Supplementary information

Cholesterol-Containing Ionizable Lipids for Safe and Efficient mRNA Delivery via Lipid Nanoparticles

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Additional Experimental Details

Materials and instrumentation

All reagents and solvents for compound synthesis were obtained from Bidepharm (Shanghai, China) or Energy Chemical (Shanghai, China). Basic biological reagents, including hematoxylin, eosin, and paraformaldehyde, were acquired from J&K Scientific (Beijing, China). Dulbecco's modified Eagle's Medium (DMEM) and phosphate-buffered saline (PBS) were sourced from Solarbio (Beijing, China), while firefly luciferase mRNA (F-Luc mRNA) was procured from GenScript (Nanjing, China). Reagents for lysis and buffer systems were sourced as follows: RIPA lysis buffer from KeyGen Biotech. Inc (Nanjing, China), RNase-free water from Cowin Biotech Co., Ltd (Jiangsu, China), and citric acid-sodium buffer (pH 4.0), tris (hydroxymethyl) aminomethane hydrochloride buffer, and Triton X-100 sodium from Yuanye Bio-Technology Co., Ltd (Shanghai, China). The RNA fluorescence (RIBOGREEN) quantitative detection kit was purchased from GenMed Scientifics Inc (USA).

^{1}H Nuclear magnetic resonance (NMR) and ^{13}C NMR spectra were acquired on an Avance NMR spectrometer (Bruker Corporation). High-resolution mass spectrometry (HRMS) was performed on a Bruker MAXIS IMPACT mass spectrometer. The hydrodynamic particle size and dispersion of the LNPs were measured with a Malvern Zetasizer Nano ZS90 DLS analyzer. For morphological characterization, samples were deposited onto ultra-thin carbon film-coated copper mesh, air-dried, and imaged on a JEOL JEM-1400 transmission electron microscope (TEM). Fluorescence imaging was performed using the AMI small animal fluorescence imaging system (Spectral Instruments Imaging Co.). Confocal laser scanning microscopy (CLSM) was conducted with an Olympus FV3000 microscope. The pain experiment was tested for mice with a Biped Balance Pain Meter.

Storage stability test of LNPs

The mRNA@LNPs were initially dispersed in PBS. For the stability study, sucrose (5 wt%) was added to the dispersion as a protectant, and the samples were stored at 4 °C. For stability studies, sucrose (5 wt%) was added to the dispersion as a cryoprotectant, and the samples were stored at 4 °C. At predetermined time points (on day 0, 1, 3, 7, and 21), aliquots (200 μL each) were withdrawn from storage at 4 °C, diluted to 1/10 of the original concentration, and equilibrated to 25 °C prior to measurement. The hydrodynamic diameter and polydispersity indexes were then determined by dynamic light scattering (DLS).

2-(p-toluidino)-6-naphthalenesulfonic acid (TNS) assay

The apparent pKa of the LNP formulations was determined by monitoring the pH-dependent fluorescence of the lipophilic dye TNS. LNPs were diluted to 100 μM in a mixed buffer system (10 mM HEPES, 10 mM MES,10 mM ammonium acetate, 130 mM NaCl). The pH of the suspensions was titrated from 3 to 11 with HCl or NaOH. TNS was added to a final concentration of 1 μM, and samples were incubated in the dark. Fluorescence was recorded at λex/λem = 321/445 nm. The apparent pKa was defined as the pH at the midpoint of the fluorescence transition curve.

Agarose gel electrophoresis

mRNA stability against nuclease degradation was evaluated by agarose gel electrophoresis. Free Fluc mRNA and the three LNP formulations (Fluc mRNA@A13 LNP, Fluc mRNA@B13 LNP, and Fluc mRNA@A12 LNP) were incubated in RNase-free buffer or with RNase A (10 μg/mL) at 37 °C for 30 min. Samples were mixed with 6× gel loading dye, loaded onto a 1% agarose gel prepared in 1× TAE buffer, and electrophoresed at 100 V for 30 min. The gel was stained with ethidium bromide (0.5 μg/mL) for 20 min, destained in water, and imaged under UV transillumination. A commercial RNA ladder served as size marker to indicate the migration positions of 5S, 18S, and 28S RNA.

Assessment on Cytotoxicity of LNPs in L-02 Cells

L0-2 cells (KeyGEN Biotechnology Co., Ltd.) were maintained at 37 °C in a humidified incubator with 5% CO_2. For the assay, approximately 10^5 cells were seeded into each well of a 96-well plate and allowed to attach for 10 hours before treatment. Cells were then exposed to varying concentrations of LNPs for 48 hours. Each concentration was tested in eight technical replicates, and all experiments were repeated independently three times. Following treatment, 10 μL of MTT solution (5 mg/mL in PBS) was added to each well and incubated for 4 hours at 37 °C in the dark. The medium was carefully removed, and 150 μL of DMSO was added to dissolve the formazan crystals, followed by gentle shaking for 10 minutes to ensure complete solubilization. Absorbance at 570 nm was measured using a Thermo MK3 microplate reader. Cell viability was calculated relative to the untreated control group, which was defined as 100% viability.

mRNA@LNPs transfection in vitro

HEK293T cells ($1.0–2.0 \times 10^5$ cells per well) were seeded onto 20-mm glass-bottom culture dishes. After cell attachment, 2.5 μg of each mRNA-eGFP@LNP formulation was added to the wells, and the plates were gently agitated to ensure even distribution of the complexes. Cells were incubated for an additional 10 hours, after which eGFP expression was examined using a confocal laser scanning microscope (CLSM). The CLSM fluorescence microscopic imaging was performed by Jinyu Bio-Technology Co.

Endosomal escape observation

Endosomal escape observation was evaluated by confocal laser scanning microscopy using Cy5-labeled OVA mRNA-loaded LNPs. HePG2 Cells were co-stained with mRNAova@Lbb LNP or mRNAova@SM-102 LNP for 1 or 2 h at 37 °C. 15 min before the end of co-incubation, LysoTracker Green (50 nM) was added and incubated for 15 min at 37°C in the dark. The culture medium was then discarded, and

the cells were gently washed twice with PBS. Hoechst 33342 (1 μg/mL) was then added and incubated for 5 min at 37°C. The confocal imaging was then performed immediately.

Quantification of Cellular uptake and transfection of LNPs by flow cytometry.

HePG2 cells were seeded into 6-well plates at a density of 1×10^6 cells per well and cultured overnight in an incubator at 37 °C with 5% CO_2. After the cells had fully adhered and grown stably, subsequent treatment experiments were carried out. First, the original complete medium in each well of the 6-well plate was aspirated, and the cells were gently washed three times with sterile phosphate-buffered saline (PBS) to thoroughly remove residual medium components and cellular metabolic impurities, thereby avoiding interference with subsequent experiments. Then, fresh reduced-serum Opti-MEM medium containing eGFP mRNA-loaded LNPs was added to each well. A PBS control group was also included. The dosing system for eGFP mRNA-loaded LNPs in all groups was standardized as follows: a total volume of 1 mL per well, containing 0.5 μg of eGFP mRNA and 50 μL of LNP formulation, with the final mRNA concentration strictly controlled at 2 μg/mL. The prepared 6-well plates were returned to the incubator and incubated for four different time points: 0 h, 1 h, 4 h, and 6 h. After each incubation time point, cells from each well were carefully collected and analyzed using flow cytometry. The in vitro transfection efficiency of different LNP formulations was quantitatively evaluated by analyzing the percentage of eGFP-positive cells and the mean fluorescence intensity (MFI).

Animal experiments

All animal experiments in this study were conducted in strict compliance with the "Regulations on the Administration of Laboratory Animals" (issued by in 1988 and revised in 2017) and the "Guidelines for Welfare of Laboratory Animals" (issued in 2006) by the State Science and Technology Commission of China, and were approved by the Animal Experiment Ethics Committee of South China Agricultural University (Approval Number: 2025-D011).

The BALB/c nude mice (6-7 weeks old) were obtained from Guangdong Yaokang Biotechnology Co., Ltd. (Foshan, China) and maintained under specific pathogen-free (SPF) conditions. The animals were housed in sterile cages within laminar flow hoods with the following environmental parameters: constant temperature control at 24 ± 1 ℃, relative humidity at 45-65%, 12-hour light/12-hour dark cycle, Ad libitum access to sterilized autoclaved chow and water. Before the experiment, the mice were randomly allocated into experimental groups for subsequent experimental studies. For lethal experimental procedures, the gradient CO_2 asphyxiation method was used, where the mice would be euthanized by being exposed to gradually increasing carbon dioxide gas.

In vivo bioluminescence imaging

LNP-encapsulated mRNA-Luc ($mRNA^{F\text{-}Luc}$@LNPs) was administered either intramuscularly into the hind limb or intravenously via the tail vein. Bioluminescence was monitored at 6, 24, and 48 hours post-injection using an AMI small-animal imaging system. To minimize background fluorescence and improve signal detection, the fur over the target region (hind limbs or abdomen) was shaved and disinfected with alcohol 24 hours before imaging. Before imaging, mice were anesthetized with 2% isoflurane delivered through a nose cone and injected intraperitoneally with 3 mg D-fluorescein. Imaging was performed 10 minutes after administration to allow for full systemic distribution, using an exposure time of 60 seconds. Bioluminescence intensity was quantified based on regions of interest (ROI), and background levels were determined from the average photon flux of the untreated hind limb in each animal.

H&E staining analysis

Gastrocnemius muscle tissues were collected immediately after the in vivo imaging studies and fixed in 4% paraformaldehyde to preserve cellular and structural integrity. The fixed samples were then processed for paraffin embedding and sectioned at a

thickness of 3 μm. Tissue sections were stained using a standard hematoxylin and eosin (H&E) protocol. Stained slides were examined and imaged using a microscope.

Static weight-bearing test

The static weight-bearing test was employed to assess unilateral pain-related behavior in mice. Briefly, each mouse was placed in a confined chamber with a pressure-sensitive floor grid. During a defined recording period, the weight (in grams) borne by each hind limb was measured simultaneously while the animal was in a stationary, standing position. The weight-bearing asymmetry was calculated as the difference in weight between the injured (ipsilateral) and non-injured (contralateral) limbs. A significant reduction in weight borne on the ipsilateral limb was interpreted as an indicator of pain and discomfort.

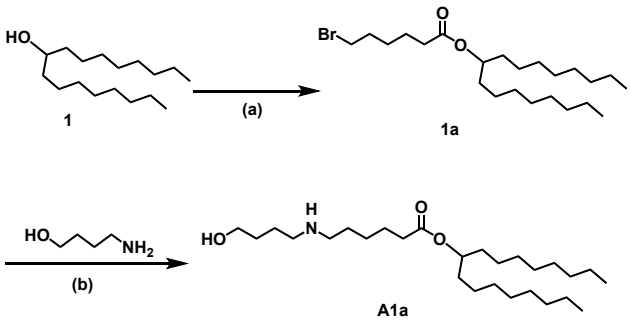
Evaluation of muscle damage after LNP treatment

A 1% (w/v) Evans blue dye (EBD) solution was prepared in PBS (pH 7.5). Following the treatment with LNPs, the mice were injected intravenously with the solution of Evans blue dye (EBD) in sterile saline at a dose of 10 mg EBD per kg body weight. The dye was allowed to circulate for 24 hours. Mice were then euthanized and subjected to transcardial perfusion with phosphate-buffered saline (PBS) to remove intravascular EBD. Target muscles were then dissected and harvested.

Statistical analysis

All quantitative data are presented as the mean ± standard deviation (SD). For spectral characterization (fluorescence or absorption), measurements were derived from three independent replicates (n=3). Cell viability was assessed through three independent experiments (n=3), with each concentration tested in eight technical replicates. In vivo animal experiment data are based on a sample size of n=3 per group. Statistical significance was determined via one-way analysis of variance (ANOVA) followed by Tukey HSD test, and statistical analyses were conducted using GraphPad Prism 9.0. Statistical significance is denoted as follows: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. ns (not significant): $p > 0.05$.

