Supporting Information for

Effect of the number of sugar units on the interaction between diosgenyl saponin and membrane lipid

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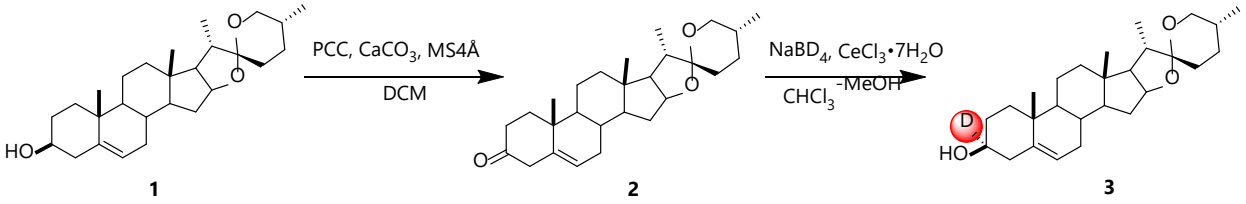
Additional Materials and Methods

Synthesis of deuterated TRL at the 3 position.

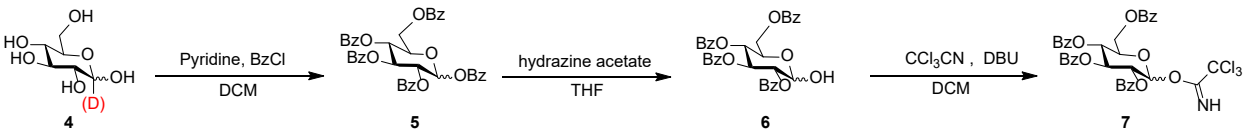
3-d_1-Diosgenin (**3**) was synthesized as previously described and as shown in Scheme 1. The same scheme was used to synthesize *3-d_1*-Chol**.** Under Ar atmosphere, pyridinium chlorochromate (PCC, 2.17 mmol), $CaCO_3$ (0.724 mmol) and MS4Å were suspended in dry CH_2Cl_2 (3 mL). To this mixture, diosgenin (0.724 mmol) dissolved in dry CH_2Cl_2 (3 mL) was added. The solution was stirred for 30 mins at 0 ºC under Ar, and the resulting slurry was poured/filtered through Celite. The filtrate was then concentrated and purified by column chromatography using silica gel with hexane/EtOAc (10:1) as eluant to afford compound **2** (0.168 mmol, 83%).

To a solution of **2** (0.266 mmol) in $CHCl_3$/MeOH (2:1) (5 mL), $NaBD_4$ (0.354 mmol) and $CeCl_3 \cdot 7H_2O$ (0.399 mmol) were added and the solution was stirred for 30 mins in Ar atmosphere at 0 ºC. After which, the solution was concentrated and purified by column chromatography using silica gel with hexane/EtOAc (18:1) as eluant to afford compound **3** (0.258 mmol, 97%). The structure identity of 3*d*-diosgenin was confirmed by ^{1}H NMR spectrum in comparison with that of diosgenin.



Scheme 1. Synthesis of deuterated TRL and 1' positions.

Synthesis of glycosyl donor -- 2,3,4,6 tetra-O-benzoyl-D-glucopyranosyl trichloroacetimidate

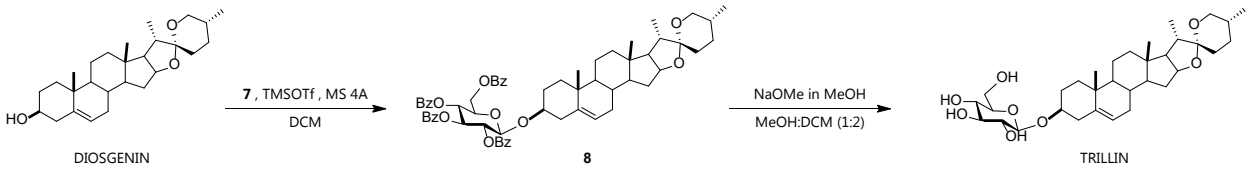


Benzoyl Chloride was slowly added to an ice-cooled solution of glucose **4** dissolved in anhydrous pyridine under Ar. The mixture was stirred overnight, followed by quenching with MeOH. The solvents were evaporated in vacuo and the residue was dissolved in EtOAc and washed with dilute HCl solution, saturated $NaHCO_3$ and brine sequentially and finally added solid Na_2SO_4 to dry. The solvents were removed in vacuo and the residue was purified by flash column chromatography (H/A 10:1) to give a white solid, **5** (Rf = 0.56 (H/A 2:1)).

