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Azido glycoside primer: a versatile building block for the biocombinatorial synthesis of glycosphingolipid analogues

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Abstract

A lactoside primer, 12-azidododecyl β-lactoside, was synthesized via the Koenigs–Knorr method by glycosylation of 1,12-dodecyldiol with perbenzoylated lactosyl bromide. The presence of the 2-*O*-acyl substituent in the donor gave the β-lactoside, and an excess of acceptor ensured monoglycosylation of the diol. Mesylation of the ω-hydroxyl group in the aglycon, followed by displacement of the mesylate with azide and subsequent O-debenzoylation gave the desired ω-azidododecyl β-lactoside. The azido glycoside primer was examined in mouse B16 melanoma cells for its feasibility as a building block for oligosaccharide biosynthesis. Uptake of the azido glycoside primer by B16 cells resulted in the sialylation of the galactose residue of the primer to give a glycosylated product having the same glycan as in ganglioside GM3. After 24 h incubation of B16 cells with the primers, the amount of sialylated ω-azidododecyl β-lactoside primer was 75% of the amount of sialylated *n*-dodecyl β-lactoside. However, after 48 h incubation, both primers gave equal amounts of the sialylated products. Interestingly, the remaining azido glycoside primer after 48 h incubation was 5.6-fold greater than that of the alkyl primer, indicating degradation of the alkyl primer to a larger extent than the ω-azido glycoside primer. The facile chemical synthesis and the efficient uptake in cells make the azido glycoside primer a versatile building block for the biocombinatorial synthesis of glycolipid oligosaccharides.

Keywords: Lactosyl ceramide analogue; Oligosaccharide library; Oligosaccharides, biocombinatorial synthesis; Azido glycoside primer; Saccharide primer, glycosylation

1. Introduction

Glycopolymers are an important class of bioactive macromolecules that have found a variety of biomedical applications in tumor diagnosis, human vaccines, detection and trapping of viruses and toxins, and targeted

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drug delivery systems [1]. Increasing attention has been paid to synthetic polymers, carrying various pendant mono- and oligosaccharides as biomaterials useful in applications utilizing an oligosaccharide-recognizing functional group. For example, various types of polymers that incorporate certain sialic acid residues have been synthesized in an attempt to inhibit the influenza virus [2]. Moreover, lactose-containing polystyrene and polypeptides are useful for culture substrates and as artificial antigens [3].

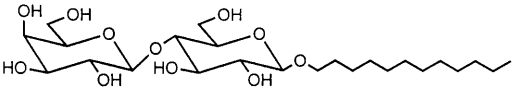
The usual synthetic strategy for the construction of glycopolymers involves polymerization or co-polymerization of a biologically important oligosaccharide unit [4]. However, chemical synthesis of oligosaccharides remains a laborious task requiring intricate methods of protection, glycosylation, and deprotection steps. We embarked on a biocombinatorial synthesis to prepare glycopolymers without these shortcomings.

Biocombinatorial synthesis has the potential to become an important tool for generating libraries of oligosaccharides and glycopolymers with desirable biological activities. The strategy involves the preparation of oligosaccharides or their glycosides by combining chemical synthetic methods with cellular biosynthetic processes. From a single building block, a large number of oligosaccharides can be constructed by using a variety of cells [5]. The ideal building blocks are synthetically accessible glycoside-based primers that act as substrates for additional glycosylation after incorporation into the cells. Moreover, such glycosides must possess functional groups amenable to further manipulation to enable preparation of glycopolymers.

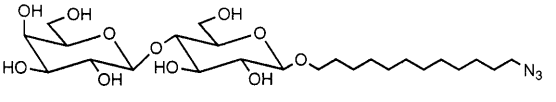
In our work in biocombinatorial synthesis to construct an oligosaccharide library, the building blocks used were amphipathic glycosides, a hydrophilic lactosyl moiety with hydrophobic aglycons of various alkyl chain lengths (e.g., a C_n alkyl lactoside and a C_n amide lactoside, where $n = C_8$, C_{12}, C_{14}, C_{16}). Incubation of amphipathic lactosides, appropriately referred to as primers, with different cell types primed the synthesis of oligosaccharides of different structures depending on the cells used. Direct glycosylation, which was shown to occur at the terminal residue of the lactoside, proceeded with the involvement of innate glycosyltransferases in the cell.

