Design and Facile Synthesis of Neoglycolipids as Lactosylceramide Mimetics and Their Transformation into Glycoliposomes

Yoichiro Harada, Takeomi Murata, Kazuhide Totani, Tetsuya Kajimoto, Shah Md. Masum, Yukihiro Tamba, Masahito Yamazaki, and Taichi Usui

Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Shizuoka 422-8529, Japan

CREST, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan

Kyoto Pharmaceutical University, 5 Misasagi-Nakauchicho, Yamashinaku, Kyoto 607-8414, Japan

Department of Physics, Faculty of Science, Shizuoka University, 836 Ohya, Shizuoka 422-8529, Japan

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Neoglycolipids composed of disaccharide glycoside and phospholipid were designed and prepared as mimetics of lactosylceramide. The lactosyl- and N-acetyllactosaminyl-phospholipids (Lac-DPPE and LacNAc-DPPA) were enzymatically synthesized from lactose and LacNAc respectively by cellulase-mediated condensation with 1,6-hexanediol, followed by conjugation of the resulting glycosides and dipalmitoylphosphatidylcholine (DPPC) mediated by Streptomyces phosholipase D. Alternatively, allyl β-lactoside was ozonolysed to give an aldehyde, which was condensed with dipalmitoylphosphatidyl ethanolamine to afford a second type of glycolipid (Lac-DPPE). NMR spectroscopy indicated that the neoglycolipids behave differently in different solvent systems. X-ray diffraction clearly showed that multilamellar vesicles (MLVs) of Lac-DPPE and Lac-DPPA-MLV are in the bilayer gel phase at 20 °C, whereas those of Lac-DPPE-MLV were in the lamellar liquid-crystalline phase at 50 °C. Differential scanning calorimetry showed that Lac-DPPE-MLV had complex thermotropic behavior depending on the incubation conditions. After a long incubation at 10 °C, endothermic transitions are observed at 39.6, 42.3 °C, and 42.9 °C. These neoglycolipids have the ability to trap calcine, a chelating derivative of fluorescein, in MLVs and showed specific binding to lectin in plate assays using fluorescently labeled compounds.

Key words: cellulase; liposome; neoglycolipid; phospholipase D; lectin

Glycoconjugates containing galactose are involved in a variety of biological events. For example, lactosylceramide (LacCer), a pivotal intermediate in the degradation and synthesis of many complex glycosphingolipids, including gangliosides,1–3) is known to be involved in cell-cell and cell-matrix interactions and in signaling events linked to cell differentiation, development, apoptosis, and oncogenesis.4,5) These applications, however, appear to have limited applicability, due to the difficulties in producing naturally occurring glycolipids such as LacCer in large quantities and in purified forms. The commercial production of liposomes containing such naturally occurring substance is therefore unfeasible. The role of galactose in cell-surface recognition has also been investigated, and such studies have led to the development of neoglycolipids both as potential carriers for transporting molecules to the liver and as inhibitors of cell adhesion.6–10) Many researchers have reported that neoglycolipids are useful not only as tools and probes in searches for carbohydrate recognition molecules but also as glycomaterials (nanomaterials).11–15)

In general, liposomes can be formed by suspending a suitable lipid in aqueous media through a self-assembly process. Glycoliposomes appear to represent a special type of multivalent cluster glycoside, because glycolipids accommodated in the bilayer of vesicles can exhibit rapid lateral mobility. Owing to their rapid lateral mobility, glycoliposomes may be useful for the delivery of molecules to target tissues or cells. For these reasons, synthetic glycolipids might be effective in the practical
application of liposomes to the delivery of molecules to target tissues or cells, especially in terms of ease of handling, of quantitative controls for liposome preparation, and of definitive identification of the saccharide determinant, because it is likely that they can be produced in large quantities.

In this paper, efficient enzymatic and chemo-enzymatic procedures for synthesizing two types of neoglycolipid as LacCer mimetics, which are made up of disaccharide and lipid parts connected by a linker, are described. Transformation of the neoglycolipids into glycoliposomes, their specific binding to lectin, and the structural features and properties of the glycoliposomes are also described.

Materials and Methods

Materials. Crude cellulase (EC 3. 2. 1. 4) preparation (cellulase XL-552) from Trichoderma reesei was purchased from Nagase Chemtex; 1,2-Dipalmitoylphosphatidylcholine (DPPC) and 1,2-dipalmitoylphosphatidyl ethanolamine (DPPE) were purchased from Wako Pure Chemical Industries. N-Acetylglucosamine, allyl β-lactoside, and 3-hydroxypropyl β-lactoside were prepared by previously described methods.16,17) Calcein (2,7′-bis(carboxymethyl)-amino)methyl-fluorescein) and 1-palmitoyl-2-[12-[7-nitro-2-1,3-benzoxadiazol]amino]dodecyl-sn-glycero-3-phosphatidyl choline (NBD-PC) were purchased from Molecular Probes, and Avanti Polar lipid, respectively. RCA120 (Ricinus communis agglutinin) and fluorescein-isothiocyanate-labeled RCA (FITC-RCA120) were purchased from Seikagaku. All other reagents were of the highest quality commercially available and were used without further purification.

Analytical methods. Condensation reactions between 1,6-hexanediol and either lactose or N-acetylglucosamine mediated by cellulase were performed and then analyzed on a TLC plate (Merck Kieselgel 60/F254) by the orcinol-sulfuric acid method. FAB-mass analysis was carried out in the positive ion mode using a JEOL JMS DX-303HF mass spectrometer coupled to a JEOL DAB-800 mass data system. An accelerating voltage of 10 kV and a mass resolution of 1,000 was used. A sample of 1 μl in H2O or CH3OH was loaded with 1 μl of glycerol as a matrix.18) and 13C-NMR spectra of each sample in D2O, CD3OD, CDC13/CD3OD, or CDCl3 were recorded on a JEOL JNM-LA 500 or JEOL JNM-EX 270 spectrometer at 30°C. Chemical shifts were expressed in δ relative to sodium 3-(trimethylsilyl)-propionate (TPS) as an external standard.