To a solution of **5** (1.0 g) dissolved THF (15 mL), H_2NNH_2•AcOH (1.5 eq) was added. The reaction was refluxed at 55 ºC for 7 h. The reaction mixture was then cooled and diluted with EtOAc, washed with brine and dried in Na_2SO_4. The solution was concentrated and then purified by flash column chromatography (H/A 8:1) to give the hemiacetal, **6** as a white solid.

A mixture of compound **6**, CCl_3CN and DBU were placed in a flask, cooled on ice. Dry CH_2Cl_2 was added to the mixture and the solution was stirred at 0ºC for 1.5 h followed by quenching with saturated NH_4Cl and then dried in Na_2SO_4. The solution was concentrated and then purified by flash column chromatography (H/A 6:1) to give the imidate, **7** as a white foam (Rf = 0.78 (H/A 2:1)). For the C1'-deuterated TRL, glucose-1-d_1 was used as the starting material and the above procedures were used.

Glycosylation of diosgenin to provide TRL



For the C3-deuterated TRL, compound **3** was used. Compound **3** and commercial diosgenin were coevaporated with toluene 3x before use. A mixture of imidate (1.2 eq), diosgenin (1.0 eq) and MS4A in dry CH_2Cl_2 (DCM) was cooled to -40ºC for 30 mins under Ar. TMSOTf (0.1eq) was added, stirred for 1 hr at -40ºC and quenched with Et_3N. This was then diluted with CH_2Cl_2 and washed 2x with H_2O and once with brine, then further dried in Na_2SO_4. The solution was concentrated and purified by flash column chromatography (H/A 12:1) to give a white solid, **8** (Rf=0.89 (H/A 2:1)).

Finally, for the deprotection, 30% NaOMe in MeOH was added to a solution of **8** in MeOH: CH_2Cl_2 (1:2). The solution was stirred overnight at room temperature and then neutralized with Dowex-50 (H^+) resin. After the resin was filtered off, the solvents were evaporated and the final residue was washed with EtOAc, filtered and dried in vacuo.

Confocal microscopy of GUVs

Preparation of Giant Unilamellar Vesicles (GUVs). Potential morphological changes induced by DGN to phase-separated GUVs was observed by confocal fluorescence microscopy. Using the electroformation method, GUVs composed of POPC/PSM/Chol 1:1:1 (total of 1mg/mL) were prepared as described previously by Angelova and Dimitrov (*Faraday Discuss. Chem. Soc.* **1986**. 81, 303‑311). Lipid stock solutions were prepared in 2:1 (v/v) $CHCl_3$/MeOH and appropriate volumes of each preparation were mixed. Labeling was carried out by pre-mixing the desired fluorescent probe (BODIPY-FL and 594neg-SSM, (Kinoshita, et al, *J. Cell Biol.* **2017,** *216*, 1183‑1204)) with the lipids in organic solvent. The concentrations were 0.6 mol % for BODIPY and 0.23 mol % for 594neg-SSM. The samples were deposited onto the surface of platinum (Pt) wires attached to glass slides which were placed under vacuum for a minimum of 2 h to completely remove the organic solvent. The glass slide was then added with 400 μL of sucrose solution, prepared with Milli-Q water. The alternating current field was applied to the Pt wires with a frequency of 10 Hz and an amplitude of 10 mV for 60 min. The temperatures used for GUV formation were over the gel to liquid phase transition in all cases, 55 ℃. After GUV formation, the glass slide was set onto an inverted confocal fluorescence microscope (Olympus Fluoview FV1000D). The excitation wavelength was 458 nm for FL-BODIPY and 559 nm for 594neg-SSM. Emission was recovered between 478-520 nm for BODIPY and 570-670 nm for 594neg-SSM. Image treatment and quantitation were performed using the FV10-ASW 4.2 (Olympus) software. Then, DSN at a final concentration of 30 μM was introduced to the glass slide and viewed from 5 to 60 min.

