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Novel fluorescent analogues for transmembrane movement study of polyprenyl phosphates

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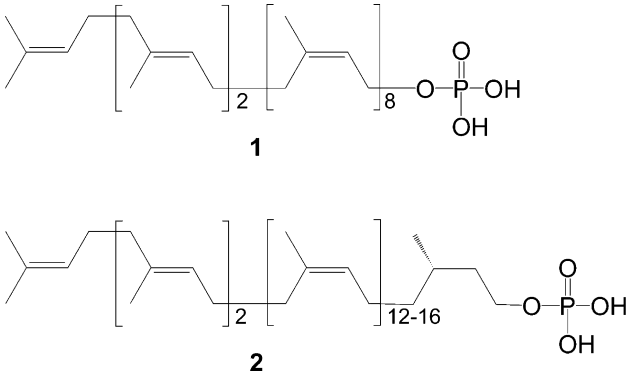
Abstract—In order to investigate the transmembrane movement of polyprenyl phosphate across biological membranes, NBD (7-nitrobenz-2-oxa-1,3-diazol-4-yl)-labeled polyprenyl phosphate analogues were prepared. These analogues proved to be possible tools for a direct observation of the transmembrane flip-flop movement of polyprenyl phosphates by use of a sodium dithionite-quenching procedure.

cis-Polyprenyl phosphates with carbon chain lengths of C_{55}–C_{120} function as glycosyl carrier lipids in the biosynthesis of cell wall of bacteria or archaea and in the biosynthesis of glycoproteins in eukaryotes.[1–3] Undecaprenyl phosphate (C_{55}–P, **1**, Scheme 1) is a key carrier lipid of various precursors involved in bacterial cell wall biosynthesis. The hydrophobic undecaprenyl moiety acts as the anchor into the membrane to mediate the proper location and processing of the hydrophilic pentapeptide oligosaccharide unit of the mature precursor of peptidoglycan (undecaprenyl pyrophosphate-MurNAc-GlcNAc-pentapeptide, the so-called Lipid II) at the inner side of the plasma membrane and to transport the pentapeptide-sugar unit from inside to outside of the membrane across the hydrophobic environment of phospholipid bilayer of plasma membrane.[4] After releasing the pentapeptide-sugar unit for the peptidoglycan construction in periplasm, the carrier lipid **1** is proposed to be recycled by the other flip-flop mechanisms into the inner leaflet of bacterial plasma membrane in order to maintain such a rapid cell division cycle.[4]



Scheme 1. Structures of undecaprenyl phosphate **1** and dolichyl phosphate **2**.

Although the flip-flop recycling of **1** is quite interesting from mechanistic and physiological viewpoints, there has been no procedure on the direct observation of the flip-flop movement of **1** in bacterial cells. Similar flip-flop transmembrane recycling of the carrier lipid, dolichyl phosphate ($C_{80–120}$–P, **2**, Scheme 1), has been thought to exist in eukaryotic endoplasmic reticulum (ER) membrane in the biosynthesis of glycoproteins.[4] Previously, the synthesis of spin-labeled polyprenyl phosphates and the transmembrane movement of these analogues were reported.[5,6] But direct proofs of the flip-flop movement in both prokaryotic plasma membrane and eukaryotic ER membrane have not yet been obtained. Radiolabeled (^{3}H-labeled) compounds were also used for clarifying the flip-flop mechanism.[7] However, lots of preparation steps are needed to discriminate the distribution of the probes in the inner/outer leaflet of phospholipid membrane. On the other hand, various fluorescent phospholipid probes for the flip-flop movement of phospholipids are now commercially available, leading to the biochemical and biophysical understanding of the transbilayer movement

