Supporting Information for

Passive translocation of phospholipids in asymmetric model membranes: Solid-state ^{1}H NMR characterization of flip-flop kinetics using deuterated sphingomyelin and phosphatidylcholine

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Effect of Pr^{3+} on ^1H NMR relaxation time

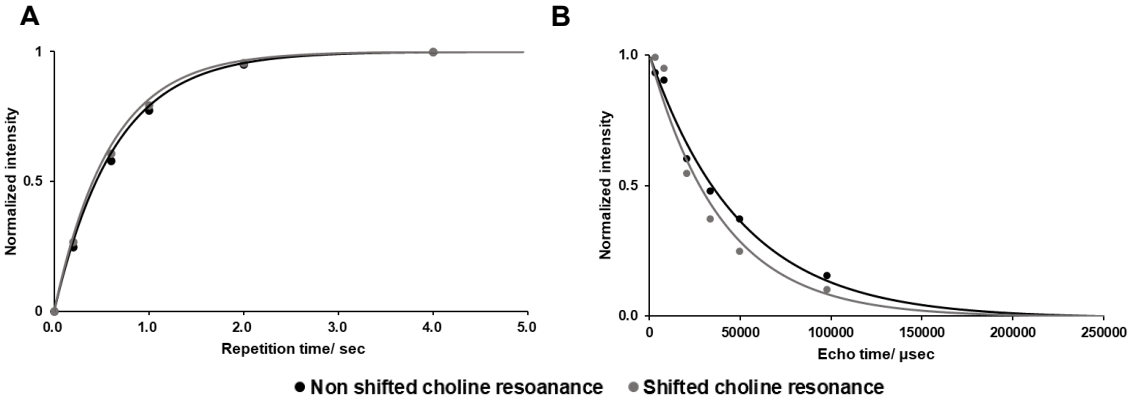


Figure S1. ^1H NMR relaxation time measurements of the -NMe_3 signal in symmetric POPC/Cho (7/3) LUVs with (black trace) or without (gray trace) Pr^{3+}. A: saturation recovery experiments for T1. B: spin echo experiments for T2. The lipid concentration of samples in the NMR insert was 75 mM.

DPH anisotropy of aLUVs and MLVs composed of PSM

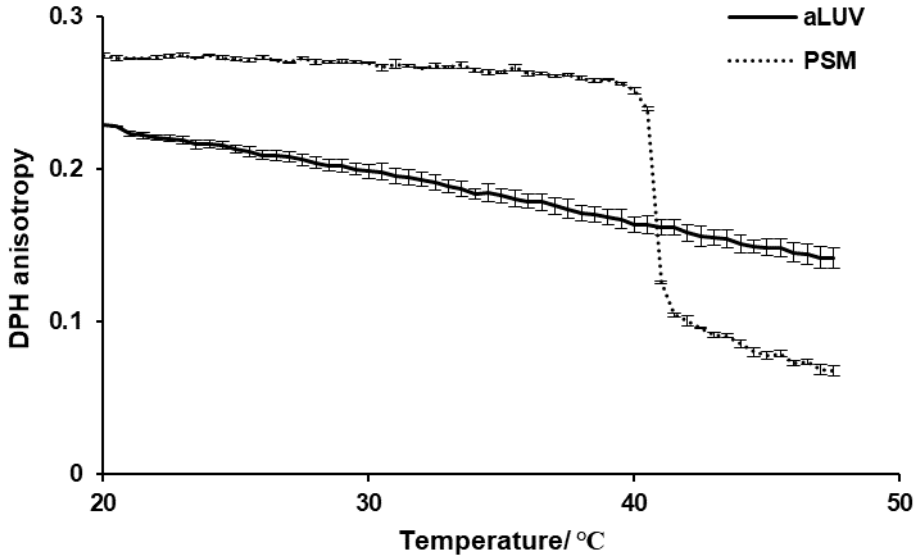


Figure S2 DPH anisotropy in aLUVs (*solid line*) and MLVs composed of PSM (*dot line*) with increasing temperatures. Error bars mean ± SE. Average and SE values were obtained from 3 preparations. The lipid concentration of samples in the cuvette was roughly 100 µM.

Estimation of the amount of donor MLV contaminated in aLUVs

Table S1. Estimation of the amount of MLVs present in aLUVs suspensions based on Phospholipids and NBD-cholesterol concentrations in aLUV sample

Total phospholipid/ μM	NBD-Cho/ μM
1638 ± 292	1.55 ± 0.41

The total concentration of the phospholipids was determined using the phospholipid quantification assay kit (Phospholipids C Test Wako Kit, FUJIFILM), based on the manufacture's protocol. NBD-Cho (1 mol%) was included in the donor MLVs. The concentration of NBD-Cho in the resultant aLUVs was determined by the UV-vis absorbance to estimate the amount of residual MLVs after centrifugation (see Additional Experimental Section for details).

