Impact of Lipid Tail Length on the Organ Selectivity of mRNA-Lipid Nanoparticles

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Section A. Materials and Methods

Materials

DSPC and DMG-PEG2k were purchased from NOF Corporation (Tokyo, Japan). Cholesterol was purchased from Nacalai Tesque (Kyoto, Japan). 3,3'-Dioctadecyloxacarbocyanine perchlorate (DiO) was purchased from MedChemExpress (Monmouth Junction, NJ, USA). Unless otherwise mentioned, mRNA molecules were purchased from TriLink BioTechnologies (San Diego, CA, USA) and uridine was substituted with 5-methoxyuridine (5moU). All antibodies were purchased from BioLegend (San Diego, CA, USA). All chemicals were purchased from Tokyo Chemical Industry (Tokyo, Japan) or FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan) and used without further purification.

Symmetrical branched fatty acid synthesis

Symmetrical branched fatty acids were synthesized according to reported procedures[1]. In brief, NaH (24 mmol) was suspended in tetrahydrofuran (THF) (24 mL) and stirred at 0°C for 10 minutes. Dimethyl malonate (10 mmol) was added, then the mixture was again stirred at 0°C for 10 minutes. An iodoalkane (30 mmol) was then added and allowed to react overnight at 25°C. After quenching with acetic acid, the mixture was washed with brine. After evaporation, the residue was dissolved in ethanol (20 mL) and hydrolyzed using an 8 N NaOH solution (6.25 mL). This reaction was performed overnight at 60°C. The mixture was washed with brine after quenching with 6 N HCl. The organic phase was dried over Na_2SO_4. After removing the solvent, the crude was heated at 160°C for 2 hours for decarboxylation. The residue was purified using the Biotage Selekt automated chromatography system (Biotage, Uppsala, Sweden) (SiO2, hexane/EtOAc) to obtain the branched fatty acid as a colorless oil.

Ionizable lipid synthesis

Tertiary amino alcohols, such as 7-(4-dipropylamino)butyl)tridecane-1,7,13-triol (CL4), 7-(4-(piperidin-1-yl)butyl)tridecane-1,7,13-triol (CL6), 5,11-duhydroxy-5-(6-hydroxyhexyl)undecyl 1-methylpiperidine-4-carboxylate (CL15), 5,11-dihydroxy-5-(6-hydroxyhexyl)undecyl 1-methylpyrrolidine-3-carboxylate (CL16), and 5,11-dihydroxy-5-(6-hydroxyhexyl)undecyl 1-isopropylpiperidine-4-carboxylate (CL17), were synthesized as previously described[1,2]. Symmetrical branched fatty acid (2.4 mmol) was added to tertiary amino alcohol (1.0 mmol) in anhydrous dichloromethane (DCM) (5 mL). Dimethylaminopyridine (DMAP) (0.1 mmol) and 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI-HCl) (3.0 mmol) were added

to the mixture, then the reaction mixture was stirred at 25°C overnight. After evaporating the solvent, the residue was suspended in EtOAc and washed with a 0.5 N NaOH solution and then brine. The organic phase was dried over Na_2SO_4. After removing the solvent, the crude was purified using the Biotage Selekt automated chromatography system [ODS, H_2O (0.1% trifluoroacetic acid)/acetonitrile:isopropanol = 50:50 (0.1% TFA) and (SiO_2, DCM/MeOH)] to obtain the branched ionizable lipid as a colorless oil.

mRNA/LNP formulation

The mRNA/LNPs were formulated by NanoAssemblr Benchtop Systems (Vancouver, BC, Canada). Briefly, the lipid mixture and mRNA solution (50 mM citrate buffer pH 3.50) were mixed through microfluidic cartridges. The resulting LNP solution was diluted with 20 mM HEPES 9% sucrose buffer (pH 7.45), then concentrated and purified using ultrafiltration (Amicon Ultra-15, MWCO 100 kDa; Millipore, Billerica, MA, USA). The LNPs were filtered through a 0.2 μm polyethersulfone (PES) filter and stored in liquid form at 4°C.

mRNA/LNP characterization

The particle size and zeta potential were evaluated using a Zetasizer Nano ZSP instrument (Malvern Instruments, Worcestershire, UK). The pKa and encapsulation efficiency values were estimated using the 6-*p*-toluidino-2-naphthalenesulfonic acid (TNS) assay and Ribogreen assay, as previously described.[1]

Transmission electron microscopy (TEM) imaging of mRNA/LNPs

Sample preparation and observation were performed by Tokai Electron Microscopy, Inc. (Nagoya, Japan). Briefly, LNP solutions without sucrose were dispersed on the carbon-coated Cu grid, then negative staining was performed with a 2% phosphor tungstic acid solution (pH 7.0) for 30 s. The TEM image was acquired using a transmission electron microscope (JEM 1400Plus, JEOL Ltd., Tokyo, Japan) with a charge-coupled device (CCD) camera (EM-14830RUBY2, JEOL Ltd.).

Membrane hydration assay

Fifty microliters of 3.75 mM LNP was added with 0.15 μL of 2.5 mM Laurdan in DMSO to generate 0.2 mol% Laurdan against total lipid. After shaking incubation at 700 rpm for 5 hours at 25°C, the samples were diluted 6.7× with 20 mM HEPES buffer. The fluorescence intensity was then measured using a Varioskan LUX plate reader (Ex: 340 nm, Em: 360–600 nm; Thermo Fisher Scientific, Waltham, MA, USA). The generalized

polarization (GP) value was calculated by (I440-I490)/(I440+I490), where I440 and I490 are the emission intensities at 440 nm and 490 nm, respectively.

In vitro mRNA/LNP cellular uptake

HepG2 cells or HuH7 cells were seeded at a density of 1.0×10^5 cells/well in a 12-well cell-culture plate containing DMEM supplemented with 10% FBS (Gibco, Grand Island, NY, USA #10270106), penicillin (100 U/mL) and streptomycin (100 μg/mL) (Gibco #15140122) at 37 °C in 5% CO_2. The cells were transfected with 500 ng Cy5 FLuc mRNA:FLuc mRNA (= 1:1) in LNPs per well 24 hours after seeding. Three hours after transfection, the cells were detached using trypsin-EDTA and flow cytometry analysis was performed. The data were analyzed using FlowJo v10.8.1 software (FlowJo, LLC, Ashland, OR, USA).

In vitro mRNA/LNP delivery

Cells were seeded at a density of 0.40×10^4 cells/well for luciferase assays in a 96-well plate. The cells were transfected with Cy5 FLuc mRNA:FLuc mRNA (= 1:1) in LNPs 24 hours after seeding. Twenty-four hours later, 300 μg/mL Beetle Luciferin (Promega, Mannheim, Germany) in D-PBS was added to each well (100 μL/well), then luciferase activity was measured using an EnSight Multimode Plate Reader (PerkinElmer, Waltham, MA, USA). To examine any cell toxicity associated with the mRNA/LNPs, the total protein from each well was quantified using a BCA assay. Briefly, the cells were washed with D-PBS and incubated in 100 μL/well Lysis Buffer M at 4°C for 20 minutes. The total protein concentration of each lysate was quantified using the BCA protein assay kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

Animals

C57BL/6, Balb/c, and C.KOR/StmSlc-Apoe^{shl} female mice were sourced from Japan SLC (Shizuoka, Japan) or Charles River Laboratories (Kanagawa, Japan). Ai14 male and female mice were sourced from Jackson Laboratory (Bar Harbor, ME, USA). All mice were acquired at 6–9 weeks old. The mice were housed in groups (five per cage) in a specific animal facility at Hokkaido University or Nitto Denko Corporation with a 12-hour day/night cycle. Water and a pelleted mouse diet (5053, LabDiet, St. Louis, MO, USA) were provided ad libitum. All experimental procedures were approved by the Hokkaido University Animal Care Committee (approval number: 20-0176) and Nitto Denko Corporation Animal Care and Use Committee (approval number: C22-Q). All procedures followed the Guidelines for the Care and Use of Laboratory Animals.

Pharmacokinetic (PK) profile of mRNA/LNPs

Cy5 FLuc mRNA/LNPs were intravenously (i.v.) injected into mice at a dose of 0.4 mg/kg. Blood samples were collected and diluted 10× with 1% SDS solution at each time point. Then, Cy5 fluorescence intensity from 200 μL of each sample was measured using a Varioskan LUX plate reader (Ex: 643 nm; Em: 667 nm).

In vivo luciferase assay

FLuc mRNA/LNPs were i.v. injected into mice at a dose of 0.1 mg/kg. Six hours after LNP administration, luciferin was i.v. injected. Three minutes later, each organ was collected. Luminescence from each organ was measured by IVIS.

Confocal laser scanning microscopy (CLSM) observations

For *in vitro* experiments, the cells were seeded in glass bottom dishes at a density of 1.0 × 10^5 cells/well 24 hours before CLSM observations. DiO-labeled LNPs carrying Cy5 FLuc mRNA:FLuc mRNA (= 1:1) were added to dishes at a dose of 500 ng/well and incubated for 3 hours. The cellular uptake of each LNP was observed using an LSM 900 (Zeiss, Germany) after the cells were stained with Hoechst 33342 for 10 minutes.

For *in vivo* experiments, fluorescently labeled mRNA/LNPs or Cre mRNA/LNPs were i.v. injected into mice. Liver tissues were collected 10 minutes after the blood vessels were stained by i.v. administration of 40 μg fluorescence-conjugated Lycopersicon esculentum Lectin. The samples were treated with 2 μg/mL Hoechst 33342 and observed using a Nikon A1 microscope.

In vivo mRNA expression profile at the cellular level

Spleen tissues were cut into small pieces (~0.5 cm^3) and disassociated with gentleMACS Octo Dissociator with Heaters (Miltenyi Biotec, Bergisch Gladbach, Germany) 24 hours after 0.5 mg/kg Cre mRNA/LNPs was injected into the mice. The splenocytes were filtered through a 40 μm cell strainer, washed with RPMI 1640 medium (Gibco, Grand Island, NY, USA), and centrifuged at 300×g for 7 minutes at 4°C. The pellet was treated with ACK Lysing buffer (Thermo Fisher Scientific) for 4 minutes at 25°C and washed with RPMI medium. The obtained splenocytes (1 × 10^6 cells/tube) were blocked with 5 μL TruStain FcX (anti-mouse CD16/32) antibody (BioLegend) on ice for 15 minutes and labeled with 5 μL fluorophore-conjugated antibodies (see Supplementary Figure S6) on ice for 30 minutes. The cells were washed in autoMACS Running Buffer (Miltenyi Biotec), then treated with 5 μL 7-AAD Viability Staining Solution (BioLegend) for 10

minutes. After filtration, the cells were analyzed using FACSymphony A1 (BD Biosciences San Jose, CA, USA). The gating strategy is shown in Figure S6.

