Supporting Information

Ando et al. 10.1073/pnas.1418088112

SI Materials and Methods

Experimental Procedures for Raman/Fluorescence Imaging and Spectroscopy.

Experimental procedures used for Fig. 2. Supported monolayer membranes of single lipid composition, including SM, diyne-SM, DOPC, and chol, were prepared on a quartz substrate (0.17-mm thickness; Starbar Japan), fixed in a metal chamber (Attofluor; Life Technologies), and placed on the sample stage of the microscope (Raman-11; Nanophoton). For Raman spectroscopic measurement, the 532-nm laser was focused at a point on the membrane with a water immersion objective lens (CFI Plan Apo IR 60XWI; Nikon). The laser intensity at the sample plane was 340 mW. Exposure time was 6 s. Slit width for the spectrophotometer was set as 50 μm. Raman measurement was performed 15 times at different positions on the same membrane. Averaged Raman spectra of each membrane are shown in Fig. 2. The Raman peak of diyne-SM appeared at 2,263 cm^{-1}.

Experimental procedures used for Fig. 3. Supported monolayer membrane of diyne-SM/DOPC/chol ternary monolayer with the composition ratio 1:1:1 was prepared on a quartz substrate (0.17-mm thickness; Starbar Japan). The membrane was fixed in a metal chamber (Attofluor; Life Technologies) and placed on the sample stage of the microscope (Raman-11; Nanophoton). For Raman spectroscopic measurement, the 532-nm laser was focused at a point on the membrane with a water immersion objective lens (CFI Plan Apo IR 60XWI; Nikon). Focus drift was suppressed during imaging by using a real-time feedback system (PFS; Nikon). The laser intensity at the sample plane was 360 mW. Exposure time was 6 s/pixel. Slit width for the spectrophotometer was set as 50 μm. Each Raman spectrum was smoothed using a moving average method. Raman images were reconstructed using peak intensity of diyne at 2,264 cm^{-1} and peak bottom intensity at 2,222 cm^{-1}. The Raman image of diyne was obtained after subtraction of the signal intensity between 2,264 and 2,222 cm^{-1}, whereas the Raman image of the peak bottom (2,222 cm^{-1}) was obtained after subtraction of the signal intensity between 2,222 and 2,180 cm^{-1}.

Experimental procedures used for Fig. 4. Supported monolayer membranes of diyne-SM/DOPC/chol ternary monolayer with composition ratios of 1:1:1, 3:7:3, and 1:0:0 were prepared on a quartz substrate (0.17-mm thickness; Starbar Japan). Each membrane on the substrate was fixed in a metal chamber (Attofluor; Life Technologies) and placed on the sample stage of the microscope (Raman-11; Nanophoton). For Raman spectroscopic measurement, a 532-nm laser was focused as a point on the membrane with a water immersion objective lens (UPLSAPO 60XW; Olympus). The laser intensity at the sample plane was 370 mW. Exposure time was 6 s/pixel. Slit width for the spectrophotometer was set as 50 μm. Smoothing of Raman data was done using a moving average. Raman images were reconstructed using peak intensity of diyne at 2,263 cm^{-1} after subtraction of peak bottom intensity.

Experimental procedures used for Fig. 5. The experimental setup was the same as used previously (1). Briefly, a frequency-doubled Nd:YVO_4 laser (Verdi; Coherent Inc.) was used as the excitation laser source. A line-shaped laser beam formed by a cylindrical lens was focused on the sample by a water immersion objective lens (CFI Plan Apo IR 60XWI; Nikon). Scattering light from the sample was collected by the same objective lens, chromatically separated by an edge filter (LP03-532RU-25; Semrock) to transmit Raman scattering light, and focused on the spectrophotometer slit (MK-300; Bunko Keiki) for detection with a cooled CCD camera (Pixis 400B; Princeton Instruments). Laser scanning was performed with a single-axis galvanometer mirror (710-745825, 000-3014016; GSI Lumonics).

For Raman imaging in Fig. 5*A*, a supported 1:1:1 ternary monolayer membrane of diyne-SM/DOPC/chol was prepared on a quartz substrate (0.17-mm thickness; Starbar Japan). The membrane on the substrate was fixed in a metal chamber (Attofluor; Life Technologies) and placed on the sample stage of the home-built slit-scanning Raman microscope. For Raman spectroscopic measurement, a 532-nm laser was focused as a line on the membrane with a water immersion objective lens (CFI Plan Apo IR 60XWI; Nikon). Focus drift was suppressed during imaging by using a real-time feedback system (PFS; Nikon). The laser intensity at the sample plane was 10.5 mW/$μm^2$. Exposure time was 100 s per line. Slit width for the spectrophotometer was set as 50 μm. The scanning number was 240 lines for Raman imaging, with an image size of 240 × 400 pixels. For noise reduction, the obtained Raman data were subjected to singular value decomposition (SVD) (1). We used a spectral region (1,799–2,301 cm^{-1}) in the calculation procedure of SVD. We chose four loading vectors that significantly contributed to the image contrast. After SVD processing, Raman images were reconstructed using the peak intensity of diyne at 2,262 cm^{-1} based on the averaged peak top intensity between 2,259 and 2,265 cm^{-1} after subtraction of the averaged peak bottom intensity between 2,223 and 2,229 cm^{-1}. To compensate for aspect ratio, the image in the scanning (horizontal) direction was extended 1.72 times by linear interpolation. The final image consists of 412 × 400 pixels.

For Raman and fluorescence imaging in Fig. 5*B*, a supported 1:1:1 ternary monolayer membrane of diyne-SM/DOPC/chol containing 0.2 mol% Bodipy-PC was prepared on a quartz substrate (0.17-mm thickness; Starbar Japan). The membrane on the substrate was fixed in a metal chamber (Attofluor; Life Technologies) and placed on the sample stage of the home-built slit-scanning Raman microscope. For both Raman and fluorescence imaging, a 532-nm laser was focused as a line on the membrane with a water immersion objective lens (CFI Plan Apo IR 60XWI; Nikon). First, fluorescence imaging was performed, and second, Raman imaging was performed at the same area of the same sample. Fluorescence background was suppressed during Raman imaging by photobleaching of Bodipy-PC under 532-nm laser exposure. Focus drift was suppressed during imaging by using a real-time feedback system (PFS; Nikon). The laser intensity at the sample plane was 14.1 mW/$μm^2$, and the exposure time was 60 s per line for Raman imaging. The laser intensity for fluorescence imaging was 0.3 mW/$μm^2$, and the exposure time was 0.5 s per line. Slit width of the spectrophotometer was set as 50 μm. The scanning number was 225 lines for both Raman and fluorescence imaging, with the image size of 225 × 250 pixels. For noise reduction, the obtained Raman data were subjected to SVD. We used a spectral region (2,000–2,314 cm^{-1}) in the calculation procedure of SVD. We chose four loading vectors that significantly contributed to the image contrast. After SVD processing, the Raman image was reconstructed using the peak intensity of diyne at 2,264 cm^{-1} based on the averaged peak top intensity between 2,261 and 2,267 cm^{-1} after subtraction of the averaged peak bottom intensity between 2,225 and 2,231 cm^{-1}. Fluorescence images were reconstructed using average intensity at 542–603 nm. To compensate for aspect ratio, the image in the scanning (horizontal) direction was extended 1.72 times by linear interpolation. The final image consists of 387 × 250 pixels.