Hydrolitic activities. To measure Lacβ-pNP hydolyzing activities, a mixture containing 25 μl of substrate (10 mM), 70 μl of 50 mM sodium acetate buffer (pH 5.5), and 5 μl of enzyme solution was incubated in a 96-well microplate for 30 min at 37°C. An aliquot of 10 μl of the reaction mixture was added to 190 μl of 1 mM sodium bicarbonate to terminate the reaction. The p-nitrophenol released was measured spectrophotometrically at 405 nm in a microplate reader (Biolumin 960, Amersham Biosciences). One unit of enzyme was defined as the amount releasing 1 μmol of p-nitrophenol per min.

Enzymatic synthesis of disaccharide glycocides. (a) 6-Hydroxyhexyl β-lactosidase (Lacβ-H). The crude cellulase from T. reesei was partially purified by removing unwanted β-D-galactosidase by our previously described method.19) A mixture containing 5 mmol of lactose, 20 mmol of 1,6-hexanediol, and partially purified enzyme (150 U of Lacβ-pNP hydrolytic activity) in 5 ml of 50 mM sodium acetate buffer (pH 5.0) was incubated at 40°C with vigorous stirring for 119 h. The reaction was terminated by heating at 100°C for 5 min, and the supernatant obtained from centrifugation (6,010 g, 20 min) was loaded onto a charcoal-Celite column (φ5.0 × 45 cm) equilibrated with 5-liter of 10% ethanol. After the column was washed with 10% ethanol, the adsorbed portion was eluted with a linear gradient of 10 to 50% ethanol in a total volume of 6-liter with a fraction size of 45 ml/tube. The neutral sugar contents of the eluted fractions were measured at 485 nm by the phenol-sulfuric acid method. The chromatogram showed one peak (F-1, tubes 80–108) in an adsorbed fraction. The corresponding fractions were pooled, concentrated, and lyophilized to afford 6-hydroxyhexyl β-lactosidase (185 mg, 8.4% yield on the basis of Lac). 6-Hydroxyhexyl β-lactoside, m/z 443 (M+H)+, and 465 (M+Na)+, 1H NMR data (in D2O): δ 4.47 (d, 1H, J 8.3 Hz, H-1), δ 4.44 (d, 1H, J 7.8 Hz, H-1′), δ 1.62 (m, 2H, 6-hydroxyhexyl group-2), δ 1.55 (m, 2H, 6-hydroxyhexyl-5), δ 1.37 (m, 4H, 6-hydroxyhexyl group-3,4). The 13C NMR data in D2O are summarized in Table 1.

(b) 6-Hydroxyhexyl β-N-acetylactosaminidase (LacNacβ-H). A reaction mixture containing 2.6 mmol of N-acetylactosamine, 10.3 mmol of 1,6-hexanediol, and partially purified enzyme (78 U of Lacβ-pNP hydrolytic activity) in 2.6 ml of 50 mM sodium acetate buffer was incubated at 40°C with vigorous stirring for 118 h. The reaction was terminated by heating at 100°C for 5 min. The supernatant obtained from centrifugation (6,010 g, 30 min) was loaded onto a charcoal-Celite column (φ5 × 45 cm) equilibrated with 5-liter of distilled water. The column was eluted with a linear gradient of 0–50% ethanol in a total volume of 6-liter, and the flow-through was monitored by measuring the absorbance at 210 nm (N-acetyl group) and 485 nm. The chromatogram showed two peaks, a sharp peak (F-1, tubes 33-61) and a broad peak (F-2, tubes 97–157). F-2 was evaporated to dryness, dissolved in 1 ml of CHCl3/MeOH/H2O (6:4:1, by volume), and loaded onto a Silica gel 60N column (φ2.5 × 50 cm). The column was eluted with the same solvent into 15-ml fractions at a
flow rate of 10 mL/min, and the flow-through was monitored at 210 and 485 nm. Fractions containing the eluted sample (tubes 23–37) were evaporated to dryness. In a manner similar to that described above, the resulting syrup was further dissolved in 1 mL of CHCl₃/MeOH/H₂O (7:3:0.5, by volume) and developed on the silica gel 60N column with the same solvent. Fractions containing the eluted sample (tubes 61–80) were evaporated to dryness. The desalted product was further purified by silica gel 60N chromatography (φ2.0 x 27 cm, CHCl₃/MeOH/H₂O, 7:3:0.5, by volume) into 8-mL fractions. The fractions that stained as described above were pooled, concentrated and lyophilized to afford Lac-DPPA (87 mg) with a yield of 36% on the basis of Lacβ-HD added. Lac-DPPA, m/z 1111 (M + K)+ and 1071 (M – H)+ (matrix; thioglycerol), 1H NMR data in (CDCl₃/CD₂OD (1:1)): δ 4.21 (d, 1H, J 8.0 Hz, Glc-1), δ 4.28 (d, 1H, J 7.6 Hz, Gal-1), δ 1.58 (m, 2H, 6-hydroxyhexyl-2), δ 1.41 (m, 2H, 6-hydroxyhexyl-5), δ 1.23 (m, 4H, 6-hydroxyhexyl-3, 4). δ 2.29 (dd, 4H, α-CH₂ of palmitoyl), δ 1.58 (m, 4H, β-CH₂ of palmitoyl), δ 1.23 (m, 48H, –CH₂– of palmitoyl), δ 0.85 (t, 6H, CH₃ of palmitoyl). The 13C NMR data in CD₂O are summarized in Table 1.