Synthesis

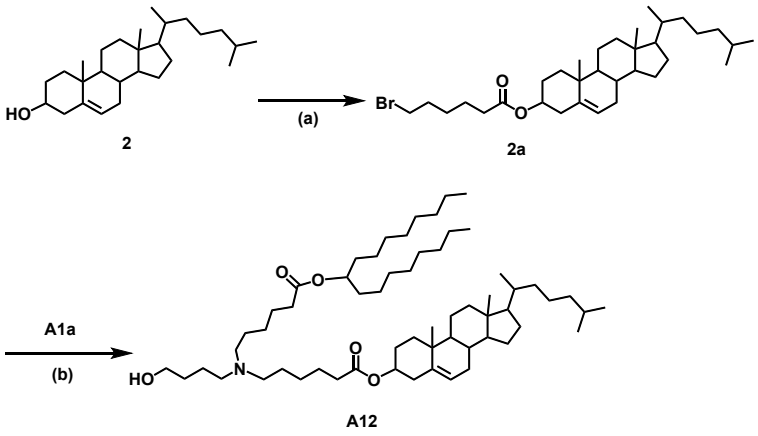


(a) Et_3N, 6-bromohexanoyl chloride, DCM, 0 °C, N_2, 16 h; (b) Potassium carbonate, potassium iodide, ACN, CMPE, 90 °C, N_2, 16 h.

Scheme S1. Synthetic route of compound **A1a**.

Synthesis of compound 1a: Compound 1 (9-Heptadecanol, 5.00 mmol, 1.76 g) and triethylamine (1.43 mL, 10.00 mmol) were dissolved in anhydrous dichloromethane (32 mL) in an ice bath at 0 °C. The system was maintained in a nitrogen atmosphere with continuous stirring. Then, 6-bromohexanoyl chloride (1.25 mL, 8.00 mmol) was added dropwise to the above solution over approximately 20 minutes while maintaining the temperature at 0 °C. After completion of the reaction, the ice bath was removed and the reaction mixture was allowed to naturally warm to room temperature and stirred for over 16 hours. The reaction mixture was transferred to a separatory funnel, and the mixture was extracted with dichloromethane (100 mL) and water (100 mL) three times. The solution was dried with anhydrous Na_2SO_4. The solvent was removed under reduced pressure to obtain the crude product. The crude product was purified by silica gel column chromatography (eluent: PE: EA = 30 :1), and the target product **1a** (2.35 g, 5.43 mmol, 79.32% yield) was obtained as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.87 (t, J = 6.3 Hz, 1H), 3.40 (t, J = 6.7 Hz, 2H), 2.31 (t, J = 7.4 Hz, 2H), 1.90 – 1.86 (m, 2H), 1.68 – 1.64 (m, 2H), 1.52 – 1.46 (m, 6H), 1.31 – 1.26 (m, 24H), 0.88 (t, J = 6.7 Hz, 6H).

Synthesis of compound A1a: In a 100 mL round-bottom flask, compound **1a** (2.00 mmol, 0.87 g), potassium carbonate (5.00 mmol, 0.69 g), potassium iodide (1.60 mmol, 0.27 g) and anhydrous acetonitrile (24 mL) were stirred under nitrogen atmosphere at room temperature. 4-Amino-1-butanol (A, 36.00 mmol, 3.32 mL) and cyclopentyl methyl ether (30.20 mmol, 3.51 mL) were then added into the flask successively. Afterwards, the reaction system was heated to 90 ℃ and stirred continuously for 16 h under reflux. After cooling the mixture to room temperature, the mixture was transferred to a separatory funnel, and 30 mL ethyl acetate and 30 mL water were added for extraction (three times). The solution was dried with anhydrous Na_2SO_4. The solvent was removed under reduced pressure to obtain the crude product. The crude product was purified by silica gel column chromatography (eluent: dichloromethane: methanol = 25:1), and the target product **A1a** (0.49 g, 1.10 mmol, 54.93% yield) was obtained as a pale white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.01 – 5.00 (m, 1H), 3.58 – 3.55 (m, 2H), 2.66 – 2.59 (m, 4H), 2.29 (t, *J* = 7.5 Hz, 2H), 1.67 – 1.61 (m, 8H), 1.52 – 1.26 (m, 31H), 0.88 (t, *J* = 6.7 Hz, 6H).

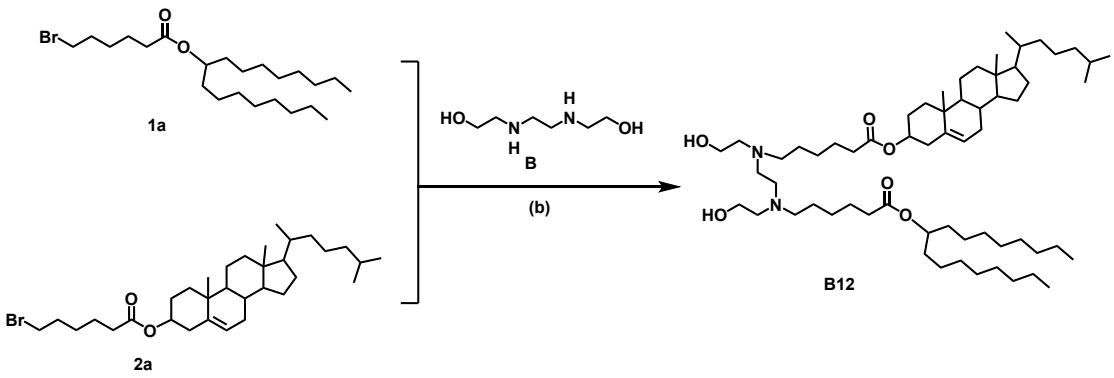


(a) Et_3N, 6-Bromohexanoyl chloride, DCM, 0 °C, N_2, 16 h; (b) Potassium carbonate, potassium iodide, ACN, CMPE, 90 °C, N_2, 16 h;

Scheme S2. Synthetic route of compound **A12.**

Synthesis of compound A12: **2a** (white solid) was prepared following the same synthetic route as compound **1a**, except that raw material 1 was replaced with 2 (cholesterol). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.40 – 5.35 (m, 1H), 4.65 – 4.57 (m, 1H), 3.41 (t, J = 6.8 Hz, 2H), 2.32 – 2.28 (m, 4H), 2.02 – 1.95 (m, 2H), 1.88 – 1.84 (m, 4H), 1.67 – 1.10 (m, 26H), 1.02 (s, 3H), 0.92 – 0.85 (m, 9H), 0.68 (s, 3H).

A12 (yellow liquid) was synthesized using a procedure analogous to that of compound **A1a**, with **1a** and 4-aminobutanol being replaced by **2a** and **A1a**, respectively. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.37 (d, J = 5.0 Hz, 1H), 4.87 – 4.85 (m, 1H), 4.62 – 4.60 (m, 1H), 3.58 – 3.54 (m, 2H), 2.43 (dd, J = 10.0, 6.0 Hz, 5H), 2.31 – 2.26 (m, 6H), 2.02 – 1.99 (m, 2H), 1.87 – 1.84 (m, 4H), 1.68 – 1.62 (m, 9H), 1.56 – 1.43 (m, 15H), 1.37 – 1.26 (m, 32H), 1.16 – 1.08 (m, 8H), 1.02 (s, 3H), 0.91 (d, J = 6.5 Hz, 3H), 0.89 – 0.86 (m, 12H), 0.68 (s, 3H). ^{13}C NMR ($CDCl_3$) δ 173.50, 173.11. 139.69, 122.62, 74.21, 73.79, 62.66, 56.69, 56.13, 54.66, 53.53, 50.02, 42.31, 39.73, 39.52, 38.16, 36.99, 36.60, 36.18, 35.80, 34.63, 34.59, 34.14, 32.70, 31.91, 31.87, 29.55, 29.52, 29.26, 28.24, 28.02, 27.81, 27.21, 27.12, 26.33, 25.68, 25.34, 25.02, 24.92, 24.29, 23.83, 22.83, 22.68, 22.57, 21.03, 19.33, 18.72, 14.13, 11.86. HR-MS (ESI, positive mode, m/z) $[M + H]^+$ $[C_{60}H_{110}NO_5]^+$ calcd. 924.8384 found 924.8327.

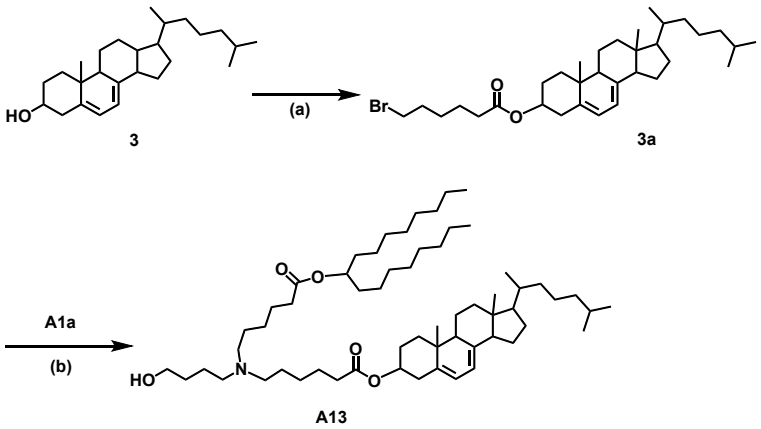


(b) potassium carbonate, potassium iodide, ACN, CMPE, 90 °C, N_2, 16 h;

Scheme S3. Synthetic route of compound **B12.**

Synthesis of compound B12: **B12** (yellow liquid) was also synthesized using a

procedure analogous to that of compound **A1a**, but using **1a** and **2a** in place of **1a**, and N,N′-bis(2-hydroxyethyl)ethylenediamine (B) instead of 4-aminobutanol. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.37 (d, *J* = 5.1 Hz, 1H), 4.62 – 4.60 (m, 1H), 4.06 (t, *J* = 6.6 Hz, 1H), 3.61 – 3.17 (m, 4H), 2.62 – 2.48 (m, 8H), 2.32 – 2.28 (m, 8H), 2.02 – 1.95 (m, 4H), 1.89 – 1.81 (m, 8H), 1.50 - 1.49 (m, 5H), 1.30 - 1.26 (m, 50H), 1.02 (s, 3H), 0.88 – 0.87 (m, 9H), 0.86 (d, *J* = 2.3 Hz, 7H), 0.68 (s, 3H). ^{13}C NMR ($CDCl_3$) δ 173.08, 139.56, 122.59, 74.21, 73.80, 60.10, 56.70, 56.14, 55.62, 52.57, 50.03, 42.32, 39.74, 39.53, 38.17, 37.00, 36.61, 36.19, 35.81, 34.62, 34.15, 31.94, 31.92, 31.88, 29.72, 29.67, 29.56, 29.53, 29.38, 29.27, 28.24, 28.03, 27.83, 27.10, 25.35, 24.29, 23.84, 22.83, 22.71, 22.68, 22.57, 21.04, 19.33, 14.13, 11.87. HR-MS (ESI, positive mode, m/z) $[M + H]^+$ $[C_{62}H_{115}N_2O_6]^+$ calcd. 983.8755, found 983.8732.



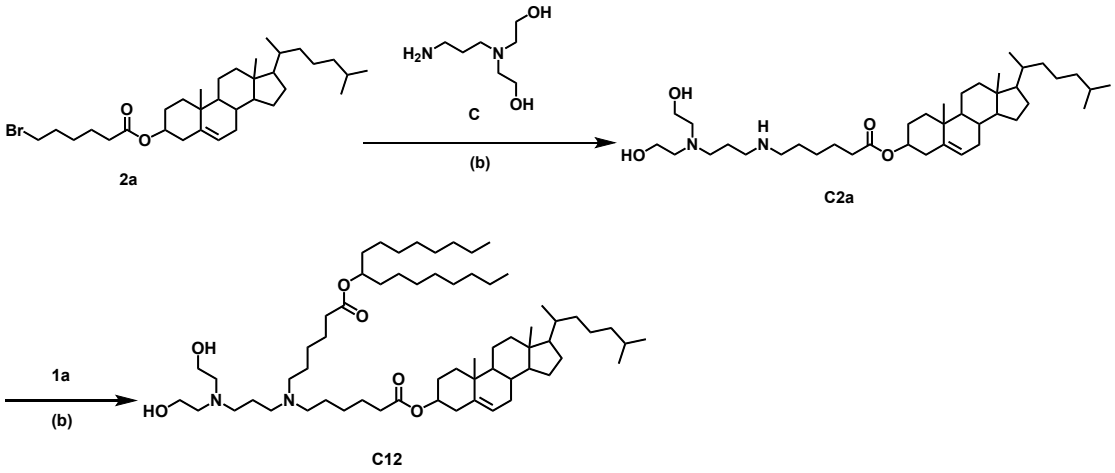
(a) Et_3N, 6-Bromohexanoyl chloride, DCM, 0 °C, N_2, 16 h; (b) Potassium carbonate, potassium iodide, ACN, CMPE, 90 °C, N_2, 16 h.