Whereas incorporation of the lactoside primers into B16 cells afforded an α-(2→3) sialyl lactoside derivative with the same oligosaccharide structure as that of GM3, the main ganglioside synthesized by B16 cells [5], incubation of the same lactoside primers with PC12 cells gave α-Gal-(1→4)- and α-Gal-(1→3)-α-Gal-(1→4) lactosides. *N*-Acylaminoethyl lactosides were also taken up and glycosylated by B16 cells to give the GM3 analogue likewise [6].

As part of our work on the biocombinatorial synthesis for the construction of an oligosaccharide library, we report in this paper the chemical synthesis of another building block, 12-azidododecyl β-lactoside primer (**2**), and its uptake by B16 cells for the synthesis of glycosphingolipid analogues. The efficiency of 12-azidododecyl β-lactoside (**2**) to prime oligosaccharide synthesis was also compared with that of the *n*-dodecyl β-lactoside primer (**1**). The introduction of an azido functional group in the aglycon allows its facile conversion into an amino group for specific incorporation into desired glycopolymers for future work.

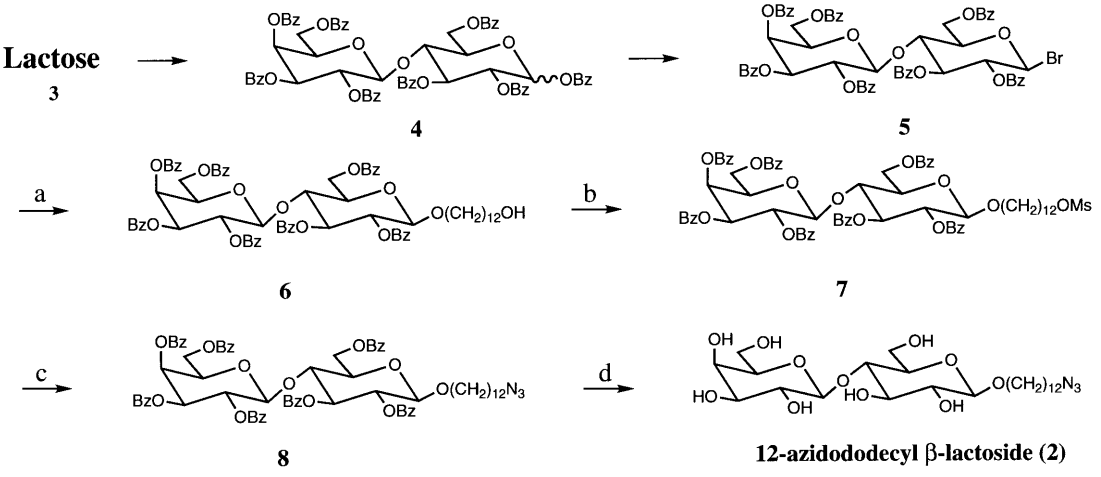


n-Dodecyl β-lactoside (alkyl glycoside primer)
1



12-Azidododecyl β-lactoside (azido glycoside primer)
2

Employing biocombinatorial synthesis of oligosaccharides, it is possible to construct a glycolipid-specific saccharide library using different glycoside primers in combination with a wide variety of cells. Using primers of diverse structures, it may be further possible to obtain a variety of N-linked glycans, *O*-glycans, and glycosaminoglycan–saccharides, leading to construction of oligosaccharide libraries that will pave the way for better understanding of



Scheme 1. Reagents and conditions: (a) $HO(CH_2)_{12}OH$, AgOTf, THF, rt, 4 h; (b) MsCl, pyridine, rt, 4 h; (c) NaN_3, DMF, 60 °C, 4 h then 12 h at rt; (d) MeOH–NaOMe, rt, overnight.

the biological function of glycoconjugates in biological systems.

2. Results

Chemical synthesis of the azido lactoside primer.—The chemical synthesis of 12-azidododecyl β-lactoside (**2**) is shown in Scheme 1. The glycosyl donor, perbenzoyllactosyl bromide (**5**), was readily prepared in two steps from lactose following a known procedure [7]. Glycosylation of 1,12-dodecyldiol using silver triflate as catalyst in dichloromethane (under Koenigs–Knorr conditions) yielded a partial analog of ceramide glycoside. Although there are two possible sites for glycosylation in dodecyldiol, the major product was **6**. The excess glycosyl acceptor (3.4 equivalents) resulted in mostly the monoglycosylation product, and the presence of the 2-*O*-benzoyl group led to the desired β-lactoside via neighboring-group participation. Purification by silica gel chromatography afforded the ω-hydroxyldodecyl β-benzoyl lactoside (**6**). The β-anomeric configuration of the glycosylation product was confirmed by its NMR spectrum. The relatively large coupling constant for $J_{1,2}$ (7.9 Hz) indicates a β configuration of newly formed glycosidic linkage.