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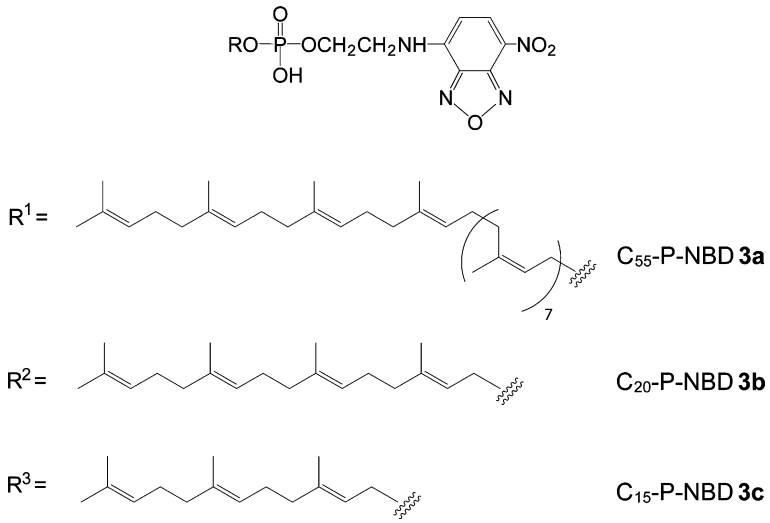
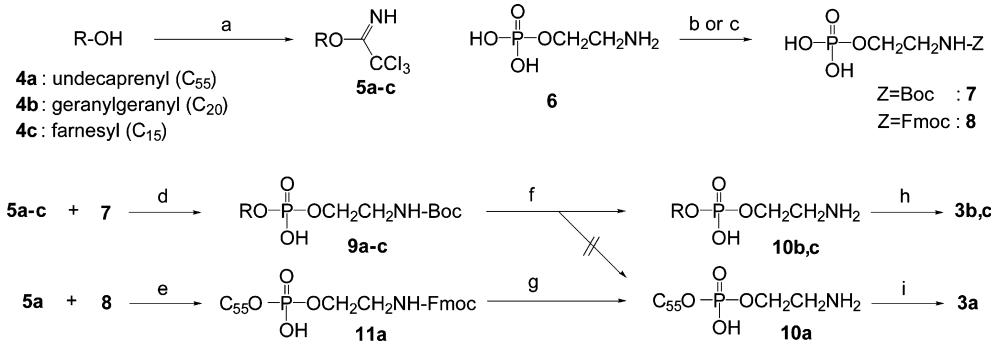
of phospholipids in cell membrane in vivo and in vitro. But fluorescent probes for the flip-flop of **1** and **2** are not commercially available.

The NBD (7-nitrobenz-2-oxa-1,3-diazol-4-yl) group is an extensively used fluorophore in biophysical, biochemical, and cell biological studies. For examples, NBD-labeled phospholipids[8] and glycosphingolipids[9] are potent fluorescent probes, leading to the biochemical and biophysical understanding of the protein-assisted transbilayer movement in membrane. Dithionite reduction of the fluorophore allows the rapid quantitative discrimination between inner and outer NBD probes in the membrane, affording the determination of inner/outer NBD probe distribution. For understanding the flip-flop of **1**, NBD-labeled polyprenyl phosphate analogues would be useful tools. In order to demonstrate the direct flip-flop motion of **1** in phospholipid membranes, we exploited the synthesis of NBD-polyprenyl phosphate analogues **3a**–**c** (Scheme 2).

The fluorescent analogues **3a**–**c** were prepared according to the procedure described in Scheme 3. DBU (1,8-diazabicyclo[5.4.0]undec-7-ene)-catalyzed reaction of polyprenols from **4a**–**c** with trichloroacetonitrile gave the trichloroacetimidates **5a**, **-b**, and **-c** in 85%, 90%, and 78% yield, respectively.[10,11] The corresponding phosphodiesters were obtained in two pathways. Amino group protection of 2-aminoethyl dihydrogen phosphate **6** with Boc_2O provided the *N*-Boc compound **7** in 77% yield.[12] The trichloroacetimidates **5a**–**c** were subjected to the coupling reaction with **7** in $CHCl_3$ to afford **9a**, **-b**, and **-c** in 47%, 45%, and 53% yield, respectively.[13] The relatively low yields are due to partial decomposition of the trichloroacetimidates. Deprotection of the *N*-Boc residue from **9b**, **c** with NaOH afforded the corresponding amines **10b** and **-c** in 37% and 45% yield, respectively.[14] But deprotection of the *N*-Boc group from **9a** was not successful under the similar conditions. To obtain **10a**, we selected *N*-(9-fluorenylmethoxycarbonyloxy)succinimide (Fmoc-OSu) for protection of the amino group of **6** to afford **8** in 89% yield.[15] The trichloroacetimidate **5a** was subjected to the coupling reaction with **8** to afford **11a** in 53% yield.[16] Deprotection of the *N*-Fmoc group from **11a** with 20% piperidine in CH_2Cl_2 provided the corresponding amine **10a** in 84% yield.[17] Reaction of **10a**–**c** with NBD-Cl in the presence of triethylamine in $CHCl_3$ or in the presence of Na_2CO_3 in 1,4-dioxane/water (1:1) gave the fluorescent analogues **3a**, **-b**, and **-c** in 89%, 20%, and 11% yields, respectively.[18,19] Total yield of