From the values in Table S1, we estimated the amount of lipids in donor MLVs that were mixed into aLUVs used for NMR experiments. First, assuming that NBD-Cho is not transferred to aLUV by MαCD (as is the case with Cho) and that the ratio of phospholipids to NBD-Cho in MLVs is not affected by translocation of PSM and DOPC between MLVs and aLUV during the lipid exchange, the amount of residual MLVs residing in an aLUV preparation should be 100 molar times the amount of NBD-Cho. As a result, contamination of the MLVs increased the amount of phospholipids (PSM+DOPC) by 9.5 mol% (in average) in the aLUVs preparation. This value means that the unshifted trimethylammonium peaks in the presence of Pr^{3+}, which is usually assigned as PSM present in the inner leaflet of aLUV, is not really the case but due to the residual MLVs. For example, the peak area at 3.2 ppm in Fig. 4C indicates that approximately 16 mol% of PSM resides in any layers inside the MLVs other than their outermost leaflet. This estimation is reasonable by the following reasons. PSM is estimated to consist about 90 mol% of the total MLV lipids (PSM and DOPC) at the beginning of incubation (the rest of lipid could be DOPC transferred from acceptor LUVs, see below). Thus, the amount of PSM in the contaminating MLV is about 8–9 mol% of the total lipids in the aLUV preparation. PSM in the contaminating MLVs mostly distributes in any layers inside the MLVs, and thus only a small fraction is likely to be present in the outermost leaflet of the MLV showing a peak shifted at 3.4 ppm. Ignoring this fraction, the molar ratio of PSM to total PSM in contaminated MLVs is estimated to be 17 mol% from the PSM/DOPC ratio of 38:37 determined by solution NMR. This value is very close to the percentage of PSM in the inner leaf (16 mol%) obtained from the ss-NMR results.

In addition, since DOPC translocated from aLUVs is present in the outer-most leaflet of the residual MLVs, and since this fraction of DOPC affects the interpretation of the NMR experiment, we estimated the percentage of DOPCs present in the residual MLVs. That is, assuming that the same amount of

DOPC translocated to MLVs as PSM translocated to aLUVs according to the above assumptions, and that the PSM-DOPC ratio in the residual MLVs is equal to that of the total donor MLVs, the DOPC transferred into MLVs was estimated to be less than 2.6 mol% of total phospholipids in the aLUVs preparation (we believe that the actual value is lower than this). Therefore, it was assumed that the NMR measurement of Flip-Flop rate was not significantly affected by this DOPC fraction, which should be included in the margin of error.

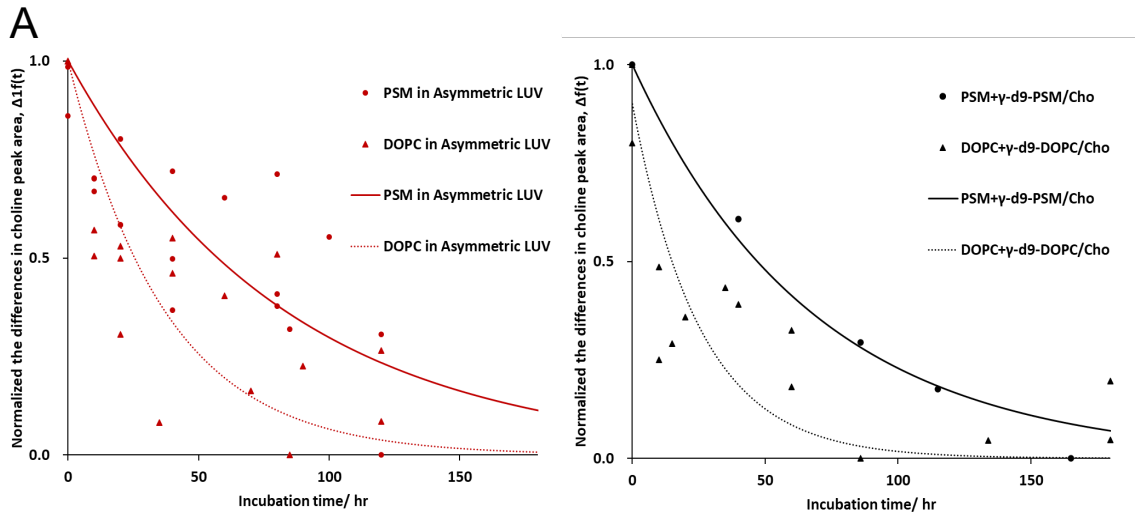
Based on the above discussion, we assumed the lipid composition of the aLUVs used in this experiment (minus the contribution of MLVs) to be PSM/DOPC/Cho = 34 ± 1.4 : 39 ± 2.1 : 27 ± 0.6 (see text), with PSM and DOPC accounting for almost 100 mol% of the phospholipids in the outer and outer lobes, respectively to interpret the experimental results.