Enzymatic synthesis of artificial glycolipid. (a) Lac-DPPA. Lacβ-HD (100 mg, 0.3 mmol) was dissolved in 0.5 mL of 40 mM sodium phosphate buffer (pH 5.5) containing 6.8 mM CaCl₂. To the solution was successively added DPPC (332 mg, 0.7 mmol) in 2 mL of chloroform and PLD from Streptomyces sp. (34.2 U) with vigorous stirring, and the mixture was stirred at 30°C according to previously reported procedures. 14LD enzyme (34 U) was added again after 22 and 44 h, and the reaction was continued for 80 h. Four milliliters of methanol was then added to the reaction mixture, and the supernatant obtained from centrifugation (4,800 g, 20 s) was loaded onto a Sep-Pak Accell QMA column (10 cc; CHCl₃/MeOH/H₂O, 30:60:8, by volume). After the column was washed with 150 mL of solvent, the adsorbed portion was eluted stepwise in 20-mL fractions with the same solvent containing 0.2 M (200 mL) and 1.0 M (75 mL) of sodium acetate. Each eluted fraction was checked by TLC (CH₃Cl/MeOH/H₂O, 6:4:1, by volume) and the fractions that stained both with Dittmer reagent and with orcinol-sulfuric acid were collected and concentrated to dryness. The resulting sample was desalted by a Sephadex LH-20 column (φ1.5 x 43 cm; CHCl₃/MeOH/H₂O, 5:5:1, by volume). The desalted product was further purified by silica gel 60N chromatography (φ2.0 x 27 cm, CHCl₃/MeOH/H₂O, 7:3:0.5, by volume) into 8-mL fractions. The fractions that stained as described above were pooled, concentrated and lyophilized to afford Lac-DPPA (87 mg) with a yield of 36% on the basis of Lacβ-HD added. Lac-DPPA, m/z 1111 (M + K)+ and 1071 (M – H)+ (matrix; thioglycerol), 1H NMR data in (CDCl₃/CD₂OD (1:1)): δ 4.21 (d, 1H, J 8.0 Hz, Glc-1), δ 4.28 (d, 1H, J 7.6 Hz, Gal-1), δ 1.58 (m, 2H, 6-hydroxyhexyl-2), δ 1.41 (m, 2H, 6-hydroxyhexyl-5), δ 1.23 (m, 4H, 6-hydroxyhexyl-3, 4). δ 2.29 (dd, 4H, α-CH₂ of palmitoyl), δ 1.58 (m, 4H, β-CH₂ of palmitoyl), δ 1.23 (m, 48H, –CH₂– of palmitoyl), δ 0.85 (t, 6H, CH₃ of palmitoyl). The 13C NMR data in CD₂O are summarized in Table 1.

(b) LacNac-DPPA. LacNac-DPPA was prepared by PLD-mediated phosphatidyl transfer from a DPPC donor (152 mg, 0.2 mmol) to a LacNacβ-HD acceptor (50 mg, 0.1 mmol) using a procedure similar to that used to prepare Lac-DPPA. The target compound was obtained as a lyophilized powder of 37 mg (33% yield on the basis of the donor added). LacNac-DPPA, m/z 1136 (M + Na)+ and 1112 (M – H)+ (matrix; thioglycerol), 1H NMR data in (CDCl₃/CD₂OD (4:1)): δ 4.23 (d, 1H, J 8.0 Hz, GlcNac-1), δ 4.26 (d, 1H, J 7.6 Hz, Gal-1), δ 1.92 (s, 3H, NHCOCH₃), δ 1.44 (m, 2H, 6-hydroxyhexyl group-2), δ 1.10 (m, 4H, 6-hydroxyhexyl group-3, 4), δ 1.24 (m, 2H, 6-hydroxyhexyl-5). δ 2.15 (dd, 4H, α-CH₂ of palmitoyl), δ 1.44 (m, 4H, β-CH₂ of palmitoyl), δ 1.10 (m, 48H, –CH₂– of palmitoyl), δ 0.72 (t, 6H, CH₃ of palmitoyl). The 13C NMR data in CDCl₃/CD₂OD are summarized in Table 2.

(c) 1-Palmitoyl-2-[12-[7-nitro-2-1,3-benzoxadiazol]-amino[dodecyl]-phosphatidylhexyl β-lactoside (LacNBD-PA). LacNBD-PA was also prepared by PLD-mediated phosphatidyl transfer from an NBD-PC donor (30 mg, 0.04 mmol) to a Lacβ-HD acceptor (100 mg, 0.2 mmol) using a procedure similar to that used for Lac-
### Table 2. $^{13}$C Chemical Shifts of Neoglycolipids Containing Disaccharides in CDCl$_3$/CD$_3$OD Solution

1,2-Dipalmitoyl-phosphatidyl hexyl $\beta$-lactoside (Lac$\beta$-DPPA)

<table>
<thead>
<tr>
<th>Chemical Shifts (s)</th>
<th>C-1</th>
<th>C-2</th>
<th>C-3</th>
<th>C-4</th>
<th>C-5</th>
<th>C-6</th>
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<th>C-$\beta$</th>
<th>C-$\gamma$</th>
<th>C-$\delta$</th>
<th>C-$\epsilon$</th>
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<td>Glc</td>
<td>103.0</td>
<td>73.6</td>
<td>75.0</td>
<td>80.4</td>
<td>75.2</td>
<td>61.4</td>
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<tr>
<td>Gal</td>
<td>104.2</td>
<td>71.4</td>
<td>73.8</td>
<td>69.6</td>
<td>76.0</td>
<td>62.0</td>
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<td>Phosphatidic acid</td>
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<td>23.0~32.3</td>
<td>25.2~25.3</td>
<td>34.5~34.6</td>
<td>174.0~174.4</td>
<td>63.1</td>
<td>70.9</td>
<td>63.8</td>
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$^{13}$C Chemical Shifts of Neoglycolipids Containing Disaccharides in CDCl$_3$/CD$_3$OD Solution

