Supplemental Data

Occurrence of Bacterial Membrane Microdomain at the Cell Division Site Enriched in Phospholipids with Polyunsaturated Hydrocarbon Chain

Sho Sato, Jun Kawamoto, Satoshi B. Sato, Bunta Watanabe, Jun Hiratake, Nobuyoshi Esaki, and Tatsuo Kurihara

Supplementary Experimental Procedures

Synthesis of fluorescent probes

Unless otherwise specified, materials were obtained from commercial suppliers—Sigma-Aldrich Co., Tokyo Chemical Industry Co., Ltd., and Wako Pure Chemical Industries, Ltd.—at the highest level of purity available and were used without further purification. Dry solvents were purchased from Wako Pure Chemical Industries, Ltd., and used in the form supplied. Flash chromatography was carried out using silica gel 60N (spherical and neutral, particle diameter: 40–50 μm; Kanto Chemical Co., Inc.). Thin-layer chromatography (TLC) was performed on glass-backed precoated silica gel plates (Merck silica gel 60 F_{254} and 60). Spots were detected with a UV hand lamp at 254 nm or by staining with either iodine vapor or 0.65% (w/v) molybdate dissolved in H_2SO_4/H_2O (10:90 v/v). Synthesized compounds were characterized by NMR (^{1}H and, if appropriate, ^{31}P) and/or MS. NMR spectra were recorded using a Bruker UltraShield^{TM} Advance 400 (400 MHz, ^{1}H; 160 MHz, ^{31}P) spectrometer or JEOL JNM-AL-300 (300 MHz, ^{1}H) FT NMR system spectrometer. The instrument was calibrated using residual undeuterated solvent and tetramethylsilane as internal references. Mass spectra were recorded by electrospray ionization mass spectrometry

(ESI-MS) with an Applied Biosystems API3000 LC/MS/MS system.

Diacyl-EPA and Diacyl-OLA were synthesized by introducing the NBD group into phosphatidylethanolamine containing EPA (OEPE) and OLA (DOPE), respectively, by the same method described for compound **14**. OEPE and DOPE were synthesized as described previously (1). The synthetic route for other fluorescent probes used in this study is summarized in Scheme S1. The methods for the synthesis of individual compounds are described below.

Compound **3**

To a magnetically stirred mixture of oleic acid (**1**, 2.51 g, 8.90 mmol) in 40 ml dry tetrahydrofuran (THF) on ice was added $LiAlH_4$ (1.01 g, 26.7 mmol) by bits. The reaction mixture was stirred at room temperature for 1 h and then cooled on ice. Excess $LiAlH_4$ was quenched with 1 ml of water and 4 ml of 10% (w/v) aqueous NaOH solution, and the mixture was dried over magnesium sulfate. The mixture was then suction-filtered, and the filtrate was evaporated to afford the crude product (2.49 g) as a colorless oil, which was used for the subsequent synthesis without further purification. *Rf* = 0.15 (benzene). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 5.42-5.28 (m, 2H), 3.64 (dt, 2H, *J* = 5.4 and 5.4), 2.12-1.96 (m, 4H), 1.57 (tt, 2H, *J* = 7.2 and 7.2), 1.43-1.19 (m, 22H), 0.89 (t, 3H, *J* = 6.9).

Compound **4**

Using a procedure similar to that described for the preparation of **3**, crude compound **4** (0.826 g) was obtained from EPA (0.765 g, 2.53 mmol). *Rf* = 0.15 (benzene). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 5.62-5.27 (m, 10H), 3.65 (dt, *J* = 6.0 and 6.0, 2H), 2.92-2.74

(m, 8H), 2.18-2.02 (m, 4H), 1.62-1.52 (m, 2H), 1.51-1.39 (m, 2H), 0.98 (t, 3H, *J* = 7.4).

Compound **5**

Compound **3** (2.35 g, 8.75 mmol) and triethylamine (2.70 g, 26.7 mmol) were dissolved in 30 ml of dry benzene at room temperature under a nitrogen atmosphere. To this solution was added methanesulfonyl chloride (3.06 g, 26.7 mmol). After stirring for 2 h, the reaction mixture was suction-filtered, and subsequent removal of solvent of the filtrate under reduced pressure afforded a crude product, which was purified by flash column chromatography (eluted with hexane/ethyl acetate = 95:5 to 80:20) to yield compound **5** (2.45 g, 7.07 mmol) as a colorless oil. *Rf* = 0.35 (benzene). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 5.45-5.30 (m, 2H), 4.22 (t, 2H, *J* = 6.0), 3.01 (s, 3H), 2.08-1.96 (m, 4H), 1.75 (tt, 2H, *J* = 6.8 and 6.8), 1.60-1.10 (m, 22H), 0.89 (t, 3H, *J* = 7.5).

Compound **6**

Using a procedure similar to that described for the preparation of **5**, compound **6** (1.13 g, 3.08 mmol, 93%) was obtained. *Rf* = 0.35 (benzene). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 5.46-5.26 (m, 10H), 4.23 (t, 2H, *J* = 6.5), 3.00 (s, 3H), 2.88-2.75 (m, 8H), 2.16-2.03 (m, 4H), 1.83-1.70 (m, 2H), 1.54-1.44 (m, 2H), 0.98 (t, 3H, *J* = 7.4).

Compound **8**

To a magnetically stirred mixture of NaH (0.136 g, 3.41 mmol) in 5 ml dry DMSO at room temperature was added (*R*)-(2,2-dimethyl-1,3-dioxolan-4-yl)methanol (**7**, 0.300 g, 2.27 mmol) of dissolved in 5 ml dry DMSO dropwise. To the resulting solution, compound **5**

(0.866 g, 2.50 mmol) dissolved in 10 ml dry DMSO was slowly added, and the reaction mixture was stirred for 20 h. The reaction mixture was then poured into water (250 ml) and extracted with hexane (250 ml). The combined organic layers were concentrated under reduced pressure to obtain compound **8** (0.578 g, 1.51 mmol, 67%), which was used for the subsequent synthesis without further purification. *Rf* = 0.54 (hexane/ethyl acetate = 9:1). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 5.40-5.28 (m, 2H), 4.26 (tt, 1H, *J* = 5.6 and 5.6), 4.06 (dd, 1H, *J* = 8.1 and 6.6), 3.73 (dd, 1H, *J* = 8.3 and 6.5), 3.54-3.37 (m, 4H), 2.06-1.94 (m, 4H), 1.60-1.56 (tt, 2H, *J* = 6.0 and 6.0), 1.42 (s, 3H), 1.36 (s, 3H), 1.24-1.34 (m, 22H), 0.82-0.85 (t, 3H, *J* = 7.2).

Compound **9**

Compound **8** (0.578 g, 1.51 mmol) was dissolved in dry $CHCl_3$ (15 ml) at room temperature under a nitrogen atmosphere. To the mixture was added 0.5 N HCl in 15 ml methanol. After stirring for 1 h, subsequent removal of the solvent under reduced pressure afforded **9** (0.535 g, 1.51 mmol, 100%), which was used for the subsequent synthesis without further purification. *Rf* = 0.07 (hexane/ethyl acetate = 3:2).