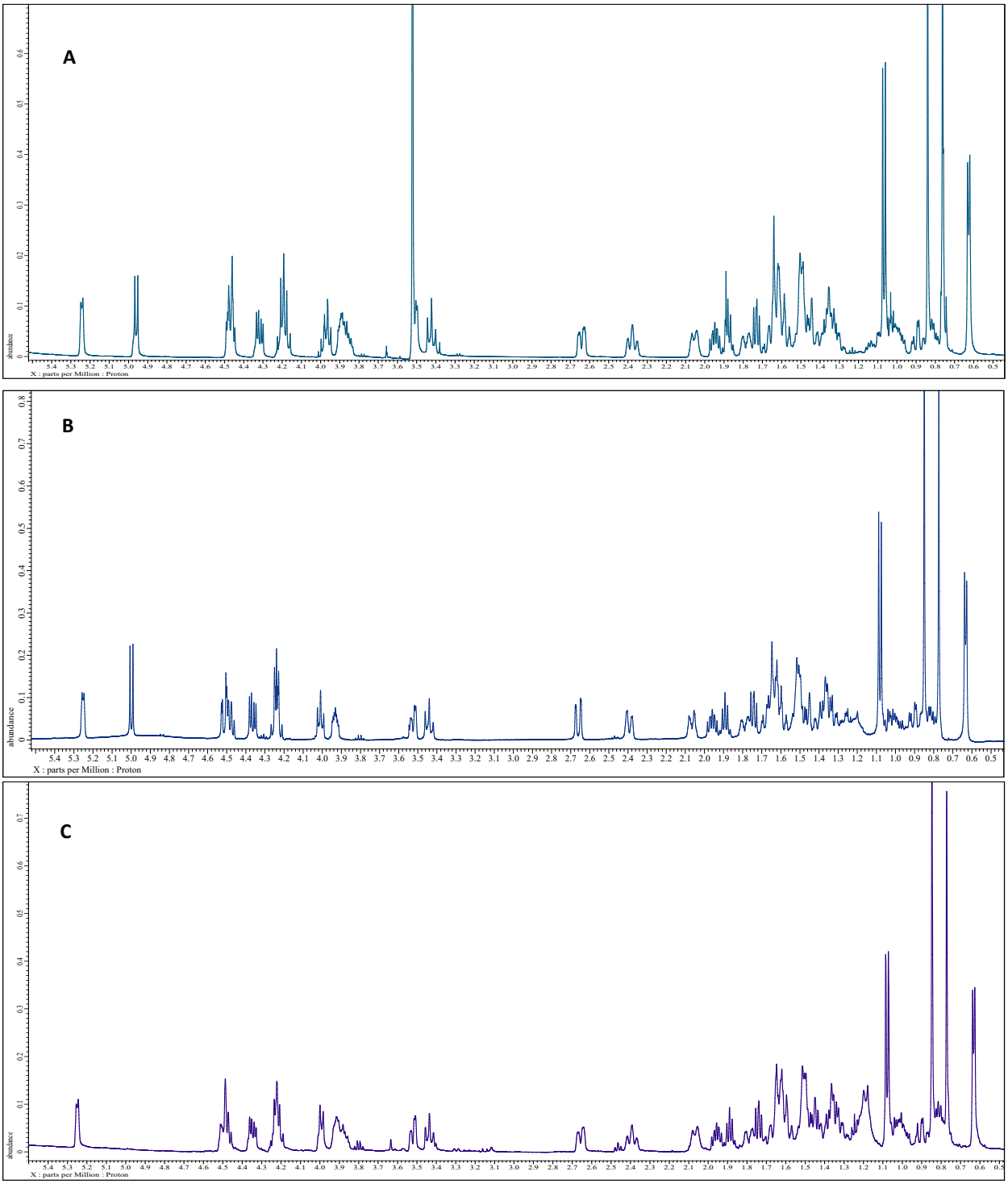


Figure S1. ^{1}H NMR spectra of TRL (A), 3*d*-TRL (B) and 1'*d*-TRL (C).