1,2-Dipalmitoyl-phosphatidyl hexyl $\beta$-N-acetyllactosaminide (LacNAc$\beta$-DPPA)

<table>
<thead>
<tr>
<th>Chemical Shifts (s)</th>
<th>C-1</th>
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<th>C-3</th>
<th>C-4</th>
<th>C-5</th>
<th>C-6</th>
<th>C-$\alpha$</th>
<th>C-$\beta$</th>
<th>C-$\gamma$</th>
<th>C-$\delta$</th>
<th>C-$\epsilon$</th>
<th>C-$\xi$</th>
<th>$\text{CH}_2\text{CO}$</th>
<th>$\text{CO}$</th>
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<td>GlcNAc</td>
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<td>56.1</td>
<td>74.1</td>
<td>80.4</td>
<td>75.6</td>
<td>61.4</td>
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<td></td>
<td>23.2</td>
<td>174.3</td>
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<tr>
<td>Gal</td>
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<td>72.0</td>
<td>73.5</td>
<td>69.7</td>
<td>76.4</td>
<td>62.1</td>
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<td>Allyl alcohol</td>
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<tr>
<td>Phosphatidic acid</td>
<td>14.3</td>
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<td>25.5~25.6</td>
<td>34.7~34.9</td>
<td>174.3~174.7</td>
<td>63.2/63.3</td>
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<td>64.1</td>
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*Chemical shifts are shown in ppm downfield from internal TPS.
DPPA. The target compound was obtained as a lyophilized powder of 0.9 mg (1.9% yield on the basis of the donor added). Lac-NBD-PA, m/z 1193 (M – H)\(^-\) (matrix; thioglycerol), \(^1\)H-NMR data (in CDCl\(_3\)/CD\(_3\)OD = 1/1): \(\delta\) 4.21 (d, 1H, J 7.7 Hz, Glc-1), \(\delta\) 8.43 (d, 1H, J 7.8, nitrobenzoxadiazol-m), \(\delta\) 6.19 (d, 1H, J 7.8, nitrobenzoxadiazol-o), \(\delta\) 4.28 (d, 1H, J 7.6 Hz, Gal-1\(^\prime\)), \(\delta\) 1.53 (m, 2H, 6-hydroxyhexyl-2), \(\delta\) 1.37 (m, 2H, 6-hydroxyhexyl-5), \(\delta\) 1.21 (m, 4H, 6-hydroxyhexyl-3,4), \(\delta\) 2.23 (dd, 4H, \(\alpha\)-CH\(_2\) of palmitoyl and aminodecyl-11), \(\delta\) 1.53 (m, 4H, \(\beta\)-CH\(_2\) of palmitoyl and aminodecyl-10), \(\delta\) 1.70 (t, 2H, aminodecyl-1), \(\delta\) 1.34 (m, 2H, \(\beta\)-CH\(_2\) of palmitoyl), \(\delta\) 1.18 (m, 2H, \(-\text{CH}_2\)- of palmitoyl), \(\delta\) 0.79 (t, 3H, CH\(_3\)- of palmitoyl).

**Chemical synthesis of artificial glycolipid.** Lac-DPPE. Lac\(\beta\)-allyl (50 mg, 0.1 mmol) was dissolved in MeOH (45 ml). The solution was cooled to \(-78^\circ\text{C}\) and treated with bubbling through nitrogen for more than 12 h. 20-wt% lipid concentration of Lac-DPPA in non-excess water or a 20-wt% lipid concentration of Lac-DPPE in excess water or a matrix (thioglycerol), lyophilized powder of 0.9 mg (1.9% yield on the basis of the donor added). The mixtures were added to the dry thin lipid film to obtain a 50 mM lipid concentration of Lac-DPPE in excess water or a 20-wt% lipid concentration of Lac-DPPE in non-excess water.

**Table 3.** \(^{13}\)C Chemical Shifts of 1,2-Dipalmitoyl-Phosphatidyl Ethanolaminyl \(\beta\)-Lactoside in CDCl\(_3\)/CD\(_3\)OD Solution

<table>
<thead>
<tr>
<th>12-Dipalmitoyl-phosphatidyl ethanolaminyl (\beta)-lactoside</th>
<th>Chemical Shifts ((\delta))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glc</td>
<td>103.0 C-1 C-2 C-3 C-4 C-5 C-6 Glc-O-CH(_2)-</td>
</tr>
<tr>
<td>Gal</td>
<td>104.2 C-1 C-2 C-3 C-4 C-5 C-6 Glc-O-CH(_2)-</td>
</tr>
<tr>
<td>Ethanolaminyl-ethyl</td>
<td>71.4 71.8 80.4 80.4 72.2 76.0</td>
</tr>
<tr>
<td>Phosphatidic acid</td>
<td>134.9 153.4 153.4</td>
</tr>
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</table>

\(\delta\) values are shown as ppm downfield from internal TSP.