Compound **10**

Compound **9** (0.535 g, 1.51 mmol) and diisopropylethylamine (0.293 g, 2.27 mmol) were dissolved in 3.1 ml of dry dichloromethane at room temperature under a nitrogen atmosphere. To this solution was added triphenylmethyl chloride (0.462 g, 1.66 mmol). After stirring for 14 h, the reaction mixture was concentrated under reduced pressure to afford a crude product, which was purified by flash column chromatography (eluted with hexane/

ethyl acetate = 98:2 to 95:5) to yield compound **10** (0.861 g, 1.47 mmol, 97%) as a colorless oil. *Rf* = 0.31 (hexane/ethyl acetate = 9:1). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.48-7.22 (m, 15H), 5.35-5.29 (m, 2H), 3.95-3.91 (m, 1H), 3.56-3.40 (m, 4H), 3.21 (dd, 1H, *J* = 9.3 and 5.7), 3.17 (dd, 1H, *J* = 9.3 and 5.4), 2.40 (d, 1H, *J* = 4.8), 2.04-1.98 (m, 4H), 1.59-1.49 (m, 2H), 1.39-1.32 (m, 22H), 0.88 (t, 3H, *J* = 6.9).

Compound **11**

Compound **10** (0.585 g, 1.00 mmol) and powdered KOH (0.272 g, 4.12 mmol) were dissolved in dry benzene (21 ml) at room temperature under a nitrogen atmosphere. Compound **6** (0.367 g, 1.00 mmol) dissolved in 6 ml dry benzene was slowly added to the resulting solution, and the reaction mixture was stirred for 2 h at 40˚C. The mixture was poured into water, and the product was extracted with hexane. After the solvent was removed under the reduced pressure, the crude product was purified by flash column chromatography (eluted with hexane/ethyl acetate = 99:1 to 98:2) to yield compound **11** (0.235 g, 0.275 mmol, 28%) as a colorless oil. *Rf* = 0.63 (hexane/ethyl acetate = 4:1). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.48-7.22 (m, 15H), 5.42-5.29 (m, 12H), 3.60-3.35 (m, 7H), 3.25-3.15 (m, 2H), 2.87-2.75 (m, 8H), 2.12-1.95 (m, 8H), 1.65-1.40 (m, 6H), 1.39-1.21 (m, 22H), 0.97 (t, 3H, *J* = 7.7), 0.88 (t, 3H, *J* = 6.6).

Compound **12**

Compound **11** (0.334 g, 0.457 mmol) was dissolved in dry $CHCl_3$ (25 ml) at room temperature under a nitrogen atmosphere. To the mixture was added 0.5 N HCl in 25 ml methanol. After stirring for 1 h, subsequent removal of the solvent under reduced pressure

afforded the crude product, which was purified by flash column chromatography (eluted with hexane/ethyl acetate = 97:3 to 9:1) to yield compound **12** (0.176 g, 0.287 mmol, 63%) as a colorless oil. *Rf* = 0.26 (hexane/ethyl acetate = 4:1). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 5.45-5.25 (m, 12H), 3.78-3.68 (m, 1H), 3.67-3.48 (m, 6H), 3.48-3.38 (t, 2H, *J* = 6.6), 2.85-2.75 (m, 8H), 2.18-1.90 (m, 8H), 1.63-1.56 (m, 4H), 1.43 (tt, 2H, *J* = 7.7 and 7.7), 1.32-1.26 (m, 22H), 0.98 (t, 3H, *J* = 7.7), 0.88 (t, 3H, *J* = 6.6).

Compound **13**

Phosphorous oxychloride (65.9 mg, 0.431 mmol) was dissolved in 0.45 ml of hexane and stirred at 5˚C. After the addition of triethylamine (43.5 mg, 0.431 mmol) dissolved in 0.9 ml of trichloroethylene, stirring was continued, and a solution of compound **12** (0.176 g, 0.287 mmol) in 3.5 ml of trichloroethylene was added dropwise over 30 min. After 1 h, the reaction mixture was filtered. Then 1 ml of toluene was added to the filtrate, and removed under the reduced pressure to afford the crude oil. To a magnetically stirred solution of the crude oil and triethylamine (116 mg, 1.15 mmol) in 4.3 ml of dry THF at 0˚C was added a solution of 2-aminoethanol (21.9 mg, 0.359 mmol) in 0.5 ml of dry THF. Then, the mixture was warmed to room temperature and kept stirring for 30 min. After an additional 30 min, the reaction mixture was suction-filtered. The filtrate solvent was removed under the reduced pressure to give the white crude oil. To a solution of the white crude oil in 5.7 ml of 2-propanol was added 2.9 ml of acetic acid/water (1:4), and then the reaction mixture was stirred magnetically for 2 h. The mixture was poured into 30 ml of chloroform/methanol/water (1:1:0.9), and the lower layer was collected and evaporated to afford the white solid, which was purified by flash column chromatography (eluted with

chloroform/methanol = 8:2 to 7:3) to yield compound **13** (0.187 g, 0.254 mmol, 89%) as a white solid. *Rf* = 0.44 (chloroform/methanol/28% NH_3 solution = 65:25:4). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.68-8.42 (br s, 2H), 5.48-5.26 (m, 12H), 4.15-4.04 (br s, 2H), 3.91 (dd, 1H, *J* = 9.2 and 5.3), 3.87 (dd, 1H, *J* = 9.3 and 5.3), 3.63-3.35 (m, 7H), 3.23-3.05 (br s, 2H), 2.88-2.76 (m, 8H), 2.12-1.97 (m, 8H), 1.62-1.48 (m, 4H), 1.40 (m, 2H), 1.35-1.20 (m, 22H), 0.97 (t, 3H, *J* = 7.6), 0.88 (t, 3H, *J* = 7.0). ^{31}P-NMR (160 MHz, $CDCl_3$): δ 0.960. ESI-MS: 734.6 $[M-H]^-$ ($C_{43}H_{78}O_6PN$).

Compound **14**

Into a brown-colored flask was placed a chloroform solution (3.5 ml) of compound **13** (36.8 mg, 0.050 mmol), 4-fluoro-7-nitrobenzofurazan (NBD-F) (Dojindo Laboratories, Kumamoto, Japan) (30.2 mg, 0.165 mmol), and triethylamine (17.2 mg, 0.170 mmol) under a nitrogen atmosphere. The mixture was stirred at room temperature for 1 h and subsequent removal of solvent under reduced pressure afforded a crude product, which was purified by flash column chromatography (eluted with benzene/methanol = 9:1) to yield 36.9 mg of compound **14** (36.9 mg, 0.041 mmol, 82 %). *Rf* = 0.73 (chloroform/methanol/28 % NH_3 solution = 65:25:4). ESI-MS: 897.6 $[M-H]^-$. ($C_{49}H_{79}O_9PN_4$).