Activation of the hydroxyl group of compound **6** by reaction with methanesulfonyl chloride (MsCl), followed by displacement of the resulting mesylate with azide (with sodium azide in DMF) afforded compound **8** in 64% yield in two steps. Usual O-deacylation with NaOMe–MeOH and purification by silica gel chromatography afforded the 12-azidododecyl β-lactoside (**2**).

Effect of amphipathic lactoside primers on cell growth.—Mouse melanoma cells (B16) were cultured in the absence or in the presence of 50 μM azido or alkyl lactoside primer. The growth of cells cultured for 2 days in the presence of the primers was similar to that of the control (Fig. 1). The primers did not express toxic activity toward the cells at this concentration.

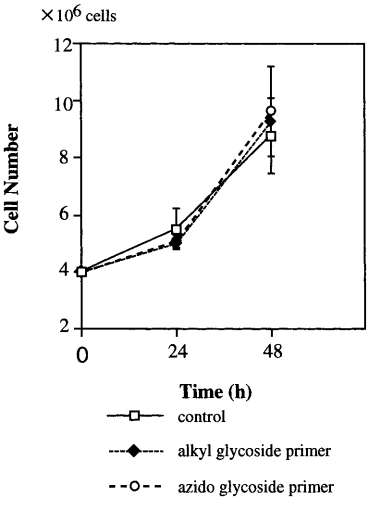


Fig. 1. Effect of amphipathic lactoside primers on cell growth. B16 cells (4.0×10^6, 100 mm dish) were incubated for 48 h in serum-free 1:1 DMEM–F12 supplemented with transferrin and insulin (TI–DF) in the absence (control) or presence of 50 μM lactoside primer.

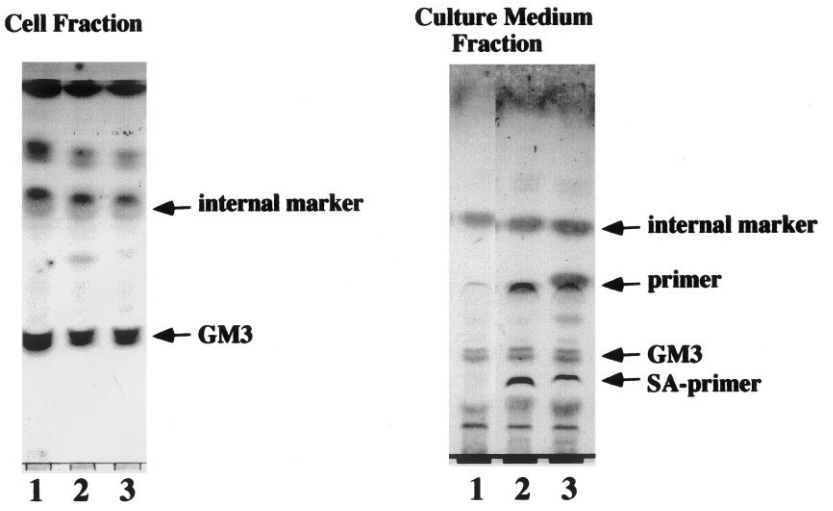


Fig. 2. Glycosylated lactosides produced by adding amphipathic lactoside primers to cells. B16 cells (3.0×10^6, 100 mm dish) were incubated for 24 h in serum-free 1:1 DMEM–F12 supplemented with transferrin and insulin (TI–DF) in the presence of 50 μM alkyl **1** or azido glycoside **2** primer. The cells were washed with PBS (−) and harvested. Lipids were extracted, purified, separated by HPTLC, and analyzed as described in the Experimental Section. The left and right panels show the glycosylated products accumulated in the cell fraction and culture medium fraction, respectively; Lane 1, control (TI–DF only); Lane 2, alkyl primer **1**; Lane 3, azido primer **2**.

Glycosylation of the azido lactoside primer.—To examine the versatility of the 12-azidododecyl β-lactoside (**2**) as a building block for the synthesis of glycosphingolipid-type oligosaccharide, B16 cells expressing high levels of GM3 were fed with 50 μM of the primer. After incubation for 24 h, culture media were collected and the lipids were purified using SepPak C_{18} column. Lipids from the cell homogenates were extracted with chloroform–methanol and then with chloroform–2-propanol–water. Analysis by HPTLC of the lipid extracts from the cell homogenates and the culture media indicated a band corresponding to a putative glycosylated lactoside primer (Fig. 2, Lane 3).