SARS-CoV-2 mRNA delivery

Spike SARS-CoV-2 (E484K; N501Y) mRNA (5moU) was purchased from OZ Biosciences（Marseille, France). Eight-week-old female BALB/c mice (Charles River Laboratories) were intramuscularly administered 5 μg of SARS-CoV2 mRNA-loaded LNPs into the left thigh muscles two times at two-week intervals. Two weeks post-boost, blood samples were collected in a MiniCollect device from the heart under isoflurane anesthesia to collect serum.

For antibody titer assays against the SARS-CoV-2 spike receptor-binding domain (RBD) protein, the nunc immunoplate (Thermo Fisher Scientific) was pre-coated with His tag-conjugated SARS-CoV-2 spike RBD protein with E484K and N501Y mutations (GeneTex, Irvine, CA, USA) at 50 ng per well in 100 μL of 50 mM carbonate-bicarbonate coating buffer (pH 9.6) overnight at 4°C. The plate was washed three times with PBS containing 0.05% Tween-20 (PBS-T) and blocked with 10 g/L Block ACE (UKB80, KAC Co., Kyoto, Japan) at 4°C overnight. After the plate was washed with PBS-T, 50 μL of diluted serum with 4 g/L Block ACE was added to each well and incubated at 37°C for 1 hour. The plate was then washed three times with PBS-T and incubated with 100 μL of diluted horseradish peroxidase（HRP)-conjugated goat anti-mouse IgG-Fc fragment antibody (A90-131P, Bethyl Laboratories, Montgomery, TX, USA) in 4 g/L Block ACE at room temperature for 45 minutes. Any unbound HRP-conjugated antibodies were removed by three washes with PBS-T. The colorimetric signal was developed after adding a chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB) (Nacalai Tesque). To evaluate the immunodominant neutralizing antibodies targeting the SARS-CoV-2 spike RBD protein, the nunc immunoplate was pre-coated with human ACE2 extracellular domain with a C-terminal human IgG1 Fc tag (InvivoGen, San Diego, CA, USA) at 100 ng per well in 100 μL of 100 mM carbonate-bicarbonate coating buffer (pH 9.6) overnight at 4°C. The plate was washed three times with PBS-T, blocked with 10 g/L Block ACE, and incubated at 4°C overnight. To react the neutralizing antibodies with the SARS-CoV-2 spike RBD protein, 0.001 ng/μL of His tag-conjugated SARS-CoV-2 spike RBD protein was pre-incubated with the test serum diluent for 1 hour at 37°C. His tag-conjugated SARS-CoV-2 spike RBD protein and test serum diluent were mixed at a 1:1 ratio. Then, 50 μL of the mixture was added to the washed human ACE-Fc-coated plate for 1 hour at 37°C. Any of the mixture that was unbound to human ACE-Fc was removed using five washes with PBS-T, followed by incubation for 1 hour at

room temperature with 100 µL of diluted HRP-conjugated anti-His Tag Antibody (BioLegend) in 4 g/L Block ACE. Unbound HRP-conjugated antibodies were removed with three PBS-T washes, followed by colorimetric signal development with TMB.

Section B. Supplementary Figures and Tables

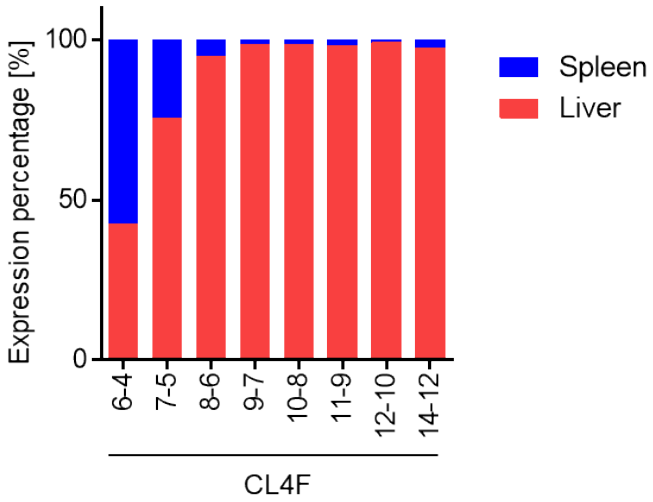
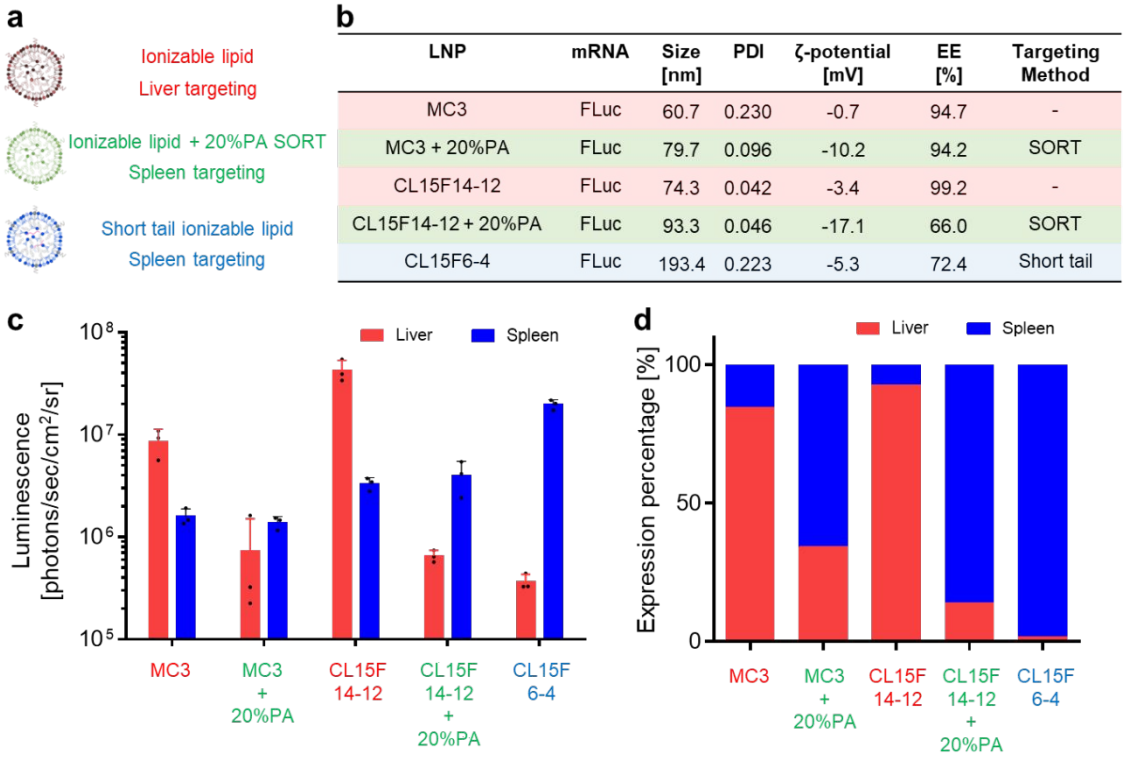


Figure S1. Preferential expression of CL4F LNPs in the liver and spleen.
Impact of the CL4F lipid tail length on *in vivo* mRNA delivery to the liver and spleen. Here, 0.1 mg/kg FLuc mRNA was i.v. delivered into mice (n = 3 Balb/c mice per group).



LNP	mRNA	Size [nm]	PDI	ζ-potential [mV]	EE [%]	Targeting Method
MC3	FLuc	60.7	0.230	-0.7	94.7	-
MC3 + 20%PA	FLuc	79.7	0.096	-10.2	94.2	SORT
CL15F14-12	FLuc	74.3	0.042	-3.4	99.2	-
CL15F14-12 + 20%PA	FLuc	93.3	0.046	-17.1	66.0	SORT
CL15F6-4	FLuc	193.4	0.223	-5.3	72.4	Short tail

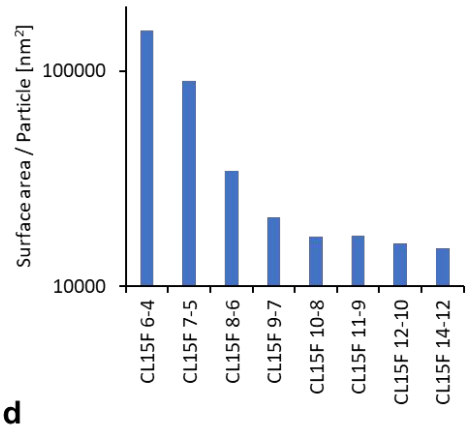
Figure S2. Experimental comparison between LNPs containing short-tail ionizable lipid and the selective organ targeting (SORT) method.

a) Schematic illustration of three different formulations. Spleen targeting was achieved by adding a SORT molecule (green) or using a short-tail ionizable lipid (blue) from the liver targeting formulation (red). b) FLuc mRNA was encapsulated in LNPs. The number mean size, PDI, and ζ-potential were evaluated using the Zetasizer. The encapsulation efficiency was evaluated using the Ribogreen assay. c) Effect of the formulation strategy on *in vivo* mRNA delivery to the liver and spleen. Here, 0.1 mg/kg FLuc mRNA was i.v. injected into mice (n = 3 Balb/c mice per group). d) Spleen selectivity was clearly improved by both formulation strategies.