Scheme 2. Fluorescent polyprenyl phosphate analogues **3a**–**c**.



Scheme 3. Synthesis of fluorescent polyprenyl phosphate analogues **3a**–**c**. Reagents and conditions: (a) Cl_3CCN, DBU, CH_2Cl_2, rt, 9 h; (b) Boc_2O, Na_2CO_3, 1,4-dioxane:H_2O (1:1), 0 °C → rt, 17 h; (c) Fmoc-OSu, Na_2CO_3, 1,4-dioxane:H_2O (3:2), rt, 1 h; (d) CH_2Cl_2, rt, 3 h; (e) 1,4-dioxane, rt, 15 min; (f) 50% NaOH, 1,4-dioxane, reflux, 1 h; (g) piperidine, CH_2Cl_2, rt, 5 min; (h) NBD-Cl, Na_2CO_3, 1,4-dioxane:H_2O (1:1), rt, 20 h; (i) NBD-Cl, Et_3N, $CHCl_3$, rt, 2 h.

3a–**c** from the starting material **4a**–**c** were 34%, 3%, and 2%, respectively. The maximum UV absorption spectrum of these fluorescent analogues **3a**–**c** in MeOH was observed at the wavelength of 463 nm. The fluorescence excitation and emission wavelengths of **3a**–**c** in MeOH were found to be 462 and 542 nm, respectively.

In order to evaluate whether the NBD-labeled polyprenyl phosphate analogues **3a**–**c** would work as probes for the flip-flop movement, we prepared large unilamellar vesicle (LUV) liposomes of egg phosphatidylcholine (PC) in the presence of these analogues by freeze–thawing and extrusion techniques.[20] We found that the intensity of the fluorescence emission of **3a**–**c** in egg PC-LUV liposome depended linearly on the concentration of **3a**–**c** incorporated into the liposome (Fig. 1). These spectra indicate