Scheme S4. Synthetic route of compound **A13.**

Synthesis of compound A13: **3a** (white solid) was prepared following the same synthetic route as compound **1a**, except that raw material 1 was replaced with 3. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.57 (d, J = 5.6 Hz, 1H), 5.38 (d, J = 3.7 Hz, 1H), 4.72 – 4.70 (m, 1H), 3.41 (t, J = 7.5 Hz, 2H), 2.32 – 2.30 (m, 2H), 2.10 – 1.99 (m, 2H), 1.91 – 1.58 (m, 13H), 1.50 – 1.02 (m, 16H), 0.95 – 0.93 (m, 6H), 0.88 – 0.86 (m, 6H), 0.62

(s, 3H).

A13 (yellow liquid) was synthesized using a procedure analogous to that of compound **A1a**, with **1a** and 4-aminobutanol being substituted by **3a** and **A1a**, respectively. ^{1}H NMR (400 MHz, DMSO-d_6) δ 5.57 – 5.52 (m, 1H), 5.39 – 5.33 (m, 1H), 4.61 – 4.53 (m, 2H), 3.43 (t, J = 6.2 Hz, 2H), 2.51 – 2.49 (m, 8H), 2.33 – 2.27 (m, 4H), 1.87 – 1.83 (m, 4H), 1.64 – 1.09 (m, 63H), 0.87 – 0.84 (m, 18H), 0.58 (s, 3H). ^{13}C NMR ($CDCl_3$) δ 173.48, 173.10, 141.60, 138.56, 120.19, 116.28, 74.30, 74.21, 62.61, 55.88, 54.60, 54.47, 53.51, 53.48, 46.04, 42.91, 39.50, 39.14, 37.93, 37.09, 36.69, 36.16, 36.12, 34.61, 34.56, 34.14, 34.12, 31.87, 29.54, 29.51, 29.25, 28.15, 28.10, 28.02, 27.19, 27.11, 25.62, 25.33, 25.00, 24.90, 24.77, 23.89, 23.02, 22.82, 22.67, 22.56, 21.04, 18.85, 16.19, 14.11, 11.83. HR-MS (ESI, positive mode, m/z) [M + H]$^+$ $[C_{60}H_{108}NO_5]^+$ calcd. 922.8228, found 922.8261.

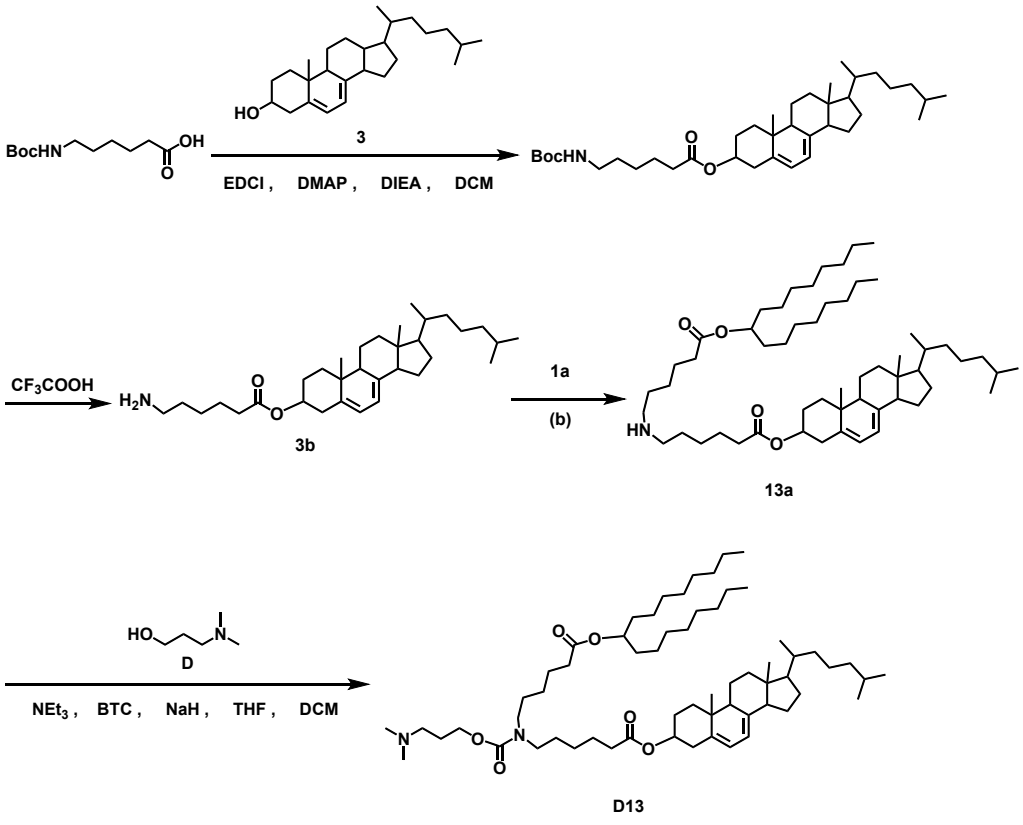


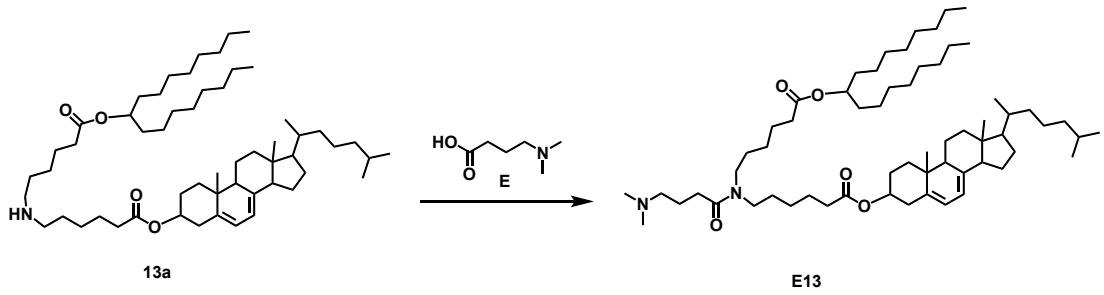
Scheme S5. Synthetic route of compound **C12.**

Synthesis of compound C12: **C2a** (yellow liquid) was synthesized using a procedure analogous to that of compound **A1a**, with **1a** and 4-aminobutanol being substituted by **2a** and 2,2'-((3-aminopropyl)azanediyl)bis(ethan-1-ol) (C), respectively. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.39 – 5.36 (m, 1H), 4.66 – 4.56 (m, 1H), 3.65 (t, J = 5.1 Hz, 4H), 2.81 (t, J = 5.8 Hz, 2H), 2.67 – 2.59 (m, 8H), 2.53 (q, J = 7.2 Hz, 4H), 2.31 – 2.25 (m, 4H), 1.99 (s, 2H), 1.84 – 1.73 (m, 6H), 1.64 – 1.47 (m, 12H), 1.36 – 1.33 (m,

4H), 1.05 – 1.01 (m, 10H), 0.91 (d, J = 6.5 Hz, 3H), 0.86 (dd, J = 6.6, 1.8 Hz, 6H), 0.68 (s, 3H).

C12 (yellow liquid) was synthesized using a procedure analogous to that of compound **A1a**, with **1a** and 4-aminobutanol being substituted by **1a** and **C2a**, respectively. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.37 (d, J = 5.1 Hz, 1H), 4.86 (t, J = 6.2 Hz, 1H), 4.62 – 4.59 (m, 1H), 3.61 (t, J = 5.2 Hz, 4H), 2.61 – 2.53 (m, 12H), 2.49 (t, J = 6.6 Hz, 2H), 2.32 – 2.25 (m, 4H), 2.02 – 1.96 (m, 2H), 1.86 – 1.84 (m, 4H), 1.63 – 0.85 (m, 80H), 0.68 (s, 3H). ^{13}C NMR ($CDCl_3$) δ 173.58, 173.20, 139.69, 122.63, 74.26, 73.84, 59.97, 56.69, 56.13, 55.91, 53.39, 53.35, 51.99, 51.11, 50.02, 46.12, 42.31, 39.73, 39.52, 38.16, 36.99, 36.60, 36.19, 35.80, 34.62, 34.13, 31.91, 31.87, 29.55, 29.51, 29.26, 28.23, 28.02, 27.82, 27.22, 27.12, 25.43, 25.33, 25.02, 24.93, 24.29, 24.17, 23.83, 22.82, 22.67, 22.57, 21.03, 19.33, 18.72, 14.12, 11.86. HR-MS (ESI, positive mode, m/z) $[M + H]^+$ $[C_{63}H_{117}N_2O_6]^+$ calcd. 997.8912, found 997.8927.





Scheme S6. Synthetic route of compound **D13** and **E13.**

Synthesis of compound 3b: A mixture of Boc-6-aminocaproic acid (4.32 mmol, 1.00 g) and compound 3 (7-dehydrocholesterol, 1.66 g, 4.32 mmol) was dissolved in anhydrous dichloromethane (12 mL) under nitrogen atmosphere. To this stirred solution were successively added 4-dimethylaminopyridine (DMAP, 0.11 g, 0.87 mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI, 1.08 g, 5.62 mmol) and N,N-diisopropylethylamine (DIEA, 3.01 mL, 17.30 mmol) under nitrogen protection and stirring condition. Afterwards, the reaction system was maintained at room temperature and stirred for over 16 hours. The reaction mixture was then transferred to a separatory funnel. Dichloromethane (100 mL) and water (100 mL) were used to extract the mixture three times. The organic phases were dried over anhydrous sodium sulfate and concentrated under reduced pressure to afford a white solid product.

The crude product was dissolved in pre-cooled anhydrous dichloromethane (21.5 mL, 0 °C), and the resulting solution was transferred to a two-neck flask. The system was kept under a nitrogen atmosphere with continuous stirring. Trifluoroacetic acid (TFA, 3.21 mL, 43.23 mmol) was then added dropwise over 20 minutes while maintaining the reaction temperature at 0 °C. After that, the ice bath was removed, and the reaction mixture was allowed to warm naturally to room temperature and stirred for additional 4 hours. The mixture was concentrated under reduced pressure to remove dichloromethane, yielding an oily residue. The residual TFA in the mixture was neutralized by adding an excess of saturated sodium bicarbonate solution. The resulting orange solution was transferred to a separatory funnel and extracted with

ethyl acetate (100 mL) and water (100 mL) three times. The organic phases were dried over anhydrous sodium sulfate and concentrated under reduced pressure to give an orange foamy crude product. The crude product was then purified via silica gel column chromatography (eluent: CH_2Cl_2/MeOH = 20:1) to afford the target compound 3b as an orange viscous liquid (1.37 g, 2.75 mmol, 63.66% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.58 – 5.54 (m, 1H), 5.42 – 5.37 (m, 1H), 4.77 – 4.69 (m, 1H), 2.72 – 2.66 (m, 2H), 2.31 – 2.27 (m, 4H), 1.91 – 1.88 (m, 5H), 1.70 – 1.12 (m, 26H), 0.95 – 0.93 (m, 6H), 0.87 (d, J = 6.6 Hz, 6H), 0.62 (s, 3H).

Synthesis of compound 13a: **13a** (yellow liquid) was synthesized using a procedure analogous to that of compound **A1a**, with **1a** and 4-aminobutanol being substituted by **1a** and **3b**, respectively. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.13 (dd, *J* = 9.8, 3.1 Hz, 2H), 5.23 (dd, *J* = 9.8, 2.1 Hz, 2H), 2.30 – 2.27 (m, 14H), 1.51 – 1.49 (m, 14H), 1.38 – 1.36 (m, 13H), 1.26 – 1.25 (m, 24H), 0.95 – 0.93 (m, 8H), 0.88 – 0.87 (m, 13H), 0.86 (d, *J* = 1.3 Hz, 9H).