Identification of the glycosylated lactoside.—To elucidate the structure of the glycan unit modified in cells, the silica gel zone corresponding to the putative glycosylated lactoside was scraped from the HPTLC plate and extracted. Analysis of the MALDI–TOF mass spectrum (Fig. 3) revealed a peak at m/z 842.9 corresponding to a monosialylated lactoside primer. To establish whether the sialylated lactoside is α-(2→3)- or α-(2→6)-linked, the purified product was hydrolyzed with endoglycosylceramidase [6]. HPTLC analysis of the released product alongside those from sialyl *n*-dodecyl lactoside standards [5] and authentic GM3 confirmed that the glycosylated product is an α-(2→3)-sialylated lactoside — an analogue of GM3, which is the glycosphingolipid predominantly expressed on the cell surface of B16 melanoma cells (Scheme 2) [5,8]. To confirm whether reduction of the azide to an amine proceeded during incubation with cells, the HPTLC plate was sprayed with ninhydrin reagent. The absence of the characteristic stain confirmed that the azido function remained intact even after glycosylation. Thus, the azido glycoside primer entered the glycosphingolipid (GSL) biosynthetic pathway and functioned as an acceptor for the

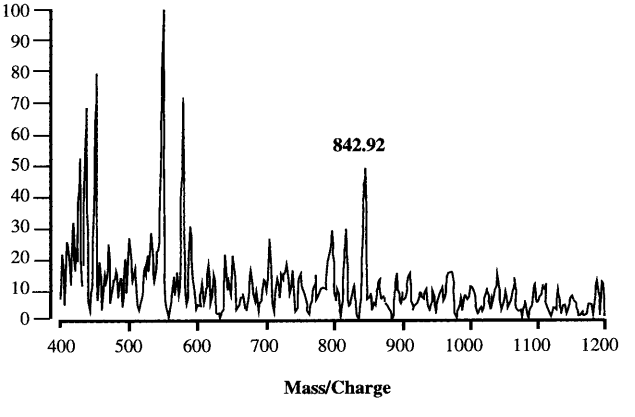
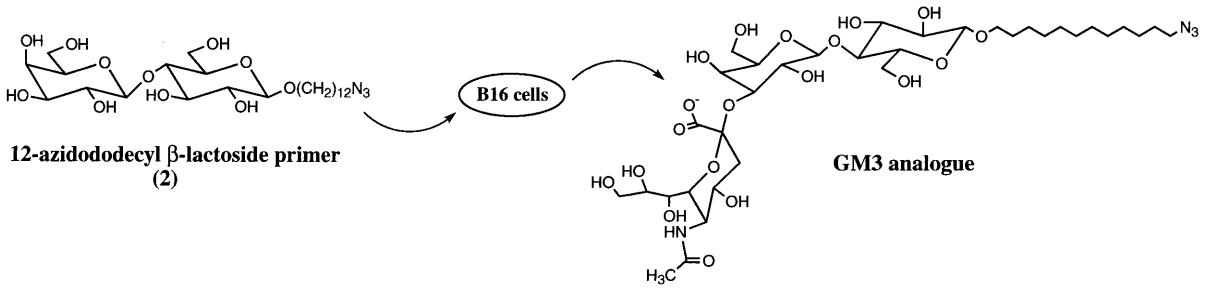


Fig. 3. MALDI-TOF MS of the glycosylated product obtained from the incubation of cells with 12-azidododecyl β-lactoside (**2**) for 24 h.



Scheme 2. Biocombinatorial synthesis of GM3 analogue after uptake of 12-azidododecyl β-lactoside primer (**2**) into B16 cells.

GM3 synthase, α-(2→3)-sialyl transferase, resulting in direct glycosylation occurring at the terminal galactosyl residue of the lactose moiety subsequent to uptake by cells.

Comparison of priming ability between the alkyl glycoside primer and azido glycoside primer.—In our previous report, the ability of different amphipathic lactosides with various alkyl aglycons [5] to prime the synthesis of glycolipid-type oligosaccharides was examined. Among the lactosides tested, *n*-dodecyl lactoside (the alkyl glycoside primer) was found to be the best acceptor for sialylation in B16 melanoma cells.

To determine whether the priming ability of the azido glycoside primer behaves similarly as the dodecyl glycoside primer, the primers were incubated for 24 and 48 h with B16 melanoma cells. The lipid fractions were extracted from the respective cell and medium fractions, and analyzed by HPTLC. As shown in Fig. 2, the glycosylated primers were mostly secreted into the culture medium.