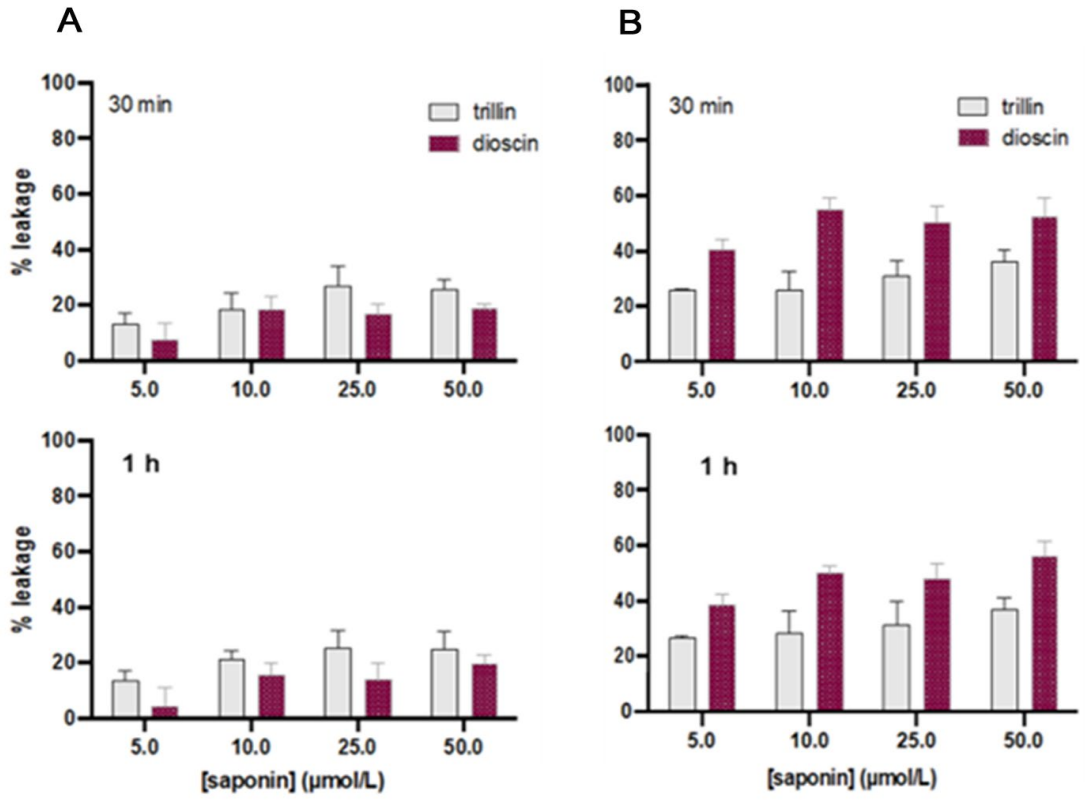


Figure S2. Calcein leakage from LUVs consisting of POPC(**A**), and POPC-Chol 9:1, (**B**) induced by TRL(gray) and DSN (maroon or dark) for 30 min and 1 h with different concentrations of TRL or DSN. The leakage data for 5 min are presented in Fig. 3. The % leakage values for the control without TRL and DSN were the same as those for 5.0 μmol/L TRL for both lipid compositions, respectively. A possible reason for the increased leakage activity in the TRL-added group for a longer period of time is thought to be due to domain formation in membranes by saponin. It is known that the addition of saponin causes liposome deformation after several tens of minutes [31]. The sizable amount of saponin adsorbed on the liposome surface in addition to the saponin that inserts into the bilayer interior. This surface-bound saponin disturbs the lipid packing structure and causes leakage probably by a toroidal mechanism. The purpose of this study was to investigate the function of saponin inserted into bilayers, as indicated by the use of saponin-bound bilayers in the NMR experiments, so it was necessary to suppress the effect of increased membrane permeability [31] caused by binding to this surface. This is the reason why we adopted the results obtained with a short incubation of 5-minute.