Compound **16**

To a stirred mixture of NaH (1.09 g, 25.2 mmol) in dry THF (38 ml) was added dropwise (*S*)-(2,2-dimethyl-1,3-dioxolan-4-yl)methanol (**15**, 2.50 g, 18.9 mmol) at 0°C. After it was stirred at room temperature for 30 min and then cooled on ice, *p*-methoxybenzyl chloride (2.96 g, 18.9 mmol) was slowly added to the resulting solution. After refluxing at

70˚C overnight, the solvent was removed under reduced pressure. The residue was redissolved in hexane and suction-filtered. To the filtrate washed with the same volume of water twice was added magnesium sulfate followed by the second suction-filtration. Subsequent removal of the solvent under reduced pressure afforded the crude product **16**, which was used for the subsequent synthesis without further purification. *Rf* = 0.41 (hexane/ethyl acetate = 4:1). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.26 (d, 2H, *J* = 8.4), 6.88 (d, 2H, *J* = 8.4), 4.52 and 4.49 (d, 1H, *J* = 9.9), 4.28 (tt, 1H, *J* = 6.3 and 6.3), 4.04 and 3.72 (dd, 1H, *J* = 8.3 and 6.3), 3.80 (s, 3H), 3.53 and 3.44 (dd, 1H, *J* = 9.7 and 5.4), 1.42 and 1.36 (s, 3H).

Compound **17**

To a magnetically stirred mixture of **16** (5.79 g, 22.9 mmol) dissolved in dry THF (45 ml) on ice was added dropwise 1 N HCl aqueous solution (45 ml). After stirring at room temperature for 10 h, 5 N NaOH aqueous solution was gently added to adjust to pH 8 on ice. The mixture was poured into saturated NaCl aqueous solution, and the product was extracted with ethyl acetate. After the solvent was removed under the reduced pressure, the crude product was purified by flash column chromatography (eluted with chloroform/methanol = 90:10 to 85:15) to yield compound **17** (4.52 g, 21.3 mmol, 93%) as a colorless oil. *Rf* = 0.13 (hexane/ethyl acetate = 3:2). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.25 (d, 2H, *J* = 7.8), 6.89 (d, 2H, *J* = 8.7), 4.48 (s, 2H), 3.86 (tt, 1H, *J* = 5.0 and 5.0), 3.81 (s, 3H), 3.73-3.48 (m, 4H), 2.67 (d, 1H, *J* = 5.1), 2.20 (dd, 1H, *J* = 6.6 and 5.7).

Compound **18**

Compound **17** (0.213 g, 1.00 mmol), NaH (0.159 g, 4.00 mmol), and dry DMSO

(15 ml) were placed in a round-bottomed flask under a nitrogen atmosphere. Compound **5** (0.693 g, 2.00 mmol) dissolved in 2 ml of dry DMSO was then added, and the reaction mixture was stirred at room temperature for 24 h. To the reaction mixture was added water. The mixture was then extracted with ethyl acetate. After the combined organic layers were concentrated under reduced pressure, the crude product was purified by flash column chromatography (eluted with hexane/ethyl acetate = 97:3 to 95:5) to give compound **18** (0.320 g, 0.449 mmol, 45%) as a colorless oil. *Rf* = 0.33 (benzene). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.25 (d, 2H, *J* = 8.7), 6.87 (d, 2H, *J* = 8.7), 5.40-5.29 (m, 4H), 4.48 (s, 2H), 3.80 (s, 3H), 3.60-3.36 (m, 9H), 2.04-1.98 (m, 8H), 1.60-1.47 (m, 4H), 1.40-1.20 (m, 44H), 0.88 (t, 6H, *J* = 6.6).

Compound **19**

Compound **18** (71.3 mg, 0.100 mmol) was dissolved in a mixture of 10 ml of dichloromethane and 133 μl of 0.1-M phosphate buffer solution (pH 7.2), and the resulting solution was stirred at 0˚C for 1 h. To the resulting solution was added of 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (DDQ, 45.4 mg, 0.200 mmol), followed by additional stirring at room temperature for 2 h. The mixture was extracted with ethyl acetate. The solvent of the combined organic layers was then removed under reduced pressure. The resulting crude product was purified by flash column chromatography (hexane/ethyl acetate = 95:5), yielding compound **19** (45.2 mg, 76.1 μmol, 76%) as a colorless oil. *Rf* = 0.52 (hexane/ethyl acetate = 4:1). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 5.40-5.30 (m, 4H), 3.77-3.67 (m, 1H), 3.66-3.35 (m, 8H), 2.01 (dd, 8H, *J* = 5.4 and 5.4), 1.62-1.52 (m, 4H), 1.40-1.20 (m, 44H), 0.88 (t, 6H, *J* = 6.6).

Compound **20**

Using a procedure similar to that described for the preparation of **13**, compound **20** (23.1 mg, 32.3 μmol, 19%) was obtained. *Rf* = 0.44 (chloroform/methanol/28% NH_3 solution = 65:25:4). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.68-8.42 (br s, 2H), 5.48-5.26 (m, 4H), 4.15-4.04 (br s, 2H), 3.91 (dd, 1H, *J* = 9.2 and 5.3), 3.87 (dd, 1H, *J* = 9.3 and 5.3), 3.63-3.35 (m, 7H), 3.23-3.05 (br s, 2H), 2.12-1.97 (m, 8H), 1.62-1.48 (m, 4H), 1.35-1.20 (m, 44H), 0.88 (t, 6H, *J* = 7.0). ^{31}P-NMR (160 MHz, $CDCl_3$): δ 0.961. ESI-MS: 714.6 $[M-H]^-$ ($C_{41}H_{82}O_6PN$).

Compound **21**

Using a procedure similar to that described for the preparation of **14**, compound **21** (6.1 mg, 6.94 μmol, 69%) was obtained. *Rf* = 0.35 (benzene). *Rf* = 0.44 (chloroform/methanol/28% NH_3 solution (65/25/4, v/v/v)). ESI-MS: 877.6 $[M-H]^-$ ($C_{47}H_{83}O_9PN_4$).

Compound **22**

Compound **17** (1.26 g, 5.95 mmol) and diisopropylethylamine (1.15 g, 8.93 mmol) were dissolved in 13 ml of dry dichloromethane at room temperature under nitrogen atmosphere. To this solution was added triphenylmethyl chloride (1.83 g, 6.54 mmol). After stirring overnight, the reaction mixture was concentrated under reduced pressure. The residue was redissolved in benzene and purified by flash column chromatography (eluted with benzene/methanol = 98:2 to 9:1) to yield compound **22** (2.61 g, 5.72 mmol, 96%). *Rf* = 0.23 (hexane/ethyl acetate = 4:1). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.45-7.18 (m, 17H), 6.86 (d, 2H,

J = 8.4), 4.46 (s, 2H), 3.96 (tt, 1H, *J* = 5.3 and 5.3), 3.80 (s, 3H), 3.57 (dd, 1H, *J* = 9.5 and 4.3), 3.52 (dd, 1H, *J* = 9.5 and 6.6), 3.22 (dd, 1H, *J* = 9.0 and 5.7), 3.18 (dd, 1H, *J* = 8.9 and 5.0), 2.39 (d, 1H, *J* = 4.8).