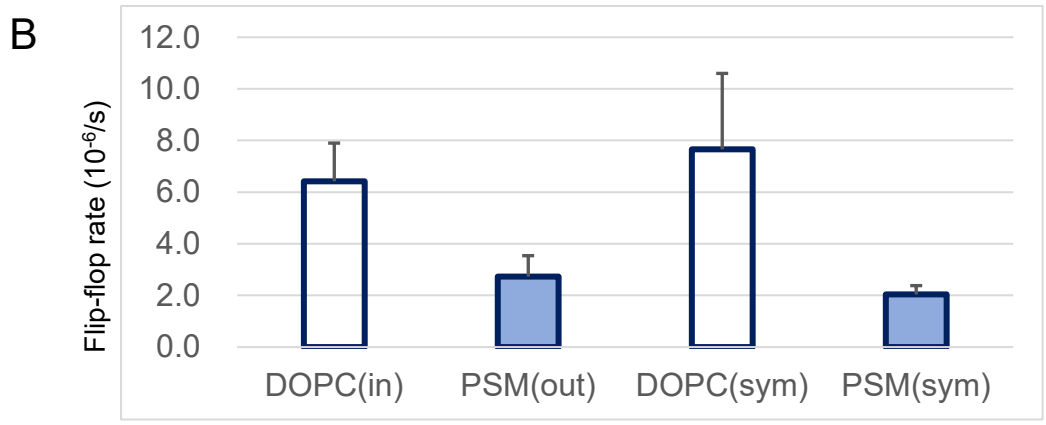
Estimation of Cho content in sLUVs by solution ^{1}H NMR

Table S2. The estimated lipid composition of sLUVs which was composed of PSM, γ-d_9-PSM and Cho, or DOPC, γ-d_9-DOPC, and Cho by solution ^{1}H NMR experiment.

PSM^{out}/γ-d_9-PSM/Cho	/ %
PSM+γ-d_9-PSM	72 ± 1.7
Cho	28 ± 1.7
γ-d_9-DOPC^{out}/DOPC/Cho	/ %
DOPC+γ-d_9-DOPC	79 ± 0.4
Cho	21 ± 0.4

Evaluation of phospholipid translocations by MAS-ss-^{1}H NMR





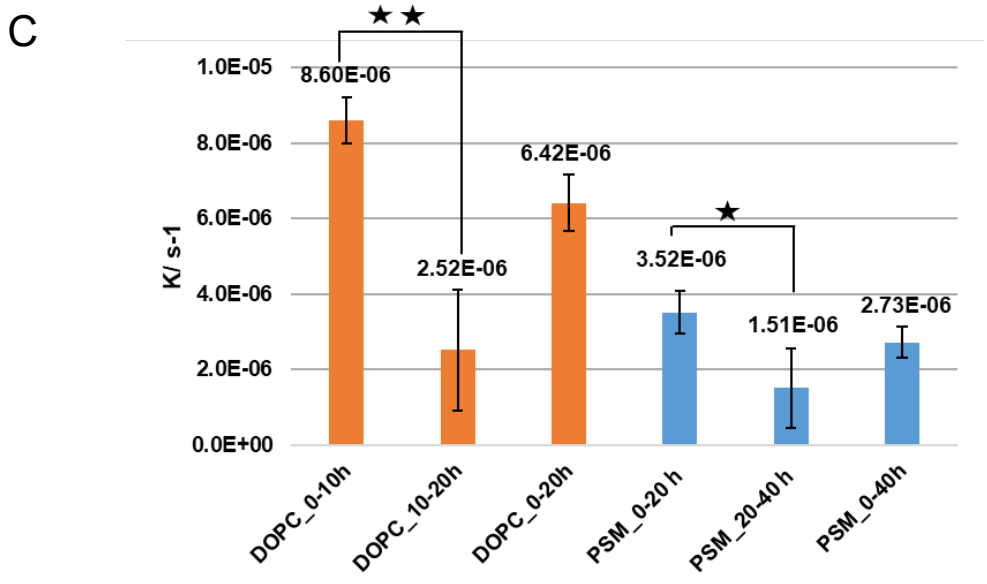


Figure S3. A: Time-dependent decay of the normalized ^{1}H NMR peak intensity afforded by phospholipid translocations in aLUVs (left) and sLUVs (right). The plots show normalized differences in the choline peak area of PSM (*circle*) and DOPC (*triangle*). The fitting curves of PSM (*solid line*) and DOPC (*dashed line*) were obtained using equations 3 and 4. **B:** The graph represents the flip-flop rates of asymmetric bilayers (left two) obtained from the data in the area above 0.5 and below 1.0 along the *y*-axis in panel A and the rate of symmetric bilayers (right two) obtained from the data in the whole area (numeric data are shown in Table 1). **C:** The flip-flop rates of DOPC and PSM in aLUVs in each incubation periods. ★★: 95% confidential interval. ★: 90% confidential interval.

Evaluation of the membrane properties dependent on Cho content based on DPH anisotropy