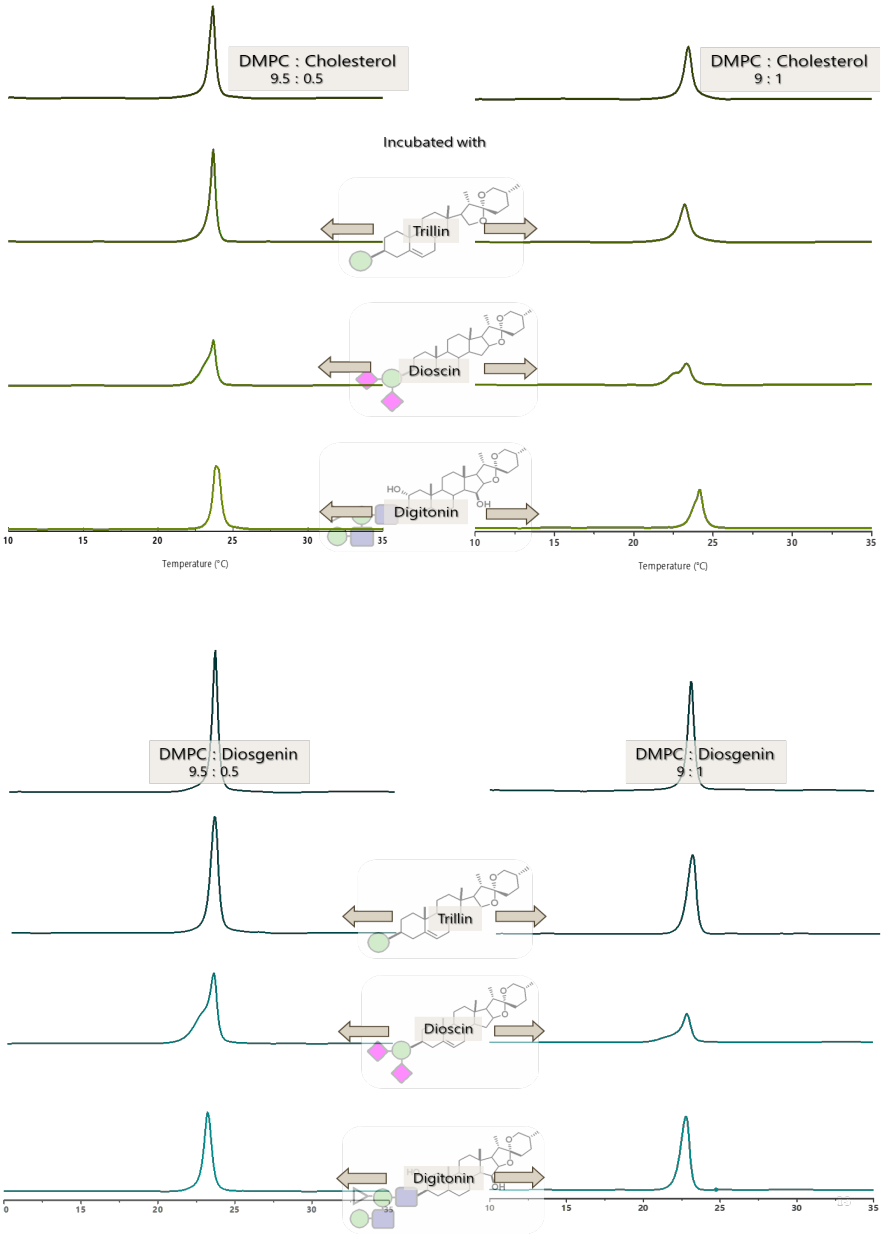


Figure S3. DSC thermograms of DMPC-Chol and DMPC-diosgenin membranes incubated with TRL, DSN and digitonin.

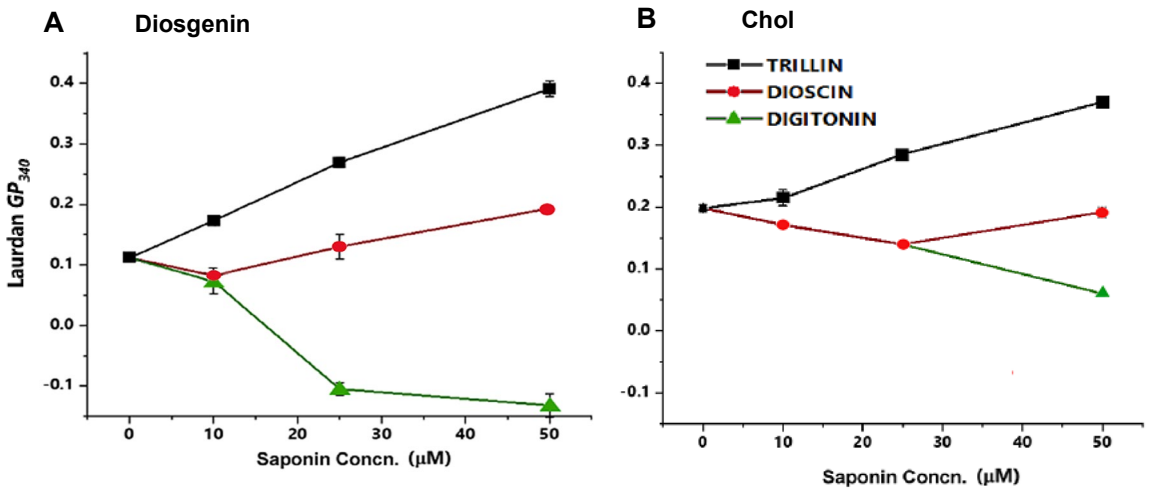


Figure S4. Membrane hydrations estimated by laurdan GP_{340} values of POPC-diosgenin 9:1 (A) and POPC-Chol 9:1 (B) at 37 °C with TRL (black line with square symbols), DSN (red line with circle symbols), and digitonin (green line with triangle symbols) at 5, 10, 25, and 50 μM, which were added to the LUVs (50 μM) containing 1 mol% laurdan. The POPC-Chol graph is the same as that in Fig. 5.