Compound **23**

Compound **22** (1.36 g, 3.00 mmol), NaH (0.263 g, 6.57 mmol), and dry DMSO (22 ml) were placed in a round-bottomed flask under a nitrogen atmosphere. Compound **5** (0.520 g, 1.5 mmol) dissolved in 3 ml of dry DMSO was then added, and the reaction mixture was stirred at room temperature for 5 h. To the reaction mixture was added water. The mixture was then extracted with hexane. After the combined organic layers were concentrated under reduced pressure, the crude product was purified by flash column chromatography (eluted with hexane/ethyl acetate = 98:2 to 9:1) to give compound **23** (0.688 g, 0.977 mmol, 65%) as a colorless oil. *Rf* = 0.56 (benzene). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.47-7.41 (m, 6H), 7.32-7.15 (m, 11H), 6.83 (d, 2H, *J* = 8.7), 5.41-5.27 (m, 2H), 4.46 and 4.42 (d, 1H, *J* = 12.0), 2.93 (s, 3H), 3.62-3.47 (m, 5H), 3.19 (d, 2H, *J* = 4.2), 2.00 (m, 4H), 1.56 (tt, 2H, *J* = 6.9 and 6.9), 1.38-1.20 (m, 22H), 0.88 (t, 3H, *J* = 6.6).

Compound **24**

Compound **22** (0.326 g, 0.718 mmol) and powdered KOH (0.201 g, 3.05 mmol) were dissolved in dry benzene (15 ml) at room temperature under a nitrogen atmosphere. Compound **6** (0.263 g, 0.718 mmol) dissolved in 2 ml dry benzene was slowly added to the resulting solution, and the reaction mixture was stirred for 12 h at 40˚C. The mixture was poured into water, and the product was extracted with hexane. After the solvent was removed

in vacuo, the crude product was purified by flash column chromatography (eluted with hexane/ethyl acetate = 97:3 to 95:5) to yield compound **24** (0.216 g, 0.303 mmol, 42%) as a colorless oil. *Rf* = 0.63 (hexane/ethyl acetate = 4:1). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.36-7.18 (m, 6H), 7.17-7.07 (m, 11H), 6.83 (d, 2H, *J* = 8.4), 5.44-5.29 (m, 10H), 4.45 and 4.42 (d, 1H, *J* = 12.0), 3.79 (s, 3H), 3.63-3.50 (m, 5H), 3.19 (d, 2H, *J* = 3.9), 2.88-2.78 (m, 8H), 2.14-2.02 (m, 4H), 1.64-1.55 (m, 2H), 1.43 (tt, 2H, *J* = 7.1 and 7.1), 0.97 (t, 3H, *J* = 7.5).

Compound **25**

Compound **23** (0.678 g, 0.961 mmol) was dissolved in dry $CHCl_3$ (75 ml) at room temperature under a nitrogen atmosphere. To the mixture was added 0.5 N HCl in 75 ml methanol. After stirring for 1 h, subsequent removal of the solvent under reduced pressure afforded the crude product, which was purified by flash column chromatography (eluted with hexane/ethyl acetate = 9:1 to 8:2) to yield compound **25** (0.317 g, 0.703 mmol, 73%) as a colorless oil. *Rf* = 0.26 (hexane/ethyl acetate = 4:1). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.25 (d, 2H, *J* = 8.1), 6.88 (d, 2H, *J* = 8.4), 5.40-5.29 (m, 2H), 4.47 (s, 2H), 3.80 (s, 3H), 3.75-3.46 (m, 7H), 2.13-1.95 (m, 4H), 1.63-1.51 (m, 2H), 1.45-1.20 (m, 22H), 0.88 (t, 3H, *J* = 6.6).

Compound **26**

Using a procedure similar to that described for the preparation of **25**, compound **26** (137 mg, 0.291 mmol, 91%) was obtained. *Rf* = 0.26 (hexane/ethyl acetate = 4:1). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.25 (d, 2H, *J* = 8.4), 6.88 (d, 2H, *J* = 8.7), 5.48-5.25 (m, 10H), 4.47 (s, 2H), 3.80 (s, 3H), 3.75-3.46 (m, 7H), 2.92-2.76 (m, 8H), 2.16-2.00 (m, 4H), 1.66-1.50 (m, 2H), 1.42 (tt, 2H, *J* = 7.5 and 7.5), 0.97 (t, 3H, *J* = 7.5).

Compound **27**

To a magnetically stirred mixture of **25** (105 mg, 0.228 mmol), dicyclohexylcarbodiimide (127 mg, 0.616 mmol), and dimethylaminopyridine (40.1 mg, 0.296 mmol) dissolved in 4.5 ml of dry chloroform was added oleic acid (142 mg, 0.502 mmol) under a nitrogen atmosphere at 0˚C. Subsequent removal of the solvent under reduced pressure after stirring for 11 h at room temperature afforded the crude product, which was purified by flash column chromatography (eluted with a mixture of hexane and ethyl acetate (98/2 to 95/5, v/v)) to yield compound **27** (156 mg, 0.220 mmol, 94%) as a colorless oil. *Rf* = 0.70 (hexane/ethyl acetate = 4:1). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.25 (d, 2H, *J* = 8.7), 6.87 (d, 2H, *J* = 8.4), 5.41-5.26 (m, 4H), 4.48 (s, 2H), 4.23 (dd, 1H, *J* = 11.7 and 4.2), 4.11 (dd, 1H, *J* = 11.7 and 5.7), 3.80 (s, 3H), 3.64 (tt, 1H, *J* = 5.4 and 5.4), 3.58-3.46 (m, 4H), 2.29 (t, 2H, *J* = 7.5), 2.08-1.94 (m, 8H), 1.68-1.48 (m, 4H), 1.39-1.22 (m, 42H), 0.88 (t, 6H, *J* = 6.6).

Compound **28**

Using a procedure similar to that described for the preparation of **27**, compound **28** (143 mg, 0.192 mmol, 95%) was obtained. *Rf* = 0.53 (hexane/ethyl acetate = 4:1). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.25 (d, 2H, *J* = 8.7), 6.87 (d, 2H, *J* = 8.4), 5.46-5.28 (m, 12H), 4.47 (s, 2H), 4.23 (dd, 1H, *J* = 11.6 and 4.4), 4.11 (dd, 1H, *J* = 11.4 and 5.7), 3.80 (s, 3H), 3.64 (tt, 1H, *J* = 5.0 and 5.0), 3.58-3.47 (m, 4H), 2.90-2.76 (m, 8H), 2.29 (t, 2H, *J* = 7.5), 2.14-1.96 (m, 8H), 1.68-1.52 (m, 4H), 1.41 (m, 2H), 1.36-1.24 (m, 20H), 0.97 (t, 3H, *J* = 7.5), 0.88 (t, 3H, *J* = 6.6).

Compound **29**

Using a procedure similar to that described for the preparation of **19**, compound **29** (136.5 mg, 0.226 mmol, 56%) was obtained. *Rf* = 0.37 (hexane/ethyl acetate = 4:1). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 5.44-5.28 (m, 4H), 4.26-4.12 (m, 2H), 3.74-3.46 (m, 5H), 2.32 (t, 2H, *J* = 7.5), 2.08-1.94 (m, 8H), 1.72-1.52 (m, 4H), 1.42-1.22 (m, 42H), 0.88 (t, 6H, *J* = 6.6).

Compound **30**

Using a procedure similar to that described for the preparation of **19**, compound **30** (60.0 mg, 95.7 μmol, 48%) was obtained. *Rf* = 0.33 (hexane/ethyl acetate = 4:1). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 5.38-5.21 (m, 12H), 4.10 (dd, 2H, *J* = 4.2 and 2.4), 3.65-3.41 (m, 5H), 2.78 (t, 4H, *J* = 4.8), 2.75 (t, 4H, *J* = 5.4), 2.29 (t, 2H, *J* = 7.5), 2.14-1.88 (m, 8H), 1.62-1.46 (m, 4H), 1.36 (m, 2H), 1.36-1.10 (m, 20H), 0.91 (t, 3H, *J* = 7.5), 0.81 (t, 3H, *J* = 6.6).