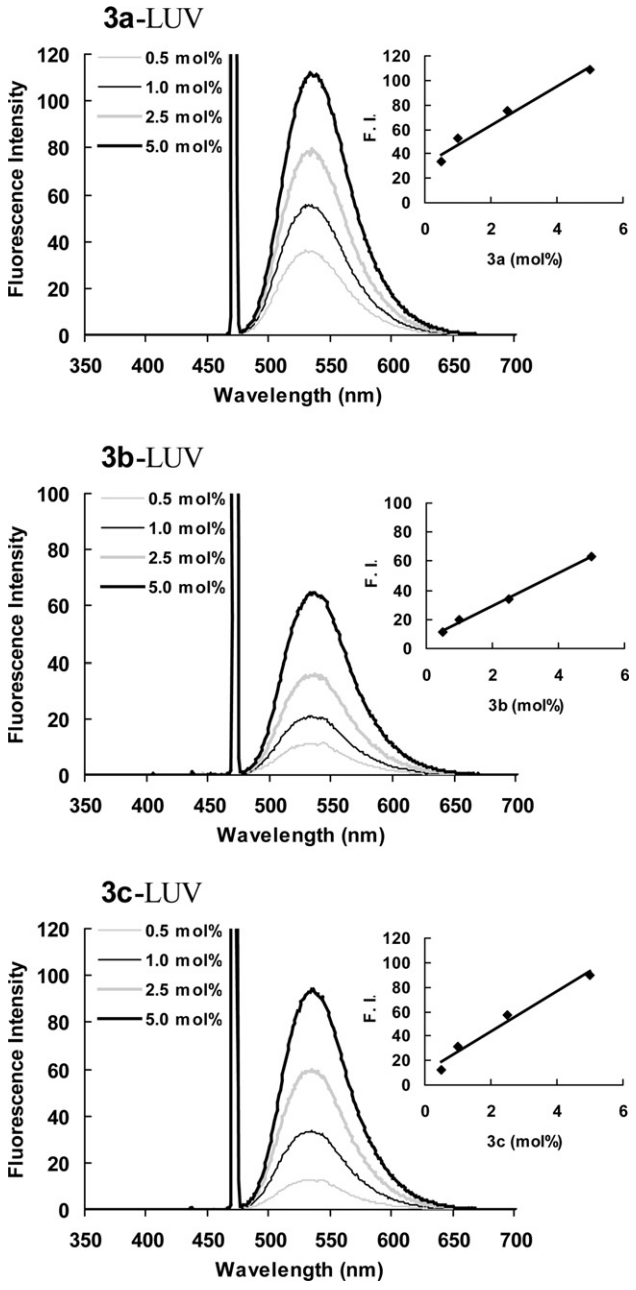


Figure 1. Fluorescence emission spectra of **3a**–**c** in egg PC-LUV.

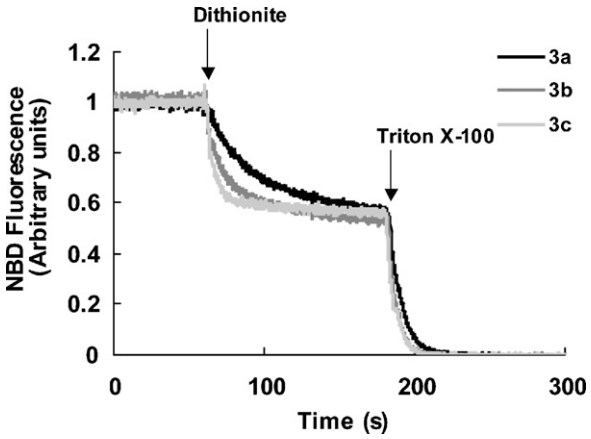


Figure 2. Fluorescence quenching experiment of NBD-labeled analogues in egg PC-LUV.

that the amount of NBD-analogues can be quantitatively determined in phospholipid liposome by monitoring the fluorescence intensity of the analogues. The distribution of the direction of **3a**–**c** in the phospholipid bilayer membrane can also be determined by quenching the NBD group headed to the outside of the membrane by sodium dithionite ($Na_2S_2O_4$), which is impermeable to the membrane. As shown in Figure 2, approximately half of the total fluorescence intensity of **3a**–**c** in egg PC-LUV was quenched after the addition of dithionite.[21] These declines of the fluorescence level correspond to the dithionite-mediated reduction of the analogues localized in the outer leaflet of the liposome. The relative differences in the profile of fluorescence declines by dithionite among **3a**–**c** in the liposome depend on the polyprenyl chain length of these NBD-analogues. Then, the residual fluorescence intensity was diminished rapidly by addition of a detergent Triton X-100, indicating the amount of the analogues localized in the inner leaflet of the liposome. Taking the fluorescence intensity data in the dithionite quenching assay into account, in spite of the differences in the profile of fluorescence declines, these NBD-analogues **3a**–**c** can be reasonably assigned to be distributed equally into both of the leaflets of the liposome. This is a first report that polyprenyl phosphate analogues labeled with NBD-fluorophore are expected to be potent functional probes for flip-flop measurement of polyprenyl phosphate in biological membrane.

In summary, we prepared fluorescent analogues **3a**–**c**. The intensity of the fluorescence emission of **3a**–**c** in the egg PC-LUV liposome was linearly dependent on the concentration of **3a**–**c** incorporated into the liposome. The distribution of the fluorescent analogues in phospholipid membrane was almost symmetrical. We conclude that these analogues could be employed as probes for analyzing the transbilayer movement of polyprenyl phosphates in biological membranes.