Line profiles in Fig. 5*C* were calculated from the average intensity of 4 pixels along the vertical direction, which is indicated

by a red dotted line in the Raman image in Fig. 5*B*, *Upper* and a gray dotted line in the fluorescence image in Fig. 5*B*, *Lower*. The profile from the Raman image was smoothed by use of the moving average.

Experimental procedures used for Fig. S5. In Fig. S5*A*, the experimental setup was the same as used in Fig. 5. For Raman imaging, a supported membrane of diyne-SM monolayer was prepared on a quartz substrate (0.17-mm thickness; Starbar Japan). The membrane on the substrate was fixed in a metal chamber (Attofluor; Life Technologies) and placed on the sample stage of the home-built slit-scanning Raman microscope. For Raman spectroscopic measurement, a 532-nm laser was focused as a line on the membrane with a water immersion objective lens (CFI Plan Apo IR 60XWI; Nikon). Focus drift was suppressed during imaging by using a real-time feedback system (PFS; Nikon). The laser intensity at the sample plane was 13.9 mW/μm^2. Exposure time was 60 s per line. Slit width for the spectrophotometer was set as 50 μm. The scanning number was 200 lines for Raman imaging, with an image size of 200 × 190 pixels. For noise reduction, the obtained Raman data were subjected to SVD (1). We used a spectral region (1,800–2,310 cm^{-1}) in the calculation procedure of SVD. We chose four loading vectors that significantly contributed to the image contrast. After SVD processing, Raman images were reconstructed using the peak intensity of diyne at 2,263 cm^{-1} based on the averaged peak top intensity between 2,260 and 2,266 cm^{-1} after subtraction of the averaged peak bottom intensity between 2,212 and 2,218 cm^{-1}. To compensate for aspect ratio, the image in the scanning (horizontal) direction was extended 1.72 times by linear interpolation. The final image consists of 344 × 190 pixels. In Fig. S5*B*, the image was extracted from lower left portion in Fig. 5*A*, which consist of 344 × 190 pixels. In Fig. S5*C*, line profiles were calculated from the average intensity of 7 pixels along the vertical direction, which is indicated by a dotted lines.

Experimental procedures used for Fig. S6. A supported monolayer membrane of 1:1:1 diyne-SM/DOPC/chol ternary monolayer containing 0.2 mol% Bodipy-PC was prepared on a quartz substrate (0.17-mm thickness; Starbar Japan). The membrane on the substrate was fixed in a metal chamber (Attofluor; Life Technologies) and placed on the sample stage of the microscope (Raman-11; Nanophoton). For both fluorescence and Raman spectroscopic measurement, a 532-nm laser was focused as a point on the membrane with a water immersion objective lens (CFI Plan Apo IR 60XWI; Nikon). Focus drift was suppressed during imaging by using a real-time feedback system (PFS; Nikon). For fluorescence imaging, the laser intensity at the sample plane was 0.5 mW, and the exposure time was 1 s/pixel. For Raman imaging, the laser intensity at the sample was 360 mW, and the exposure time was 6 s/pixel. First, fluorescence imaging was performed, and second, Raman imaging was performed at the same area of the same sample. Slit width for the spectrophotometer was set as 50 μm. For reconstruction of fluorescence images, average intensity between 555 and 605 nm was used. Smoothing of Raman data was done using a moving average. Raman images were reconstructed using peak intensity of diyne at 2,264 cm^{-1} after subtraction of peak bottom intensity.

DSC Thermograms of Diyne-SM Bilayers. The thermal-phase behavior of SM bilayers was examined with a nanodifferential scanning calorimeter (Calorimetry Science Corp.). Bilayer samples were prepared by a conventional method. Briefly, SM dissolved in chloroform/methanol (4:1) was dried under a flow of nitrogen and then, reduced pressure for at least 24 h. The resulting lipid film was dispersed into distilled and deionized water (Simplicity UV; Merck Millipore) and incubated for ∼30 min at 60 °C with intermittent vortexing. The final concentration was 2.47 mM. Then, 330 μL sample was placed in the DSC immediately before measurement. A scanning rate of 0.5 °C/min was used. The main transition temperatures of diyne-SM and SM bilayers were found to be 39.5 °C and 44.5 °C, respectively, as shown by the arrows in Fig. S7.

π-*A* Isotherm Measurements and Supported Monolayer Preparation. Monolayers of lipid mixtures were prepared on a computer-controlled Langmuir film balance (USI System) calibrated using stearic acid (Sigma Aldrich). The subphase, which consisted of distilled, freshly deionized water, was obtained using a Milli-Q System. The sample solution was prepared by mixing the appropriate amount of each lipid solution in a microvial. A total of 30 μL lipid dissolved in chloroform/methanol (4:1 vol/vol; 1 mg/mL) was spread onto the aqueous subphase (100 × 290 mm^2) using a glass micropipette (Drummond Scientific Company). After an initial delay period of 10 min for evaporation of the organic solvent, the monolayers were compressed at a rate of 20 mm^2/s. The subphase and ambient temperatures were controlled at 25.0 °C ± 0.1 °C and 25 °C ± 2 °C, respectively. The π-*A* isotherm measurements were repeated three to five times under the same conditions. These measurements provided the molecular area at a corresponding pressure within an error of ∼ ±1 Å^2. The influence of oxidation on the unsaturated chains at the air–water interface was checked by intentionally exposing pure SM and pure DOPC monolayers to air for 10–30 min before compression. The change in the isotherm after prolonged exposure of SM or DOPC monolayer to air was within the error described above.

Analysis. In Fig. 6, we evaluated the intermolecular interaction in lipid binary mixtures at the surface pressure of 5 mN/m on the basis of the deviations of experimentally obtained mean molecular areas (A_{mean}) from those of ideal mixtures (A_{12}):

$$A_{12} = A_1(1-x) + A_2 x,$$

where A_1 and A_2 are the molecular areas of pure components 1 and 2 (e.g., SM and chol), respectively, and x is the molar fraction of component 2. Thus, the value of A_{12} corresponds to the mean molecular area in the mixture constituted of noninteractive or completely immiscible molecules. According to previous literature (2), PMAs of components 1 (A^1_{PMA}) and 2 (A^2_{PMA}) can be defined as

$$A^1_{\mathrm{PMA}} = \left(\frac{\partial N A^1_{\mathrm{mean}}}{\partial N_1}\right)$$

and

$$A^2_{\mathrm{PMA}} = \left(\frac{\partial N A^2_{\mathrm{mean}}}{\partial N_2}\right),$$

where N, N_1, and N_2 are the total amounts of all constituents and components 1 and 2, respectively. On the basis of the additivity rule, the A_{mean} also can be expressed as

$$A_{\mathrm{mean}}(x) = (1-x)A^1_{\mathrm{PMA}}(x) + xA^2_{\mathrm{PMA}}(x).$$

Here, denoting derivatives with respect to x by prime yields, the following equations are obtained:

$$A^1_{\mathrm{PMA}}(x) = A_{\mathrm{mean}}(x) - xA'_{\mathrm{mean}}(x)$$

and

$$A^2_{\mathrm{PMA}}(x) = A_{\mathrm{mean}}(x) + (1-x)A'_{\mathrm{mean}}(x).$$

Areal compressibility (C_s) at the surface pressure of 5 mN/m was calculated from the π-A isotherm using

$$C_s = -\frac{1}{A_{mean}}\left(\frac{\partial A_{mean}}{\partial \pi}\right)_{\pi}.$$