Synthesis of compound D13: Compound 13a (0.234 mmol, 0.200 g) and triethylamine (0.098 mL, 0.711 mmol) were dissolved in pre-cooled anhydrous dichloromethane (6 mL) at 0 °C under a nitrogen atmosphere with continuous stirring. A separate solution was prepared by dissolving triphosgene (0.028 g, 0.094 mmol) in anhydrous dichloromethane (6 mL). This triphosgene solution was added slowly to the first mixture via a syringe while maintaining the temperature at 0 °C. The addition rate was controlled to prevent an excessive temperature rise. Upon completion of the addition, the ice bath was removed, and the reaction mixture was allowed to gradually warm to room temperature. Stirring was continued for 1 hour. The reaction mixture was then concentrated under reduced pressure to remove the solvent, yielding the product as a yellow liquid.

Subsequently, under a nitrogen atmosphere with continuous stirring, sodium hydride (0.031 g, 1.287 mmol) and compound D (3-dimethylamino-1-propanol, 0.133 g, 1.287 mmol) were dissolved in anhydrous tetrahydrofuran (THF, 10 mL) that had been pre-

cooled to 0 °C. The resulting mixture was then transferred to a dropping funnel and added dropwise to the reaction system containing the yellow liquid product obtained in the previous step. Throughout the addition, the temperature was maintained at 0 °C. After complete addition, the ice bath was removed and the reaction system was allowed to naturally warm gradually to room temperature with continued stirring for >1 hour, yielding an orange-yellow solution. The reaction was quenched by addition of excess saturated ammonium chloride solution (20 mL), and the resulting yellowish mixture was transferred to a separatory funnel. The mixture was extracted with ethyl acetate (100 mL) and water (100 mL), and the extraction process was repeated three times. The combined organic phases were dried over anhydrous sodium sulfate and concentrated under reduced pressure to afford an orange crude product. The crude product was purified using silica gel column chromatography (eluent: dichloromethane: methanol = 40:1), and the target product **D13** (0.141 g, 0.144 mmol, 61.28% yield) was obtained as an orange viscous liquid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.30 (s, 2H), 4.86 (t, J = 6.2 Hz, 4H), 2.39 – 2.25 (m, 16H), 1.86 (t, J = 11.0 Hz, 6H), 1.68 – 1.08 (m, 61H), 0.96 – 0.83 (m, 21H). ^{13}C NMR ($CDCl_3$) δ 173.09, 141.63, 138.57, 120.20, 116.28, 74.39, 74.24, 62.58, 55.88, 54.48, 53.44, 46.05, 42.92, 39.51, 39.15, 37.94, 37.09, 36.70, 36.17, 36.12, 34.60, 34.15, 31.88, 29.55, 29.53, 29.27, 28.16, 28.11, 28.03, 27.16, 27.08, 25.51, 25.34, 24.98, 24.87, 23.90, 23.03, 22.84, 22.68, 22.57, 21.04, 18.86, 16.21, 14.13. HR-MS (ESI, positive mode, m/z) $[M + H]^+$ $[C_{62}H_{111}N_2O_6]^+$ calcd. 979.8442, found 979.8427.

Synthesis of compound E13: Under a nitrogen atmosphere with continuous stirring, the following reagents were sequentially added to the reaction vessel: compound **13a** (0.353 mmol, 0.300 g), compound E (4-(dimethylamino)butanoic acid, 0.042 g, 0.353 mmol), N,N-diisopropylethylamine (DIEA, 0.135 mL, 0.776 mmol), and 2-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU, 0.161 g, 0.423 mmol). The mixture was dissolved in anhydrous dichloromethane (1.77 mL) and stirred until completely dissolved. After complete addition, the reaction mixture was stirred at room temperature for 1 hours. The reaction mixture was

transferred to a separatory funnel. The mixture was the extracted with ethyl acetate (100 mL) and water (100 mL) three times. The organic phase was dried over anhydrous sodium sulfate and concentrated under reduced pressure to remove the ethyl acetate solvent, yielding the crude product. The crude product was purified by silica gel column chromatography (eluent: dichloromethane: methanol = 25:1), and the target product **E13** (0.288 g, 0.299 mmol, 84.70% yield) was obtained as an orange-yellow viscous liquid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.30 (s, 2H), 4.86 (t, *J* = 6.2 Hz, 2H), 2.71 – 2.63 (m, 12H), 2.31 – 2.27 (m, 8H), 1.90 – 1.82 (m, 4H), 1.66 – 1.08 (m, 61H), 0.96 – 0.83 (m, 21H). ^{13}C NMR ($CDCl_3$) δ 173.50, 173.11, 139.70, 122.62, 74.21, 73.79, 62.67, 56.70, 56.13, 53.54, 50.03, 42.32, 39.74, 39.52, 38.16, 37.00, 36.60, 36.19, 35.80, 34.63, 34.60, 34.15, 32.71, 31.91, 31.88, 29.55, 29.53, 29.26, 28.24, 28.03, 27.82, 27.21, 27.13, 26.34, 25.69, 25.34, 25.02, 24.93, 24.29, 23.83, 22.83, 22.68, 22.57, 21.04, 19.33, 18.72, 14.13. HR-MS (ESI, positive mode, m/z) $[M + H]^+$ $[C_{62}H_{111}N_2O_5]^+$ calcd. 963.8493, found 963.8474.

Supplementary Figures:

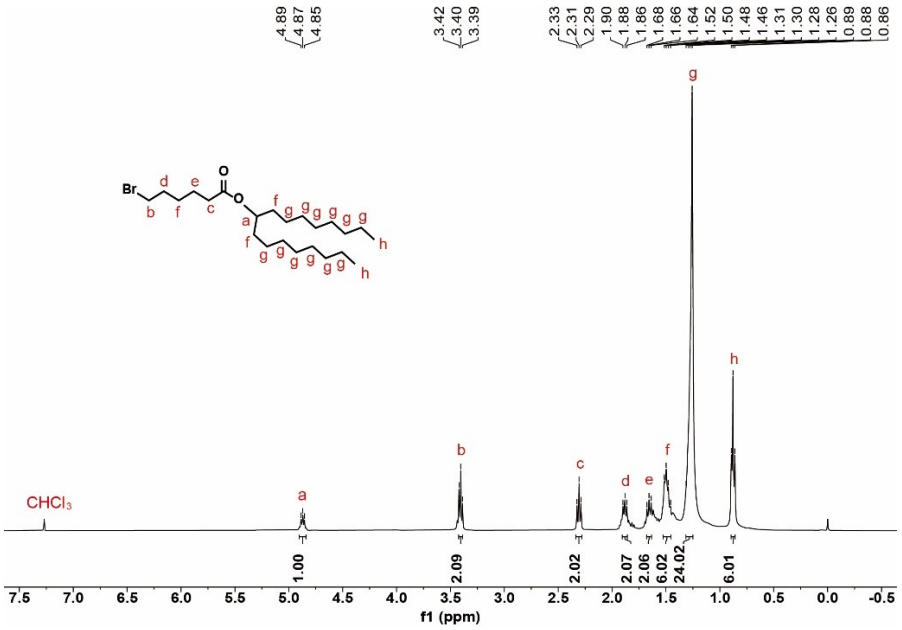


Fig. S1. ^{1}H NMR spectrum ($CDCl_3$) of compound **1a**.

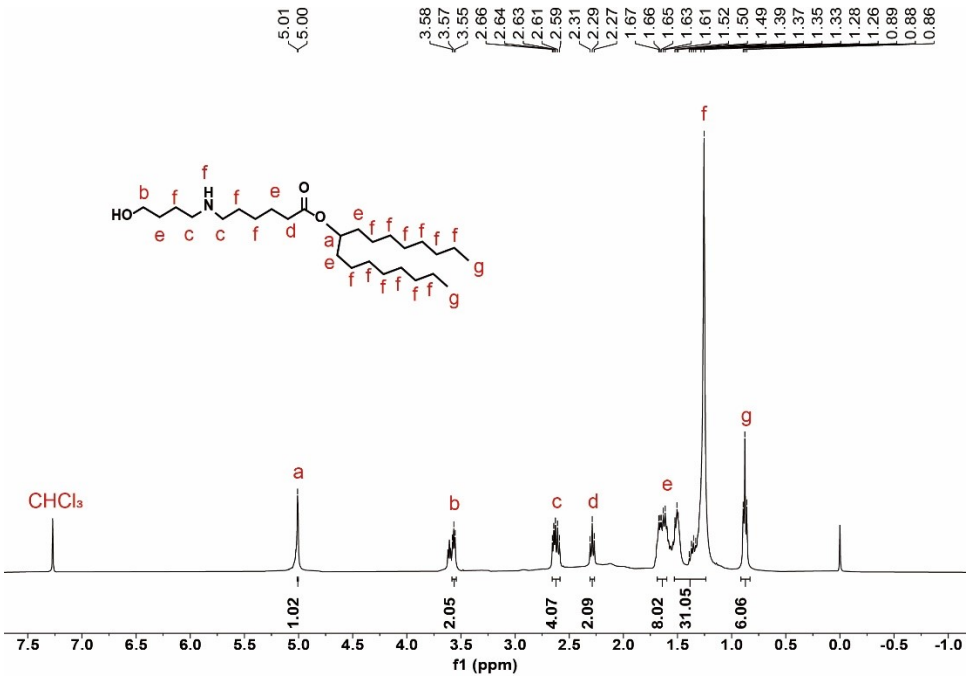


Fig. S2. ^{1}H NMR spectrum ($CDCl_3$) of compound **A1a**.

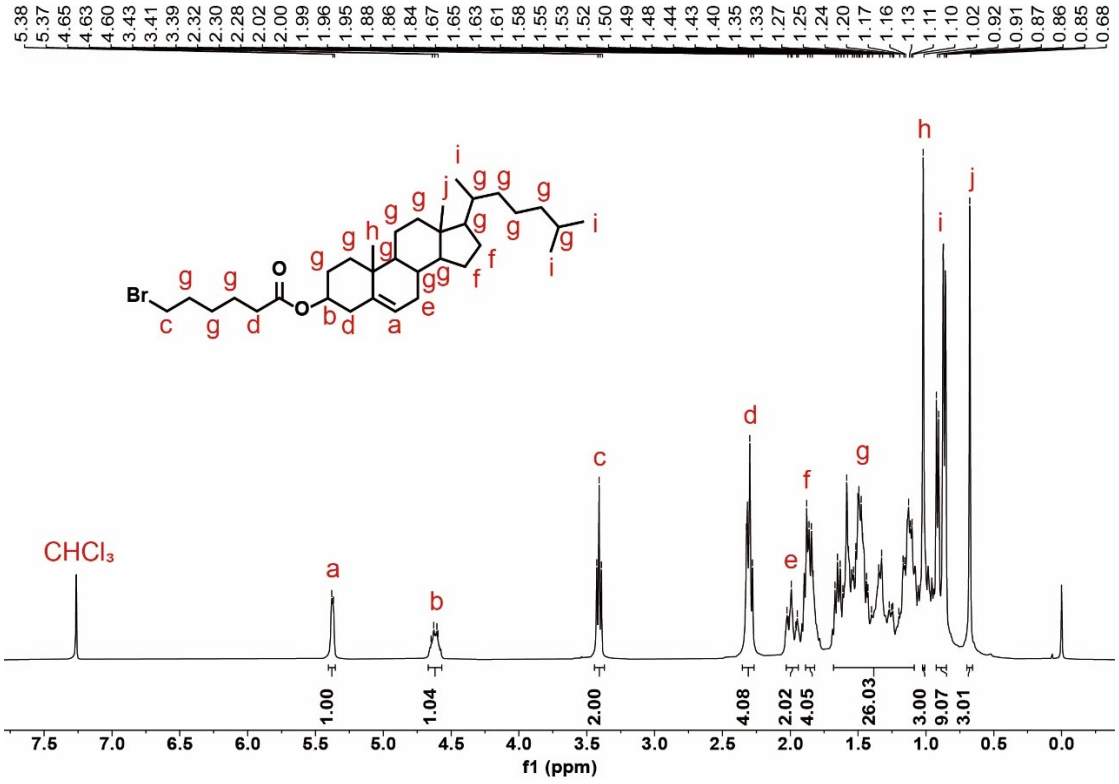


Fig. S3. ^{1}H NMR spectrum ($CDCl_3$) of compound **2a**.