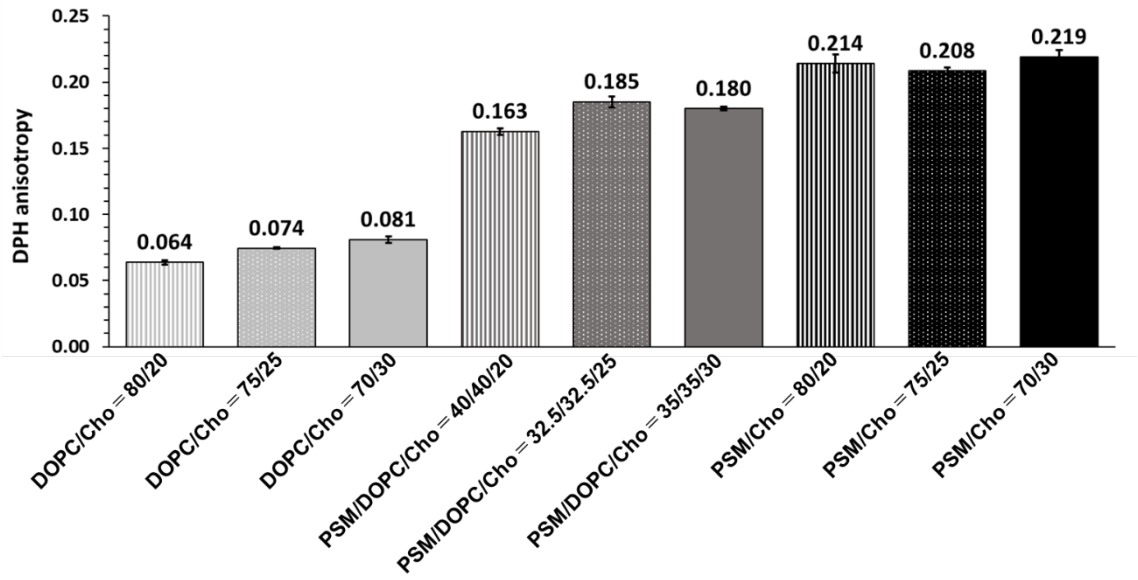


Figure S4. DPH anisotropy at 40 ℃ in MLVs that composed of DOPC/Cho, PSM/DOPC/Cho, or PSM/Cho. Error bars represent the mean ± standard error (SE) of a set of n =3. The lipid concentration of samples in the cuvette was roughly 100 μM. The Cho content of the vesicles consisting of PSM^{out}/γ-d$_9$-PSM^{in}/Cho was determined to be PSM+γ-d$_9$-P

Estimation of Cho contents in the symmetric LUVs

The Cho content of the vesicles consisting of PSM^{out}/γ-d$_9$-PSM^{in}/Cho was determined to be total PSM/Cho = 72(±1.7): 28(±1.7) in mol %, which was quite similar to that of aLUVs, and that of the vesicles consisting of γ-d_9-DOPC^{out}/DOPC^{in}/Cho was determined to be total DOPC/Cho = 79(±0.4): 21(±0.4) using

the LUVs consisting of DOPC/Cho, which was exchanged with donor DOPC MLVs. A few percent difference of the Cho content between the LUVs that composed of PSM/DOPC/Cho, PSM^{out}/γ-d_9-PSM^{in}/Cho, or γ-d_9-$DOPC^{out}$/$DOPC^{in}$/Cho may be attributed to the difference in the amount of the donor MLV contamination. Even though there is a difference in the Cho content in each LUVs, the Cho amount within the range does not significantly affect the membrane properties (Fig.S4). Hence, the differences in the flip-flop kinetics of each vesicle cannot be due to the Cho content.

Additional Experimental Section

The estimation of kinetics of lipid flip-flop

f_{PSM}^{Outer} and f_{DOPC}^{Inner} which was observed by the NMR experiments were determined using the following equations as shown in the section of *Materials and Methods*,

$$f_{PSM}^{Outer} = \frac{R_{shifted}}{R_{shifted}+R_{unshifted}} \quad (1)$$

$$f_{DOPC}^{Inner} = \frac{R_{unshifted}}{R_{shifted}+R_{unshifted}} \quad (2)$$

However, the observable value f'^{Outer}_{PSM} may contain the fraction from donor MLV contamination. Thus, equation (1) can be expressed as follows.

$$f'^{Outer}_{PSM} = \frac{R_{shifted}}{(R_{shifted}+R_{unshifted})(1-u)} = f_{PSM}^{Outer}\frac{1}{1-u} \quad (S1)$$

$$u = \frac{R_{MLV}}{R_{shifted}+R_{unshifted}} \quad (S2)$$

where R_{MLV} is the corrected area from the donor MLV contamination and the constant u is the ratio of the donor MLV contamination. Based on the experiments using symmetric LUVs, the difference of the relaxation time affected for these intensities was negligible. The observable fractions of PSM in inner-leaflet (f'^{Inner}_{PSM}) and DOPC in outer-leaflet (f'^{Outer}_{DOPC}) are represented using the following equations,

$$f'^{Inner}_{PSM} = 1 - f'^{Outer}_{PSM} \quad (S3)$$

$$f'^{Outer}_{DOPC} = 1 - f'^{Inner}_{DOPC} \quad (S4).$$

Assuming a unimolecular process, PSM translocation from the outer to the inner leaflet was represented by,

$$f'^{Outer}_{PSM} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} f'^{Inner}_{PSM} \quad (S5)$$

where the rate constants for movement from the outer leaflet to the inner leaflet or from the inner leaflet to the outer leaflet are given by k_1 and k_2, respectively. The time dependent changes of f'^{Outer}_{PSM} and f'^{Inner}_{DOPC} are given by,

$$\frac{d f'^{Outer}_{PSM}}{dt} = k_{2(\mathrm{PSM})} f'^{Inner}_{PSM} - k_{1(\mathrm{PSM})} f'^{Outer}_{PSM} \quad (S6),$$

respectively. For simplicity, assuming there is no difference between k_1 and k_2 and equating k_1 to k_2 such that $k_1=k_2=k$, Eq. S6 can be converted to

$$\frac{d f'^{Outer}_{PSM}}{dt} = -k_{PSM}(2f'^{Outer}_{PSM} - 1) \quad \text{(S7)}$$