The compressibility in ideal mixtures (C_{12}) is calculated according to Ali et al. (3):

$$C_{12} = \left(\frac{1}{A_{12}}\{(C_{s1}A_1)(1-x) + (C_{s2}A_2)x\}\right),$$

where C_{s1} and C_{s2} are the areal compressibilities of the pure components 1 and 2, respectively. They suggested that C_{12} is additive with respect to the product of C_{si} and A_i rather than C_{si} for either ideal or completely nonideal mixing. Areal compressibility (C_s) was expressed in term of areal compressional modulus (C_s^{-1}) for easy comparison with previous data.

Fluorescence Observation of Ordered Domains in Diyne-SM/DOPC/Chol and SM/DOPC/Chol Ternary Monolayers. Fig. S8 shows fluorescence images of diyne-SM/DOPC/chol (1:1:1 mol/mol/mol) (Fig. S8*A*) and SM/DOPC/chol (1:1:1 mol/mol/mol) (Fig. S8*B*) quartz-supported monolayers in the presence of 0.2 mol% Bodipy-PC at 12 mN/m and 25 °C. Fluorescence observations were conducted using a confocal laser-scanning microscope (FV1000-D IX81; Olympus) with an air objective lens with a long working distance (LUCPLFLN 60X; Olympus). A wavelength of 488 nm was used for excitation of Bodipy. A laser-scanning rate of 4.0 or 8.0 μs/pixel was used for acquisition of confocal images (1,024 × 1,024 pixels).

^{2}H NMR Measurements. A mixture of lipids comprising 10.0 μmol d_2-SM or d_2-diyne-SM (Fig. 7), 10.0 μmol chol, and 10.0 μmol DOPC was dissolved in chloroform/methanol (1:1 vol/vol). After removing the solvent in vacuo for 20 h, the dried membrane film was hydrated with 1 mL distilled water and vigorously vortexed at 65 °C to make multilamellar vesicles. The sample was frozen and thawed three times, lyophilized, and rehydrated with deuterium-depleted water to make 50% (wt/wt) water. Then, the mixture was again frozen and thawed 10 times. The sample was transferred into a glass tube (5 × 26 mm), which was sealed with epoxy glue. ^{2}H NMR measurements were recorded on a 300-MHz CMX300 Spectrometer (Chemagnetics; Varian) with a 5-mm ^{2}H static probe (Otsuka Electronics) using a quadrupolar echo sequence (4). The 90° pulse width was 2 μs, interpulse delay was 30 μs, and repetition rate was 0.5 s. The sweep width was 200 kHz, and the number of scans was around 100,000.

General Information for the Synthesis of Diyne-SM. Chemicals and solvents were purchased from Nacalai Tesque, Aldrich, TCI, or Kanto Chemicals Inc. and used without additional purification unless otherwise noted. TLC was done on Merck Precoated Silica Gel 60 F-254 Plates. Spots on TLC plates were stained with phosphomolybdic acid. NMR spectra were collected on a JEOL ECA 500 (500 MHz) using deuterated solvent as the lock. Chemical shift is given in parts per million (δ), and coupling constant (J) is in hertz. The following terms are used to designate multiplicity: singlet (s), doublet (d), triplet (t), quartet (q), quintuplet (quint), multiplet (m), and broad (b). High-resolution mass spectra (HRMS) were recorded on an LTQ-Orbitrap XL.

Synthetic Procedures for Diyne-SM and d_2-Diyne-SM.

(2S,3R,E)-(2-stearoylamino-3-hydroxyoctadec-4-en-1-yl)-{2-[(6-hydroxyhexa-2,4-diyn-1-yl)dimethylammonio]ethyl}phosphate (diyne-SM). To a solution of propargyl alcohol **S1** (300 mg, 5.35 mmol) in acetone (15 mL), *N*-bromosuccinimide (1.02 g, 5.78 mmol) and silver nitrate (91 mg, 0.54 mmol) were added at room temperature. The reaction mixture was stirred at room temperature for 2 h and then, concentrated. The residue was extracted two times with diethyl ether. The combined organic layers were dried with Na_2SO_4, filtered, and concentrated to afford **S2** (645 mg, 90% yield) as a pale yellow oil, which was used directly in the next step. ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.63 (brs, 1H), 4.30 (s, 2H). CuCl (0.3 mg, 3.1 μmol), *i*-$PrNH_2$ (5.1 μL, 0.06 mmol), and $NH_2OH{\cdot}HCl$ (1.0 mg, 14.5 μmol) were added to MeOH (1 mL) at room temperature under argon. The mixture was cooled to 0 °C, and then, **S3** (5) (compound **S3** was the intermediate for the synthesis of SM head group analogs; 40 mg, 0.05 mmol) was added, forming a yellow acetylide suspension. A solution of **S2** (21 mg, 0.16 mmol) in MeOH (0.2 mL) was added immediately. The reaction mixture was stirred at the same temperature for 30 min, concentrated, and extracted with $CHCl_3$. The combined organic layers were dried with Na_2SO_4, filtered, and concentrated. The mixture was passed through a short bed of silica gel eluting with $CHCl_3$/MeOH (5:1). The crude product was purified on a Cosmosil 5C18-AR-II column (10 × 150 mm; Nacalai Tesque) with MeOH as the eluent to give diyne-SM (5.1 mg, 12%) as a white solid. TLC: R_f = 0.18 (CH_2Cl_2/MeOH/NH_4OH 70:30:3); ^{1}H NMR (500 MHz, CD_3OD): δ 0.88 (t, J = 7.5 Hz, 6H); 1.24–1.42 (m, 50H); 1.53–1.63 (m, 2H); 2.01 (dt, J = 7.0, 7.0 Hz, 2H); 2.13–2.20 (m, 2H); 3.24 (s, 6H); 3.70 (t, J = 5.5 Hz, 2H); 3.87–3.98 (m, 2H); 4.03 (dd, J = 8.0, 8.0 Hz, 1H); 4.06–4.13 (m, 1H); 4.27 (s, 2H); 4.25–4.30 (m, 2H); 4.56 (s, 2H); 5.43 (ddt, J = 15.5, 8.0, 1.5 Hz, 1H); 5.69 (dtd, J = 15.5, 7.0, 1.0 Hz, 1H); 7.90 (d, J = 9.0 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD): δ 13.11, 22.41, 25.85, 29.15, 29.18, 29.37, 29.44, 29.74, 31.75, 32.15, 36.05, 49.55, 50.86, 53.93, 55.79, 58.90, 64.22, 64.61, 65.64, 66.54, 71.22, 75.42, 81.27, 129.89, 133.82, 174.58; HRMS (electrospray ionization) calculated for $C_{46}H_{86}N_2O_7P$ $[M + H]^+$ 809.6167, found 809.6184. ^{1}H and ^{13}C NMR spectra of diyne-SM are shown in Figs. S9 and S10. The synthetic scheme is summarized in Scheme S1.