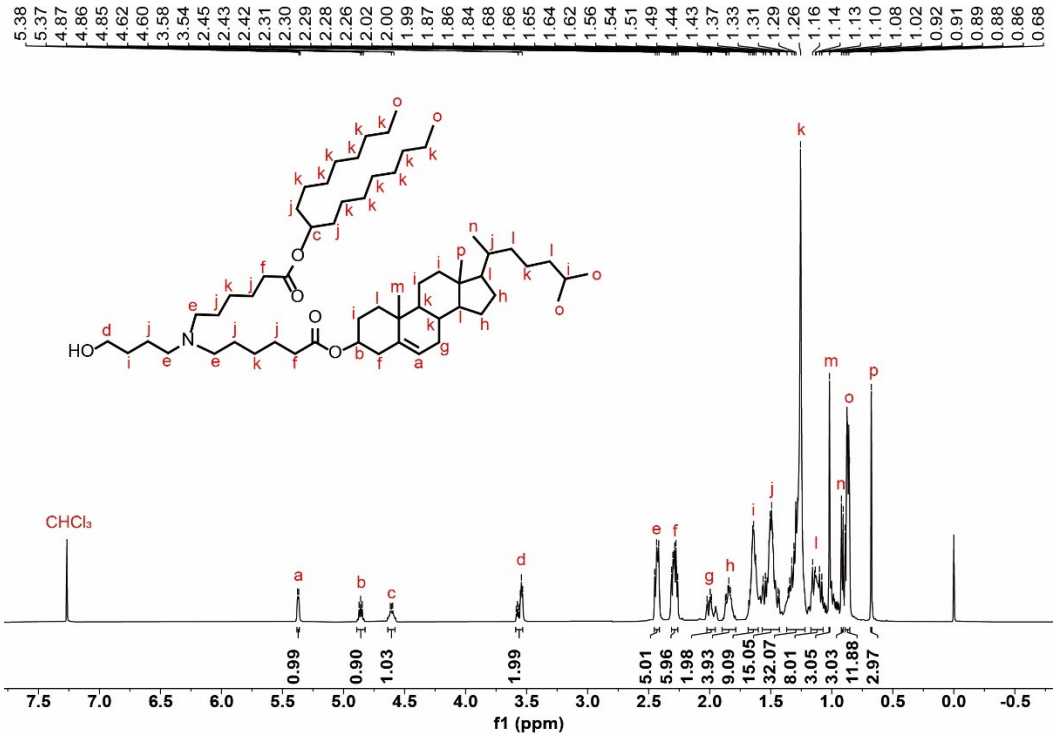


Fig. S4. ^{1}H NMR spectrum of compound **A12.**

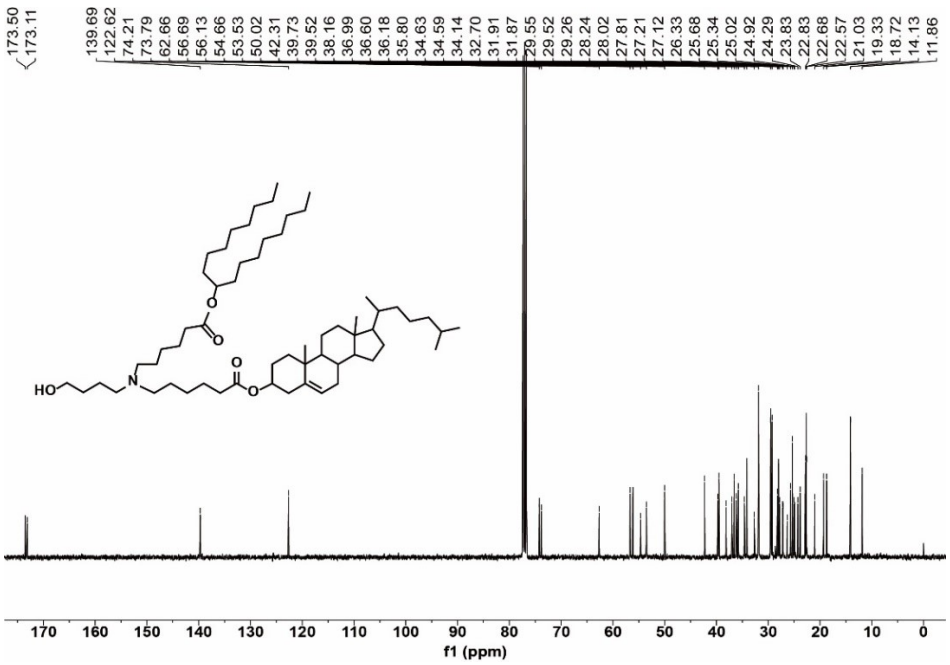


Fig. S5. ^{13}C NMR spectrum of compound **A12.**

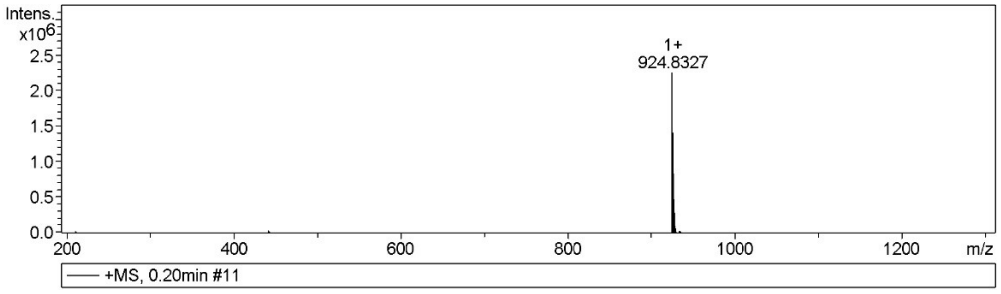


Fig. S6. HR-MS spectrum of compound **A12.**

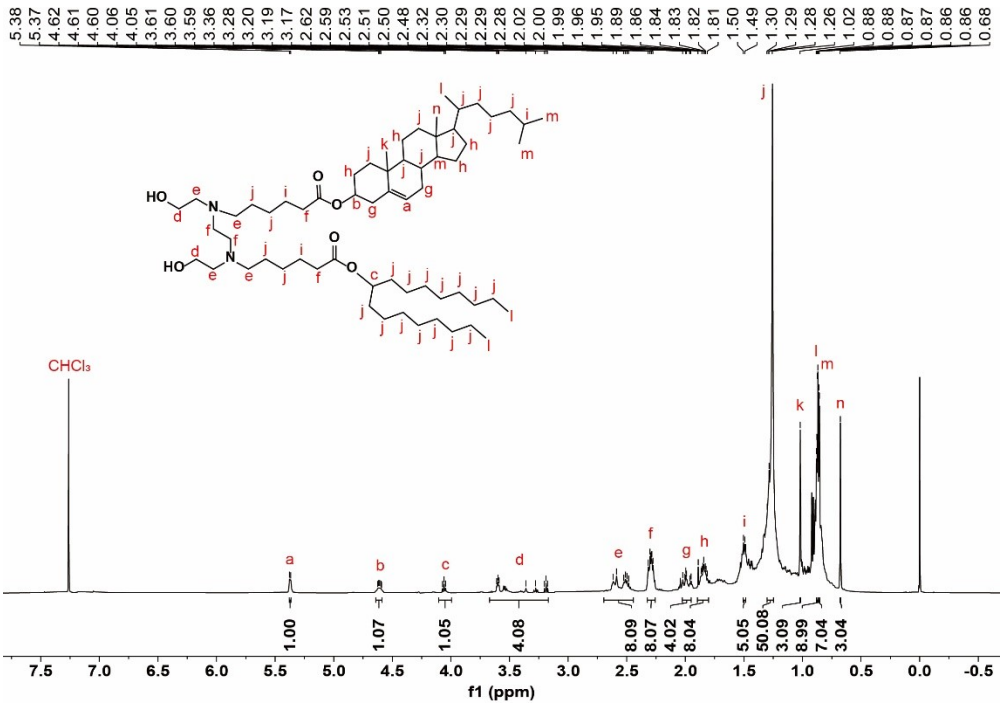


Fig. S7. ^{1}H NMR spectrum of compound **B12.**

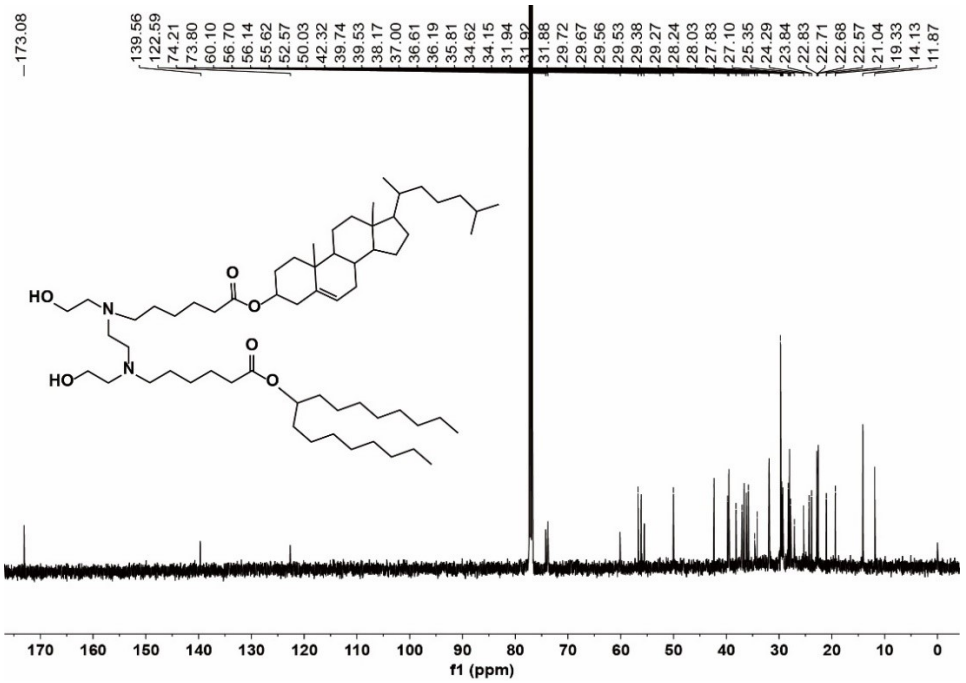


Fig. S8. ^{13}C NMR spectrum of compound **B12.**

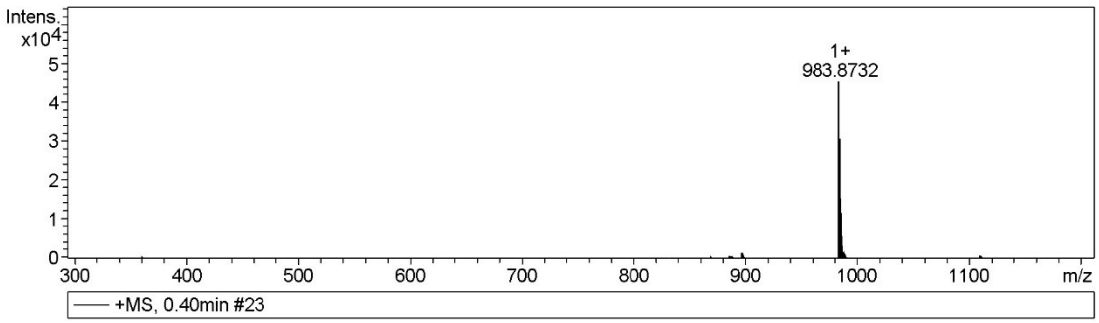


Fig. S9. HR-MS spectrum of compound **B12.**

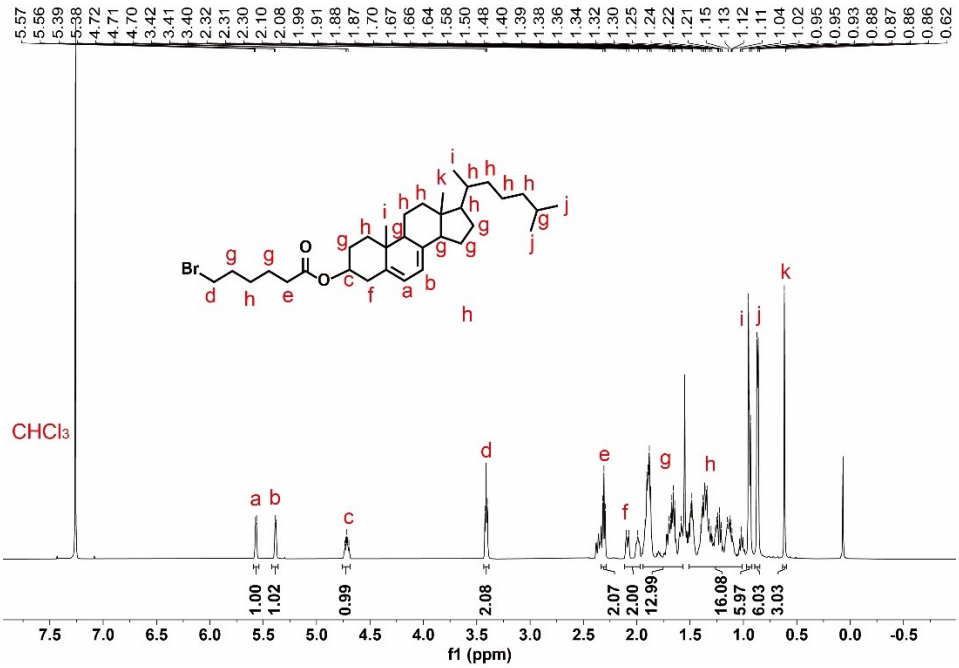


Fig. S10. ^{1}H NMR spectrum ($CDCl_3$) of compound **3a**.