a

Lipid	DSPC [%]	mRNA	Size [nm]	PDI	EE [%]	pKa	ζ-potential [mV]
CL15F 6-4	10%	FLuc	229.3	0.316	66.7	6.90	-5.87
CL15F 7-5	10%	FLuc	177.4	0.007	97.6	6.33	-4.17
CL15F 8-6	10%	FLuc	112.6	0.011	99.2	6.57	-4.64
CL15F 9-7	10%	FLuc	89.6	0.010	99.5	6.64	-4.32
CL15F 10-8	10%	FLuc	81.4	0.026	99.8	6.24	-4.18
CL15F 11-9	10%	FLuc	81.9	0.023	99.8	6.59	-3.46
CL15F 12-10	10%	FLuc	79.0	0.030	99.8	6.59	-5.04
CL15F 14-12	10%	FLuc	77.1	0.051	98.4	6.56	-7.50

b



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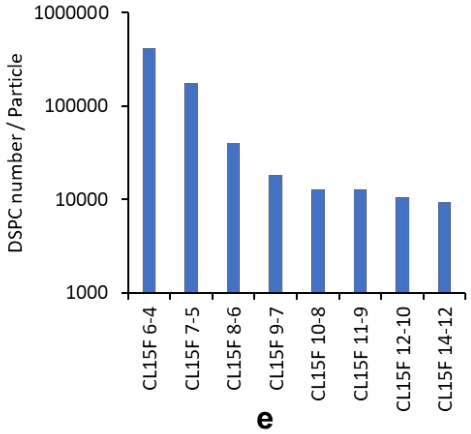


d

Lipid	DSPC [%]	mRNA	Size [nm]	PDI	EE [%]	pKa	ζ-potential [mV]
CL15F 10-8	0%	FLuc	121.6	0.046	98.4	6.80	-4.16
CL15F 10-8	5%	FLuc	108.9	0.054	98.3	6.65	-2.85
CL15F 10-8	10%	FLuc	90.56	0.029	98.8	6.56	-3.59
CL15F 10-8	20%	FLuc	70.4	0.100	98.6	6.32	-4.00
CL15F 10-8	30%	FLuc	56.6	0.109	98.3	6.20	-5.44
CL15F 14-12	0%	FLuc	147.7	0.038	98.2	6.71	-4.10
CL15F 14-12	5%	FLuc	104.1	0.050	98.7	6.62	-4.38
CL15F 14-12	10%	FLuc	86.6	0.037	98.7	6.55	-4.62
CL15F 14-12	20%	FLuc	59.0	0.085	98.3	6.35	-4.33
CL15F 14-12	30%	FLuc	49.7	0.171	98.1	6.23	-6.76

e

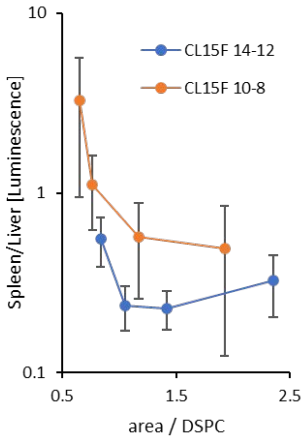


Figure S3. LNP profiling using experimental data and theoretical calculations.
a) FLuc mRNA was encapsulated in LNPs. The number mean size, PDI, and ζ-potential were evaluated using the Zetasizer. The encapsulation efficiency and pKa were evaluated using the Ribogreen and TNS assays, respectively. b) The surface area per LNP was theoretically calculated assuming that the LNPs were spherical and the thickness of the PEG layer was 4 nm.[3] A larger particle size leads to a larger surface area per particle. c) The number of DSPC molecules per particle was theoretically obtained using the same

parameters shown in a previous report.[3] The lipid volume in the LNP core was obtained by subtracting the water and RNA volumes from the core volume. The lipid monolayer was assumed to be 2.4 nm, with the lipid volume in the monolayer also calculated. These values were summed to obtain the total lipid volume per particle. From the molecular volume and molar ratio of the four component lipids, the relative volume contribution of each lipid was calculated to obtain the DSPC volume per particle and finally converted to the DSPC number. As noted, the molecular volume of water,[3] cholesterol,[4] DSPC,[5] lipid-anchoring portion of the PEG-lipid,[5] PEG unit[3,6] and RNA[3,7] values were obtained from the literature. The molecular volume of ionizable lipids was determined from the measured density. d) FLuc mRNA was encapsulated in LNPs. The lipid composition was ionizable lipid:Chol:DSPC:DMG-PEG2k = X:Y:Z:1.5, wherein Z was 0, 5, 10, 20, or 30, the X:Y molar ratio was 50:38.5, and the total was 100. The number mean size, PDI, and ζ-potential were evaluated using the Zetasizer. The encapsulation efficiency and pKa were evaluated using the Ribogreen and TNS assays, respectively. e) Relationships between the surface DSPC density and mRNA delivery with the spleen-to-liver ratio.

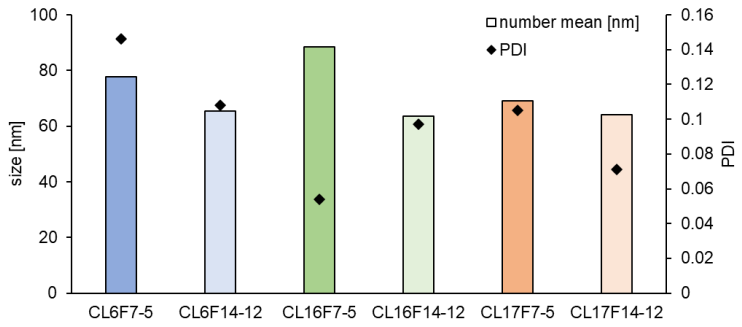


Figure S4. FLuc mRNA was encapsulated in LNPs. The number mean size and PDI were evaluated using the Zetasizer. Short-tail (7-5) ionizable lipids yielded larger particles compared with long-tail (14-12) ionizable lipids.

a

Lipid	DSPC [%]	mRNA	Size [nm]	PDI
CL15F 6-4	10%	FLuc:Cy5-Fluc=1:1	166.2	0.096
CL15F 10-8	10%	FLuc:Cy5-Fluc=1:1	92.8	0.023
CL15F 14-12	10%	FLuc:Cy5-Fluc=1:1	88.6	0.022
CL15F 10-8	30%	FLuc:Cy5-Fluc=1:1	53.6	0.118

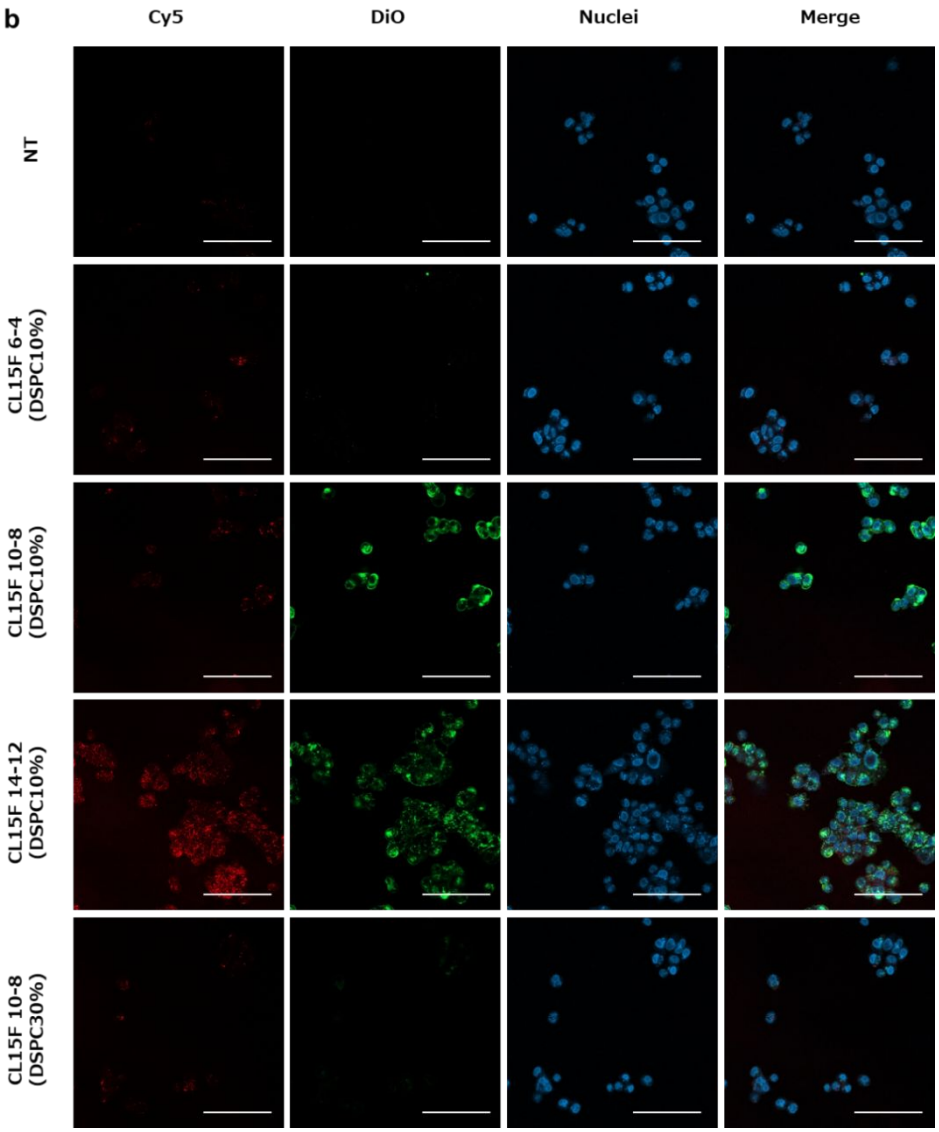


Figure S5. CLSM observation of LNP-treated HepG2 cells.

a) DiO-labeled LNPs carrying Cy5 FLuc mRNA/FLuc mRNA (= 1:1) were prepared. The number mean size and PDI were evaluated using the Zetasizer. b) Confocal laser scanning microscopic images were acquired 3 hours after HepG2 cells were transfected with 500 ng mRNA/well and stained with Hoechst 33342. The Cy5-positive areas (red), DiO-positive areas (green), and nuclei (blue) are shown. Scale bars = 100 μm.