**X-ray diffraction.** For X-ray diffraction measurements, lipid membranes in aqueous solution were prepared as follows: An appropriate lipid solution of Lac-DPPE, Lac-DPPA, or Lac-DPPA/DPPC, dissolved in CHCl\(_3\)/MeOH (1/1 by volume), was dried first in a stream of nitrogen to form a thin lipid film and then under vacuum with a rotary pump for more than 12 h. Appropriate amounts of 10 mM PIPES buffer (pH 7.0) were added to the dry thin lipid film to obtain a 50 mM lipid concentration of Lac-DPPE in excess water or a 20-wt% lipid concentration of Lac-DPPE in non-excess water.
water conditions. For the 20-mol% Lac-DPPA/80 mol% DPPC mixture, appropriate amounts of 10 mM PIPES buffer (pH 7.0) containing 100 mM NaCl were added to the dry thin lipid film to give a 50 mM lipid concentration in excess water. The suspensions were then vortexed several times for about 30 s at about 55°C. The Lac-DPPE suspension was centrifuged at 13,000 × g for 30 min at 20°C, and the resulting pellets were used as samples in excess water for X-ray diffraction. The Lac-DPPA and Lac-DPPA/DPPC suspensions were also treated in the same way. X-ray diffraction experiments were performed with nickel-filtered Cu Kα X-rays (λ = 0.154 nm) generated from a rotating anode-type X-ray generator (Rigaku, Rotaflex, RU-300) under operating conditions of 40 kV × 200 mA.21,22) Small-angle X-ray scattering (SAXS) data were recorded using a linear (one-dimensional) position-sensitive proportional counter (PSPC) (Rigaku, PSPC-5) with a camera length of 350 mm and associated electronics (multichannel analyzer, etc., Rigaku). Wide-angle X-ray scattering (WAXS) patterns were recorded using a one-dimensional PSPC with a sample-to-detector distance of 250 mm, and diffraction spacings were calibrated using polyethylene. In all cases, samples were sealed in a thin-walled glass capillary tube (outer diameter 1.0 mm) and mounted in a temperature-controlled holder whose stability was +0.2°C.

**Ultrasensitive differential scanning calorimetry (ultra-sensitive DSC).** For ultra-sensitive DSC measurements, lipid membranes in aqueous solution were prepared as follows: An appropriate lipid solution of Lac-DPPE or Lac-DPPA, dissolved in CHCl₃/MeOH (1/1 by volume), was dried first in a stream of nitrogen to form a thin lipid film and then under vacuum with a rotary pump for more than 12 h. Appropriate amounts of 20 mM phosphate buffer (pH 7.0) were added to the dry lipids to obtain a 2 mM lipid concentration in excess water, and the suspensions were vortexed several times for about 30 s at about 55°C.

DSC experiments23) were performed with an ultra-sensitive microcalorimeter (VP-DSC, Micorocal, Northampton). The feedback mode was set to high, and the filtering period was set to 5 s. Lac-DPPE-MLV or Lac-DPPA-MLV dispersed in 20 mM phosphate buffer (pH 7.0) (for the raw sample data) and buffer alone (for the reference data) were heated at a rate of 1.0 K/min from 10°C to 80°C. Corrected sample data were obtained by subtracting the reference data from the raw sample data. Under this condition, the noise level of the DSC curve was in the range of 2–3 μcal/°C. Phase-transition temperatures were determined by the peak of the endothermic curve.23)

**Inclusion ability of glycoliposomes.** The inclusion ability of neoglycolipids was analyzed by a previously reported procedure.24) Lac-DPPE or Lac-DPPA/DPPC was dissolved in a mixture of CHCl₃/MeOH (1/1 by volume). The solution was then thoroughly evaporated in vacuo to form a thin film. To this film, 0.1 mM of calcein in PBS (pH 7.4) was added (final lipid concentration: 1 mM), and the resulting solution was sonicated for 5 min with a sonicator and warmed at 80°C to form a vesicle. The sample obtained was transferred to a slide glass and 10 mM CoCl₂ was added to quench the calcein that was not incorporated into the vesicles. The vesicles were then observed under a fluorescence microscope (IX-FLA, Olympus Optical).

**Binding assay.** (a) Lectin binding assay for neoglycolipids immobilized on a microtiter plate. Neoglycolipids dissolved in a small amount of CHCl₃/CH₃OH were diluted with ethanol and pipetted onto a microtiter plate at 4.5 ng/well to 4.5 μg/well (96-well flat-bottom plastic plate, 0.36 ml/well volume). The plate was incubated at 37°C for 2 h to remove the solvent completely. The wells were blocked with 100 μl of 2% bovine serum albumin (BSA) in PBS for 2 h at 25°C and washed with PBS five times before use. Fifty microliters of FITC-RCA₁₂₀ (lectin) (10 μl/ml) was then added to the neoglycolipid-immobilized wells, followed by incubation at 4°C for 5 h. The wells were then washed with PBS five times. After the plate was washed, the fluorescence intensity from FITC-labeled lectin was measured with a microplate reader (Biolumina 960) at excitation and emission wavelengths of 485 and 520 nm respectively.

(b) Liposome binding assay on a lectin-immobilized microtiter plate. Fifty microliters of RCA₁₂₀ (10 μg/ml) in PBS was pipetted into a microtiter plate (96-well flat-bottom plastic plate, 0.36 ml/well), which was then incubated for 1 h at room temperature and allowed to stand overnight at 4°C. After removing the solution, the wells were blocked with 100 μl of 2% BSA/PBS overnight at 4°C. After being washed with PBS containing 0.05% Tween 20 three times, the wells were further washed with the same buffer immediately before use. A control experiment was done in parallel using BSA instead of RCA₁₂₀. Fifty microliters of liposomes containing various concentrations from 5.0 × 10⁻¹² to 5.0 × 10⁻⁵ (μM) of 5 mol% Lac-NBD-PA/95 mol% DPPC or 5 mol% Lac-DPPA/95 mol% DPPC as a control was pipetted into the RCA₁₂₀-immobilized plate and incubated overnight at 4°C. After removing the solution, the wells were washed with PBS five times and then 100 μl of PBS was added. The fluorescence intensity from the bound NBD-labeled liposomes was then measured as in the FITC-labeled lectin-binding assay described above.