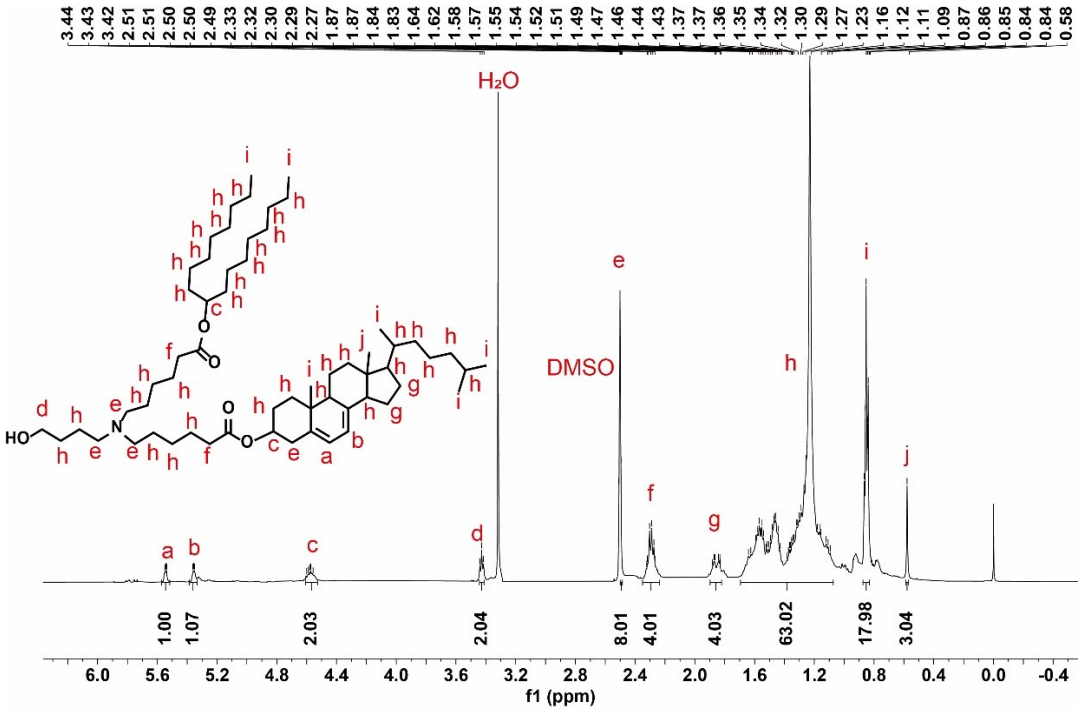


Fig. S11. [1]H NMR spectrum of compound **A13.**

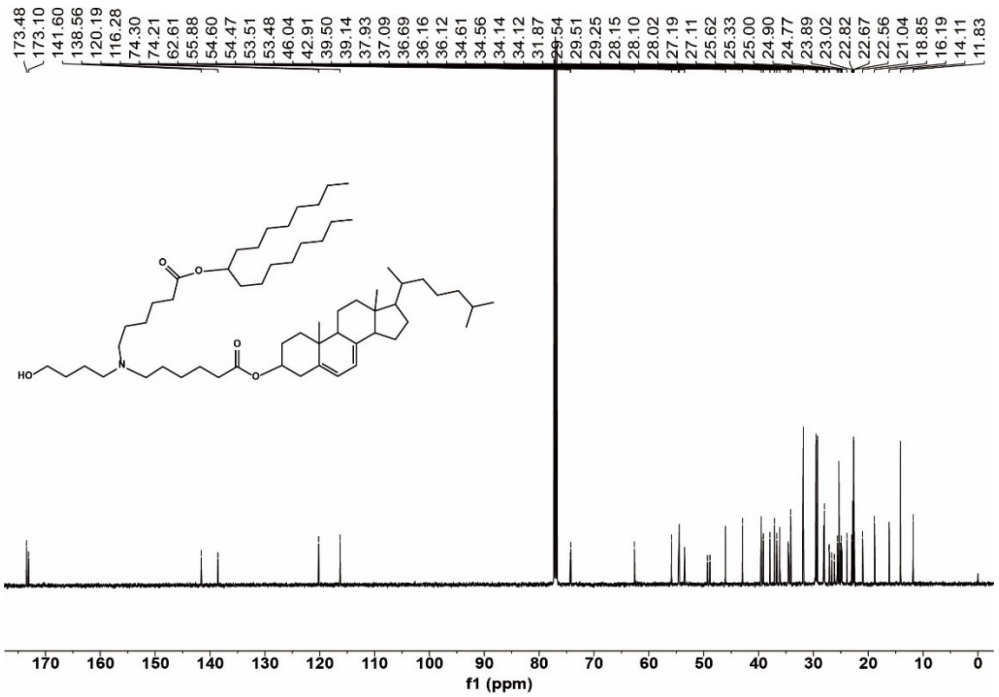


Fig. S12. [13]C NMR spectrum of compound **A13.**

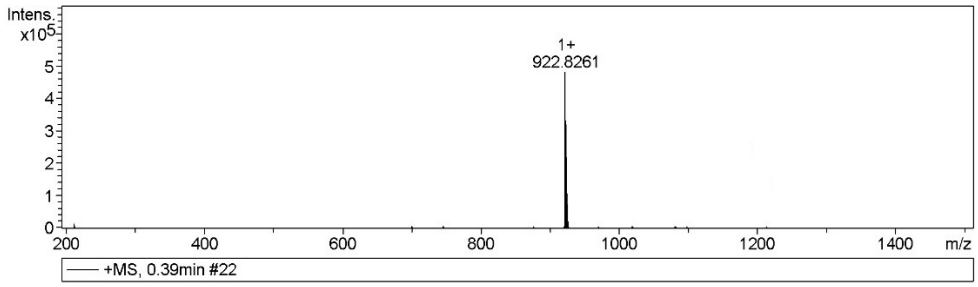


Fig. S13. HR-MS spectrum of compound **A13.**

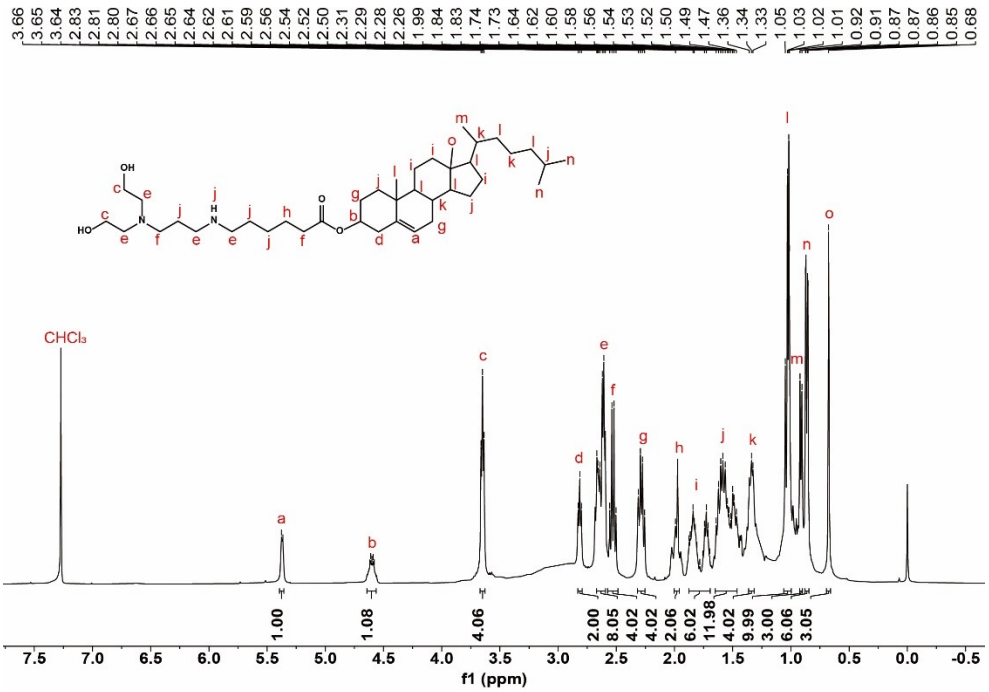


Fig. S14. ^{1}H NMR spectrum ($CDCl_3$) of compound **C2a**.