After incubation of the lactoside primers for 24 h, HPTLC of the culture medium fraction showed clearly the preference for the uptake of the alkyl glycoside primer over the azido glycoside primer as judged from the band corresponding to the respective glycosylated product (Fig. 2). Quantification of the sialylated primers showed that the amount of sialylated azido glycoside primer was 75% of the amount of sialylated alkyl glycoside primer. As shown in Fig. 4, the amounts of glycosylated product using the azido glycoside primer **2** and the alkyl glycoside primer **1** were 10.3 nmol (4.1%) and 14.0 nmol (5.6%) of the administered primers, respectively. However, after 48 h incubation, the amounts of glycosylation products accumulated in the cultures for both azido and alkyl glycoside primers were about the same (9.0 and 8.4%, respectively) (Fig. 5).

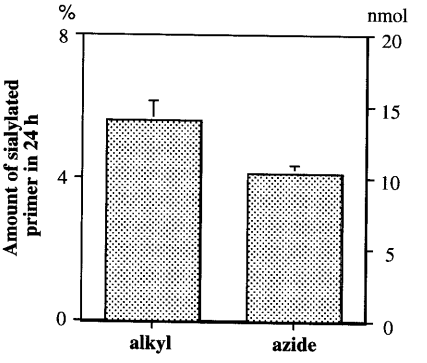


Fig. 4. Sialylated lactosides from the cell and medium fractions. B16 cells (3.0×10^6, 100 mm dish) were incubated for 24 h in serum-free 1:1 DMEM–F12 supplemented with transferrin and insulin (TI–DF) in the presence of 50 μM alkyl **1** or azido glycoside primer **2** for 24 h. The amounts of sialylated primer (nmol) from the cell and medium fractions were calculated. Each value represents the average of three determinations and the experiment was repeated five times.

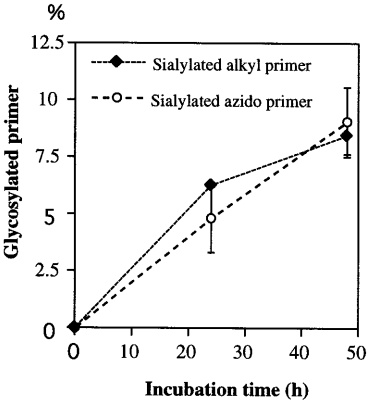


Fig. 5. Sialylated lactoside production with time. B16 cells (3.0×10^6, 100 mm dish) were incubated for 24 h in serum-free 1:1 DMEM–F12 supplemented with transferrin and insulin (TI–DF) in the presence of 50 μM alkyl **1** or azido glycoside primer **2**. The amounts of sialylated products were estimated at 24 and 48 h.

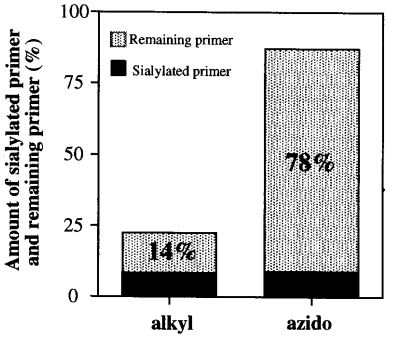


Fig. 6. Amount of remaining lactoside primer after incubation in B16 cells for 48 h. B16 cells (3.0×10^6, 100 mm dish) were incubated in serum-free 1:1 DMEM–F12 supplemented with transferrin and insulin (TI–DF) in the presence of 50 μM alkyl **1** or azido glycoside primer **2**. The amounts of remaining primers **1** or **2** were estimated after 48 h.

Interestingly, although the amounts of sialylated primers were almost the same for both primers after 48 h incubation, the remaining azido glycoside primer recovered from the culture medium was 78% of the input while that of the alkyl primer was only 14%, as shown in Fig. 6.

3. Discussion

In the study of oligosaccharide structure and cellular functions, one of the critical steps is the facile synthesis of the desired oligosaccharide analogues. We have described in this paper the synthesis of GM3 oligosaccharide by combining chemical synthesis and cellular biosynthesis. This is one of the segments in our ongoing work of building an oligosaccharide library by biocombinatorial synthesis, a novel technique using different glycoside primers and subsequent incorporation in a variety of cells. The glycosylated products thus obtained can be used for glycopolymer or other modulations by treatment with endoglycosylceramidase [9] or by further modification of the aglycon unit.