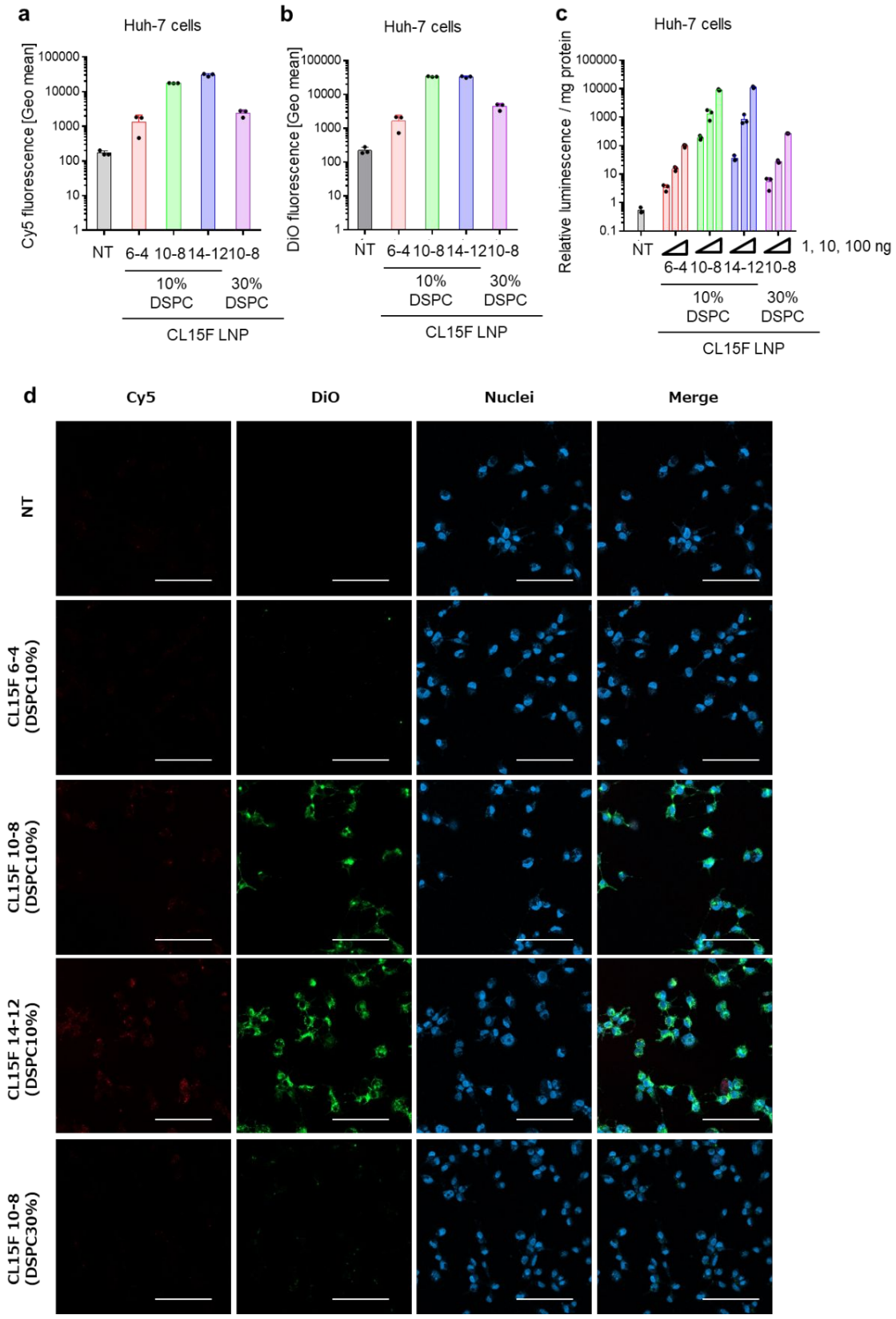


Figure S6. Cellular uptake, luciferase assay results, and CLSM observation of LNP-

treated HuH-7 cells.

a, b) *In vitro* cellular uptake of DiO-labeled LNPs carrying Cy5 FLuc mRNA/FLuc mRNA (= 1:1). Flow cytometry analysis was performed 3 hours after HuH-7 cells were transfected with 500 ng mRNA/well. The graphs in (a) and (b) indicate the Geo mean values of Cy5 and DiO, respectively. c) Luciferase assays were performed 24 hours after HuH-7 cells were transfected with LNPs carrying DiO-labeled Cy5 FLuc mRNA/FLuc mRNA (=1:1). d) Confocal laser scanning microscopic images were acquired 3 hours after HepG2 cells were transfected with 500 ng mRNA/well and stained with Hoechst 33342. The Cy5-positive areas (red), DiO-positive areas (green), and nuclei (blue) are shown. Scale bars = 100 μm.

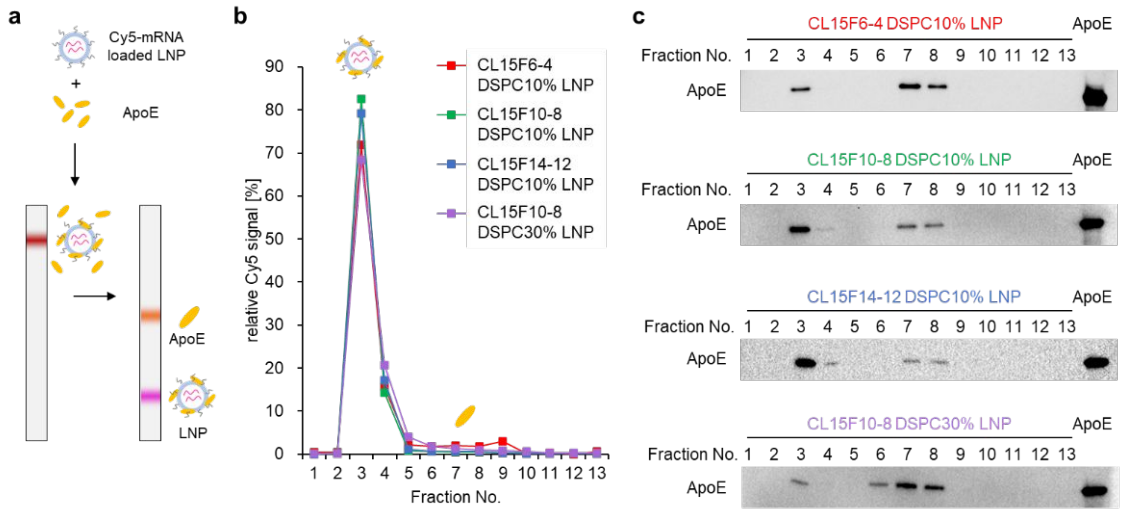


Figure S7. Interaction between mRNA/LNPs and ApoE.

a) To evaluate the interaction between the mRNA/LNPs and ApoE, 75 µL of CL15F 6-4, CL15F 10-8, or CL15F 14-12 LNPs containing 10% DSPC or CL15F 10-8 LNPs containing 30% DPSC (0.15 mg mRNA/mL) was incubated with 7.5 µg recombinant mouse apolipoprotein E (His tag) (0.5 mg/mL, ab226314, Abcam, Cambridge, MA, USA) for 1 hour at 25°C with gentle mixing at 700 rpm. After incubation, the sample was passed through size exclusion chromatography (SEC) columns and sequential fractions were collected (400 µL/fraction). This ensured the separation of ApoE bound to LNPs from unbound ApoE. As SEC columns, qEV single (35 nm, Izon, Christchurch, New Zealand) was used according to the manufacturer's instructions. b) Cy5 signals from each fraction were measured using an EnSight Multimode Plate Reader (PerkinElmer, Ex = 643 nm, Em = 667 nm). The results showed that the mRNA/LNPs eluted earlier. c) Western blot analysis was performed to quantify the ApoE protein levels in each fraction. Ten µL of each fraction or 10 µg/mL ApoE were separated by Any kD Mini-Protean TGX Stain-Free Gels (Bio-Rad, Hercules, CA, USA) and transferred to PVDF membranes (Trans-Blot Turbo Mini PVDF Transfer Packs, Bio-Rad) using the BioRad Trans-Blot Turbo Blotting system. Membranes were incubated with an anti-His-tag mAb-HRP-DirecT (D291-7, OGHis; MBL, Aichi, Japan) at 1:10,000. The signals were visualized by chemiluminescence using Clarity Western ECL Substrate (Bio-Rad) with ChemiDoc XRS Plus (Bio-Rad). The results demonstrated that LNPs with short-tail lipids adsorbed less ApoE and LNPs containing 30% DSPC had a lower affinity with ApoE than LNPs containing 10% DSPC.

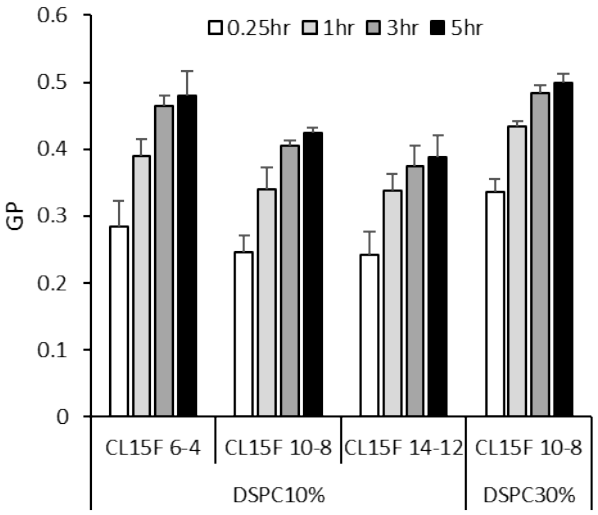


Figure S8. Relationships between the incubation time and generalized polarization (GP) value in Laurdan experiments.

Fifty μL of 60 μg/mL mRNA-LNP was combined with 0.15 μL of 2.5 mM Laurdan in DMSO to generate 0.2 mol% Laurdan against total lipid. After incubation at 25°C with shaking at 700 rpm, the samples were diluted 6.7× with 20 mM HEPES buffer. The fluorescence intensity was measured using a Varioskan LUX plate reader (Ex: 340 nm; Em: 360–600 nm). The GP value was calculated by (I440–I490)/(I440+I490), where I440 and I490 are the emission intensities at 440 nm and 490 nm, respectively.

a

Lipid	mRNA	Size [nm]	PDI	EE [%]
CL15F 6-4	Cre	237.1	0.040	90.5
CL15F 14-12	Cre	96.78	0.005	101.7

b

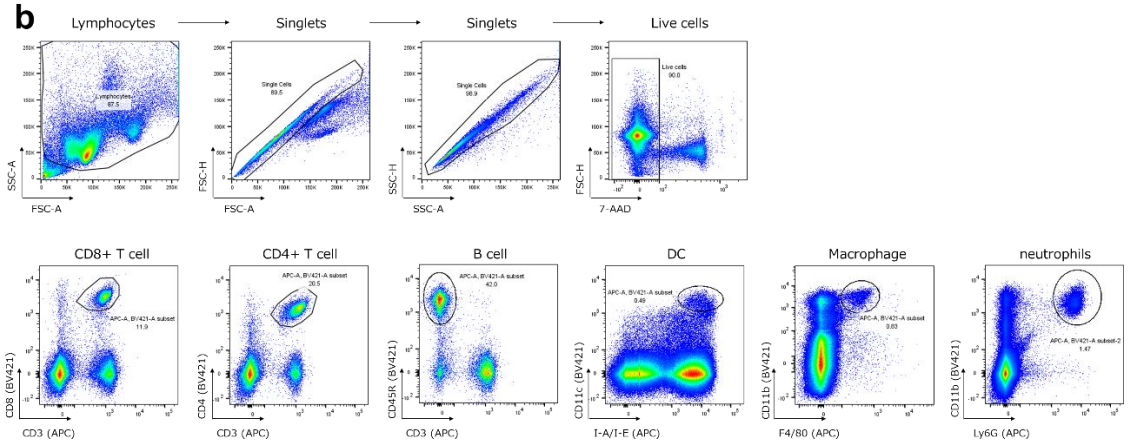


Figure S9. Gating strategy for flow cytometry analysis.