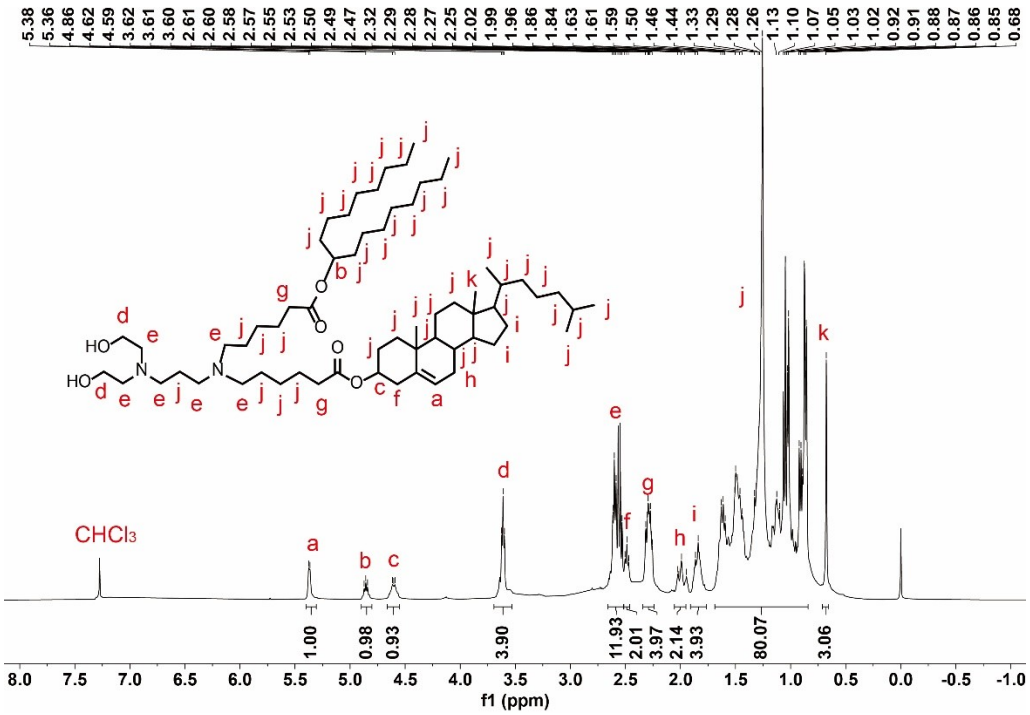


Fig. S15. ^{1}H NMR spectrum of compound **C12.**

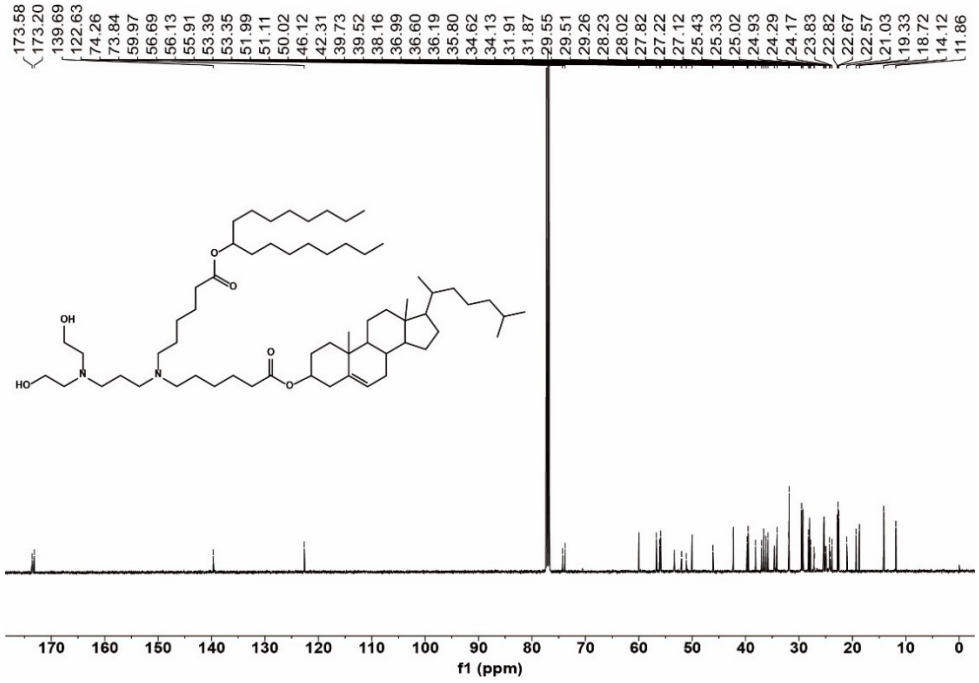


Fig. S16. ^{13}C NMR spectrum of compound **C12.**

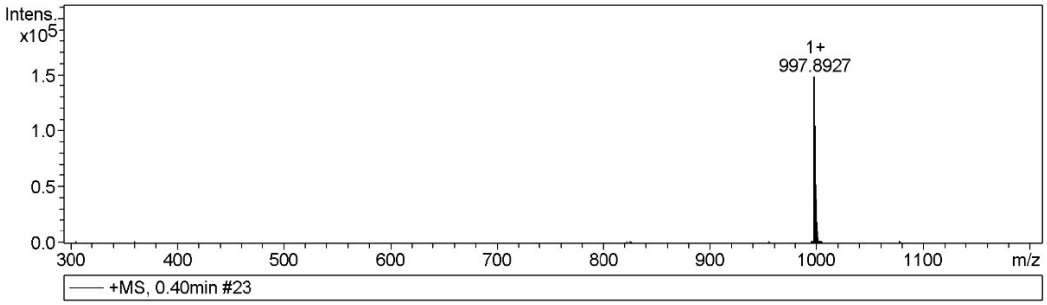


Fig. S17. HR-MS spectrum of compound **C12.**

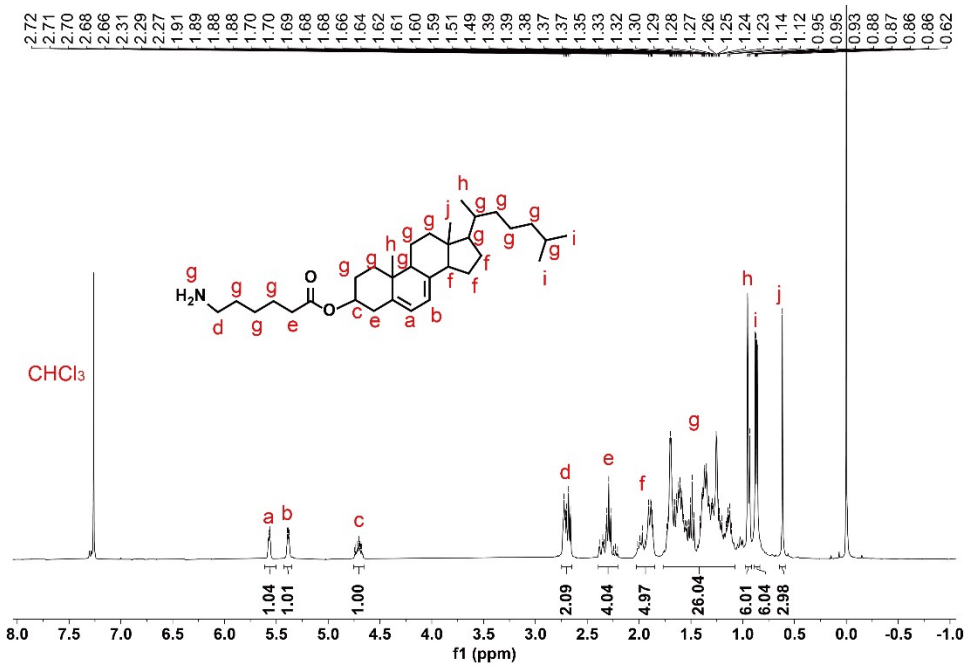


Fig. S18. ^{1}H NMR spectrum ($CDCl_3$) of compound **3b**.

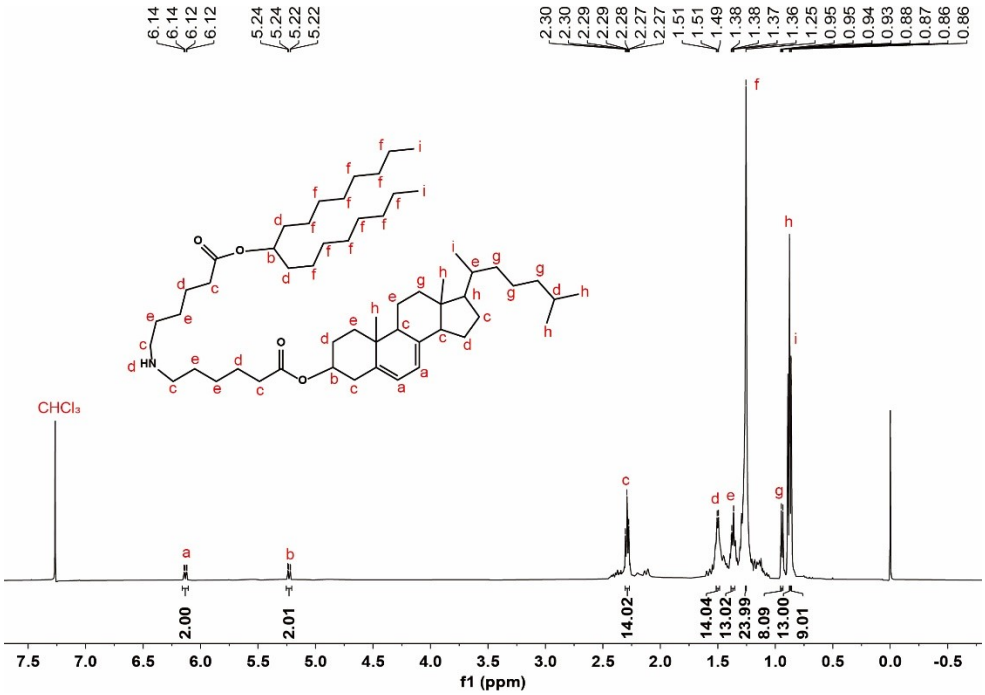


Fig. S19. ^{1}H NMR spectrum ($CDCl_3$) of compound **13a**.