Lactosylceramide (LacCer) is a common intermediate in glycosphingolipid synthesis, and the lactose structure in glycoconjugates is unique to glycosphingolipids [10]. Recently, we reported a series of artificial glycolipids consisting of a lactosyl moiety that functions effectively as a GSL primer subsequent to their incorporation into cells [5,6]. The lactosides used have hydrophobic aglycons that mimic the ceramide group of LacCer and help the lactoside reach the Golgi apparatus. It is evident that the lactosides are taken up by the cells, conveyed to the Golgi apparatus, and taken through the GSL biosynthetic pathway of glycosylation. The sialylated lactosides were mostly secreted into the culture medium.

Glycosylation was cellular GSL-specific [10], and the efficiency of glycosylation is dependent on the aglycon structure. In B16 cells, amphipathic lactosides synthesized GM3 oligosaccharide, whereas in PC12 cells, lactosides produced Gb3 and α-Gal-(1→3)-Gb3 [5,11]. Thus, the carbohydrate structure of the glycosylation product depends on the glycolipid the cells synthesize. Primers with longer hydrophobic chains hampered the release of glycosylated products from cells. Lactosides with a dodecyl aglycon were glycosylated 2–3-fold greater than hexadecyl lactosides, and 1.4 times more than the aminoethyl lactoside with the dodecanoyl chain. The presence of an *N*-acyl chain suppressed the secretion of glycosylated products. Octyl lactosides did not function as glycosylation acceptors under the conditions used in the study.

Among many lactosides tested, the dodecyl β-lactoside (**1**) appears to be the best acceptor for glycosylation in vivo [5,6]. However, for the preparation of glycopolymers, the alkyl lactoside has limited utility because the alkyl aglycon is not readily modified further. Thus, for the preparation of biocombinatorial glycopolymers, a different lactoside primer with a readily modifiable functional aglycon that is amenable to cellular uptake, glycosylation, and secretion was deemed necessary.

In this report, we synthesized 12-azidododecyl β-lactoside (**2**), and examined its feasibility for the biocombinatorial synthesis of glycosphingolipid analogues. After internalization and glycosylation by the cells, the azido function in the aglycon can be selectively and easily reduced into an amino group, enabling it for further manipulation such as incorporation into glycopolymers.

The synthesis of the 12-azidododecyl glycoside primer (**2**) was achieved under the Koenigs–Knorr conditions using 1,12-dodecyldiol and per-O-benzoylated lactosyl bro-

mide. Through mesylation, displacement of the mesylate with azide, and subsequent O-debenzoylation, the 12-azidododecyl β-lactoside (**2**) was obtained in a moderate yield. This synthetic scheme should be applicable to the preparation of other azido alkyl glycosides using diols with various chain lengths.

Both the alkyl (**1**) and azido glycoside (**2**) primers gave the GM3 oligosaccharide moiety subsequent to the uptake by B16 cells. Incubation of the lactoside primers with the cells for 24 h showed that the amount of sialylated product from the azido glycoside primer was 75% of that from the alkyl glycoside primer. However, during the 48 h incubation period, the amount of glycosylated product in both primers was almost the same. The amount of recovered azido glycoside primer after 48 h incubation was 5.6-fold greater than the recovered alkyl glycoside primer. This indicates that after incorporation into cells, the alkyl glycoside primer and/or its sialylated form possibly underwent greater degradation than the azido glycoside primer. Thus, the azido glycoside primer functions more efficiently as an acceptor in oligosaccharide synthesis in the B16 cells than the alkyl glycoside primer overall. These results provide fundamental information on the effects of aglycon functionalities and the factors important for the efficient biocombinatorial synthesis of oligosaccharides using lactoside primers.

Phenol red is a commonly used indicator to monitor pH changes during cell culture. To determine if phenol red in the culture medium interferes in the glycosylation of lactoside primers, the cells were cultured in DMEM, with or without phenol red, prior to the 24-h incubation with the primers. Treatment with the alkyl glycoside primer gave almost the same amount of sialylation product with or without phenol red in the culture medium. However, the amount of glycosylated products, 5.0 and 8.8 nmol, from the azido glycoside primer with or without phenol red, respectively, may represent a significant difference. These results revealed that phenol red, a commonly used indicator for pH changes in the culture medium, interferes with intracellular glycosylations. Although the intrinsic effect of phenol red in cells goes beyond the scope of this study, our data suggest that phenol red should be used with caution as a pH indicator in cell cultures, especially when glycosylation is under study.