Compound **31**

Using a procedure similar to that described for the preparation of **13**, compound **31** (60.0 mg, 0.124 mmol, 75%) was obtained. *Rf* = 0.44 (chloroform/methanol/28% NH_3 solution = 65:25:4). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.68-8.42 (br s, 2H), 5.45-5.28 (m, 4H), 4.28 (dd, 2H, *J* = 8.9 and 2.6), 4.15-4.04 (br s, 2H), 3.96-3.81 (br s, 2H), 3.64-3.48 (m, 3H), 3.19-3.12 (br s, 2H), 2.30 (t, 2H, *J* = 5.7), 2.12-1.97 (m, 8H), 1.62-1.50 (m, 4H), 1.36-1.20 (m, 42H), 0.88 (t, 6H, *J* = 5.3). ^{31}P-NMR (160 MHz, $CDCl_3$): δ 0.998.

Compound **32**

Using a procedure similar to that described for the preparation of **13**, compound **32**

(22.0 mg, 29.3 μmol, 30%) was obtained. *Rf* = 0.44 (chloroform/methanol/28% NH_3 solution = 65:25:4). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.68-8.42 (br s, 2H), 5.48-5.25 (m, 12H), 4.28 (dd, 2H, *J* = 8.9 and 2.6), 4.15-4.04 (br s, 2H), 3.96-3.81 (br s, 2H), 3.64-3.48 (m, 3H), 3.19-3.12 (br s, 2H), 2.88-2.76 (m, 8H), 2.30 (t, 2H, *J* = 5.7), 2.12-1.97 (m, 8H), 1.62-1.50 (m, 4H), 1.40 (m, 2H), 1.36-1.20 (m, 20H), 0.97 (t, 3H, *J* = 5.7), 0.88 (t, 3H, *J* = 5.3). ^{31}P-NMR (160 MHz, $CDCl_3$): δ 1.051. ESI-MS: 748.6 $[M-H]^-$ ($C_{43}H_{76}O_7PN$).

Compound **33**

Using a procedure similar to that described for the preparation of **14**, compound **33** (17.3 mg, 19.4 μmol, 78%) was obtained. *Rf* = 0.35 (benzene). *Rf* = 0.67 (chloroform/methanol/28% NH_3 solution (65/25/4, v/v/v)). ESI-MS: 891.6 $[M-H]^-$ ($C_{47}H_{81}O_{10}PN_4$).

Compound **34**

Using a procedure similar to that described for the preparation of **14**, compound **34** (4.66 mg, 5.10 μmol, 92%) was obtained. *Rf* = 0.35 (benzene). *Rf* = 0.67 (chloroform/methanol/28% NH_3 solution = 65:25:4). ESI-MS: 911.4 $[M-H]^-$ ($C_{49}H_{77}O_{10}PN_4$)

Compound **35**

To a stirred mixture of NaH (50.0 mg, 1.25 mmol) in dry THF (16 ml) was added dropwise compound **26** (60.3 mg, 0.125 mmol) at 0˚C. After it was stirred at room temperature for 30 min and then cooled on ice, iodomethane (285 mg, 2.01 mmol) was slowly added to the resulting solution. To the reaction mixture was added water after stirring at 8˚C

for 16 h. The mixture was then extracted with ethyl acetate. After the combined organic layers were concentrated under reduced pressure, the crude product was purified by flash column chromatography (eluted with hexane/ethyl acetate = 90:10 to 80:20) to give compound **35** (59.7 mg, 0.120 mmol, 94%) as a colorless oil. *Rf* = 0.49 (hexane/ethyl acetate = 4:1). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.25 (d, 2H, *J* = 8.4), 6.87 (d, 2H, *J* = 8.7), 5.44-5.27 (m, 10H), 4.48 (s, 2H), 3.80 (s, 3H), 3.61-3.41 (m, 7H), 3.35 (s, 3H), 2.88-2.77 (m, 8H), 2.14-2.01 (m, 4H), 1.60 (tt, 2H, *J* = 7.4 and 7.4), 1.42 (tt, 2H, *J* = 7.7 and 7.7), 0.97 (t, 3H, *J* = 7.5).

Compound **36**

Using a procedure similar to that described for the preparation of **19**, crude **36** was obtained, which was purified by flash column chromatography (hexane/ethyl acetate = 80:20), yielding compound **36** (8.0 mg, 21.2 μmol, 18%) as a colorless oil. *Rf* = 0.12 (hexane/ethyl acetate = 4:1). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 5.44-5.27 (m, 10H), 3.75-3.43 (m, 7H), 3.37 (s, 3H), 2.88-2.77 (m, 8H), 2.14-2.01 (m, 4H), 1.60 (tt, 2H, *J* = 7.2 and 7.2), 1.43 (tt, 2H, *J* = 7.8 and 7.8), 0.97 (t, 3H, *J* = 7.5).

Compound **37**

Using a procedure similar to that described for the preparation of **13**, compound **37** (4.5 mg, 9.0 μmol, 41%) was obtained. *Rf* = 0.23 (chloroform/methanol/28% NH_3 solution = 65:25:4). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.68-8.42 (br s, 2H), 5.48-5.26 (m, 10H), 4.15-4.04 (br s, 2H), 3.91 and 3.87 (dd, 1H, *J* = 9.3 and 5.3), 3.63-3.38 (m, 5H), 3.37 (s, 3H), 3.23-3.05 (br s, 2H), 2.88-2.76 (m, 8H), 2.12-2.02 (m, 4H), 1.60 (tt, 2H, *J* = 7.2 and 7.2), 1.43 (tt, 2H, *J* = 7.8 and 7.8), 0.97 (t, 3H, *J* = 7.5). ^{31}P-NMR (160 MHz, $CDCl_3$): δ 0.955. ESI-MS: 498.6

$[M-H]^-$ ($C_{26}H_{46}O_6PN$).

Compound **38**

Using a procedure similar to that described for the preparation of **14**, compound **38** (4.7 mg, 7.1 μmol, 80%) was obtained. *Rf* = 0.59 (chloroform/methanol/28% NH_3 solution = 65:25:4). ESI-MS: 661.6 $[M-H]^-$ ($C_{32}H_{47}O_9PN_4$).