(2S,3R,E)-[2-(10,10-dideuteriumstearoylamino)-3-hydroxyoctadec-4-en-1-yl]-{2-[(6-hydroxyhexa-2,4-diyn-1-yl)dimethylammonio]ethyl}phosphate (d_2-diyne-SM). This compound was prepared from **S4** (a full account of the synthesis of compound **S4** will be reported elsewhere) in 14% yield as a white solid by following the same procedure as described for **S3**. TLC: R_f = 0.18 (CH_2Cl_2/MeOH/NH_4OH 70:30:3); ^{1}H NMR (500 MHz, CD_3OD): δ 0.88 (t, J = 7.5 Hz, 6H); 1.22–1.43 (m, 48H); 1.50–1.63 (m, 2H); 2.01 (dt, J = 7.0, 7.0 Hz, 2H); 2.13–2.20 (m, 2H); 3.25 (s, 6H); 3.71 (t, J = 5.0 Hz, 2H); 3.88–3.99 (m, 2H); 4.02 (dd, J = 8.0, 8.0 Hz, 1H); 4.06–4.12 (m, 1H); 4.27 (s, 2H); 4.21–4.34 (m, 2H); 4.56 (s, 2H); 5.43 (ddt, J = 15.0, 8.0, 1.2 Hz, 1H); 5.69 (dtd, J = 15.0, 7.0, 0.6 Hz, 1H); 7.90 (d, J = 9.0 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD): δ 13.16, 22.43, 25.87, 29.18, 29.21, 29.33, 29.47, 29.52, 29.55, 29.58, 31.77, 31.79, 32.18, 36.06, 49.56, 50.87, 53.87, 53.93, 55.80, 58.90, 59.94, 64.22, 64.28, 64.61, 64.65, 65.67, 66.56, 71.21, 75.43, 81.29, 129.93, 133.79, 174.54; HRMS (electrospray ionization) calculated for $C_{46}H_{83}D_2N_2O_7PNa$ $[M + Na]^+$ 833.6112, found 833.6124. ^{1}H and ^{13}C NMR spectra of d_2-diyne-SM are shown in Figs. S11 and S12. Synthetic scheme is summarized in Scheme S2.

1. Palonpon AF, et al. (2013) Raman and SERS microscopy for molecular imaging of live cells. *Nat Protoc* 8(4):677–692.
2. Edholm O, Nagle JF (2005) Areas of molecules in membranes consisting of mixtures. *Biophys J* 89(3):1827–1832.
3. Ali S, Smaby JM, Brockman HL, Brown RE (1994) Cholesterol's interfacial interactions with galactosylceramides. *Biochemistry* 33(10):2900–2906.
4. Davis JH, Jeffrey KR, Bloom M, Valic MI, Higgs TP (1976) Quadrupolar echo deuteron magnetic resonance spectroscopy in ordered hydrocarbon chains. *Chem Phys Lett* 42(2):390–394.
5. Goretta SA, Kinoshita M, Mori S, Tsuchikawa H, Matsumori N, Murata M (2012) Effects of chemical modification of sphingomyelin ammonium group on formation of liquid-ordered phase. *Bioorg Med Chem* 20(13):4012–4019.

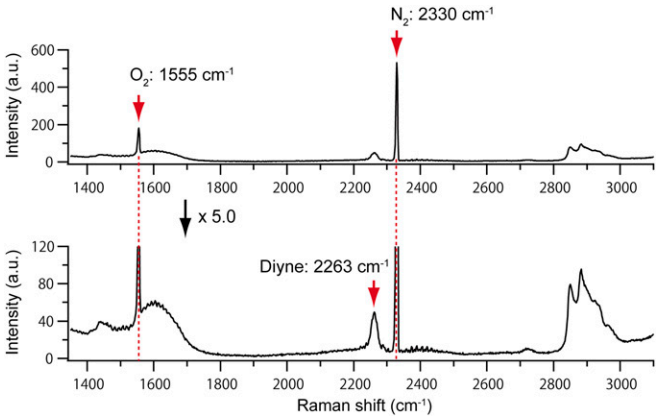


Fig. S1. Raman spectrum of the diyne-SM monolayer supported on a quartz substrate shown in Fig. 2*A*. Raman peaks of N_2, O_2, and diyne are indicated by red arrows. *Lower* is a five times enlarged view of *Upper* in terms of intensity (vertical axis). Strong Raman scattering by N_2 and O_2 in air was always detected during Raman measurement of lipid membranes. For ease of picking out the important Raman peaks, such as diyne, the two peaks of N_2 and O_2 have been removed in Figs. 2 and 4 and Figs. S3 and S4.

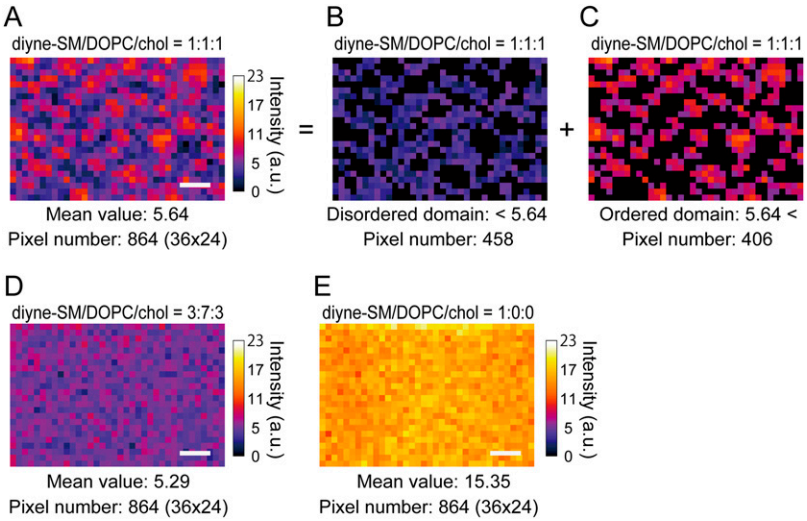


Fig. S2. (*A*) Raman image of the diyne-SM/DOPC/chol ternary monolayer with a 1:1:1 ratio reconstructed using the intensity of the diyne peak at 2,263 cm^{-1}. The image is the same as Fig. 4*A*. Mean value of the image is 5.64. The two phases were separated by taking pixel regions (*B*) below the mean value as disordered domains and (*C*) above the mean value as ordered domains. Raman images of diyne-SM/DOPC/chol ternary monolayers with (*D*) a 3:7:3 ratio and (*E*) a 1:0:0 ratio. The images are the same as Fig. 4 *B* and *C*, respectively. (Scale bar: 10 μm.)

