F8 and GN H6F6, the presence of one did not prevent the glycosylation sequence of the other. Moreover, the activity of transferases was hardly affected by the presence of perfluorinated single-tailed aglycon unit that is structurally different from the ceramide, the aglycon of the natural precursor did not affect recognition by the transferases. Both primers have different number of fluorine atoms in the aglycon unit but both were likewise elongated. Although the number of fluorine atoms in the aglycon unit seemed immaterial in this case, the hydrophilic and hydrophobic balance should be carefully considered in the design of the primer to ensure efficient incorporation of primer into the cell and subsequent release into the culture medium for separation.

Separation of the products obtained from glycosylation of the mixture of Lac H2F8 and GN H6F6 primers was also carried out. By using ordinary HPTLC plates, bands corresponding to the products, sialylated Lac H2F8 and sialylated galactosyl, were overlapping. However, successful separation of the two products was accomplished by using FluoroFlash TLC plates with F254 indicator and 80% MeOH as developing solvent as shown in Fig. 5.

The unique physicochemical properties of partially fluorinated glycosides make these compounds particularly interesting in drug delivery and other biomedical applications. The single-tailed and partially fluorinated saccharide primers are viable scaffolds for the synthesis of more complex oligosaccharides. This research exemplified a convergence of chemical and biochemical aspects towards the synthesis of important oligosaccharides using saccharide primers. By combining chemical synthetic and biosynthetic processes, the convenient preparation of oligosaccharides was accomplished by administering Lac H2F8 and GN H6F6 into B16 cells. Consequently, a library of important oligosaccharides with partially fluorinated tails can easily be achieved by the same principle of biocombinatorial synthesis using various fluorous-tagged saccharide primers and a variety of cells.

3. Conclusion

The mixture of fluorous-tagged saccharide primers (Lac H2F8 and GN H6F6) was non-cytotoxic. Each primer was recognized independently by cellular enzymes and elongated according to the biosynthetic sequence inherent to the type of cells used. Incubation of mouse melanoma B16 cells in the presence of a mixture of primers resulted to simultaneous glycosylation and afforded a GM3-type oligosaccharide and a sialylated lactosamine derivative, respectively, which were released in the culture medium and effectively separated.

4. Materials and methods

The saccharide primers (Lac H2F8 and GN H6F6) were prepared in two steps, glycosylation followed by deacylation, according to the literature cited in the text.

4.1. Cellular uptake of glycoside primers

4.1.1. General methods

Dulbecco Modified Eagle's Medium (DMEM) and Ham F12 (1:1), trypsin and insulin-transferrin-selenium (ITS-X) solution were from Gibco. The lactoside primers were dissolved in sterile Me_2SO to an initial concentration of 50 mM. SepPak C18 was from Waters. HPTLC plates were from E. Merck, Darmstadt, Germany. $CHCl_3$: MeOH: 0.25% KCl (aq) = 5:4:1 (v/v/v) was used as developing solvent for HPTLC. The mass spectrum of the products of cellular enzyme catalyzed glycosylation was recorded on a Bruker Esquire HCT Ultra ESI LC MS using MeOH: acetonitrile (1:1, v/v). Solid phase extraction cartridges were by Fluorous Technologies Inc.

4.1.2. Cell culture

Mouse B16 melanoma cells were cultured in 1:1 DMEM-F12 supplemented with 10% fetal bovine serum (FBS). Cells were detached through application of 0.25% trypsin-EDTA, passaged every 3 days and maintained in humidified atmosphere of 5% CO_2 air at 37 °C.

4.1.3. Incubation of cells with lactoside primer

Inocula of 5×10^6 cells were seeded into 150 mm culture dishes containing 20 ml of medium and incubated for 48 h. Then, the cells were washed with TI-DF without phenol red (1:1 DMEM Hams F12 with ITS-X) to remove the serum, and cells were incubated with 50 μM fluorous-tagged saccharide primer (or mixture of 50 μM of each primer) for 48 h at 37 °C. After incubation, culture media were collected and cells were washed with PBS (−), harvested with 0.25% EDTA in PBS (−), and centrifuged at 1000 rpm for 10 min.

The lipids were extracted from the cell pellet with $CHCl_3$: MeOH (2:1, v/v), then with $CHCl_3$: 2-propanol: water (7:11:2, v/v), in a sonicated bath. Lipids from the culture media were purified using SepPak C18 column. Lipids from the cell homogenate and culture medium fractions were analyzed by HPTLC with $CHCl_3$: MeOH: 0.2% aq KCl (5:4:1, v/v) as developing solvent. HPTLC plates were sprayed with resorcinol and heated to detect the glycolipids.

4.1.4. Identification of glycosylated product

The lipid extract from the culture medium fraction was separated by gradient elution with MeOH/water using fluorous solid phase extraction cartridges (FSPE) and the fractions obtained were analyzed by HPTLC. The fraction corresponding to the glycosylated products were collected and the mass spectra were obtained.

Acknowledgements

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