a) Cre mRNA was encapsulated in LNPs. The number mean size and PDI were evaluated using the Zetasizer. The encapsulation efficiency was evaluated using the Ribogreen assay. b) Doublets were excluded by plotting the area and height for forward scatter and side scatter. 7-AAD Viability Staining Solution (BioLegend) was used to exclude dead cells. $CD8^+$ T cells were identified using an anti-mouse CD3 antibody (APC, clone 17-A2, BioLegend, 100236) and an anti-mouse CD8α antibody (BV421, clone53-6.7, BioLegend, 100737). $CD4^+$ T cells were identified using an anti-mouse CD3 antibody (APC, clone 17-A2, BioLegend, 100236) and an anti-mouse CD4 antibody (BV421, clone RAM4-5, BioLegend, 100543). B cells were identified using an anti-mouse CD3 antibody (APC, clone 17-A2, BioLegend, 100236) and an anti-mouse/human CD45R/B220 antibody (BV421, clone RA3-6B2, BioLegend, 103239). DCs were identified using an anti-mouse I-A/I-E antibody (APC, clone M5/114.15.2, BioLegend, 107614) and an anti-mouse CD11c antibody (BV421, clone N418, BioLegend, 117329). Macrophages were identified using an anti-mouse F4/80 antibody (APC, clone QA17A29, BioLegend, 157305) and an anti-mouse/human CD11b antibody (BV421, clone M1/70, BioLegend, 101235). Neutrophils were identified using an anti-mouse Ly-6G antibody (APC, clone 1A8, BioLegend, 127614) and an anti-mouse/human CD11b antibody (BV421, clone M1/70, BioLegend, 101235). Rat IgG2b, κ Isotype Ctrl antibody (APC, clone RTK4530, BioLegend, 400611), Rat IgG2a, κ Isotype Ctrl antibody (BV421, clone RTK2758 BioLegend, 400535), Mouse IgG2a, κ Isotype Ctrl antibody (BV421, clone MOPC-173, BioLegend, 400259), Armenian Hamster IgG Isotype Ctrl antibody (BV421, clone HTK888, BioLegend, 400935), and Mouse IgG1, κ Isotype Ctrl antibody (APC,

clone MOPC-21, BioLegend, 400119) were used as respective isotype controls. A purified anti-mouse CD16/32 antibody (BioLegend, 101302) was used for Fc blocking.

a

Lipid	mRNA	Size [nm]	PDI
CL15F 6-4	FLuc	171.3	0.051
CL15F 14-12	FLuc	83.0	0.035

b

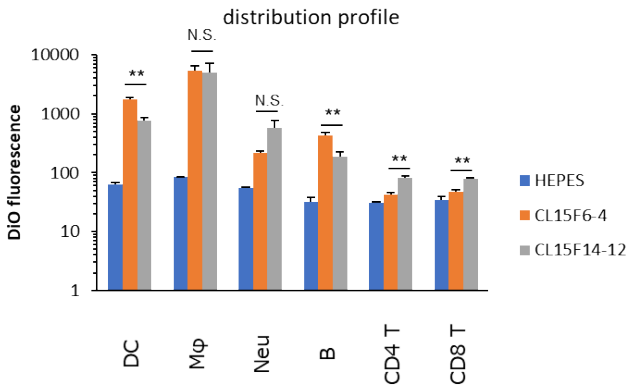
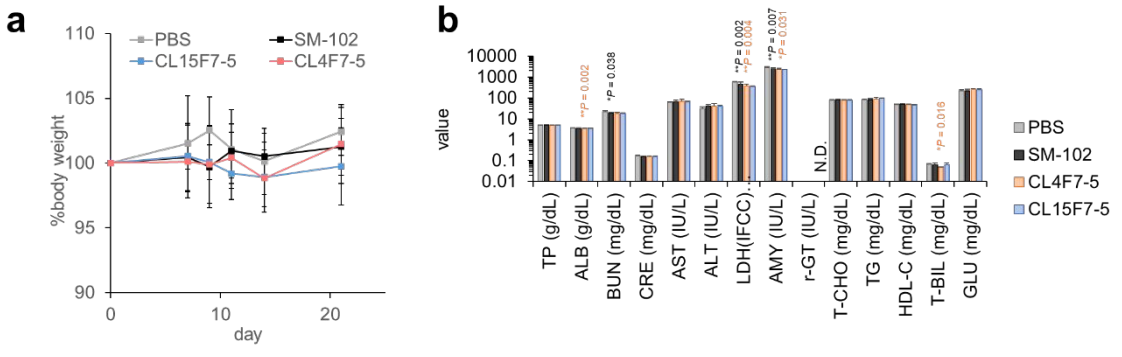
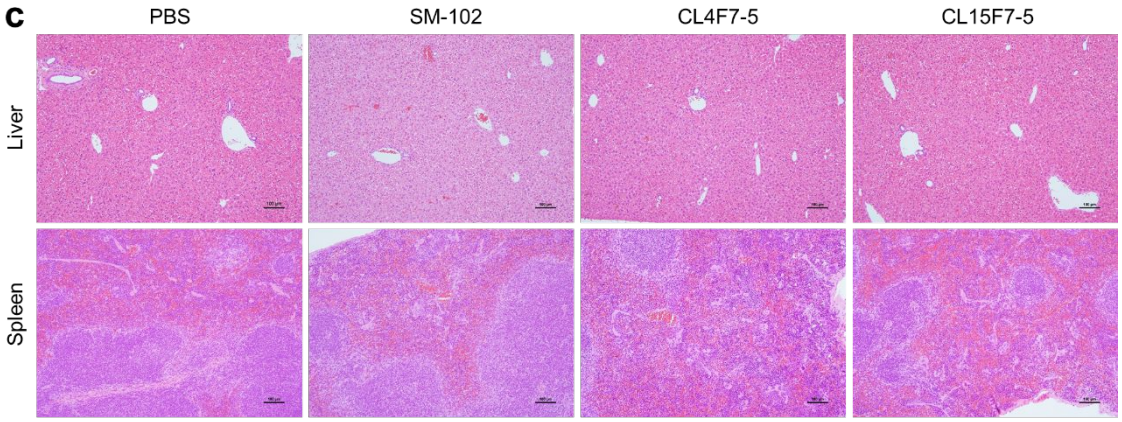


Figure S10. Distribution profile of DiO-labeled LNPs in splenocytes.

a) FLuc mRNA was encapsulated in LNPs. The number mean size and PDI were evaluated using the Zetasizer. b) 0.5 mol% DiO-labeled LNPs was i.v. injected at 0.5 mg/kg FLuc mRNA. The spleen was collected from C57BL/6 mice 24 hours after the mRNA/LNP dose was administered (n = 3 mice per group). The distribution profile of the DiO-labeled LNPs in splenocytes was evaluated by flow cytometry. The gating strategy was the same as described in Figure S6. The LNP cellular distribution was detected using DiO fluorescence. Statistical significance was determined using an unpaired Student's t-test; ** $P < 0.01$.





Group	PBS					SM-102					CL4F7-5					CL15F7-5				
Sample No.	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Liver																				
Necrosis, hep	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inflammatory cell infiltration in the portal area	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hepatocellular hypertrophy in the portal area	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Enlarged Kupffer cells	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Increased simple mitotic figures	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Vacuolar degeneration, hep	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fibrosis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hemorrhage	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Spleen																				
Extramedullary hematopoiesis	-	-	-	-	-	-	±	±	-	±	±	±	±	+	±	±	±	±	±	±
White pulp hyperplasia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lymphoid atrophy	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Necrosis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hemorrhage, Red pulp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

- : negative ± : minimal + : mild ++ : moderate +++ : marked

Figure S11. Repeated dose toxicity studies *in vivo*.

a–c) Body weight measurements (a), hematological tests (b), hematoxylin and eosin (H&E) staining of the liver and spleen, and histopathological analyses (c) were performed 1 week post-boost of PBS, SM-102 LNPs, CL4F 7-5 LNPs, and CL15F 7-5 LNPs (5 μg FLuc mRNA per mouse, two times at two-week intervals, $n = 5$ biologically independent Balb/c mice per group). a) Body weight was maintained during the experiments. b) Serum chemistry parameters were measured at Oriental Yeast Co., Ltd. (Shiga, Japan). The data are presented as the mean ± standard deviation. The data were analyzed by ordinary one-

way ANOVA with Dunnett's multiple comparisons test of each LNP-treated mouse compared with PBS-treated mice. TP: total protein, ALB: albumin, BUN: blood urea nitrogen, CRE: creatinine, AST: aspartate transaminase, ALT: alanine transaminase, LDH: lactose dehydrogenase, AMY: amylase, γ-GT: gamma-glutamyl transpeptidase, T-CHO: total cholesterol, TG: triglyceride, HDL-C: high density lipoprotein cholesterol, T-BIL: total bilirubin, GLU: glucose. c) The liver and spleen tissues were fixed in Mildform 10N and 3-μm slices were stained with H&E. Scale bars = 100 μm. Histopathological analysis of the liver and spleen was performed at the Sapporo General Pathology Laboratory Co., Ltd. (Hokkaido, Japan). Minimal or mild extramedullary hematopoiesis with megakaryocytic hyperplasia was observed in the mice that were administered mRNA/LNPs.

a

Lipid	mRNA	Size [nm]	PDI	EE [%]
SM-102	FLuc	75.11	0.066	99.5
CL15F 7-5	FLuc	169.9	0.009	98.1
CL4F 7-5	FLuc	101.3	0.023	99.3
SM-102	SARS-CoV-2 RBD	75.71	0.016	99.5
CL15F 7-5	SARS-CoV-2 RBD	166.4	0.035	97.7
CL4F 7-5	SARS-CoV-2 RBD	90.00	0.032	99.3