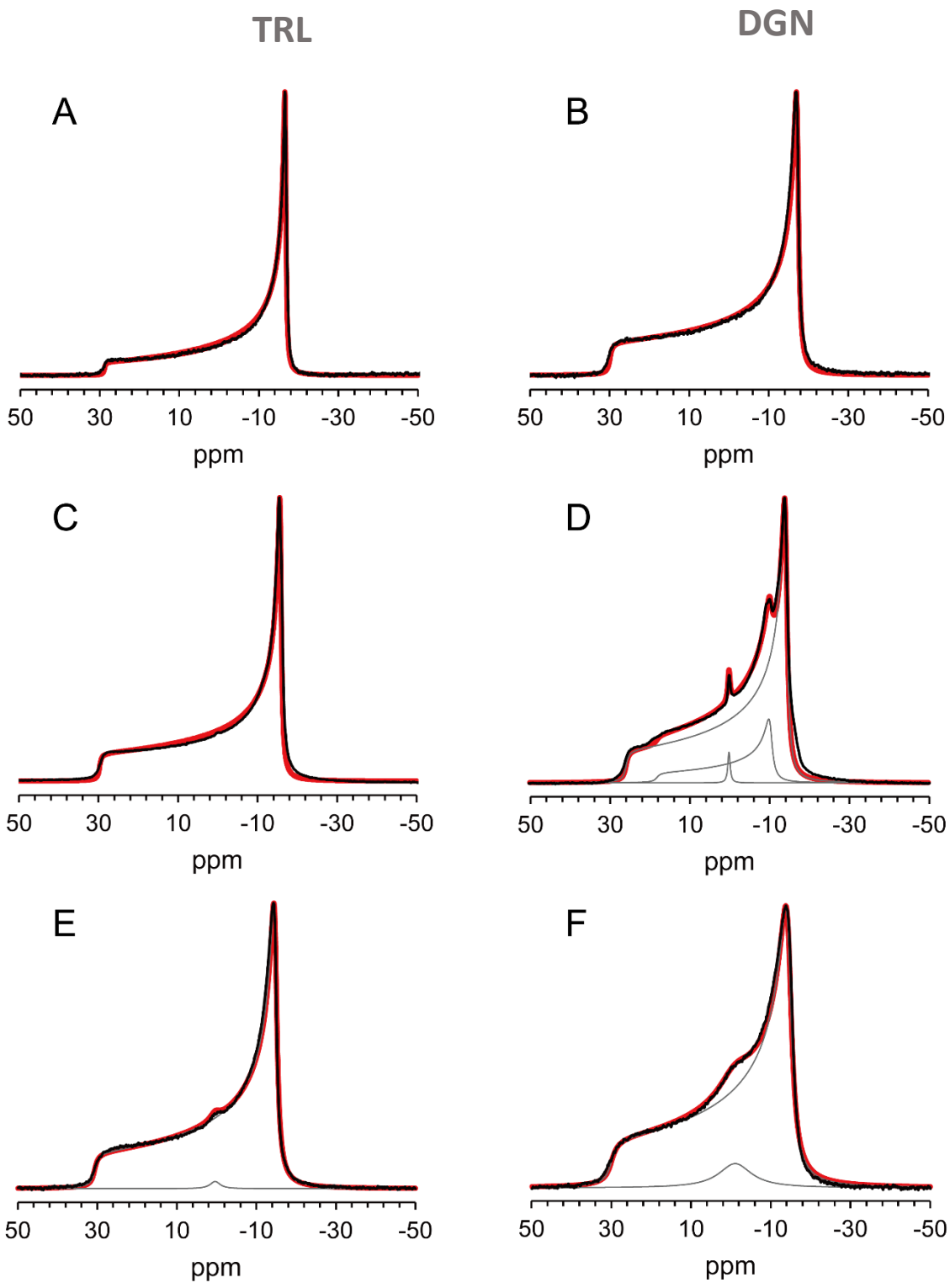


Figure S5. ^{31}P NMR Spectra of unitary POPC (panels A and B), POPC-Chol 9:1 (panels C and D), and POPC-diosgenin 9:1 (panels E and F) in the presence of 10mol% TRL (panels A, C and E) and DSN (panels B, D, and F). Experiment conducted at 30 ^{o}C. Simulation (red) and Experimental data (black). The CSA values as $\delta_{//}$ - $\delta\perp$ are shown in Table 1 in the manuscript. Panels D and F clearly show that DSN partially induced high curvature domains in the presence of Chol but the overlapping peak of a little narrower CSA demonstrates that the domains still hold the bilayer characters of MVLs.