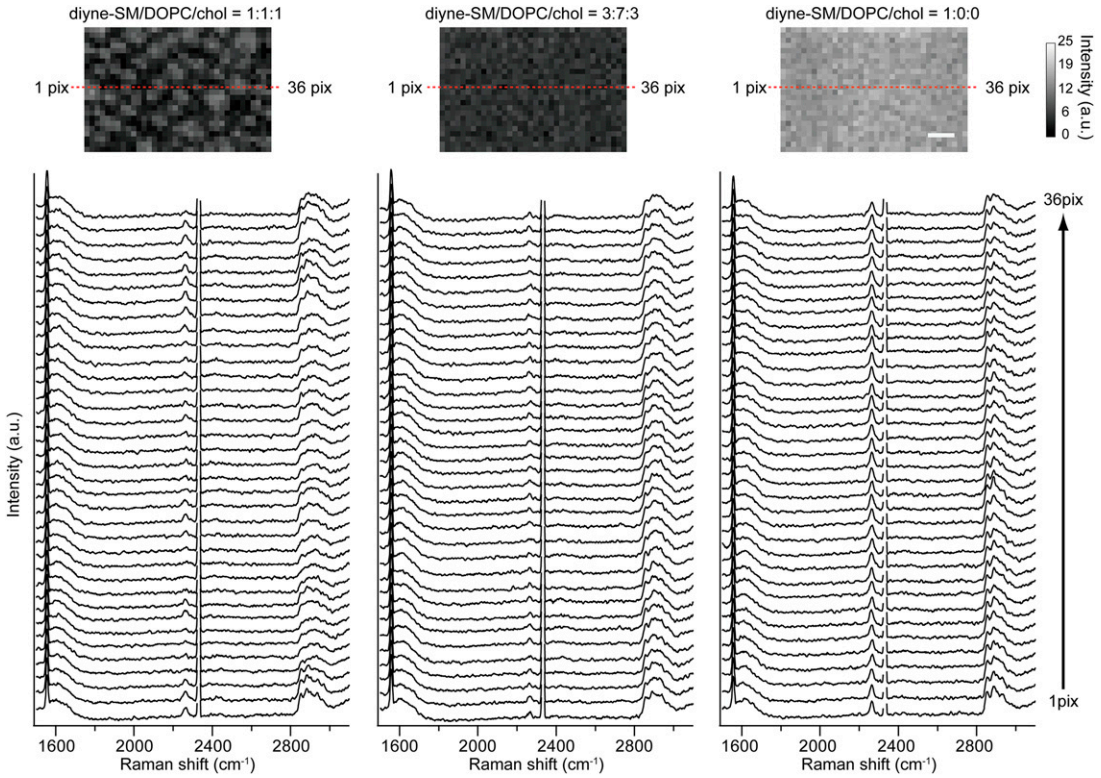


Fig. S3. Raman spectra obtained along the red lines in the Raman images of the diyne-SM/DOPC/chol ternary monolayer with 1:1:1, 3:7:3, and 1:0:0 ratios. The images are the same as in Fig. 4 *A*–*C*, with 36 × 24 pixels. Raman peak of N_2 at ~2,330 cm^{-1} was removed so that it would be easier to see Raman peaks from lipid molecules. Pix, pixel. (Scale bar: 10 μm.)

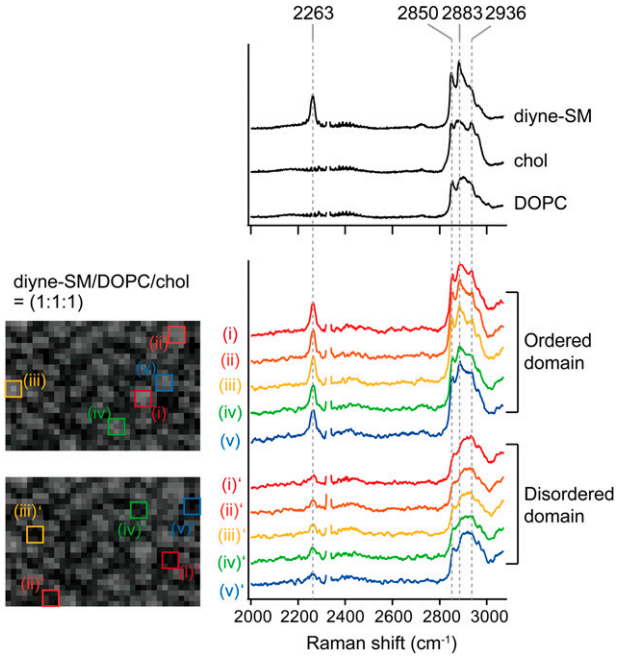


Fig. S4. Comparison between Raman spectra of supported lipid monolayers with single-component composition (diyne-SM, DOPC, or chol) and Raman spectra of diyne-SM/DOPC/chol ternary monolayer at ordered and disordered domains. The spectra of the ternary monolayer were obtained from five representative areas (3 × 3 pixels) calculated using the Raman image in Fig. 4*A*. Characteristic Raman peaks are displayed in the image, including diyne stretching vibration (2,263 cm^{-1}), CH_2 symmetric/asymmetric stretching vibration (2,850/2,883 cm^{-1}), and CH_3 symmetric stretching vibration (2,936 cm^{-1}). Raman peak of N_2 at ~2,330 cm^{-1} was removed so that it would be easier to see Raman peaks from lipid molecules.

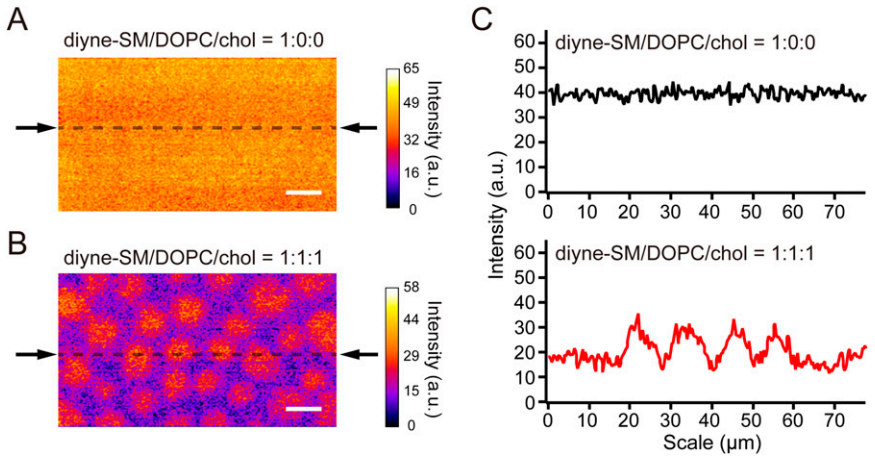


Fig. S5. (*A*) Raman image of a pure diyne-SM monolayer taken with slit-scanning Raman microscopy. The image was reconstructed using the diyne peak intensity at 2,263 cm^{-1}. Exposure time and laser power were 60 s per line and 13.9 $mW/\mu m^2$, respectively. The image consists of 344 × 190 pixels. (Scale bar: 10 μm.) (*B*) Raman image of a 1:1:1 diyne-SM/DOPC/chol ternary monolayer extracted from the lower left portion in Fig. 5*A*. The image consists of 344 × 190 pixels. (Scale bar: 10 μm.) (*C*) Line profiles of lipid membranes calculated along the dotted lines of the images in *A* and *B*.