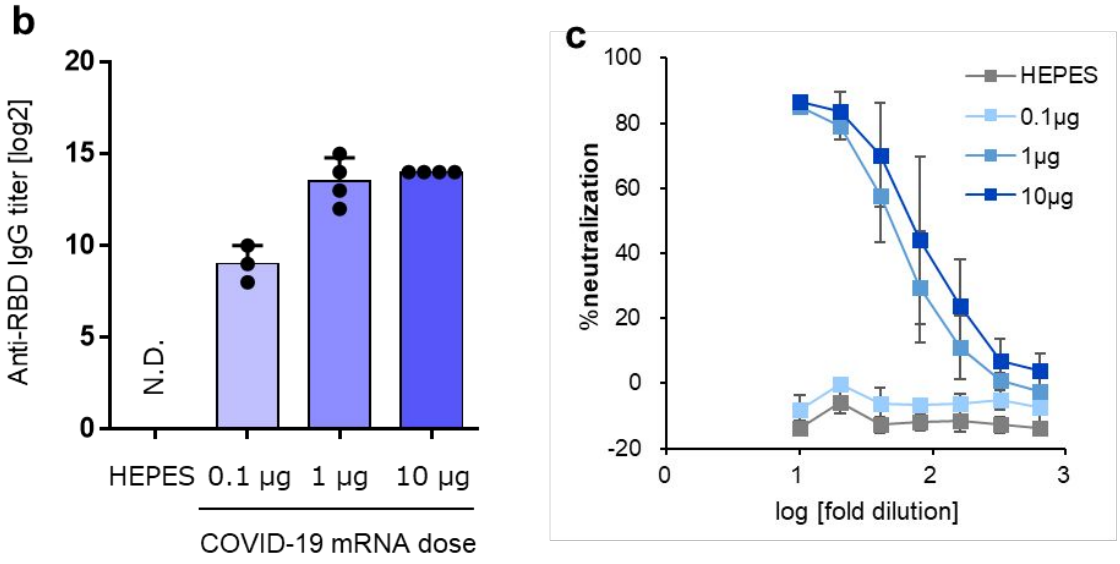


Figure S12. Dose dependency of SM-102 LNP-induced immunogenicity.

a) FLuc mRNA or RBD mRNA was encapsulated in LNPs. The number mean size and PDI were evaluated using the Zetasizer. The encapsulation efficiency was evaluated using the Ribogreen assay. b–c) Thirty μL of RBD mRNA-loaded LNP was injected into the left thigh muscles two times at two-week intervals. Two weeks post-boost, serum samples were collected from the mice (n = 3–4 C57BL/6 mice per group). (c) The circulating RBD-specific IgG antibody titer was evaluated. (d) Inhibition of the SARS-CoV-2 RBD–hACE2 interaction by mouse serum.

Table S1. FLuc mRNA was encapsulated in LNPs. The number mean size and PDI were evaluated using the Zetasizer.

Lipid	DSPC [%]	Number mean size [nm]	PDI
CL15F 6-4	10	197.1	0.100
CL15F 10-8	10	94.5	0.057
CL15F 14-12	10	82.5	0.040
CL15F 10-8	30	52.1	0.113

Section C. Characterization of Synthetic Lipids

General information

All reactions were monitored using pre-coated thin-layer chromatography (TLC) plates (Millipore) and stained with *p*-anisaldehyde solution. ^{1}H NMR spectra were obtained using JEOL ECZ400 and ECP400 (Tokyo, Japan), and chemical shifts are expressed in parts per million (ppm) with respect to the residual solvent peak. The following abbreviations express multiplication: s = singlet, d = doublet, t = triplet, and m = multiplet. All final compounds were identified using reverse-phase ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) using a Waters Acquity UPLC instrument. The following conditions were used: ACQUITY UPLC BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm, 100 mm, and a gradient of 50% to 95% isopropanol/acetonitrile (62:33) in water with 5 mM ammonium acetate over 20 minutes, then held at 95% isopropanol/acetonitrile (62:33) in water with 5 mM ammonium acetate for 20 minutes at 0.2 mL/min. The injection volume was 1.0 µL with a 60°C column temperature. Mass spectral data were acquired using an ACQUITY QDa in the positive electrospray ionization (ESI) mode. All compounds were provided as mixtures of all possible stereoisomers.

1

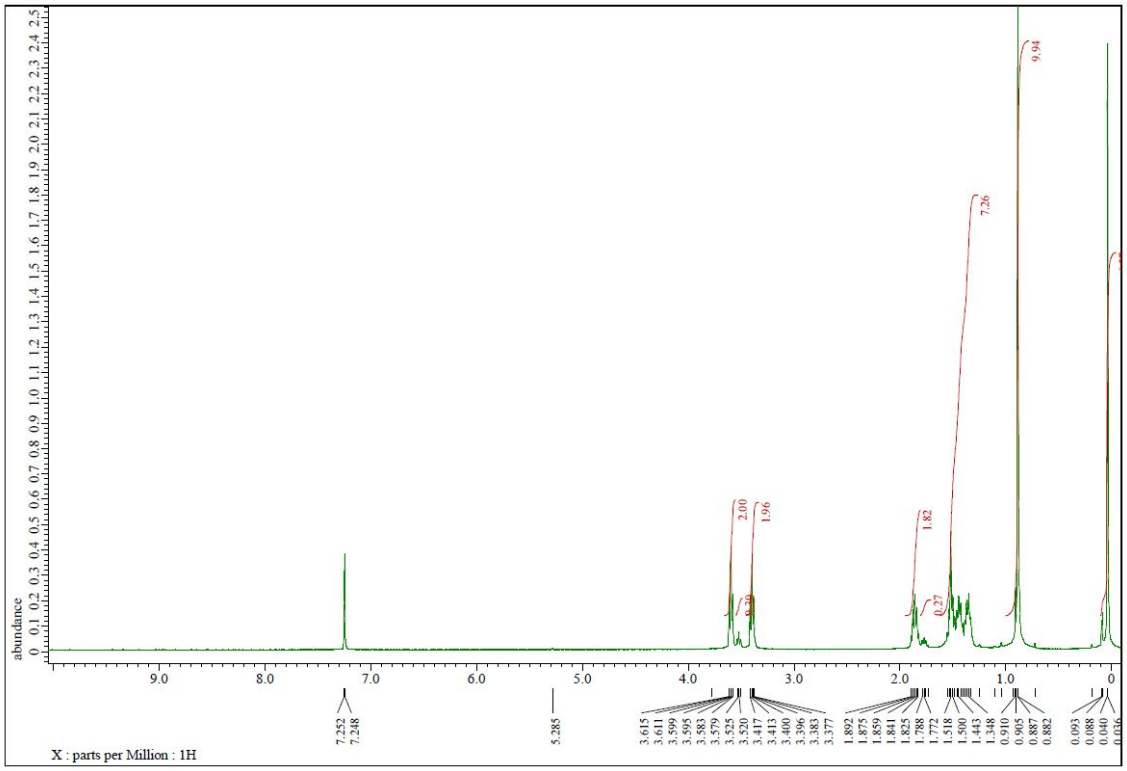
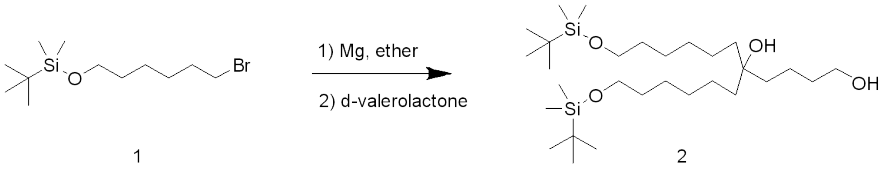


Figure S9. ((6-bromohexyl)oxy)(tert-butyl)dimethylsilane (1).

^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 0.04 (s, 6H), 0.89 (s, 9H), 1.28–1.52 (m, 6H), 1.88 (m, 2H), 3.40 (t, 2H), 3.59 (t, 2H).



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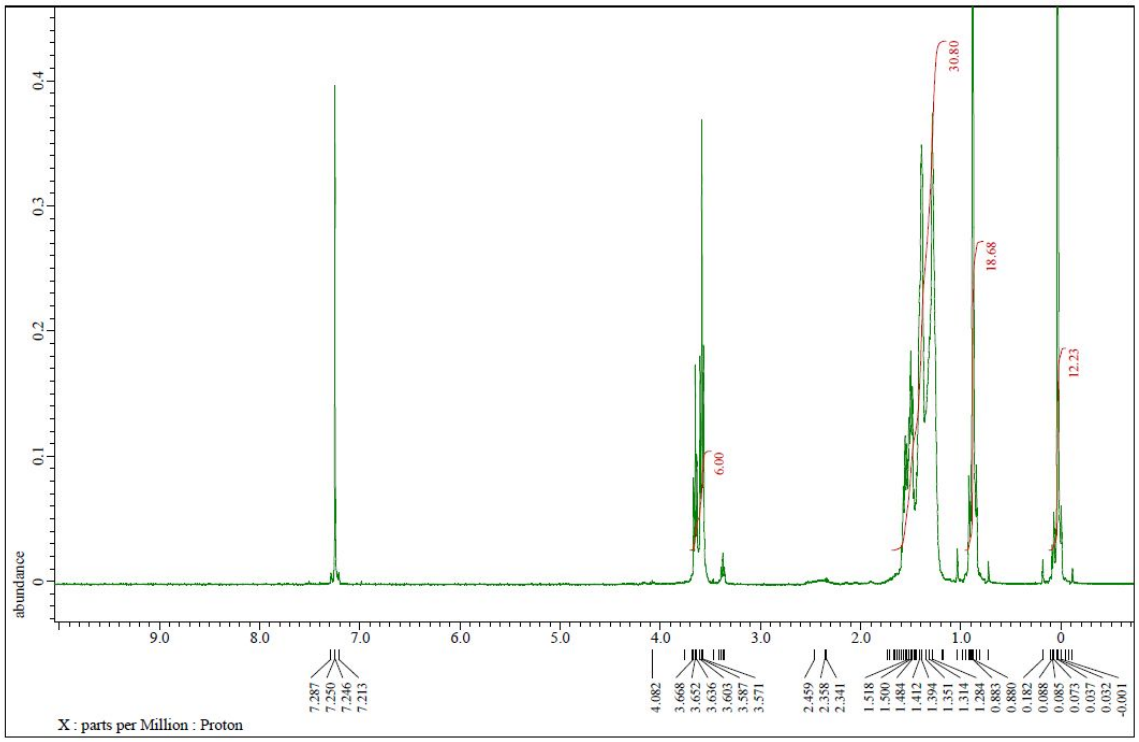
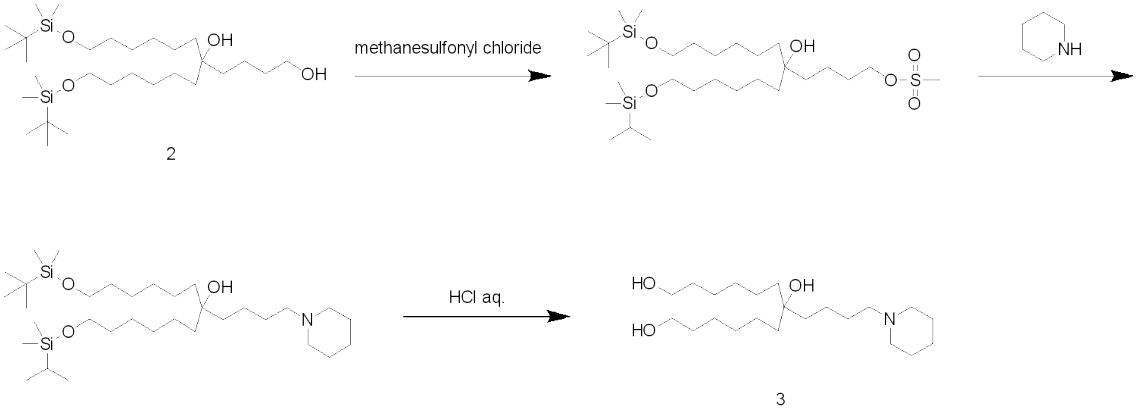