In the initial stage of incubation where the high degree of asymmetry is maintained, this assumption is secured. The integrated form is then given by,

$$2f'^{Outer}_{PSM}(t) - 1 = (2f'^{Outer}_{PSM}(0) - 1)\exp(-2k_{PSM}t) \quad \text{(S8)}$$

Where $f'^{Outer}_{PSM}(0)$ is the initial value of the shifted peak in ^{1}H NMR. Using the corrected values in equation S1 for the PSM fraction, equation S8 is transformed as follows,

$$\frac{f^{Outer}_{PSM}(t)-\frac{1-u}{2}}{f^{Outer}_{PSM}(0)-\frac{1-u}{2}} = \exp(-2k_{PSM}t) \quad \text{(S9)}$$

where $f^{Outer}_{PSM}(0)$ is the initial ratio of PSM in the outer leaflet of aLUVs. $f^{Outer}_{PSM}(t_{max})$ is defined as the value when this exponential decay reaches plateau ($t \to max$); $f^{Outer}_{PSM}(t_{max}) = (1-u)/2$. This can be applied for ideal experimental system (i.e. $u = 0$) where $f^{Outer}_{PSM}(0)$ is 1 and $(1-u)/2$ is 0.5. respectively. Therefore, eq.S9 can be transformed into equation (3) in the main text:

$$\frac{f^{Outer}_{PSM}(t)-\frac{1-u}{2}}{f^{Outer}_{PSM}(0)-\frac{1-u}{2}} = \frac{f^{Outer}_{PSM}(t)-f^{Outer}_{PSM}(t_{max})}{f^{Outer}_{PSM}(0)-f^{Outer}_{PSM}(t_{max})} = \Delta f^{Outer}_{PSM}(t) = \exp(-2k_{PSM}t) \quad \text{(S10)}$$

A similar derivation can be applied for DOPC in the inner leaflet.

Solution ^{1}H NMR experiment to determine the total lipid composition of aLUVs.

aLUV pellets obtained upon the final ultracentrifugation were dissolved in an appropriate amount of isopropanol. The organic solvent was then evaporated, and the remained lipid films were exposed to a high vacuum for at least 1 h. The residue was then dissolved in $CD_3OD/CDCl_3$=1/1 (v/v) (0.6 mL). Solution NMR was collected by ECA-500 (500 MHz) spectrometers (Tokyo, Japan) to determine the total lipid composition. The ratio of each lipid in aLUVs was quantified based on the peak areas of H4 for PSM, H9, H10, H9' and H10' for DOPC, and H18 for Cho.

Estimation of the lipid concentration in NMR insert by UPLC-ESI-MS

The estimation of the total lipid concentration in NMR insert was performed on a quadruple time-of-flight (QTOF) mass spectrometer (X500R QTOF, Sciex, Framingham, MA, USA) coupled with a UPLC (ACQUITY UPLC H-class plus, Waters, Milford, MA, USA). Sphingomyelin, phosphatidylcholine, and Cho were separated using ACQUITY UPLC BEH C8 column (2.1 mm × 100 mm, particle size 1.7 μm,

pore size 130 Å) (Waters, Milford, MA, USA), and detected on positive mode by ESI-MS. 80:20 MeOH/Water with 0.1% formic acid and 10 mM ammonium formate was used as the solvent A, and MeOH with 0.1% formic acid and 10 mM ammonium formate was used as the solvent B. The flow rate was 0.3 mL/min. The solvent condition for separation were 20% solvent B for 0-1 min, linear gradient to 100% solvent B over 1 min, and 100% solvent B was kept for 4 min, and then linear gradient to 20% solvent B from 6-6.1 min, and constant to 9 min. SM and PC were found as $[M+H]^+$, and Cho was found as 369.3514 $[M-OH]^+$ and 404.3886 $[M+NH_4]^+$. The retention time of PSM, DOPC and Cho were 4.16 min, 4.41 min, and 3.98 min, respectively.

Estimation of the remaining MLVs after the lipid exchange process

Imperfect separation of donor MLVs from LUVs during preparation and purification of aLUVs disrupt the integral ratio of the PSM choline resonances, because Pr^{3+} added into the vesicle solution cannot access most lipid layers of MLVs except for the outermost layer. Therefore, the amount of MLV contamination was evaluated based on the absorbance of 1 mol% NBD-cholesterol added in the MLVs. The small cavity of MαCD selectively accepts the hydrocarbon chain of phospholipids while it cannot accept larger molecules such as cholesterol (London, 2019; Ohtani ' et al., 1989). The NBD-Cho concentration was determined by the absorbance. After preparation for aLUVs, they were dissolved with the appropriate amount of MeOH, and then the absorbance of the solution at 470 nm was measured using the UV-vis spectrophotometer V-730 BIO (JASCO corporation) and the total concentration of the phospholipids of aLUVs was determined using the phospholipid quantification assay kit (Phospholipids C Test Wako Kit, FUJIFILM). The absorbances and the molar extinction coefficient of NBD at 470 nm (22,000 $M^{-1}cm^{-1}$) indicated the amount of donor MLVs in aLUVs was estimated to be approximately 9% in PSM^{out}/$DOPC^{in}$/Cho aLUVs. As described, 16% PSM which was not affected by Pr^{3+} (Fig.3B upper panel) was due to donor MLV contamination.