**Results.**

**Enzymatic synthesis of 1,2-dipalmitoyl-phosphatidyl hexyl β-lactoside (Lac-DPPA) and 1,2-dipalmitoyl-phosphatidyl hexyl β-N-acetyllactosaminide (LacNAC-DPPA)**
Recently we found that endo-\(\beta\)-glycosidase, which is a cellulase from \textit{T. reesei}, catalyzes two types of reaction, transglycosylation and condensation.\(^{17,18}\) This enzymatic catalysis has been harnessed for the present synthesis of lactosyl and \(N\)-acetyllactosaminyl glycosides. A condensation reaction between Lac and 1,6-hexanediol was performed using a partially purified enzyme preparation from \textit{T. reesei}. The molar ratio of lactose to 1,6-hexanediol was 1:4, and the total substrate concentration was 52% (w/v) in the reaction mixture. The reaction mixture was easily fractionated with a charcoal-Celite column. Production of the desired compound reached a maximal level at about 100 h, with a yield of 8.4% on the basis of the amount of lactose added. No glycosylation to bilational hydroxyl groups in 1,6-hexanediol was detected by HPLC analysis during the entire course of reaction (data not shown). Structural analysis confirmed that the product was Lac\(\beta\)-HD. LacN\(\text{Ac}\beta\)-HD was synthesized similarly by a condensation reaction between Lac\(\text{Ac}\) and 1,6-hexanediol. The target product was obtained by successive chromatographic steps on charcoal-Celite and Silica gel 60N columns in 7.1% yield on the basis of the amount of Lac\(\text{Ac}\) added.

PLD, which catalyzes the hydrolysis of the terminal phosphate diester bond of glycerophospholipids to release phosphatidic acid, has been shown to transfer the phosphatidyl group from phospholipids to primary alcohols.\(^{11,19}\) This phosphatidyl transfer also occurs in PLD-mediated coupling reactions with Lac\(\beta\)-HD and Lac\(\text{Ac}\beta\)-HD, as shown in Scheme 1. This transphosphatidylation was performed in a chloroform/aqueous buffer two-phase system with DPPC as a donor and Lac\(\beta\)-HD as an acceptor. The mixture was shaken at 25°C for 124 h and fresh enzyme was added twice during the course of the reaction. The resulting product was purified by successive chromatographic steps on Sep-pak Accell QMA, Sephadex LH-20, and Silica gel 60N columns to afford the target product Lac-DPPA in 36% yield on the basis of the amount of acceptor added. Similarly, PLD catalyzed the formation of Lac\(\text{Ac}\)-DPPA from a Lac\(\text{Ac}\beta\)-HD acceptor in 33% yield. When the donor was changed to fluorescently labeled NBD-PC, however, transphosphatidylation of the Lac\(\beta\)-HD acceptor resulted in a very low yield of Lac-NBDPA (1.9%).

\textit{Chemo-enzymatic synthesis of 1,2-dipalmitoyl-phosphatidyl ethanol-aminoethyl \(\beta\)-lactoside (Lac-DPPE)}

Allyl \(\beta\)-lactoside prepared by a cellulase-mediated condensation reaction, as previously described,\(^{17,19}\) was used as a starting substance. The double bond in the allyl group of this compound was ozonolyzed to give an aldehyde,\(^{20}\) which was condensed with 1,2-dipalmitoyl-phosphatidyl ethanolamine in the presence of sodium cyanotrihydroborate to afford the target compound Lac-
DPPE in a yield of 87%. In this reductive amination, it was important to heat the reaction mixture of aldehyde and DPPE at 70°C for 2 h before reduction with the reducing reagent. The synthesis of Lac-DPPE is shown in Scheme 2.

Structural investigation of neoglycolipids in solvent
Lac-DPPA and Lac-DPPE exhibited interesting structural features in different organic solvent systems. The 1H and 13C NMR spectra of Lac-DPPE are represented in Fig. 1. In CDCl3/CD3OD (1/1), both glycolipids showed clear 1H and 13C signals that reflected the structure of a single molecule. In CDCl3, however, peak broadening in the 1H NMR spectra was seen in the hydrophilic lactoside and glyceroyl portions, and peak reduction or disappearance (e.g., 174, 103, 104, and 80–66) was seen in the 13C NMR spectra. This indicates that in CDCl3 the neoglycolipids probably associate to form inverse micelles; that is, the polar ends of the phospholipids are buried inside, and a less polar shell is on the outside. By contrast, DPPC showed characteristic 1H and 13C NMR spectra in both solvent systems as a single molecule.

Membrane structure and phase behavior of neoglycolipids
We investigated the structures of MLVs of Lac-DPPE in 10 mM PIPES buffer (pH 7.0) at 20°C using X-ray diffraction. The small-angle X-ray scattering (SAXS) pattern of Lac-DPPE in excess water showed a set of three SAXS peaks with spacing (d1 = 7.9 nm) in a 1:2:3 ratio, indicating that the Lac-DPPE membrane was in a lamellar bilayer structure (Fig. 2A). This spacing is larger than that of DPPC (6.5 nm). The wide-angle X-ray scattering (WAXS) pattern of Lac-DPPE in the same buffer at 20°C consisted of a strong, broad reflection at 1/0.415 nm–1 (Fig. 2B), showing an ordered chain...
packing. These data indicate that the Lac-DPPE membrane at 20 °C in excess water was in the bilayer gel phase. At 50 °C, the SAXS pattern showed a set of three peaks with spacing \((d_1 = 7.1 \text{ nm})\) in a 1:2:3 ratio, and the WAXS pattern was very diffuse at 1/0.4–1/0.5 nm, indicating that the Lac-DPPE membrane was in a lamellar liquid-crystalline (L\(_\alpha\)) phase.

Phase transitions of 2.0 mM Lac-DPPE-MLV in 20 mM phosphate buffer (pH 7.2) were investigated by using ultra-sensitive DSC. Figure 3 shows representative DSC heating curves recorded from 10 to 80 °C at a heating rate of 1.0 °C/min using an ultra-sensitive microcalorimeter, VP-DSC. (A) First heating scan. Freshly prepared Lac-DPPE-MLV dispersion was incubated for 20 min at 10 °C before the heating scan was done; (B) Second heating scan. The sample was cooled to 10 °C after the first heating scan and incubated at 10 °C for 25 min before the second scan was done. (C) Third heating scan. The sample was cooled to 10 °C after the second heating scan and incubated at 10 °C for 17 h before the third scan was done.