Figure S10. *11-((tert-butyldimethylsilyl)oxy)-5-(6-((tert-butyldimethylsilyl)oxy)hexyl)undecane-1,5-diol* (2).

^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 0.04 (s, 12H), 0.88 (s, 18H), 1.21–1.60 (m, 26H), 3.59 (t, 4H), 3.66 (t, 2H).



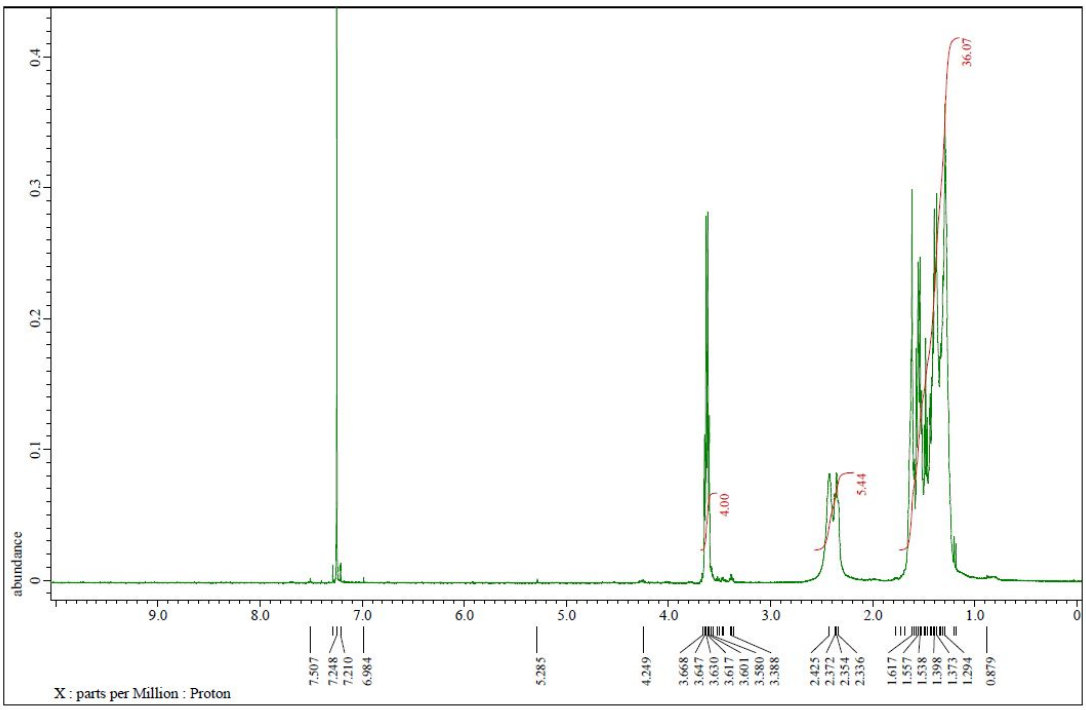
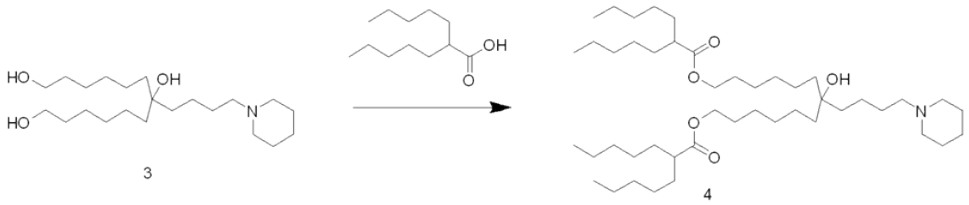


Figure S11. 7-(4-(piperidin-1-yl)butyl)tridecane-1,7,13-triol (3).

^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 1.20–1.68 (m, 35H), 2.30–2.48 (m, 6H), 3.60 (m, 4H).



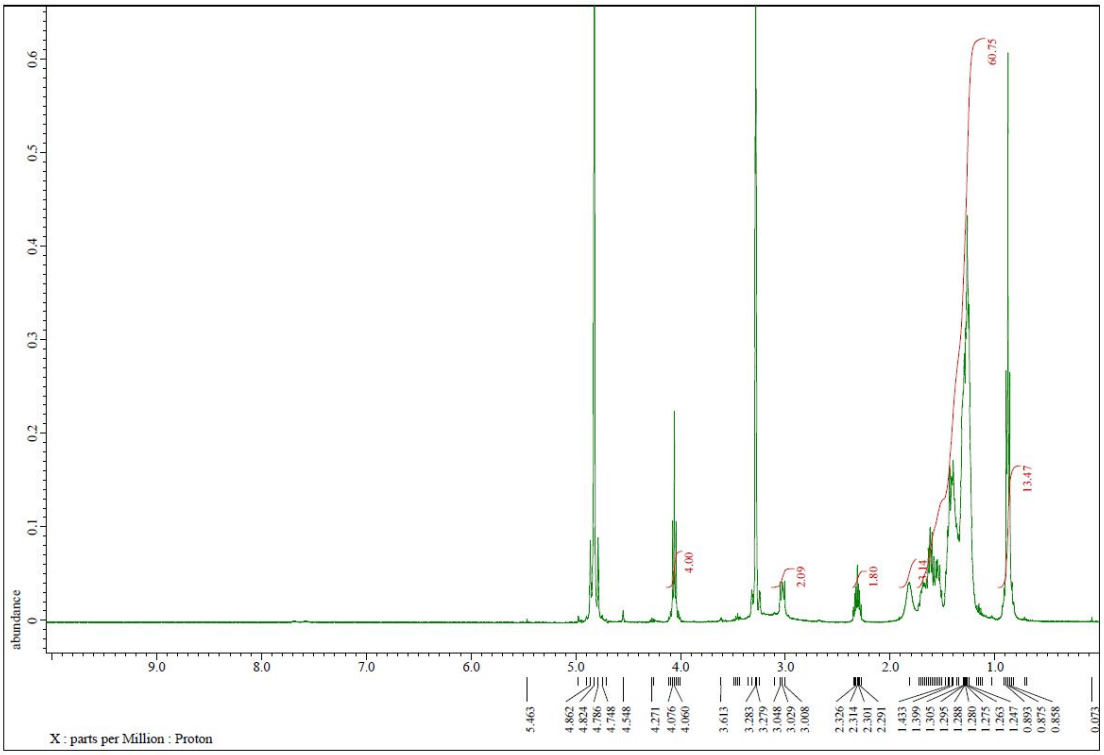


Figure S12. CL6F 7-5 (4).

^{1}H NMR (300 MHz, CD_3OD, ppm) δ: 0.88 (t, 12H), 1.18–1.88 (m, 69H), 2.31 (m, 2H), 3.03 (m, 2H), 4.06 (t, 4H).

UPLC/CAD: RT = 8.24 in. MS (ESI): m/z calcd for $C_{74}H_{146}NO_5$ $(M+H)^+$, 736.68; found, 736.95.

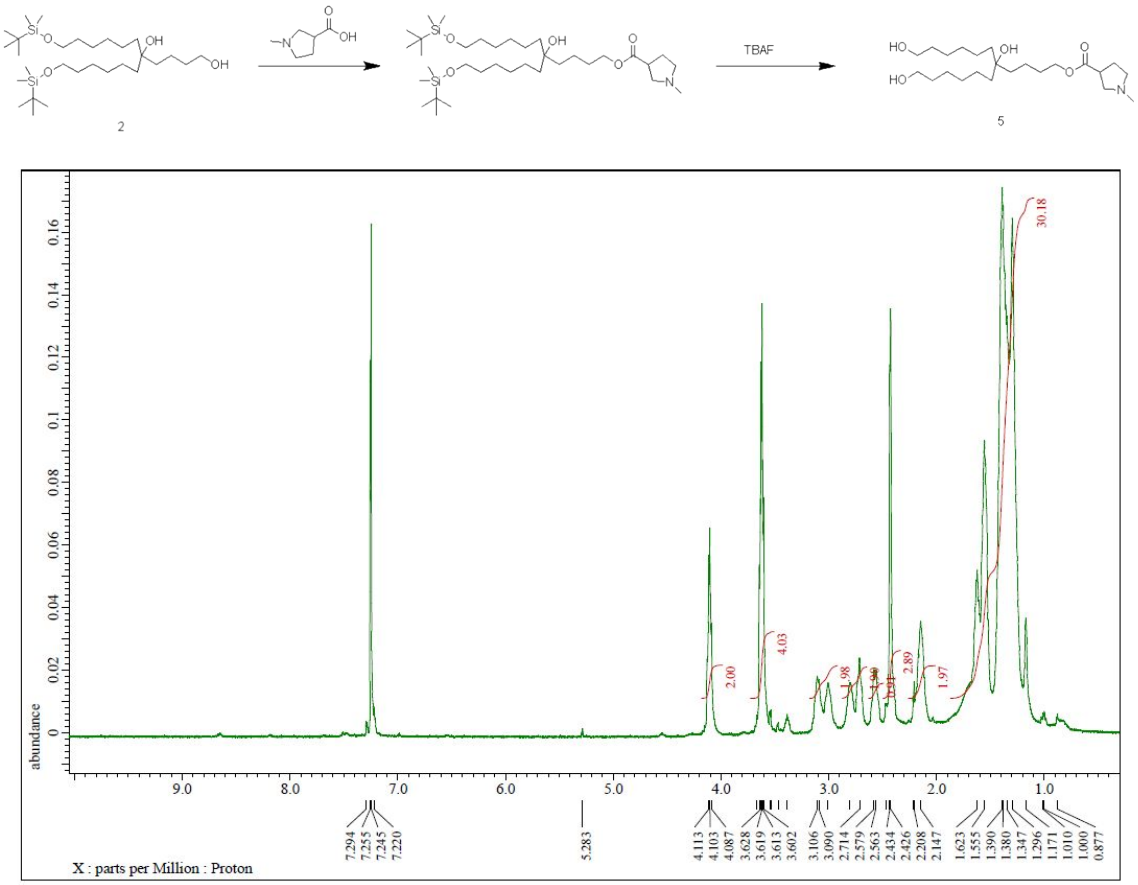
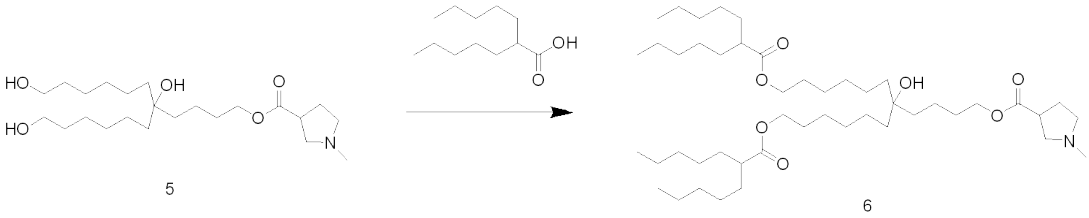