Preparation of symmetric membrane for lipid lipid-flip kinetics

LUVs containing γ-d_9-DOPC or γ-d_9-PSM in one leaflet were prepared by lipid exchange between the acceptor LUV composed of 2:1 γ-d_9-PSM/Cho (mol/mol, 6 μmol) and the donor MLV composed of PSM (12 μmol), or the acceptor LUV composed of 2:1 DOPC/Cho (mol/mol, 6 μmol) and donor MLV composed of γ-d_9-DOPC (12 μmol) as described in Materials & Methods. For isolation of LUVs by ultracentrifugation, sucrose solution (2 mL) composed of 2.5% (w/w) for the γ-d_9-$DOPC^{out}$/ DOPC/Cho LUV or 10% (w/w) for the PSM^{out}/γ-d_9-PSM/Cho LUVs was used instead of 8% (w/w) sucrose solution. The isolated LUVs were installed into the NMR insert as described in Materials & Methods and then MAS ss-^{1}H NMR was measured. In γ-d_9-$DOPC^{out}$/ DOPC/ Cho system, DOPC was remained in the outer-leaflet even after lipid exchange due to low exchange efficiency between the outer-leaflet DOPC and γ-d_9-DOPC in the acceptor LUV.

TMA-DPH or DPH anisotropy.

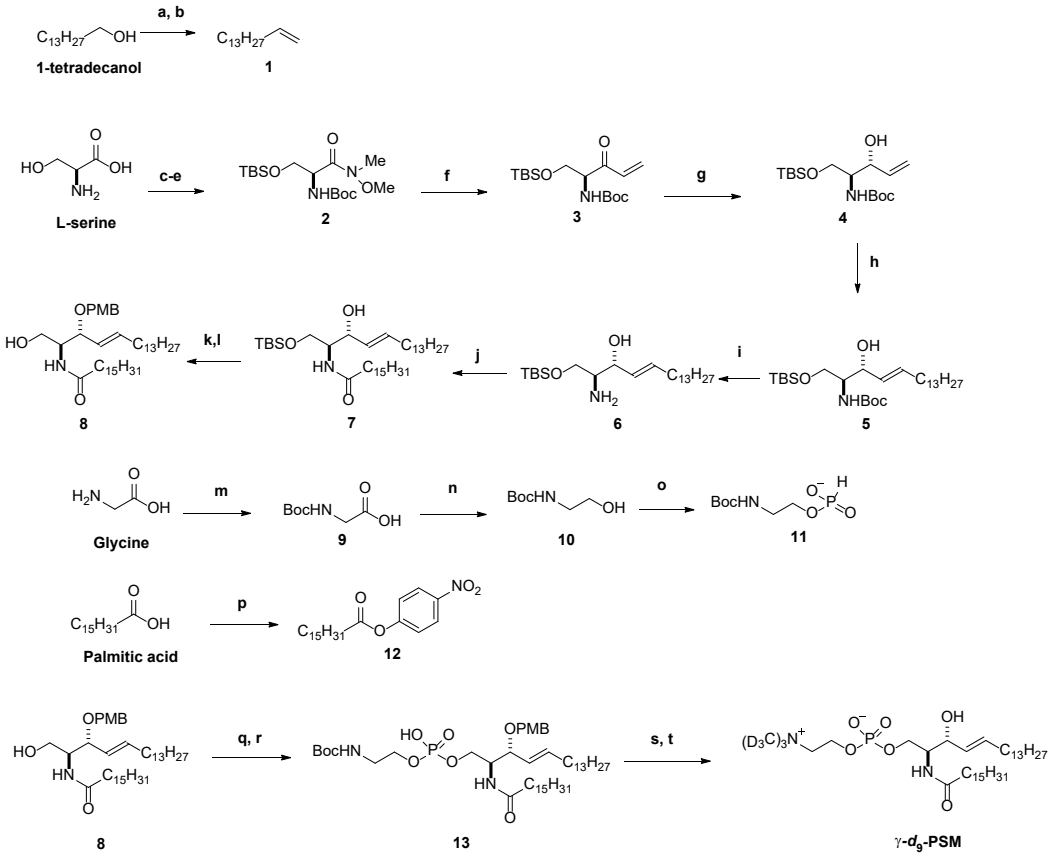
T MA-DPH: To evaluate the order of each leaflet in aLUV, the outer- or the inner-leaflet was selectively labeled with TMA-DPH(Wang & London, 2018). For the outer leaflet selective staining, 5 μL of 0.05 mg/mL TMA-DPH solution in EtOH was added to the aLUVs suspension, and the suspension was incubated for 20 min in the dark. For the inner-leaflet, 0.2 mol% of TMA-DPH was introduced to acceptor LUV when prepare the lipid film. Upon lipid exchange with MαCD, TMA-DPH was mostly distributed in the inner-leaflet because TMA-DPH was also extracted from the outer-leaflet by MαCD. The final lipid concentration in cuvette was ~100 μM and the ratio of TMA-DPH to lipids was approximately in 0.1 mol%.