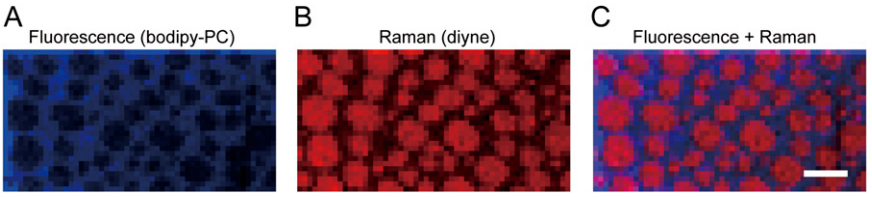


Fig. S6. (*A*) Fluorescence and (*B*) Raman images of a 1:1:1 diyne-SM/DOPC/chol ternary monolayer containing 0.2 mol% Bodipy-PC. The fluorescence image was reconstructed using the average fluorescence intensity at 555–605 nm (blue). The Raman image was reconstructed using the diyne peak intensity at 2,264 cm^{-1} (red). Both images were obtained in the same imaging area of the same sample. Fluorescence background during Raman imaging was suppressed by photobleaching of Bodipy-PC under 532-nm laser exposure. (*C*) Superimposed image of fluorescence and Raman images obtained in Fig. S5 *A* and *B*. The images consist of 54 × 28 pixels. (Scale bar: 20 μm.)

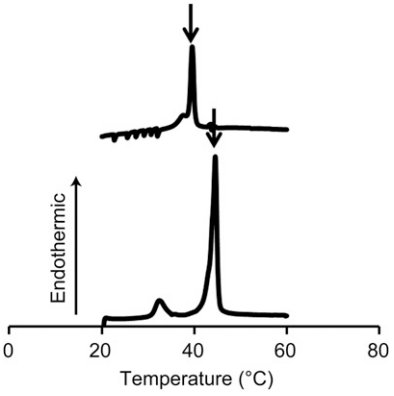


Fig. S7. DSC heating thermograms of (*Upper*) diyne-SM and (*Lower*) SM bilayers immediately after preparation. The main transitions are indicated by arrows.

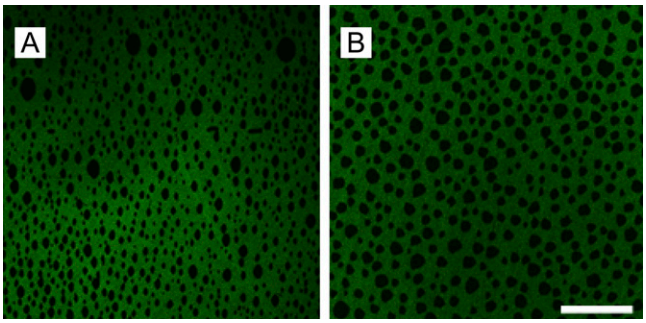


Fig. S8. Fluorescence images of (*A*) diyne-SM/DOPC/chol and (*B*) SM/DOPC/chol quartz-supported monolayers in the presence of 0.2 mol% Bodipy-PC at 12 mN/m and 25 °C. (Scale bar: 50 μm.)

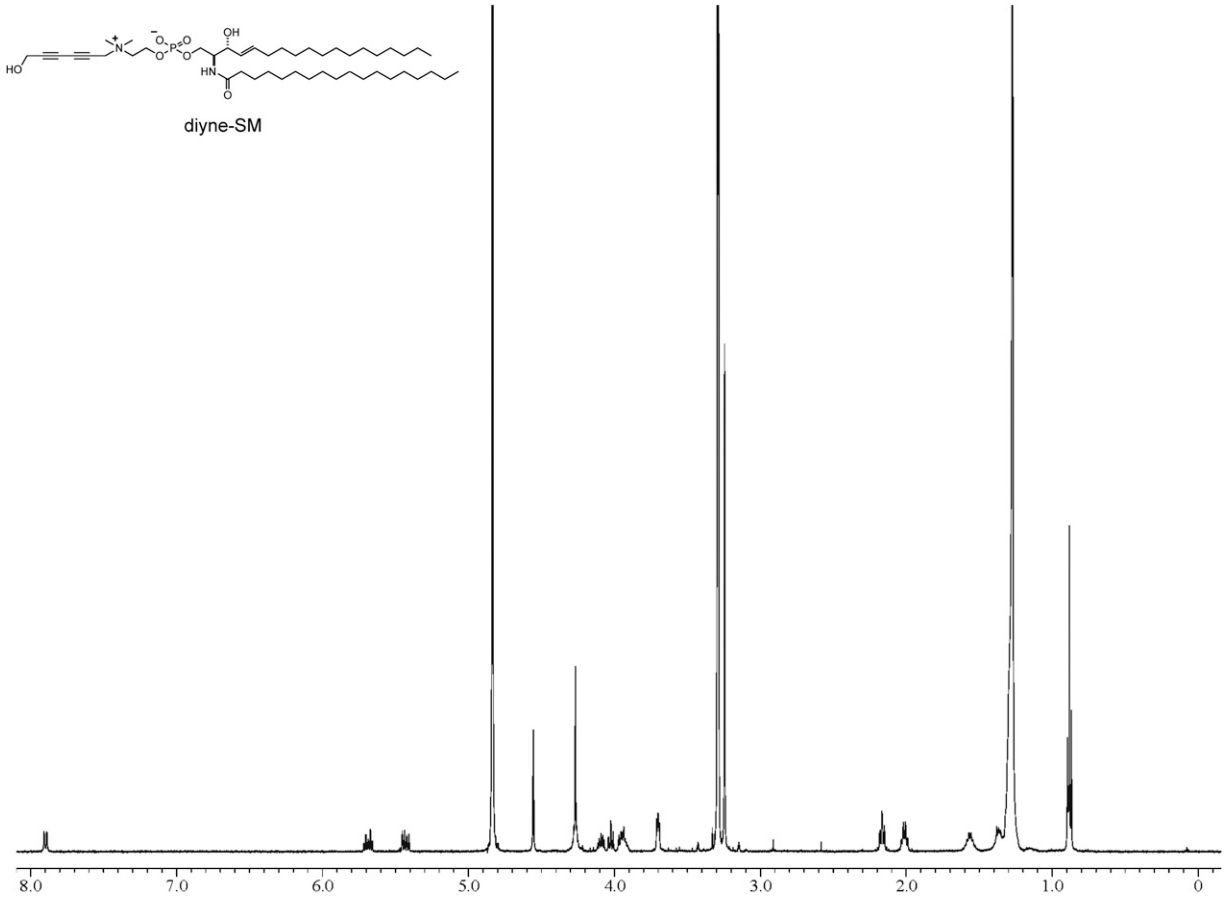


Fig. S9. ^{1}H NMR spectrum of diyne-SM.