Compound **39**

In a round bottom flask, an heterogenous mixture of compound **32** (5.0 mg, 6.6 μmol) dissolved in 0.13 ml of diisopropyl ether and phospholipase A_1 solution (0.65 ml of 50 mM sodium acetate (pH 4) containing 1.6 μl of Lecitase Ultra (phospholipase A_1 from *Thermomyces lanuginosus/Fusarium oxysporum*, Novozymes Co. Ltd)) was sonicated at 35 kHz under a nitrogen atmosphere at 35°C for 30 min by using an ultrasonic washer (SHARP, UT-105S). To the mixture after the reaction was added 1.6 ml of methanol. Then, 3.2 ml of hexane/diisopropyl ether (1:1) was added to the mixture followed by removal of the upper layer to separate compound **39** and free oleic acid. This wash was repeated three times. Then, the crude product was extracted by the method of Bligh and Dyer and used in the next step without further purification. *Rf* = 0.13 (chloroform/methanol/28% NH_3 solution = 65:25:4). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.68-8.42 (br s, 2H), 5.48-5.26 (m, 10H), 4.15-4.04 (br s, 2H), 3.89-3.85 (m, 2H), 3.81-3.71 (m, 2H), 3.63-3.38 (m, 3H), 3.23-3.05 (br s, 2H), 2.88-2.76 (m, 8H), 2.12-2.02 (m, 4H), 1.60 (tt, 2H, *J* = 7.2 and 7.2), 1.43 (tt, 2H, *J* = 7.8 and 7.8), 0.97 (t, 3H, *J* = 7.5). ^{31}P-NMR (160 MHz, $CDCl_3$): δ 0.955. ESI-MS: 483.2 $[M-H]^-$ ($C_{25}H_{43}O_6PN$).

Compound **40**

Using a procedure similar to that described for the preparation of **14** except for the reaction temperature, compound **40** (7.1 mg, 11.2 μmol, 80%) was obtained. The reaction temperature was changed from room temperature to 0˚C for specific reaction between the amino group and NBD-F. *Rf* = 0.51 (chloroform/methanol/28% NH_3 solution = 65:25:4). ESI-MS: 631.6 $[M-H]^-$ ($C_{31}H_{45}O_8PN_4$).

Fluorescence microscopic analysis

Fluorescence images of the cells were obtained using an epifluorescence microscope (E6F-RFL equipped with ×100 oil objective; Plan Fluor, Nikon, Japan) with a super-high pressure mercury lamp (Nikon model HB-10103AF). Fluorescence filters, UV-1A (Ex: 365/10, DM: 400, BA: 400) and B-2A (Ex: 450-490, DM: 505, BA: 520), were used for Hoechst 33342 fluorescence (Ex: 350 nm, Em: 460 nm) and NBD fluorescence (Ex: 467 nm, Em: 540 nm), respectively. Digital images were acquired and analyzed using ImageJ software (http://rsbweb.nih.gov/ij/).

ESI-MS analysis of phospholipids

After the fluorescence microscopic analysis, lipids were extracted from the residual sample by the Bligh and Dyer method (2). Chloroform (100 μl) containing 1.14 μM 1,2-dilauroyl-*sn*-glycero-3-phosphoethanolamine (DLPE, Avanti Polar Lipids) as the internal standard 1 was used for the quantification of phospholipids. The chloroform-soluble fraction was dried, suspended in 100 μl of acetonitrile/methanol (2:1 v/v) containing 0.1% (v/v) ammonium formate (pH 6.5) and 1.02 μM 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol

(DPPG, Nippon Oil & Fats Co. Ltd) as the internal standard 2, and subjected to ESI-MS/MS analysis (Applied Biosystems API3000 LC/MS/MS system). Lipid samples (70 μl) were applied directly to the ESI source at a rate of 5 μl/min. The data were collected in the negative ion mode from 200 to 1200 m/z. The efficiency of lipid extraction was shown to be almost identical among samples by comparing the intensities of the internal standards 1 and 2. Experiments were repeated three times and yielded almost identical spectra. The fatty acyl residues in phospholipids were analyzed in the precursor ion scan mode. To compare the amounts of different NBD-labeled phospholipids incorporated into the cells, the following value was used:

$$A_{NBD}/A_{i.s.2}/I_{ef}/A_{600} \quad \text{-----(1)}$$

where A_{NBD} is an average area of the particular NBD-labeled phospholipid(s) in the mass spectra; $A_{i.s.2}$ is an average area of DPPG (internal standard 2) in the mass spectra; I_{ef} is the efficiency of lipid extraction, which was calculated by dividing "$A_{i.s.1}$ after extraction" by "$A_{i.s.1}$ before extraction," where $A_{i.s.1}$ is an average area of DLPE (internal standard 1) in the mass spectra; and A_{600} is the absorbance of the culture at 600 nm.

Dithionite assay for measuring lipid distribution in the inner (IM) and outer (OM) membranes

To determine the distribution of NBD-labeled phospholipids in the IM and OM of the bacterium, we employed a fluorescence-quenching assay by using sodium dithionite. Dithionite quenches NBD fluorescence by reduction and hardly penetrates across the lipid bilayer membrane because of its high hydrophilicity. Thus, dithionite can be used to determine the distribution of NBD-labeled phospholipids inside and outside the membrane

compartments (3-5). For this assay, we prepared the following three samples from *S. livingstonensis* Ac10 grown at 6°C in LB medium for 250 h: non-treated, lysozyme-treated, and lysozyme-and-Triton X-100-treated cells (Fig. S1A). Because both OM and IM are intact in non-treated cells, dithionite can access only the outer leaflet of OM (OM_{out}), where the fluorescence from the NBD-labeled phospholipids is quenched. When the cells are treated with lysozyme, the OM is disrupted, and both the inner leaflet of the OM (OM_{in}) and the outer leaflet of the IM (IM_{out}), in addition to the OM_{out}, become accessible to dithionite for fluorescence quenching. When the cells are treated with lysozyme and Triton X-100, both OM and IM are disrupted, and the inner leaflet of the IM (IM_{in}) as well as the OM_{out}, OM_{in}, and IM_{out} become accessible to dithionite for fluorescence quenching. To prepare the lysozyme-treated sample, 10 mg/ml of lysozyme in buffer A was added to the cells to the final concentration of 100 μg/ml, followed by the addition of 1.5 mM EDTA in buffer A to the final concentration of 0.5 mM. The sample was incubated on ice for 40 min. To prepare the lysozyme-and-Triton X-100-treated cells, 4% (w/v) Triton X-100 in buffer A was added to the lysozyme-treated sample to the final concentration of 0.4%, and the sample was incubated on ice for 20 min.

The assay was performed by the following procedure: (i) Cells incubated with NBD-labeled phospholipids for 250 h were diluted to A_{600} = 0.032 with buffer A (0.75M sucrose, 10 mM Tris-HCl (pH 7.5)) in a fluorescence cuvette. (ii) The fluorescence emission of this solution was recorded at an excitation wavelength of 467 nm and an emission wavelength of 540 nm by using a Hitachi 850S Fluorescence Spectrophotometer (Tokyo, Japan). (iii) After the fluorescence became constant, 1.0M of sodium dithionite ($Na_2S_2O_4$) in degassed H_2O was added at a final concentration of 10 mM, and the decrease in the

fluorescence was monitored. Experiments were repeated three times; each experiment yielded almost identical spectra.

Figure S1B shows a typical result of the dithionite assay obtained with the cells grown in the presence of Acylalkyl-EPA for 250 h. The degree of fluorescence quenching was in accordance with the degree of membrane disruption, verifying the validity of this method in determining the membrane localization of the NBD-labeled phospholipids. Each curve in Fig. S1B shows that the fluorescence intensity rapidly decreased in the initial stage of the reaction for about 2 min, and a very slow decrease of the fluorescence followed. The slow decrease is probably due to the slow passage of $S_2O_4^{2-}$ or SO_2^- radicals across the cell membranes and their reaction with NBD inside the membrane compartment (4). The slow reaction rate was extrapolated to time zero to determine the degree of "fast" fluorescence quenching corresponding to the reduction of the NBD group outside the membrane compartment.