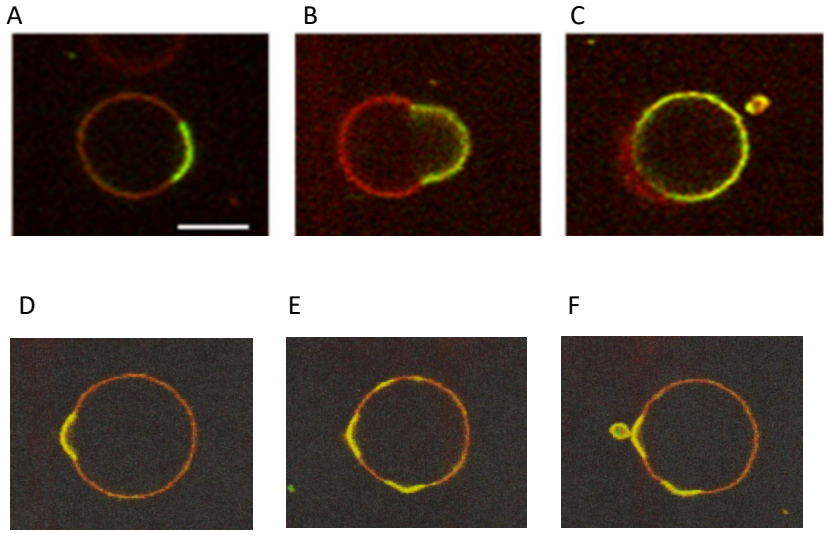


Figure S6. Time-course images of confocal microscopy of GUVs after adding 30 μM DSN from 5 min (A/D) to 60 min in a phase-separated vesicle. Panels A-C and Panels D-F are time-course images from different GUVs. The GUVs were composed of palmitoyl-sphingomyelin/POPC/Chol (1:1:1). The reddish and yellowish or green areas were visualized by 594neg-SSM and BodipyFL-PC (ref. 42), respectively, corresponding to Lo and Ld phase. The Ld areas became greater (panels B and E), and bilayer budding and fission occurred in the Ld areas (panels C and F). The lipid composition of the Ld phase of GUV (green areas in Fig. S9) is estimated to be PSM-POPC-Chol 17:75:8 according to a reported phase diagram (de Almeida et al. *Biophys. J.* **2003,** *85,* 2406−2416). The scale bar in panel A is 5 μm. See Additional Materials and Methods for more detail.

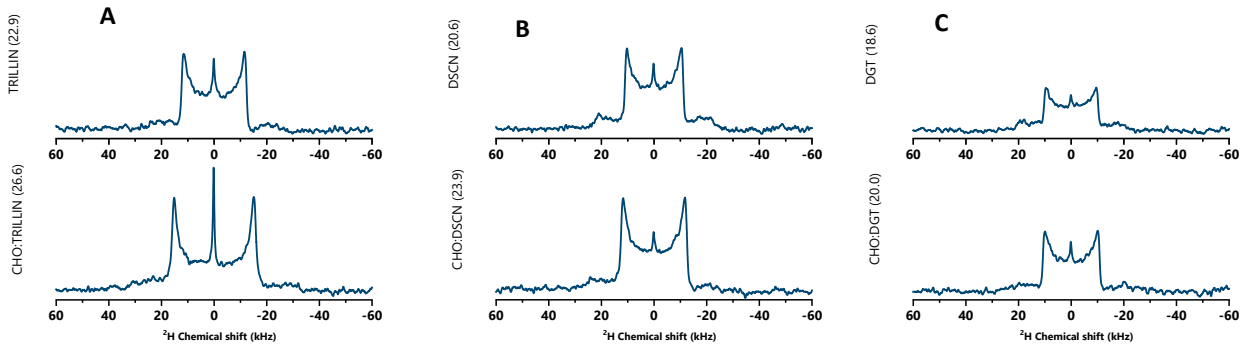


Figure S7. NMR spectra of 10',10'-d_2-POPC in POPC (upper panels) and POPC:Chol (9:1, lower panels) bilayers mixed with 10 mol% of saponin at 30 °C; TRL (A), DSN (B) and digitonin (C). The Δν values of 10',10'-d_2-POPC in unitary POPC and POPC:Chol (9:1) bilayers in the absence of saponin were approximately 19 and 25 kHz, respectively. Apparent decreases in signal intensity in panel C were due to bilayer-disrupting effect of digitonin. These spectra were measured in a quantitative way. Thus, reduction in the peak intensity of 10',10'-d_2-POPC in the presence of digitonin may be due to disruption of bilayer structure (ref. 29 in the manuscript).

Table S1. Quadrupolar splitting values (kHz) of ^{2}H-labeled diosgenin and Chol (10 mol%) for saponin (10 mol%) in bilayers.

	d-Chol in POPC	*d*-Diosgenin in POPC
w/o saponin	43.8	41.1
TRL	41.0	41.1
DSN	38.7	38.9
digitonin	ND*	39.8

*no splitting peak detected [29].