Next, we investigated structures and phase transition of MLVs of Lac-DPPA. The SAXS pattern of Lac-DPPA-MLV in 10 mM PIPES buffer (pH 7.0) at a low water content (20 wt% liquid concentration, which is not an excess water condition) at 20 °C showed two SAXS peaks with spacing \((d_1 = 8.5 \text{ nm})\) in a 1:2 ratio, and its WAXS pattern consisted of two broad reflections at about 1/0.42 nm\(^{-1}\) and 1/0.39 nm\(^{-1}\), showing an ordered chain packing. These data indicate that the Lac-DPPA membrane at 20 °C was in the bilayer gel phase. At 50 °C, the SAXS pattern consisted of a broad strong peak, and the WAXS pattern was very diffuse at 1/0.4–1/0.5 nm, indicating a melted \((i.e.,\) disordered\)) chain packing such as L\(_\alpha\) phase. In the first DSC heating scan of 2.0 mM Lac-DPPA-MLV in 20 mM phosphate buffer (pH 7.2), a sharp endothermic transition was observed at 34.4 °C. In a second scan done after cooling the Lac-DPPA-MLV sample to 10 °C and incubating it at 10 °C for 25 min, however, there was no endothermic transition. This hysteresis shows that the phase transition from the structure with melted chains to the bilayer gel phase requires a long time. More-detailed studies on the structure with the melted chains at a high temperature and phase transition are necessary to characterize Lac-DPPA-MLV further. We also examined the structure of a membrane of a mixture of 20 mol% Lac-DPPA/80 mol% DPPC mixture in 10 mM PIPES buffer (pH 7.0) containing 100 mM NaCl (in excess water) at 20 °C. The SAXS pattern showed two peaks with spacing \((d_1 = 7.9 \text{ nm})\) in a 1:1 ratio, indicating that the membrane was in a lamellar bilayer structure.
Inclusion ability of calcein by glycoliposomes

The ability of glycoliposomes to trap calcein, a chelating derivative of fluorescein, in vesicles was analyzed by fluorescence microscopy. When calcein is used as a marker of the aqueous compartment, any calcein that is not trapped inside the liposome does not need to be removed because the addition of a quenching cobalt compound suffices to eliminate the fluorescence of the external phase. Liposomes consisting of various compositions of glycolipid and DPPC were prepared in PBS (pH 7.4) containing 0.1 mM calcein. After the fluorescence from free calcein had been eliminated by the addition of cobalt, the liposomes were observed under a fluorescence microscope, as shown in Fig. 4. Glycoliposomes containing 5–100 mol% Lac-DPPE, 5–40 mol% Lac-DPPA, and 5–80 mol% LacCer showed the ability to trap calcein.

Lectin binding assay

(a) Lectin binding assay with neoglycolipids immobilized on microtiter plates

In order to confirm the specificity of neoglycolipids binding to lectin, a binding assay of FITC-RCA₁₂₀ to glycolipids coated on the solid phase was performed. The neoglycolipids Lac-DPPE and Lac-DPPA, as well as LacCer as a positive control, were directly immobilized on microtiter plates (96 wells each), and then FITC-RCA₁₂₀ was added to the plates. The fluorescence intensity from bound lectin increased linearly with increasing amounts (4.5 ng to 4.5 μg per well) of immobilized neoglycolipid on the plates for Lac-DPPE but not for Lac-DPPA, as in Fig. 5. It was possible to detect lectin binding to Lac-DPPE, Lac-DPPA, and LacCer immobilized at concentrations of 4.5 ng/well, 45 ng/well, and 4.5 μg/well, respectively. Thus, the ability to bind lectin in this plate assay increased in the order: LacCer < Lac-DPPA << Lac-DPPE.

(b) Liposome binding assay with lectin immobilized on microtiter plates

Fluorescence-labeled glycoliposomes containing 5% Lac-NBD-PA in DPPC obtained by small unilamellar vesicle preparation were added to RCA₁₂₀-immobilized plates. Non-labeled liposomes containing 5% Lac-DPPA/DPPC and BSA-immobilized plates were used as control samples. Figure 6 shows the binding of fluorescently labeled glycoliposomes to RCA₁₂₀ immobilized in the solid phase. The fluorescence intensity from Lac-NBD-PA liposomes bound to RCA₁₂₀ showed a dependence on glycoliposome concentrations in the range of 0.1 μM to 0.1 mM. These results show that, compared to the control assays using BSA, the fluorescently labeled glycoliposomes binds specifically to lectin.

Discussion

Our interest was first directed towards establishing a method for the efficient synthesis of lactosyl glycoside as a precursor of neoglycolipid in sufficient amounts. The enzymatic process for obtaining Lacβ-HD and LacNAcβ-HD, which are formed by a condensation reaction mediated by Trichoderma cellulase, is simple, and the yield is sufficiently high to make the method practical. It is worth noting that the glycosylation of 1,6-hexanediol is selective for the unilateral hydroxy group and does not target the other hydroxyl.