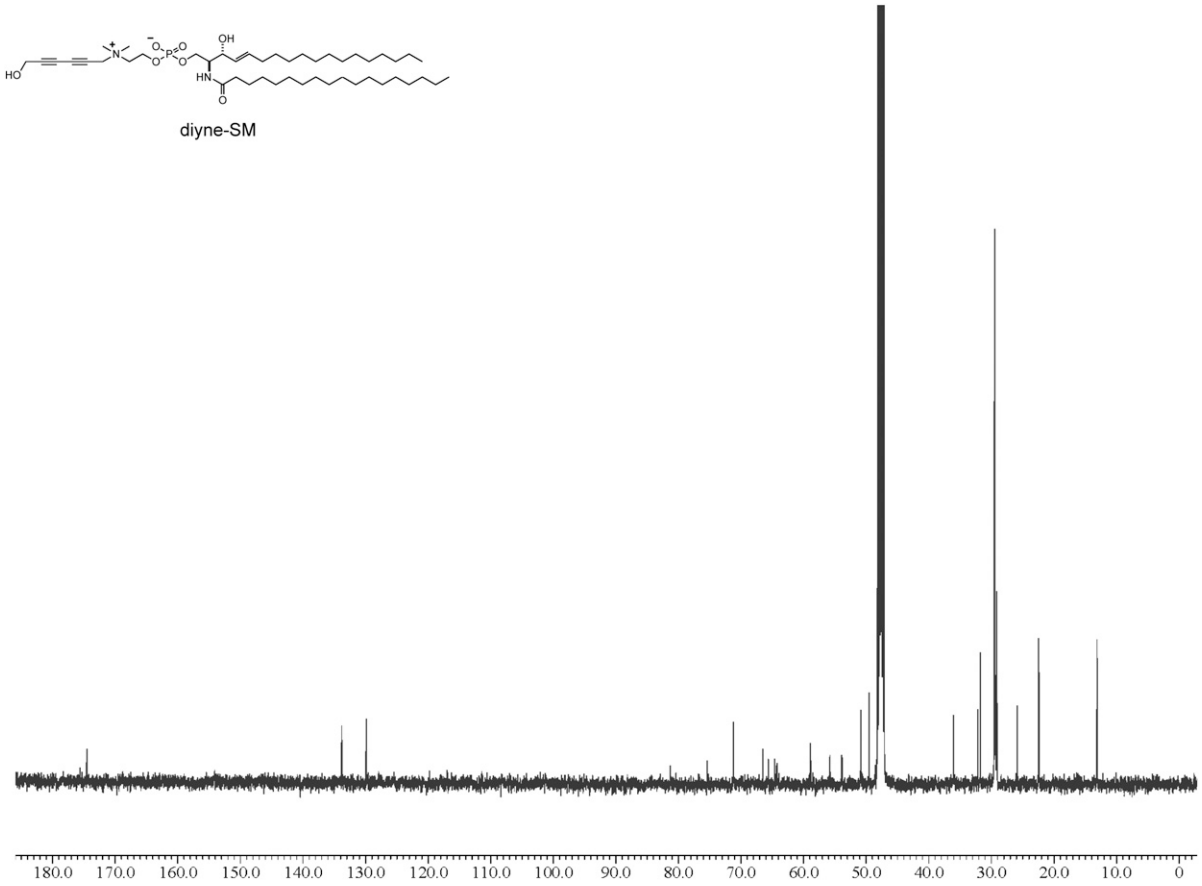


Fig. S10. ^{13}C NMR spectrum of diyne-SM.

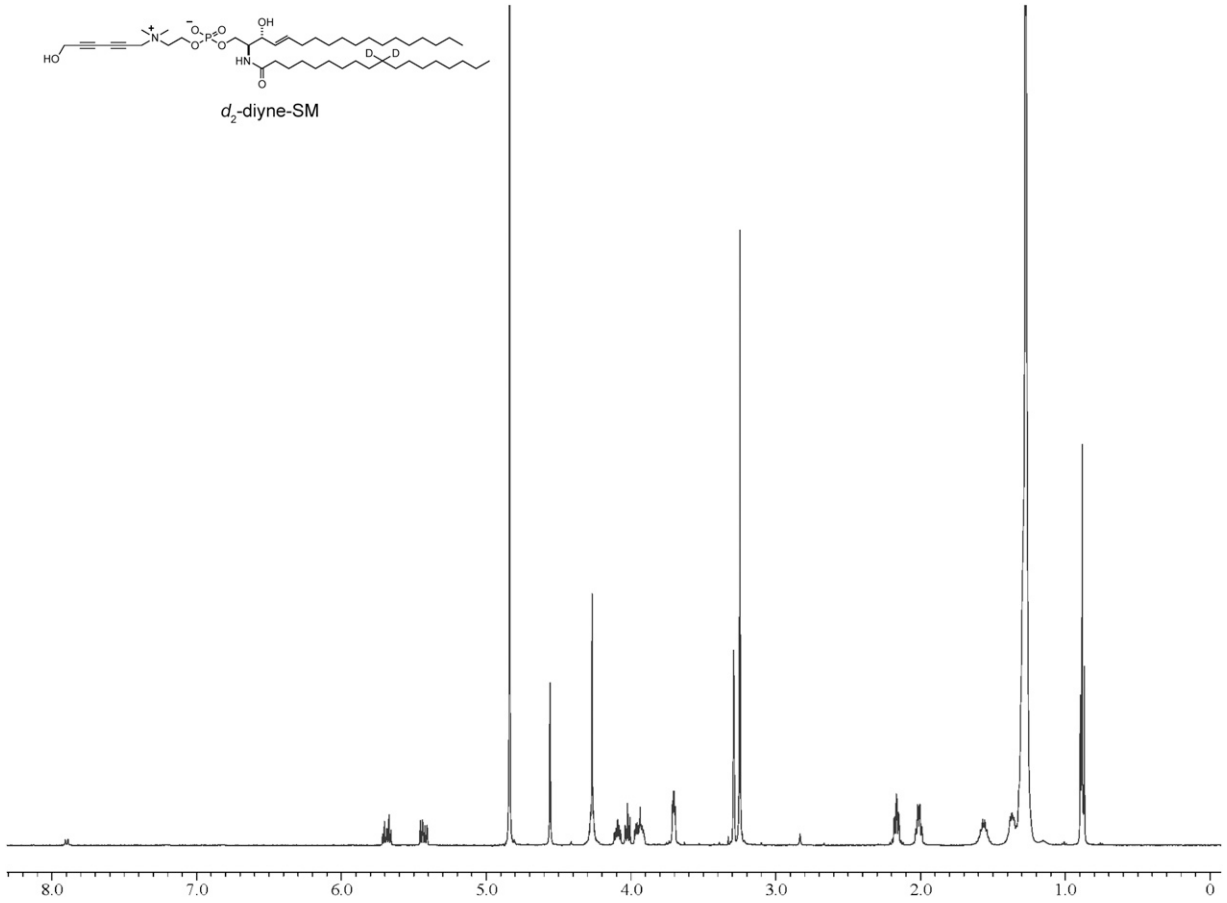


Fig. S11. ^{1}H NMR spectrum of d_2-diyne-SM.