Acknowledgments

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nol purified from silkworm feces. We also thank professor Kazue Kurihara in our institute for helpful discussions. This work was supported by Nagase Science and Technology Foundation and by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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11. Compound **5a**: To a solution of undecaprenol **4a** (117 mg, 0.152 mmol) in dry CH_2Cl_2 (12 mL) at room temperature was added CH_2Cl_2 (2 mL) solution of DBU (39.0 mg, 0.256 mmol). After stirring at room temperature for 20 min, a solution of Cl_3CCN (127 mg, 0.880 mmol) in CH_2Cl_2 (2 mL) was added dropwise to the mixture. The resultant solution was stirred at room temperature for 9 h. The reaction mixture was concentrated and dissolved in *n*-hexane/ethyl acetate (2:1, 20 mL), and then washed with H_2O and saturated NaCl. The organic layer was concentrated and the residue was purified by flash chromatography on silica gel with *n*-hexane/ethyl acetate (5:1) to afford **5a** as yellow oil (118 mg, yield 85%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.55–1.65 (m, 12H), 1.68 (s, 21H), 1.79 (s, 3H), 1.9–2.2 (m, 40H), 4.78 (dd, J = 0.9, 7.1 Hz, 2H), 5.13 (m, 10H), 5.49 (t, J = 7.1 Hz, 1H), 8.24 (s, 1H). Compound **5b**: **5b** was prepared from **4b** in the similar manner as described for **5a**. Yield 90%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.60 (s, 9H), 1.68 (s, 3H), 1.74 (s, 3H), 1.9–2.2 (m, 12H), 4.84 (d, J = 6.8 Hz, 2H), 5.10 (m, 3H), 5.48 (t, J = 6.8 Hz, 1H), 8.25 (s, 1H). Compound **5c**: **5c** was prepared from **4c** in the similar manner as described for **5a**. Yield 78%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.60 (s, 6H), 1.66 (s, 3H), 1.74 (s, 3H), 1.9–2.2 (m, 8H), 4.81 (d, J = 7.1 Hz, 2H), 5.08 (m, 2H), 5.48 (t, J = 7.1 Hz, 1H), 8.25 (s, 1H).
12. Compound **7**: To a solution of 2-aminoethylphosphate **6** (300 mg, 2.13 mmol) and Na_2CO_3 (225 mg, 2.12 mmol) in 1,4-dioxane/H_2O (1:1 v/v, 100 mL) was added a solution of Boc_2O (700 mg, 3.21 mmol) in 1,4-dioxane (5 mL) at 0 °C. After stirring at room temperature for additional 17 h, the solution was concentrated. The residue was dissolved in 2-PrOH/H_2O (3:2) and purified by flash chromatography on silica gel with 2-PrOH/H_2O (3:2). The residue was dissolved in water and passed through a column of Dowex 50W-X2 (H^+ form) resin. The fractionate was adjusted to pH 7 by addition of pyridine and evaporated to give **7** as an amorphous solid (555 mg, yield 82%). ^{1}H NMR (400 MHz, D_2O) δ 1.42 (s, 9H), 3.30 (t, J = 5.4 Hz, 2H), 3.90 (dd, J = 5.4, 12.0 Hz, 2H).
13. Compound **9a**: To a solution of **7** (48.2 mg, 0.150 mmol) in CH_2Cl_2 (6 mL) was added CH_2Cl_2 (2 mL) solution of **5a** (137 mg, 0.150 mmol). After stirring at room temperature for 3 h, the reaction mixture was concentrated and the residue was partially purified by flash chromatography on silica gel with $CHCl_3/CH_3OH/H_2O$ (30:8:1) to afford a crude product **9a**, which was used in the next step without further purification. Compound **9b**, **9c**: **9b** and **9c** were prepared from **5b** or **5c** in the similar manner as described for **9a**.