On the basis of this result, we embarked on the design of LacCer mimetics that carry a double-chain lipid. We chose DPPC as the lipid part of our first target molecule, because it is one of the most widely distributed lipids.
The resulting disaccharide glycosides facilitated the synthesis of novel lactosyl- and N-acetyllactosaminylphospholipids via enzymatic transphosphatidylation mediated by PLD. Thus the transphosphatidylation of Lacβ-HD and LacNAcβ-HD was regioselective for the primary alcohol of the hydroxyhexyl group on the aglycon moiety. By contrast, replacement of DPPC by a NBD-PC donor markedly diminished the efficiency of transphosphatidylation. This suggests that an N-substituted 7-nitro-2-oxa-1,3-diazoyl (NBD) group in the lipid part serves as a sterical block against the catalytic activity of PLD. As an alternative method, a neoglycolipid composed of phosphatidylethanolamine and lactosyl β-glycoside was designed and conveniently prepared as a LacCer mimetic. Thus, allyl β-lactoside was chemically transformed into a second type of neoglycolipid by the following two steps: ozonolysis of the allyl function to afford an aldehyde, followed by conjugation of this aldehyde to phosphatidylethanolamine by reductive amination. Lac-DPPE was obtained from allyl β-lactoside in a high yield (86%). From these results, we developed enzymatic and chemo-enzymatic procedures for obtaining two types of neoglycolipid, comprising disaccharide and lipid parts connected by a linker.

These neoglycolipids exhibited interesting structural features in different solvent systems, as indicated by their NMR spectra. A possible explanation for the different spectral features is that in CDCl$_3$/CD$_3$OD, Lac-DPPE and Lac-DPPE exist as single molecules, whereas in CDCl$_3$, they aggregate with the hydrophilic portion (including the lactoside and the polar ends of the phospholipids) buried inside, suggesting the formation of inverse micelles. By contrast, LacCer exhibited characteristic NMR spectra (all H and C peaks were observed) in CDCl$_3$/CD$_3$OD, and it was very difficult to detect the signals in CDCl$_3$ under the same condition, due to its poor solubility. This suggests that LacCer has less polarity and less tendency to form aggregates in CDCl$_3$. The same phenomenon has been observed in NMR spectra of phospholipid-inhibitor conjugates, as reported by Wong et al.$^{(19)}$ A full understanding of this observation will require further physical studies.

The X-ray diffraction experiments clearly showed that Lac-DPPE-MLV was in the bilayer gel phase at 20 °C and that Lac-DPPE-MLV was in the Lα phase at 50 °C. Lac-DPPE-MLV showed complex thermotropic behavior depending on the incubation conditions. In the final DSC curve of Lac-DPPE-MLV (Fig. 3C), endothermic transitions were observed at 39.6 °C, 41.3 °C, and 42.9 °C. In the first scan (Fig. 3A), the phase transition at the highest temperature was not clear, suggesting that a long incubation time is needed for complete equilibration of the sample. Freeze thawing of the sample did not change the results of the phase transition temperatures. Such a complex thermotropic behavior has been observed in many glycosphingolipids.$^{(25)}$ For example, in the DSC curve of C16:0-lactosyl-ceramide, endothermic transitions has been observed at 66 °C, 69 °C, 72 °C, and 78 °C (corresponding to the chain-melting phase transition temperature).$^{(26)}$ Intermolecular interaction between sugars, such as the lactose of lipid head groups, is one of the main causes of this complicated thermotropic behavior. At present, however, we have no detailed information on the structures of the intermediate phases or detailed schemes of the intermolecular interactions of the lactose moiety. The highest phase-transition temperature of Lac-DPPE (42.9 °C) corresponds to its chain-melting phase transition temperature, which is much lower than that of DPPE (61.5 °C). This is probably due to the large steric repulsive interaction between the large-sized head groups (i.e., lactosyl-phosphoethanolamine group).$^{(21,25)}$

The ability of glycoliposomes containing Lac-DPPE or Lac-DPPE to trap calcein was analyzed by fluorescence microscopy. In this case, it is notable that a sample containing Lac-DPPE alone self-assembled to form liposomes that possessed the ability to incorporate calcein. A sample containing more than 50 mol% Lac-DPPE, however, did not show this ability. This difference in inclusion abilities perhaps results from the difference between the electrostatic characters on the linkers. Thus, Lac-DPPE, which has an acidic phosphoryl group and a basic nitrogen atom in the linker, is apparently neutral, suggesting that it does not show electrostatic interactions with calcein containing two carboxyl groups. As a result, it readily forms liposomes that trap calcein.

Studies of the interactions between lectin and the sugar moiety in the neoglycolipids were performed by microtiter plate assays. Neoglycolipid immobilized on the microtiter plate showed a higher sensitivity than LacCer for FITC-RCA$_{120}$. It was possible to detect the

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**Fig. 6.** Binding Assay of Liposome Containing Lac-NBD-PA to RCA$_{120}$ Immobilized on a Microtiter Plate.

Between 5.0 × 10$^{-12}$ and 5.0 × 10$^{-5}$ (m) of 5 mol% Lac-NBD-PA/95 mol% DPPC was added to a microtiter plate containing immobilized RCA$_{120}$ and incubated overnight at 4 °C. After the plate was washed, the fluorescence intensity of the FITC-labeled lectin was measured with a microplate reader at excitation and emission wavelengths of 485 and 520 nm respectively. Data are expressed as the mean ± SEM. Each assay was carried out in duplicate.
lectin binding at very low concentrations (ng/well) of immobilized neoglycolipids. In this case, a difference in sensitivity between the neoglycolipids and LacCer was clearly observed. The linker present in neoglycolipids is perhaps effective for providing the mobility required for lectin interaction. To confirm the binding observed by another assay, fluorescently labeled Lac-NBD-PA was prepared and its binding to lectin immobilized on plates was assayed. As expected, the assay system showed high prepared and its binding to lectin immobilized on plates another assay, fluorescently labeled Lac-NBD-PA was

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References

9) Yamauchi, H., Kikuchi, H., Sawada, M., Tomikawa, M., and Hirota, S., Characterization and tissue distribution of liposomes containing lactose mono-fatty acid deriva-
14) Stoll, M. S., Feizi, T., Loreless, R. W., Chai, W., Lawson, A. M., and Yuen, C. T., Fluorescent neo-
glycolipids: improved probes for oligosaccharide ligand