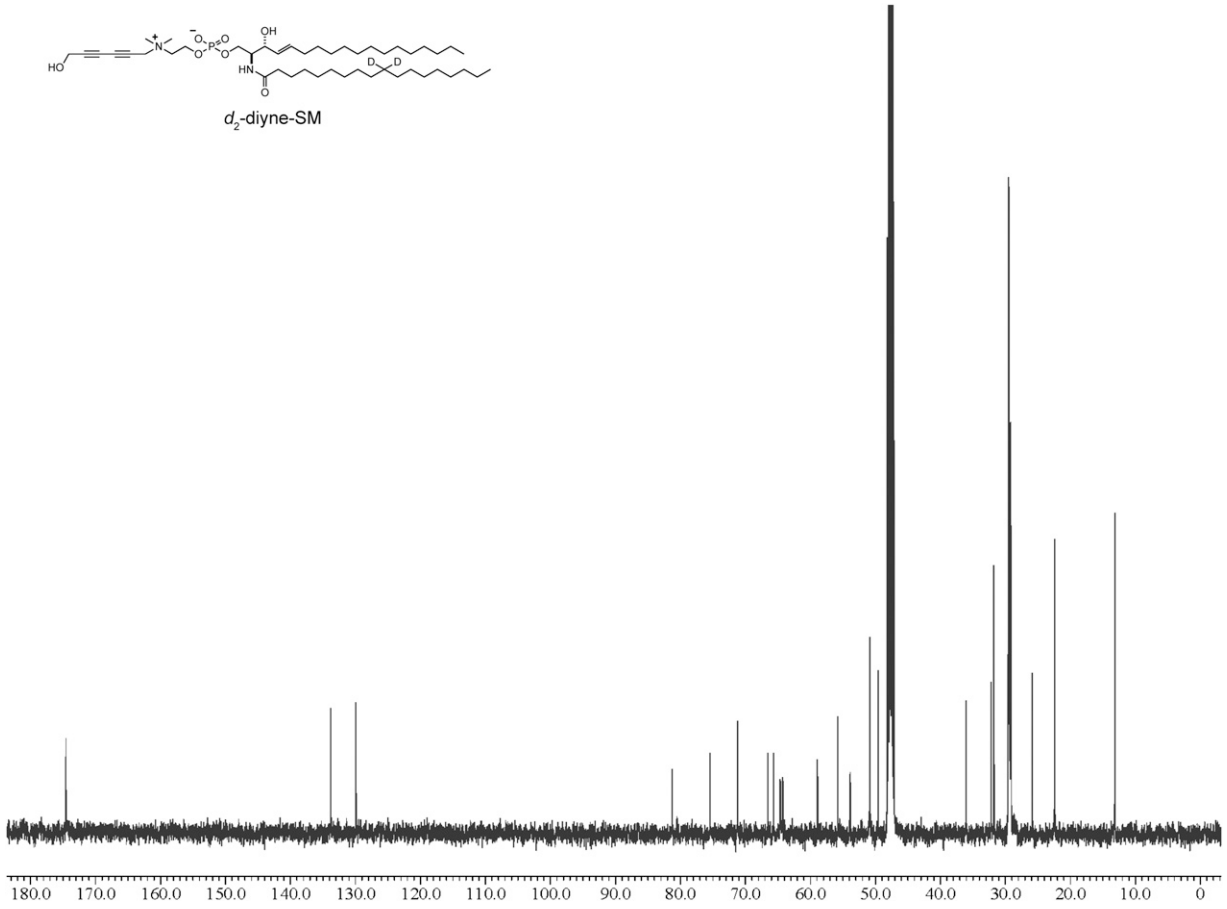
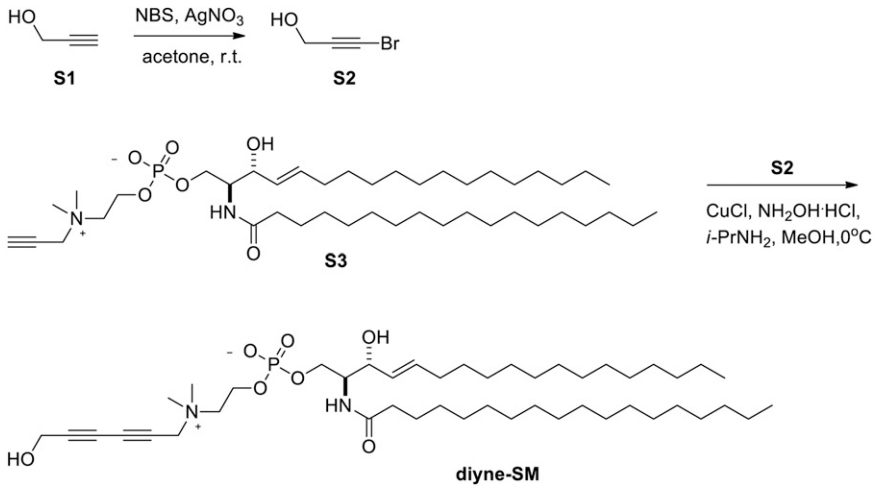
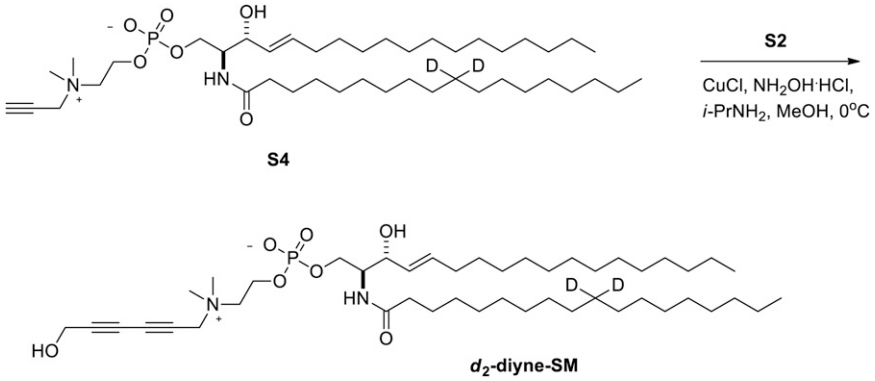


Fig. S12. ^{13}C NMR spectrum of d_2-diyne-SM.



Scheme S1. Synthesis of diyne-SM.



Scheme S2. Synthesis of d_2-diyne-SM.

Table S1. Mean value of Raman intensity of the diyne peak in diyne-SM/DOPC/chol ternary monolayer calculated from the images shown in Fig. S2

Diyne-SM/DOPC/chol	Pixel no.	Mean (± SD)	Relative, %
1:1:1 (ordered)	406	8.01 (± 1.60)	52.2
1:1:1 (disordered)	458	3.54 (± 1.23)	23.1
3:7:3	864	5.29 (± 1.60)	34.5
1:0:0	864	15.35 (± 1.48)	100

Relative value was calculated using diyne peak intensity of the membrane with a 1:0:0 ratio as 100%.