DPH: MLVs were prepared with 300 nmol of the lipids composed of the appropriate ratio of PSM/Cho, DOPC/Cho or PSM/DOPC/Cho (mol/mol) plus 0.3 nmol DPH, and the final lipid concentration in cuvette was ~100 μM and the ratio of TMA-DPH to lipids was roughly 0.1 mol%. To measure the incubation time depending change in DPH anisotropy in aLUV, aLUV suspension was incubated at 40 ℃ for 0, 20, 40, and 120 h. Then, 5 μL of 0.05 mg/mL DPH solution in EtOH was added to the vesicle suspension, and the suspension was incubated for 20 min in the dark. The final lipid concentration in cuvette was ~100 μM and the ratio of DPH to lipids was roughly 0.1 mol%. The anisotropy data was obtained using JASCO FP-8500 spectrometer with 3 mL sample in a quartz cell. TMA-DPH and DPH were excited at 375 nm and fluorescence at 430 nm was collected from 20 to 47.5 °C with an increment of 0.5 °C/min.

Synthesis of headgroup deuterated phospholipids

Materials: CD_3I was purchased from Cambridge Isotope Laboratory (CIL, UK). Dry THF and*d* CH_2Cl_2 were purchased from Kanto Chemical Co., Inc.(Tokyo, Japan). The other chemicals were purchased from Nacalai tesque (Kyoto, Japan), FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), TCI (Tokyo, Japan), and Sigma-Aldrich (St. Louis, MO, US), unless otherwise specified.

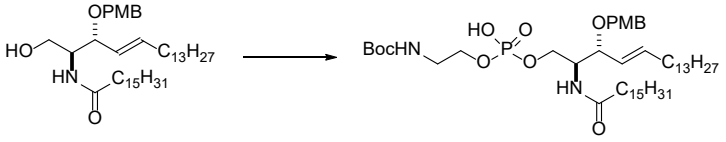
Synthesis of γ-d_9-PSM. γ-d_9-PSM was synthesized from L-serine by following our previous study (ref. 38 in the manuscript) as shown in Scheme 1.



Scheme S1. (*a*)DMSO, NEt_3, Pyr・SO_3 complex, CH_2Cl_2, 0 ℃, 2 h (88%); (*b*) $(Ph)_3PCH_3Br$, Potassium *tert*-butoxide, THF, rt, 1 h (44%); (*c*) $(Boc)_2O$, NaOH, H_2O, dioxane, rt, 2 h; (*d*) NH(Me)OMe·HCl, NMM, EDC, CH_2Cl_2, −15 °C, 4 h; (*e*) TBSCl, imidazole, DMF, rt, 1 h (86% for three steps); (*f*) vinyl MgBr, THF, rt, 1.5 h (86%); (*g*) $LiAlH(O^tBu)_3$, EtOH, −78 °C, 2 h (93%); (*h*) Compound **1**, Grubbs II catalyst, *p*-quinone, CH_2Cl_2, 55 °C, 2 h (64%); (*i*) $ZnBr_2$, CH_2Cl_2, rt., 24 h (86%); (*j*) Compound **12**, DMAP, NEt_3, THF, rt., 14 h (76%); (*k*) PMBO-C(=NH)CCl_3, $Yb(OTf)_3$, CH_2Cl_2, rt., 6 h (72%); (*l*) TBAF, THF, 0 ºC, 2 h, (66 %); (*m*) Boc_2O, 1 M KOH aq/1,4-dioxane=10/1, rt, 36 h; (*n*) $LiAlH_4$, THF, rt, 2 h, (63% for 2 steps); (*o*) 2-Chloro-4H-1,3,2-benzodioxaphosphorin-4-one, Pyr, THF, rt, 2 h, (62%); (*p*) DCC, *p*-nitrophenol, THF, rt, 17 h (75%); (*q*) Compound **11**, $(Me)_3CCOCl$, Pyr, 0 ºC, 40 min; (*r*) I_2, Pyr/H_2O=9/1, 0 ºC, 3 h, (51% for 2 steps); (*s*) TFA, CH_2Cl_2, 0

°C, 1 h, (71%); (*t*) CD_3I, 1 M KOH aq, MeOH/THF=1/1, rt, 26 h, (36%)

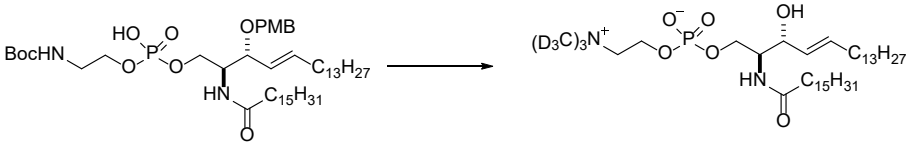
1-((*S*)-2-*N*-Boc-aminoethoxyphospho)-3-O-(*p*-methoxybenzyl)-2-*N*-palmitoyl- sphingosine 11