Figure S13. 5,11-dihydroxy-5-(6-hydroxyhexyl)undecyl 1-methylpyrrolidine-3-carboxylate (5).

^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 1.15–1.85 (m, 29H), 2.15 (m, 2H), 2.43 (s, 3H), 2.56 (m, 1H), 2.72–2.85 (m, 2H), 2.95–3.15 (m, 2H), 3.62 (m, 4H), 4.10 (m, 2H).



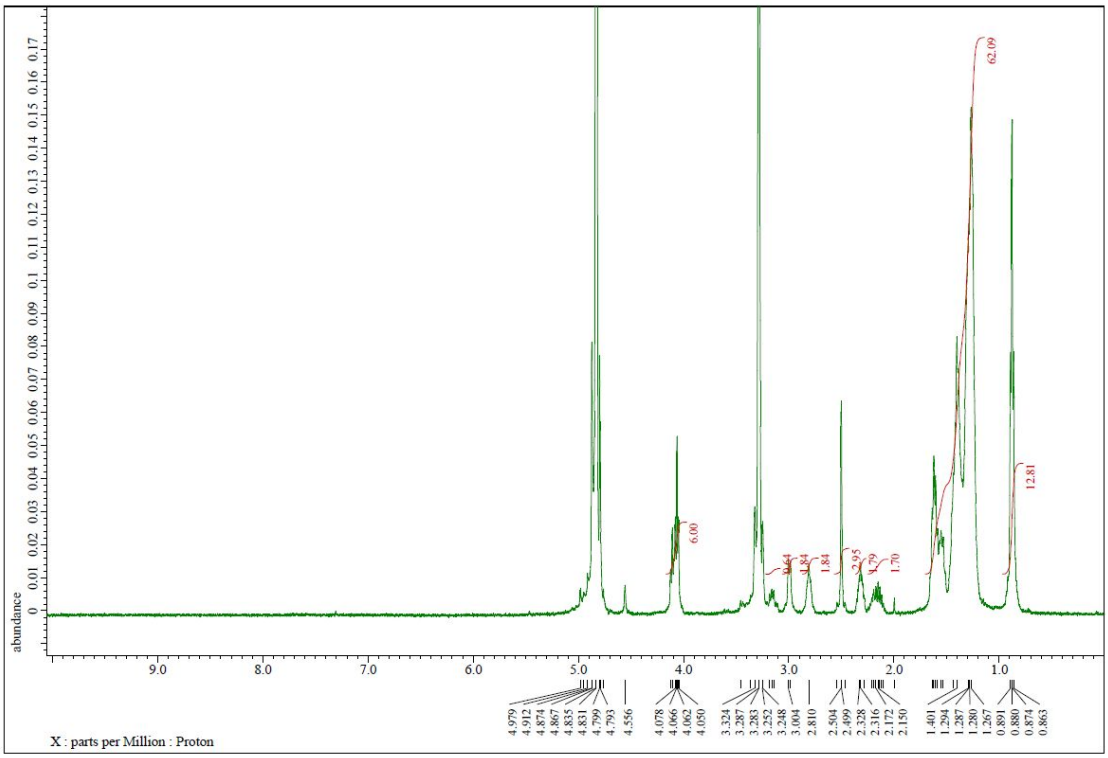
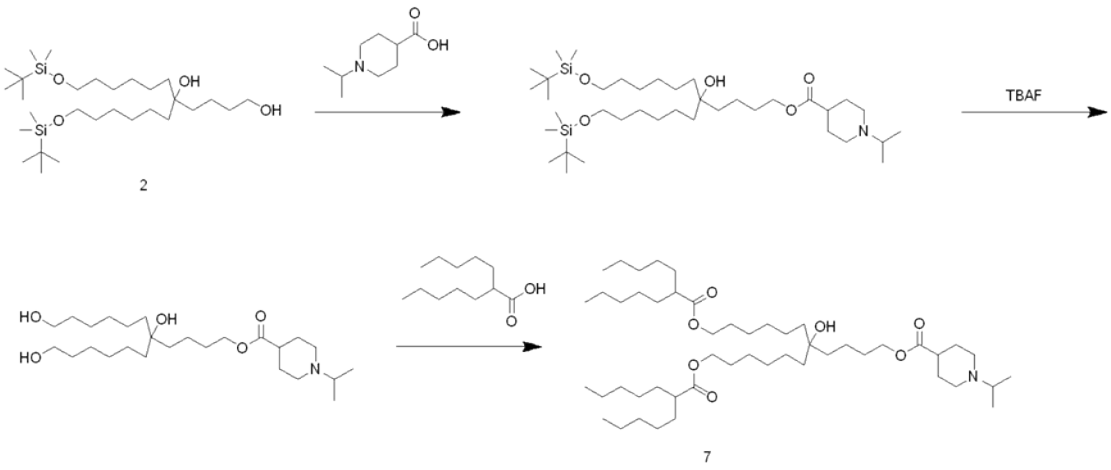


Figure S14. CL16F 7-5 (6).

^{1}H NMR (400 MHz, CD_3OD, ppm) δ: 0.88 (t, 12H), 1.20–1.65 (m, 60H), 2.17 (m, 2H), 2.32 (m, 2H), 2.50 (s, 3H), 2.81 (m, 2H), 3.00 (m, 2H), 4.06 (m, 6H).

UPLC/CAD: RT = 9.40 in. MS (ESI): m/z calcd for $C_{47}H_{90}NO_7$ $(M+H)^+$, 780.67; found, 780.75.



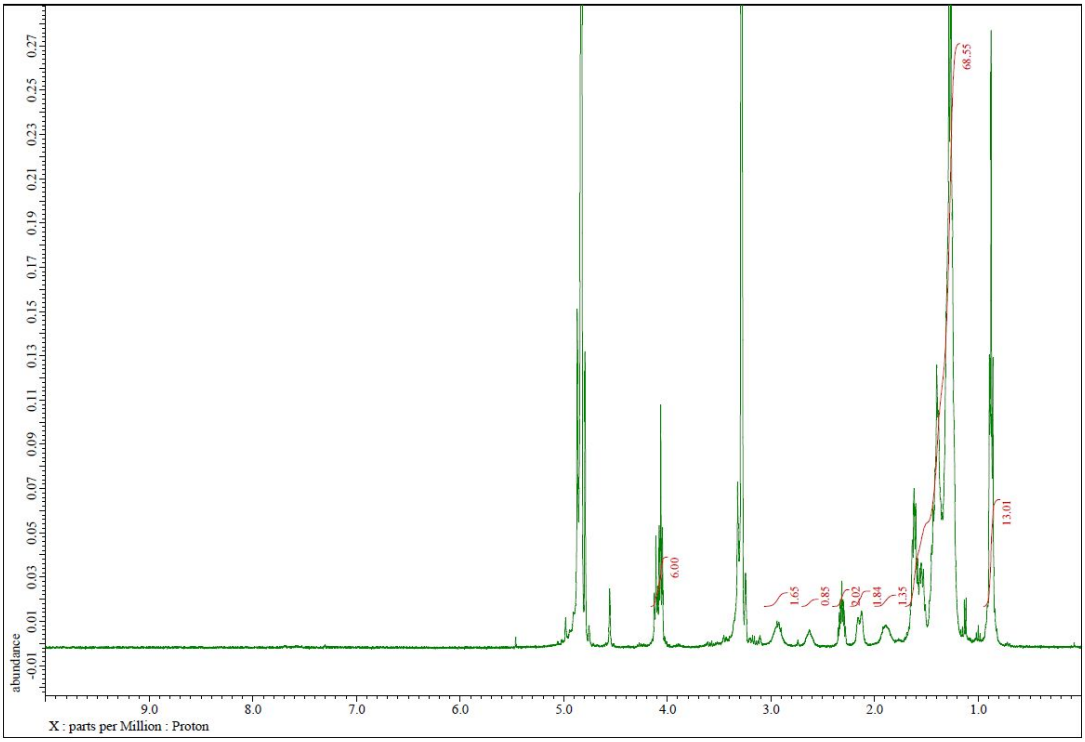


Figure S15. CL17F 7-5 (7).

^{1}H NMR (400 MHz, CD_3OD, ppm) δ: 0.88 (t, 12H), 1.21–1.68 (m, 68H), 1.87 (m, 2H), 2.12 (m, 2H), 2.32 (m, 2H), 2.62 (m, 1H), 2.92 (m, 2H), 4.08 (m, 6H).

UPLC/CAD: RT = 8.96 in. MS (ESI): m/z calcd for $C_{50}H_{96}NO_7$ $(M+H)^+$, 822.72; found, 822.78.

References

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