To calculate the amount of NBD-labeled phospholipid inside the membrane compartment, which dithionite cannot access, the following equation was used:

Percentage of the NBD-labeled phospholipid inside the membrane = $(F_r - F_{ap})/(F_0 - F_{ap}) \times 100$ -----(2)

F_r is the fluorescence after the reaction with dithionite is complete, F_{ap} is the apparent fluorescence of the cells without NBD-labeled phospholipid, and F_0 is the fluorescence of the cells with NBD-labeled phospholipid before reaction. F_r is obtained by extrapolation of the slow reaction rate to time zero.

Fluorescence microscopic analysis of the cells treated with dithionite to

quench the fluorescence of NBD-labeled phospholipids in the OM_{out}

To find out on which membrane and on which side of the membrane the fluorescent NBD-labeled phospholipids were enriched after 250 h, we combined fluorescent microscopic analysis with the dithionite quenching method. The assay was performed by the following procedure: (i) *S. livingstonensis* Ac10 was cultivated in the presence of Acylalkyl-EPA and Lysoalkyl-EPA. The culture (200 μl each) was sampled at 250 h, and the cells were treated with Hoechst 33342 (final concentration = 25 μg/ml) for nucleoid labeling, followed by two wash with 200 μl of buffer A (0.75 M sucrose, 10 mM Tris-HCl (pH 7.5)). (ii) The cells were diluted to A_{600} = 2.7 with buffer A in a tube at 4°C. (iii) Sodium dithionite (100 mM) in degassed H_2O at 4°C was added to the suspension at a final concentration of 10 mM, and the mixture was incubated at 4°C. (iv) After 10 min of the incubation, the excess amount of dithionite was inactivated by the addition of 16% (v/v) of formaldehyde aqueous solution (methanol free). (v) Fluorescence images derived from NBD and Hoechst33342 of the cells were obtained using an epifluorescence microscope (E6F-RFL equipped with ×100 oil objective; Plan Fluor, Nikon, Japan). Digital images were acquired and analyzed using ImageJ software.

Legend to Scheme in Supplemental Data

Scheme S1

Synthetic scheme of NBD-labeled ether phospholipids, Dialkyl-EPA, Dialkyl-OLA, Acylalkyl-EPA, Acylalkyl-OLA, Methylalkyl-EPA and Lysoalkyl-EPA (compound **14**, **21**, **34**, **33**, **38**, and **40**, respectively). R_1COOH; oleic acid ($18:1_{9c}$), R_2COOH; eicosapentaenoic acid ($20:5_{5,8,11,14,17\ all\ cis}$). Reagents and conditions: a. $LiAlH_4$, THF, rt; b. methanesulfonyl chloride, triethylamine, benzene, rt; c. NaH, **5** (1.1 equiv.), DMSO, rt; d. $CHCl_3$/0.5 N HCl-MeOH (1:1, v/v); e. triphenylmethyl chloride, diisopropylethylamine, dichloromethane, rt; f. powdered KOH, **6** (1.0 equiv.), benzene, 40˚C; g. $POCl_3$, triethylamine, trichloroethylene-hexane (91:9, v/v), rt; h. 2-aminoethanol, triethylamine, THF, rt; i. 2-propanol-20% (v/v) AcOH aq. (2:1, v/v), rt; j. NBD-F, triethylamine, $CHCl_3$, rt; k. *p*-methoxybenzyl chloride, NaH, THF, 70˚C; l. NaH, **5** (2.0 equiv.), DMSO, rt; m. 2,3-dichloro-5,6-dicyano-*p*-benzoquinone, dichloromethane, rt; n. **1** (2.2 equiv.), dicyclohexylcarbodiimide, dimethylaminopyridine, $CHCl_3$, rt; o. iodomethane, NaH, THF, rt; p. PLA_1 (cat.), 1:5 (v/v) ether-aqua solution (50 mM sodium acetate (pH 4)), 35˚C; q. NBD-F, triethylamine, $CHCl_3$, 0˚C.

Legends to Figures in Supplemental Data

Fig. S1

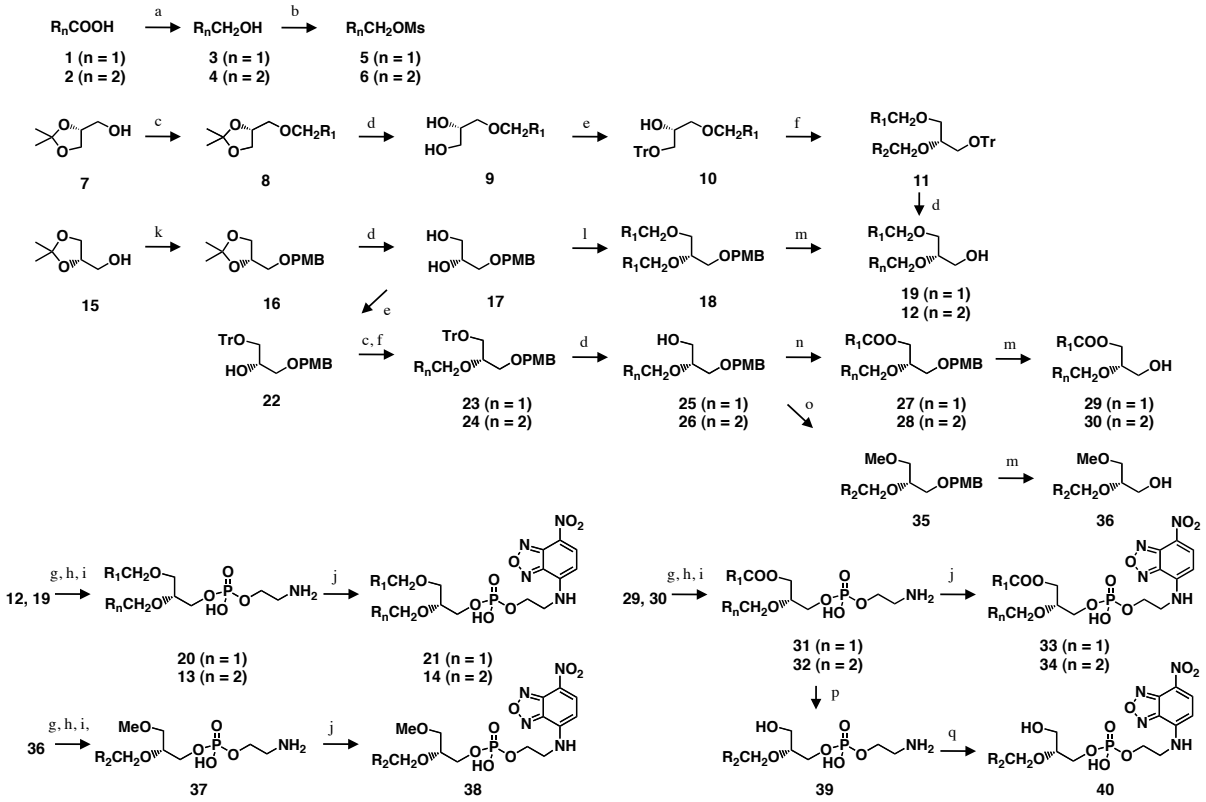
Dithionite quenching assay. (*A*) Schematic diagram of the dithionite quenching assay to determine the distribution of NBD-labeled phospholipids in the IM and OM. Dithionite hardly penetrates across the lipid bilayer, thus quenching the NBD fluorescence only outside the membrane compartments. See text for details. (*B*) Typical result of the dithionite quenching assay for the cells grown in the presence of Acylalkyl-EPA. The assay was performed for the intact cells (*black*), the lysozyme-treated cells (*red*), and the lysozyme-and-TritonX-100-treated cells (*blue*). The dashed line is the extrapolation of the slow quenching curve to time zero. F_r is the intercept of the extrapolated line at time zero, indicating the fluorescence that should remain after fast quenching of the NBD fluorescence outside the membrane compartments.