8 **13**

A solution of compound **8** (100 mg, 152 μmol) and **11** (61 mg, 274 μmol) were dissolve with pyridine (4 mL) and then evaporated. Then, the residue was dissolved with pyridine (3 mL), and $(Me)_3CCOCl$ (73 mg, 608 μmol) was added to the solution. The mixture was stirred at 0 ℃ for 40 min. After that, I_2 (77 mg, 304 μmol) in Pyr/H_2O solution (9:1=*v*/*v*, 1 mL) was added to the reaction mixture. After stirring at 0 ℃ for 3 h, The solution was quenched with sat. Na_2SO_3 aq, and extracted by $CHCl_3$ (×3). The organic phase was washed with brine (×1), and then dried over Na_2SO_4. After filtration, the organic phase was evaporated, and then purified by silica-gel chromatography ($CHCl_3$/CH_3OH=5/1) to give the compound **13** (77 mg, 87.9 μmol, 51 %).

Perdeuterated-trimethylammonium-*N*-palmitoyl-sphingomyelin

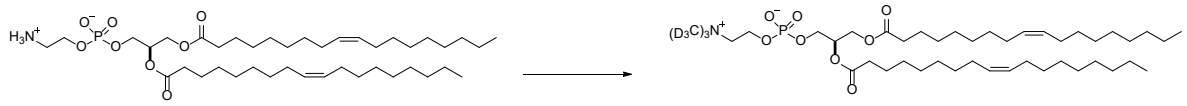


13 **γ-d_9-PSM**

To a solution of compound **13** (52 mg, 59 μmol) in CH_2Cl_2 (3 mL) at 0 ℃ added TFA (45 μL, 585 μmol) was stirred at 0 ℃ for 1 h. The reactant was then diluted with CH_3OH, and solvent was evaporated. The residue was passed through a short silica-gel column ($CHCl_3$/CH_3OH/H_2O=65:25:4) to remove debris. The residue was dissolved in THF/CH_3OH (1/1= *v*/*v*, 6 mL), and then added 1 M KOH aq (200 μL) and methyl iodide-d_3 (300 μL). The mixture was stirred at rt for 24 h, and then diluted with acetone (×1) to form a precipitate (white solid). The precipitate was further purified by reverse-phase HPLC (5C18-AR-II, 10 × 250 mm, MeOH) to give a desired γ-d_9-PSM (34 mg, 47 μmol, 36 %).

^{1}H-NMR(500MHz, CD_3OD) δ 5.78 (1H, d-t, *J* =15.0, 7.0 Hz, H5), 5.40 (1H, dd, *J* = 17.5,8.5 Hz, H4), 4.24 (2H, br, Hα), 4.10 (1H, m, H1) 4.07 (1H, d-d, *J* = 8.0, 4.0 Hz, H3), 3.97 (1H, d-d, *J* = 11.5, 3.0 Hz, H2), 3.89 (1H, m, H3), 3.61 (2H, t, Hβ), 2.17 (2H, t-d, *J* = 7.4, 2.0 Hz, H2'), 2.02 (2H, q, *J* = 7.2 Hz, H6), 1.57 (2H, m, H3'), 1.27-1.40 (46H, m, H7-H17, H4'-H15'), 0.87 (6H, t, H18, H16').
HR-ESI-MS C39H70D9N2O6PNa $[M+Na]^+$ calcd.734.6138, found 734.6135.

Preparation of γ-d_9-DOPC



γ-d_9-DOPC was prepared from DOPE (NOF, Tokyo, Japan). DOPE (104 mg, 139.9 μmol) was dissolved with methyl iodide-d_3 (1.2 mL) in the presence of 10 M KOH aq, (50 μL), and then the mixture was stirred at rt for 10 h. The solution was diluted with $CHCl_3$, and then washed with water (×1) and brine (×1), and dried over Na_2SO_4. After filtration, the organic phase was evaporated, and then purified by silica-gel chromatography ($CHCl_3$/CH_3OH/H_2O = 65:25:4). Following reverse-phase HPLC purification (column: 5C18-AR-II (Nacalai tesque), 10 × 250 mm, MeOH) gave desired γ-d_9-DOPC (60 mg, 75.8 μmol, 54 %).

^{1}H NMR (500 MHz, CD_3OD) δ 5.27 (4H, m, H9, H10, H9', H10'), 5.17 (1H, br, H*sn*-2), 4.37 (1H, dd, H*sn*-1), 4.20 (2H, br, Hα), 4.10 (1H, dd, H*sn*-1), 3.93 (2H, m, H*sn*-3), 3.57 (2H, d, Hβ), 2.26 (4H, m, H2, H2'), 1.96 (8H, m, H8, H11, H8', H11), 1.53 (4H, m, H3, H3'), 1.24 (42H, m, -CH_2-), 0.83 (6H, t, H18, H18'); HR-ESI-MS $C_{44}H_{75}D_9NO_8PNa$ $[M+Na]^+$ calcd. 817.6397, found 817.6395.