14. Compound **10b**: A solution of crude **9b** (41.8 mg) in 1,4-dioxane (4 mL) and 8 mL of 50% NaOHaq was refluxed for 1 h. After the mixture was cooled, it was extracted with 1,4-dioxane, washed with saturated NaCl/H_2O (1:1), and concentrated. The residue was purified by flash chromatography on silica gel with $CHCl_3/CH_3OH/H_2O$ (65:25:4). After the fractionate was concentrated, the residue was dissolved in 25 mM NH_4HCO_3/CH_3OH (2:3) and passed through a column of Dowex 50W-X2 (NH_4^+ form) resin to give **10b** (12.6 mg, yield 17% for two steps from **5b**).^{1}H NMR (400 MHz, $CDCl_3$) δ 1.60–1.70 (m, 15H), 1.90–2.10 (m, 12H), 3.17 (m, 2H), 4.08 (brs, 2H), 4.38 (brs, 2H), 5.10 (m, 3H), 5.37 (m, 1H), 8.46 (br, 2H). Compound **10c**: **10c** was prepared from **9c** in the similar manner as described for **10b** (yield 24% for two steps from **5c**). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.60–1.70 (s, 12H), 1.90–2.10 (m, 8H), 3.15 (brs, 2H), 4.07 (brs, 2H), 4.38 (brs, 2H), 5.08 (m, 2H), 5.35 (m, 1H), 8.43 (br, 2H).
15. Compound **8**: To a solution of 2-aminoethylphosphate **6** (100 mg, 0.71 mmol) and Na_2CO_3 (380 mg, 3.59 mmol) in 1,4-dioxane/H_2O (3:2 v/v, 25 mL) was added a solution of Fmoc-OSu (360 mg, 1.07 mmol) in 1,4-dioxane (5 mL). After stirring at room temperature for 1 h, acetone (120 mL) was added, and the precipitate was filtered. The filtrate was concentrated, dissolved in 2-PrOH/H_2O (3:2), and purified by flash chromatography on silica gel with 2-PrOH/H_2O (3:2). After the fractionate was concentrated, the residue was dissolved in water and passed through a column of Dowex 50W-X2 (H^+ form) resin to give **8** as a white solid (228 mg, yield 89%). ^{1}H NMR (400 MHz, CD_3OD) δ 3.38 (t, J = 5.6 Hz, 2H), 4.00 (dd, J = 5.6, 12.3 Hz, 2H), 4.17 (t, J = 7.1 Hz, 1H), 4.31 (d, J = 7.1 Hz, 2H), 7.29 (td, J = 1.0, 7.6 Hz 2H), 7.37 (t, J = 7.3 Hz, 2H), 7.62 (d, J = 7.6 Hz, 2H), 7.76 (d, J = 7.3 Hz, 2H).
16. Compound **11a**: To a solution of **8** (46.6 mg, 0.128 mmol) in 1,4-dioxane (3 mL) was added 1,4-dioxane (1 mL) solution of **5a** (117 mg, 0.128 mmol). After stirring at room temperature for 15 min, the pH of the mixture was adjusted to pH 9 by addition of saturated aqueous $NaHCO_3$. The reaction mixture was concentrated and the residue was partially purified by flash chromatography on silica gel with $CHCl_3/CH_3OH/H_2O$ (65:25:4) to afford a crude product **11a**, which was used in the next step without further purification.
17. Compound **10a**: To a solution of piperidine (4 mL) in CH_2Cl_2 (16 mL) was added CH_2Cl_2 (2 mL) solution of the crude **11a** (76.2 mg, 0.068 mmol). After stirring at room temperature for 5 min, water (5 mL) was added. The organic layer was separated and concentrated. The residue was purified by flash chromatography on silica gel with $CHCl_3/CH_3OH/H_2O$ (65:25:4) to afford **10a** (51.3 mg, yield 45% for two steps from **5a**). ^{1}H NMR (400 MHz, $CDCl_3$). δ 1.60 (s, 12H), 1.68 (s, 24H), 1.90–2.10 (m, 40H), 3.13 (s, 2H), 4.07 (s, 2H), 4.38 (s, 2H), 5.12 (s, 10H), 5.34 (s, 1H), 8.44 (br, 2H).