Although a more detailed pathway of the primers after uptake by cells needs to be established, the azido glycoside primer **2** is more favorable as an acceptor in oligosaccharide synthesis over the alkyl glycoside primer **1** because of the greater extent of glycosylation and the higher efficiency in the uptake of primer. The azido group of the glycosylated products can be reduced and conjugated for the synthesis of various glycoconjugates, with which the saccharide functions can be analyzed widely. Development of methods for the large-scale preparation of GM3 oligosaccharide from the azido primer **2** using cells are underway in order to produce chemical quantities with homogeneity required for application to polymer or antigen synthesis. The simple chemical synthetic route and efficient uptake by cells, coupled with the potential for the facile preparation of glycopolymers, make the azido glycoside primer a versatile building block for the biocombinatorial synthesis of glycosphingolipid analogues. Since no inherent damage to B16 melanoma cells has been brought about by the administration of the azide primer, these results open new avenues leading to further use of the azido group to different primers for the synthesis of other types of glycans.

4. Experimental

Synthesis of 12-azidododecyl β-lactoside primer (**2**)

General methods.—^{1}H NMR spectra were recorded at 200 MHz with a JEOL spectrometer in $CDCl_3$ using Me_4Si as the internal reference. All reactions were monitored by thin layer chromatography (TLC) on Silica Gel 60 F-254 (E. Merck), with detection by UV light or by visualizing with spraying with anisaldehyde–H_2SO_4 and heating. Column chromatography was performed on Silica Gel 60 (70–230 mesh, E. Merck, Darmstadt). The MALDI mass spectrum was recorded on a KOMPACT MALDI 1 mass spectrometer

(Kratos, UK) with a 2,5-dihydroxybenzoic acid (DHB) matrix. The *n*-dodecyl lactoside primer was synthesized from lactose as described [5]. The per-*O*-benzoyl lactosyl bromide (**5**) was prepared from lactose through per-O-benzoylation and subsequent treatment with HBr–HOAc [9].

12-Hydroxydodecyl 2,3,4,6-tetra-O-*benzoyl-β-*D*-galactopyranosyl-(1→4)-2,3,6-tri*-O-*benzoyl-β-*D*-glucopyranoside* (**6**).—To 30 g (26.5 mmol) BzLacBr (**5**) and 4 Å molecular sieves in THF (500 mL) were added 8.0 g of AgOTf (31.1 mmol, 1.2 equiv) and 18.0 g (88.9 mmol, 3.4 equiv) of 1,12-dodecanediol, and the resulting mixture was stirred at rt. The reaction was monitored by TLC. After the reaction was over (4 h), $CHCl_3$ was added, and the mixture was filtered. The filtrate was neutralized with aq $NaHCO_3$, washed with water, dried with anhyd Na_2SO_4 and concentrated in vacuo. Silica gel column chromatography of the residue with 1:1 toluene–EtOAc as eluent, afforded compound **6** in 72% yield. ^{1}H NMR ($CDCl_3$): 8.037–7.125 (m, 35 H, 7 Bz), 5.816 (t, 1 H, $J = 9.5$ Hz, H-3), 5.757–5.696 (m, 2 H, H-2′,4′), 5.463 (dd, 1 H, $J =$ 7.9, 9.8 Hz, H-2), 5.371 (dd, 1 H, $J = 3.4$, 10.3 Hz, H-3′), 4.870 (d, 1 H, $J = 7.9$ Hz, H-1′), 4.687 (d, 1 H, $J = 7.9$ Hz, H-1), 4.618–4.473 (m, 2 H, H-6a,b), 4.256 (t, 1 H, $J = 9.5$ Hz, H-4), 3.915–3.400 (m, 8 H, H-5,5′,6′a,b, $-CH_2OH$, and OCH_2-), 1.621–1.090 [m, 20 H, $-(CH_2)_{10}-$]. Anal. Calcd for $C_{73}H_{74}O_{19}$ (1255.36): C, 69.84; H, 5.94. Found: C, 70.13; H, 6.10.