Fig. S2

ESI-MS spectra of the phospholipids extracted from *S. livingstonensis* Ac10 grown in the presence of Diacyl-EPA (*A*) and Diacyl-OLA (*B*), in the negative ion scan mode. Spectra were measured after 36 h (purple), 60 h (blue), 105 h (green), 130 h (orange), and 166 h (red) of cultivation. Relative intensities of different samples were normalized by using the intensity of the internal standard (1,2-dilauroyl-*sn*-glycero-3-phosphoethanolamine). Phospholipids increased during cultivation were identified by MS/MS analysis (precursor ion scan and product ion scan) and are shown as X/Y-PE or –PG (X, Y = acyl chain, PE = phosphatidylethanolamine, and PG = phosphatidylglycerol).

References

1. Sato, S., Kurihara, T., Kawamoto, J., Hosokawa, M., Sato, S. B., and Esaki, N. (2008) *Extremophiles* **12,** 753-761
2. Bligh, E. G. and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37,** 911-917
3. Huijbregts, R. P., de Kroon, A. I., and de Kruijff, B. (1996) *Biochim. Biophys. Acta* **1280,** 41-50
4. McIntyre, J. C. and Sleight, R. G. (1991) *Biochemistry* **30,** 11819-11827
5. Angeletti, C. and Nichols, J. W. (1998) *Biochemistry* **37,** 15114-15119



Scheme S1

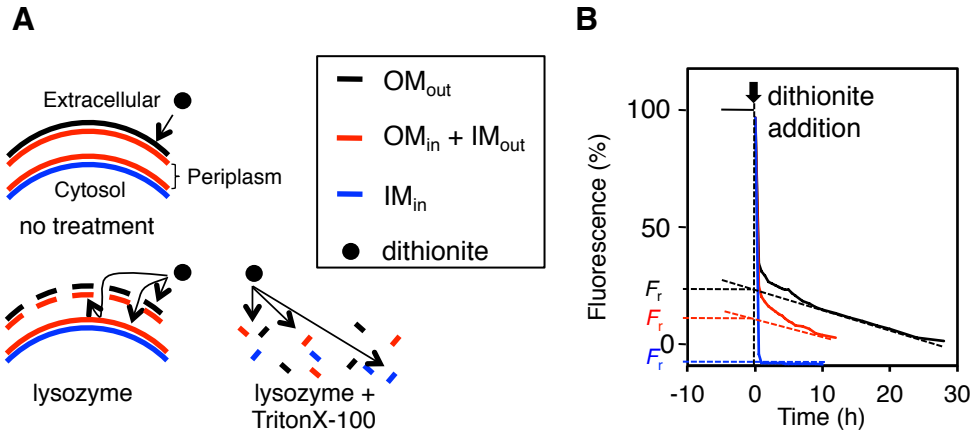

A
B
Extracellular
Periplasm
Cytosol
no treatment
lysozyme
lysozyme +
TritonX-100
OMout
OMin + IMout
IMin
dithionite
dithionite
addition
Fluorescence (%)
100
50
0
Fr
Fr
Fr
-10
0
10
20
30
Time (h)

Figure S1

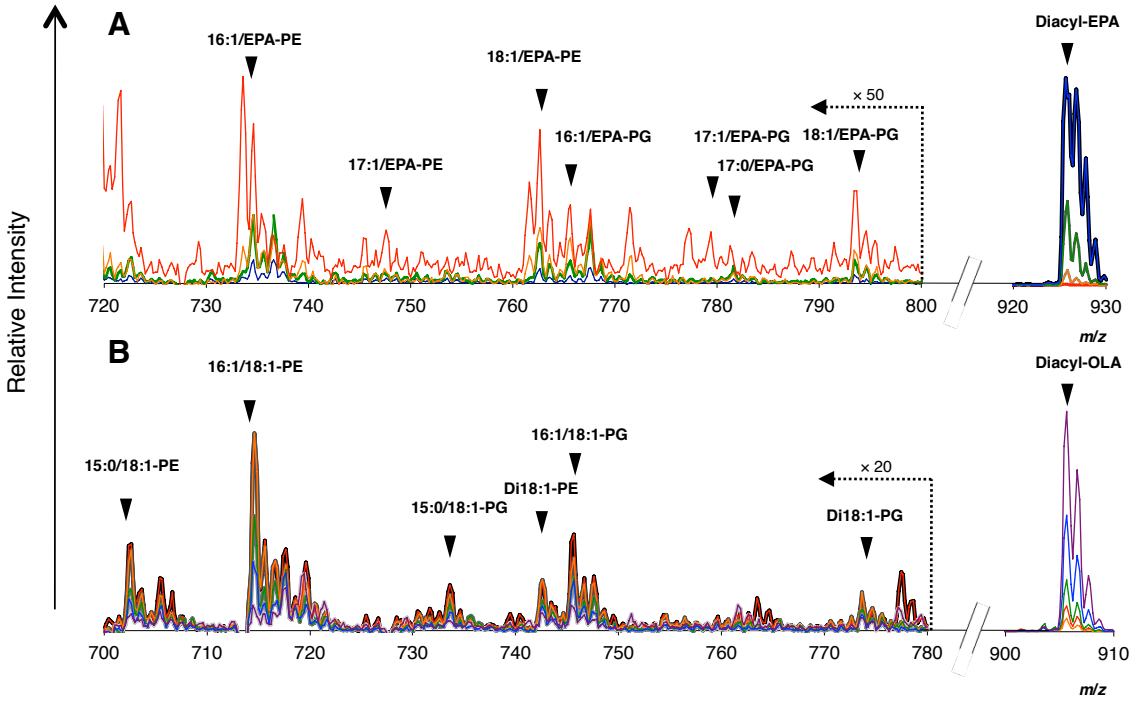

A
16:1/EPA-PE
18:1/EPA-PE
16:1/EPA-PG
17:1/EPA-PE
17:1/EPA-PG
18:1/EPA-PG
17:0/EPA-PG
× 50
Diacyl-EPA
Relative Intensity
720
730
740
750
760
770
780
790
800
920
930
m/z
B
16:1/18:1-PE
16:1/18:1-PG
15:0/18:1-PE
Di18:1-PE
15:0/18:1-PG
Di18:1-PG
× 20
Diacyl-OLA
700
710
720
730
740
750
760
770
780
900
910
m/z

Figure S2