18. Compound **3a**: To a solution of **10a** (51.3 mg, 0.058 mmol) in $CHCl_3$ (3 mL) was added triethylamine (2 μL) as a catalyst followed by dropwise addition of NBD-Cl (38.0 mg, 0.190 mmol) in $CHCl_3$ (1 mL). The reaction mixture was maintained at room temperature for 2 h. The mixture was concentrated and then dissolved in $CHCl_3$/CH_3OH. The organic phase was washed with water and 5% $NaHCO_3$, and evaporated. The residue was purified by flash chromatography on silica gel with ethyl acetate and further chromatographed using a mixture of $CHCl_3$/CH_3OH with increasing amount of CH_3OH in $CHCl_3$ (4:1, 3:1). The residue was dissolved in $CHCl_3$/CH_3OH/H_2O (65:25:4) and passed through a column of Dowex 50W-X2 (triethylammonium form) resin to give **3a** as a yellowish brown solid (228 mg, yield 89%). ^1H NMR (400 MHz, $CDCl_3$, Et_3NH^+ form) δ 1.32 (t, $J = 7.3$ Hz, 9H), 1.60 (s, 12H), 1.68 (s, 24H), 1.9–2.1 (m, 40H), 3.11 (m, 6H), 3.60 (m, 2H), 4.31 (m, 2H), 4.46 (t, $J = 6.9$ Hz, 2H), 5.12 (s, 10H), 5.39 (s, 1H), 6.05 (s, 1H), 8.43 (s, 1H). MALDI-TOF m/z $[M-H]^-$ calcd. 1051.70, found 1051.48.
19. Compound **3b**: To a solution of **10b** (15.8 mg, 0.030 mmol) in 1,4-dioxane/H_2O (1:1, 3 mL) were added 60 mM NaOH (40 μL) and Na_2CO_3 (6.3 mg, 0.059 mmol) in H_2O (0.5 mL), followed by dropwise addition of NBD-Cl (12.0 mg, 0.060 mmol) in 1,4-dioxane (0.5 mL) at 0 °C and then warming to room temperature over 20 h. The reaction mixture was concentrated, dissolved in ethyl acetate, and then was purified by flash chromatography on silica gel with ethyl acetate and further chromatographed with $CHCl_3$/CH_3OH/H_2O (30:8:1). The fractionate was dissolved in 2-propanol/25 mM aqueous NH_4HCO_3 (3:2) and passed through a column of Dowex 50W-X2 (ammonium form) resin to give **3b** as a yellowish brown solid (3.6 mg, yield 20%). ^1H NMR (400 MHz, $CDCl_3$) δ 1.50–1.70 (m, 15H), 1.9–2.1 (m, 12H), 3.49 (brs, 2H), 4.20 (brs, 2H), 4.39 (brs, 2H), 5.08 (m, 3H), 5.30 (brs, 1H), 5.87 (brs, 1H), 8.19 (brs, 1H). MALDI-TOF m/z $[M-H]^-$ calcd 575.26, found 575.18. Compound **3c**: **3c** was prepared from **10c** as a triethylammonium salt in the similar manner as described for **3b** (yield 11%). ^1H NMR (400 MHz, $CDCl_3$) δ 1.32 (t, $J = 7.3$ Hz, 9H), 1.50–1.70 (m, 12H), 1.90–2.10 (m, 8H), 3.15 (m, 6H), 3.60 (brs, 2H), 4.29 (brs, 2H), 4.47 (brs, 2H), 5.08 (brs, 2H), 5.37 (brs, 1H), 6.02 (brs, 1H), 8.43 (brs, 1H). MALDI-TOF m/z $[M-H]^-$ calcd 507.20, found 507.10.
20. Preparation of liposome: Egg PC liposome containing NBD-labeled probe was prepared as follows. Egg PC and NBD-probe (**3a**–**c**, 2.5 mmol% of egg PC) were dissolved in $CHCl_3$/MeOH (3:1). The lipid mixture was transferred to a glass tube, then evaporated under a stream of nitrogen and dried under vacuum for 2 h. The lipid film was hydrated by vortex mixing in 2 mL of 50 mM Tris–HCl (pH 7.5), then kept at room temperature overnight. The lipid suspension was rapidly frozen by submergence into liquid nitrogen, followed by melting by incubation in water at room temperature. The freeze–thawing cycle was repeated 5 times and the resultant liposomes were extruded three times through a 0.2 μm polycarbonate membrane (Anotop 10, Whatman, Maidstone, UK).
21. Dithionite quenching assay: All fluorescence measurements were performed in 50 mM Tris–HCl (pH 7.5). 1.2 mL of NBD-labeled LUV suspension was placed in a fluorescence cuvette and fluorescence was recorded at room temperature under continuous stirring using a Shimadzu RF-5300PC spectrometer. The excitation and emission wavelengths were set at 470 and 530 nm, respectively. After 60 s, 12 μL of 1 M sodium dithionite solution was injected into the cuvette (final concentration 10 mM). The concentration of dithionite was enough to diminish the fluorescence of NBD-probes localized in outer leaflet of liposome. Additionally, after 180 s, 30 μL of 20% Triton X-100 solution was added (final concentration 0.5%).