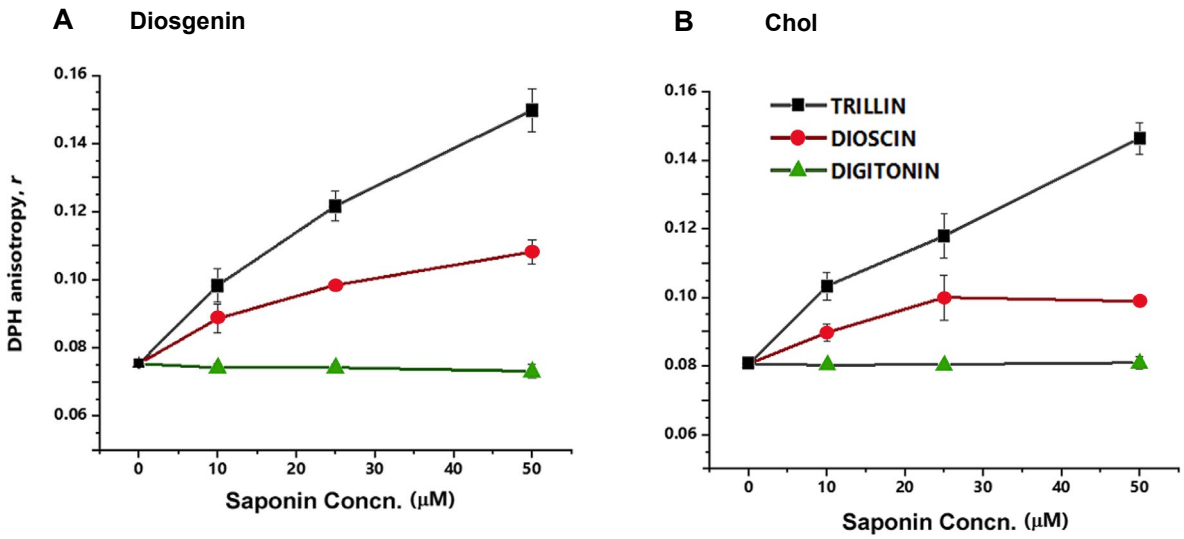


Figure S8. Fluorescent anisotropy (*r*) of DPH in POPC-diosgenin 9:1 (A) and POPC-Chol 9:1 (B) bilayers at 37 °C with TRL (black line with square symbols), DSN (red line with circle symbols), and digitonin (green line with triangle symbols) at 5, 10, 25, and 50 μM, which were added to the LUVs (50 μM) containing 1 mol% DPH. The POPC-Chol graph is the same as that in Fig. 6.

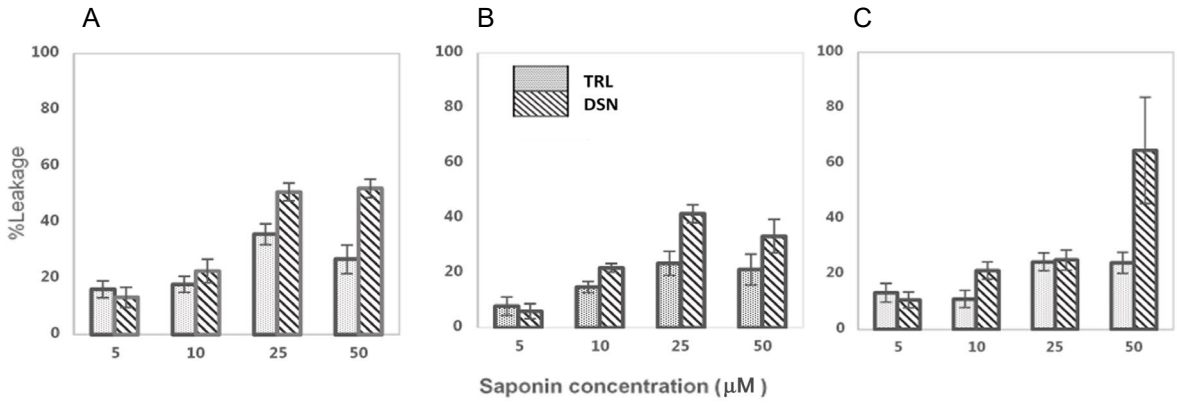


Figure S9. Calcein leakage from LUVs consisting POPC(A), POPC-Chol 9:1 (B), and POPC-diosgenin 9:1 (C) induced by TRL (gray) and DSN (hatched) at different concentrations. The LUVs were incubated for 2 h. Error bars denote SEM derived from triplicate experiments.