12-Azidododecyl 2,3,4,6-tetra-O-*benzoyl-β-*D*-galactopyranosyl-(1→4)-2,3,6-tri*-O-*benzoyl-β-*D*-glucopyranoside* (**8**).—To a solution of **6** (10 g, 7.97 mmol, in pyridine (250 mL) was added MsCl (1.82 g, 15.9 mmol, 2 equiv), and the solution was stirred for 4 h at rt. The mixture was filtered and evaporated to give a syrup, which was diluted with $CHCl_3$ and neutralized with aq $NaHCO_3$. The organic layer was dried with anhyd Na_2SO_4 and concentrated in vacuo to afford the mesylated compound **7**, which was used immediately for the preparation of **8**. To a solution of **7** in DMF (500 mL) was added NaN_3 (1.36 g, 20.9 mmol), and the solution was stirred at 60 °C for 4 h and then at rt for 12 h. The reaction mixture was diluted with $CHCl_3$, and washed with aq $NaHCO_3$ and water. The organic layer was dried with anhyd Na_2SO_4 and filtered, and the filtrate was concentrated in vacuo. Purification by column chromatography with 1:1 toluene–EtOAc as eluent afforded compound **8** in 64% yield. ^{1}H NMR ($CDCl_3$): 7.124–8.036 (m, 35 H, 7 Bz), 5.807 (t, 1 H, $J = 9.5$ Hz, H-3), 5.756–5.695 (m, 2 H, H-2′,4′), 5.462 (dd, 1 H, $J = 7.9$, 9.3 Hz, H-2), 5.367 (dd, 1 H, $J = 3.4$, 10.3 Hz, H-3′), 4.870 (d, 1 H, $J = 7.9$ Hz, H-1′), 4.683 (d, 1 H, $J = 7.9$ Hz, H-1), 4.619–4.480 (m, 2 H, H-6a,b), 4.259 (t, H, $J = 9.5$ Hz, H-4), 3.925–3.710 (m, 4 H, H-5,5′,6′a,b), 3.700–3.400 (m, 2 H, OCH_2-), 3.260 (t, 2 H, $J = 6.9$ Hz, CH_2N_3), 1.621–1.092 [m, 20 H, $-(CH_2)_{10}-$]. Anal. Calcd for $C_{73}H_{73}N_3O_{18}$ (1280.37): C, 68.48; H, 5.75; N, 3.28. Found: C, 68.69; H, 5.90; N, 3.05.

12-Azidododecyl β-lactoside (**2**).—Compound **8** (5 g, 3.48 mmol) was dissolved in dry MeOH (50 mL), and to the solution was added 5% NaOMe in MeOH. The mixture was stirred overnight at rt and then neutralized with cation-exchange resin (Dowex 50W-X2, H^+ form). After filtration, the filtrate was evaporated, and the residue was treated with toluene and evaporated repeatedly to give a white solid. Recrystallization with EtOH afforded the 12-azidododecyl β-lactoside primer (**2**) in 80% yield. ^{1}H NMR (Me_2SO-d_6 containing 5% D_2O): 4.202 (d, 1 H, $J = 7.9$ Hz, H-1′), 4.141 (d, 1 H, $J = 7.9$ Hz, H-1), 3.183 (t, 2 H, $J = 6.8$ Hz, CH_2N_3), 1.470–1.130 [m, 20 H, $-(CH_2)_{10}-$]. Anal. Calcd for $C_{24}H_{45}N_3O_{11}\cdot 0.5\ H_2O$ (560.63): C, 51.41; H, 8.11; N, 7.49. Found: C, 51.70; H, 8.40; N, 7.50.

Incubation of B16 melanoma cells with lactoside primer

General methods.—B16 cells were obtained from Riken Cell Bank (Tsukuba, Japan). Dulbecco Modified Eagles's Medium (DMEM), DMEM and Ham F12 (1:1), and fetal bovine serum (FBS) were from GIBCO. Insulin and transferrin were from Wako Pure Chemical, Tokyo. SepPak C_{18} was from Waters. HPTLC (Silica Gel 60) and preparative TLC plates were from E. Merck, Darmstadt, Germany. The lactoside primers were dissolved in Me_2SO to an initial concentration of 50 mM.

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.

Incubation of cells with lactoside primer. Inocula of 3.0×10^6 cells were seeded into 100-mm culture dishes containing 7 mL of medium and incubated for 48 h. This was followed by washing with TI–DF without phenol red (1:1 DMEM–Hams F12 containing 30 nM SeO_2, 5 μg/mL transferrin, and 50 μg/mL insulin) to remove the serum, and cells were incubated with either 50 μM of the alkyl primer **1** or 50 μM of the azido primer **2** for 24 and 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 2:1 $CHCl_3$–MeOH, then with 7:11:2 $CHCl_3$–2-propanol–water, in a sonicated bath. Lipids from the culture media were purified using a SepPak C_{18} column. Lipids from the cell homogenate and culture medium fractions were analyzed by HPTLC with 5:4:1 $CHCl_3$–MeOH–0.2% aq KCl as developing solvent. HPTLC plates were sprayed with resorcinol, and with orcinol–H_2SO_4 reagent, and heated to detect the separated glycolipids which were later quantified using BIO-PROFIL BIO Image Analyzer.

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