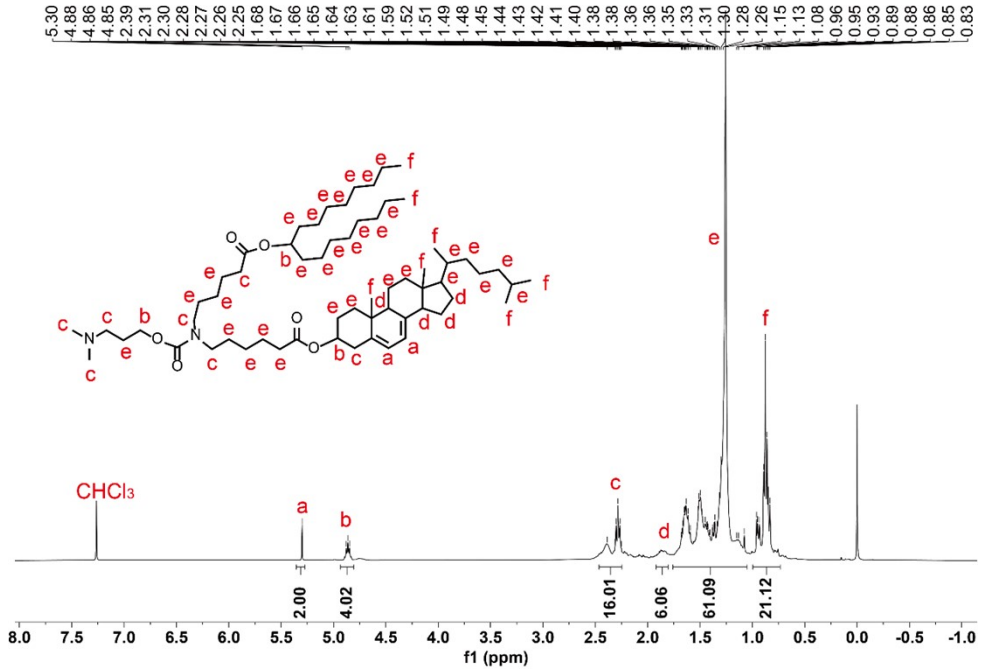


Fig. S20. ^{1}H NMR spectrum of compound **D13.**

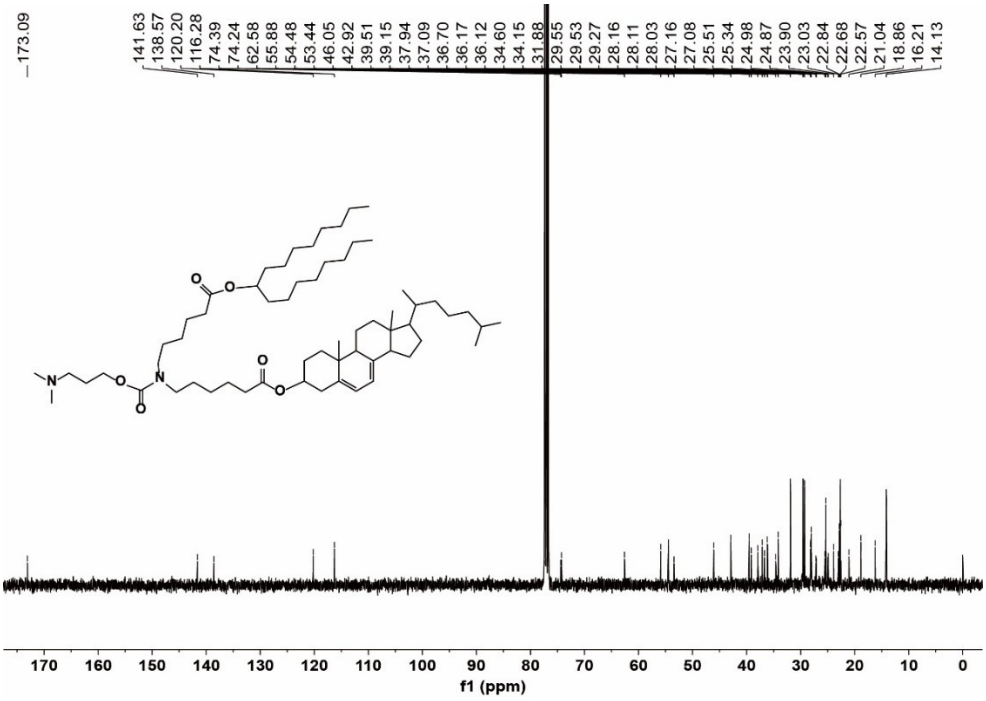


Fig. S21. ^{13}C NMR spectrum of compound **D13.**

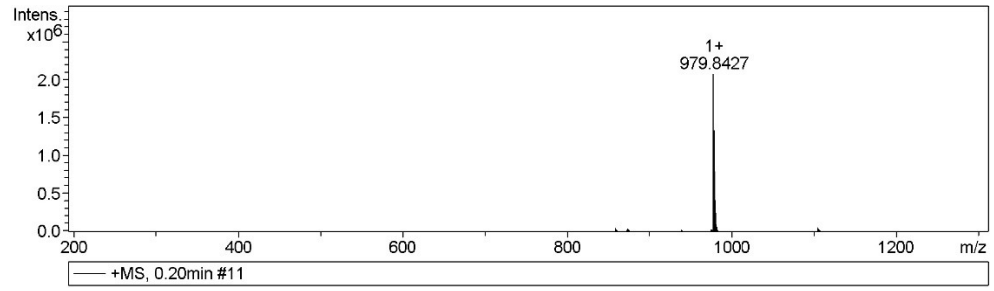


Fig. S22. HR-MS spectrum of compound **D13.**

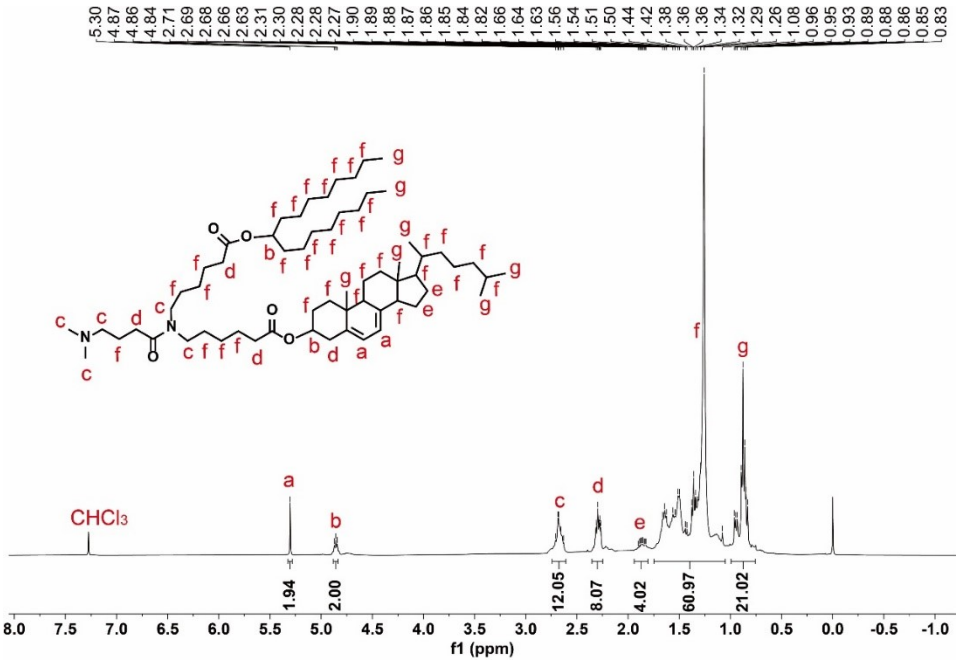


Fig. S23. ^{1}H NMR spectrum of compound **E13.**

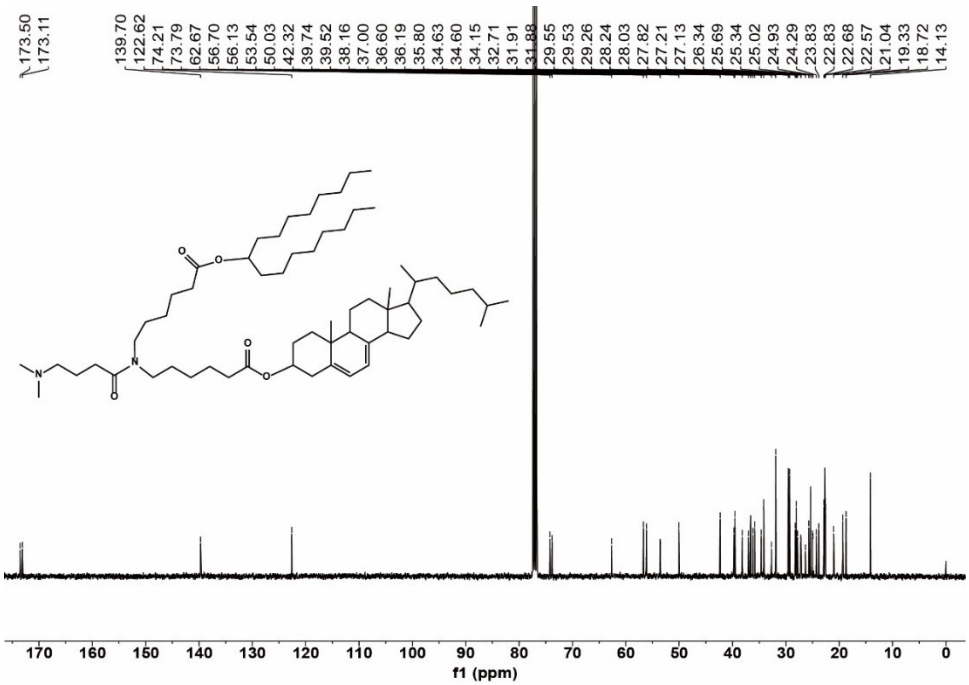


Fig. S24. ^{13}C NMR spectrum of compound **E13.**

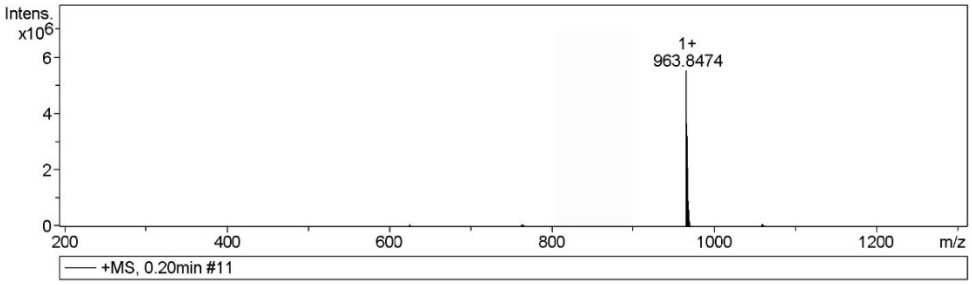


Fig. S25. HR-MS spectrum of compound **E13.**

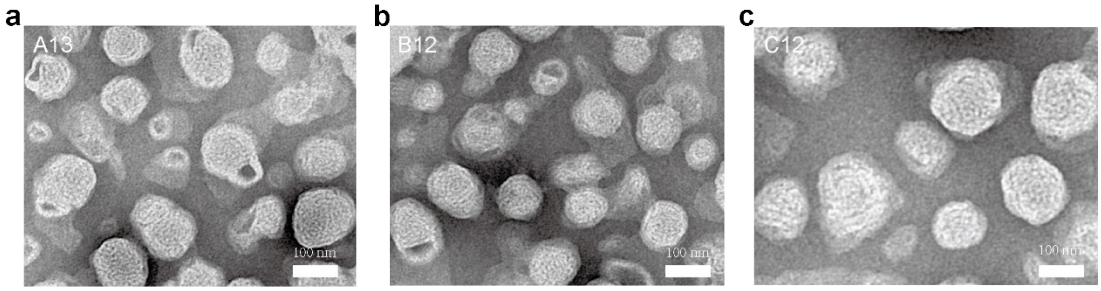


Fig. S26. TEM images of A13- (a), B12- (b) and C12-bearing LNPs. Scale bar: 100 nm.

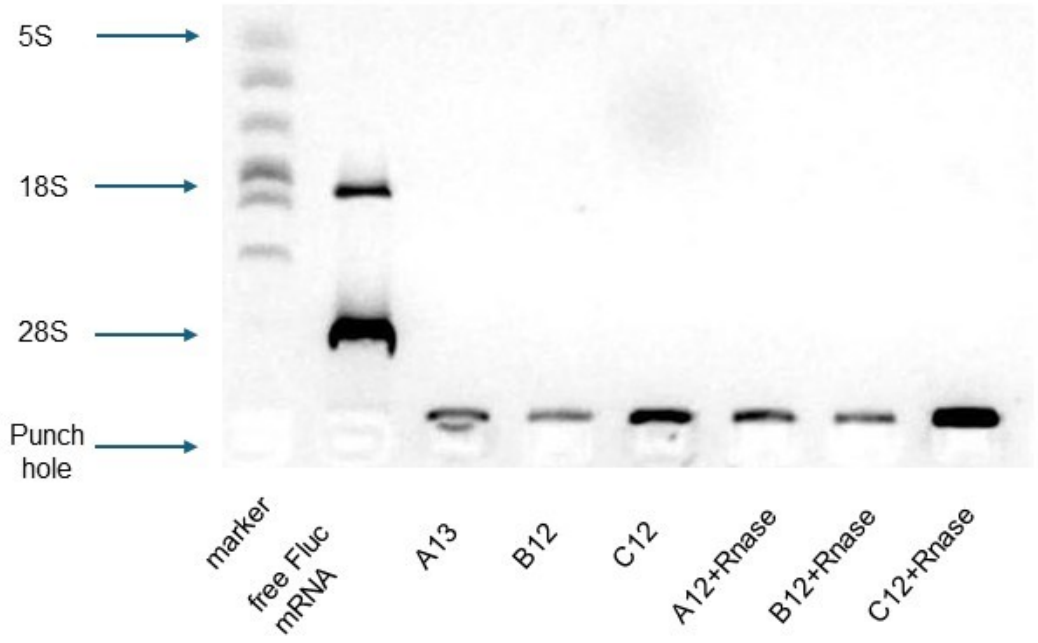


Fig. S27. Integrity analysis via gel electrophoresis for Fluc mRNA before and after LNP encapsulation. Lanes M: RNA ladder; Lane 1: free Fluc mRNA (control); Lane 2-4: Fluc mRNA@LNPs; Lane 4-6: Fluc mRNA@LNPs treated with RNase.

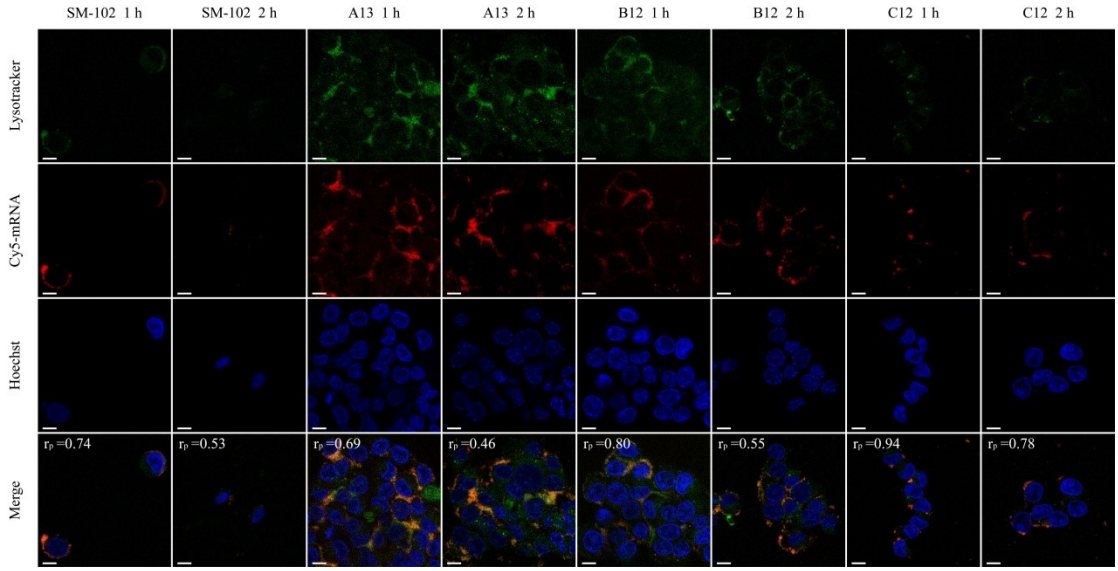
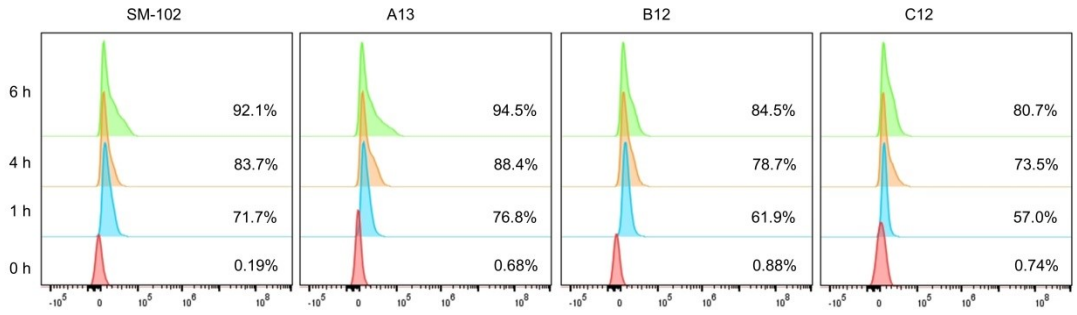
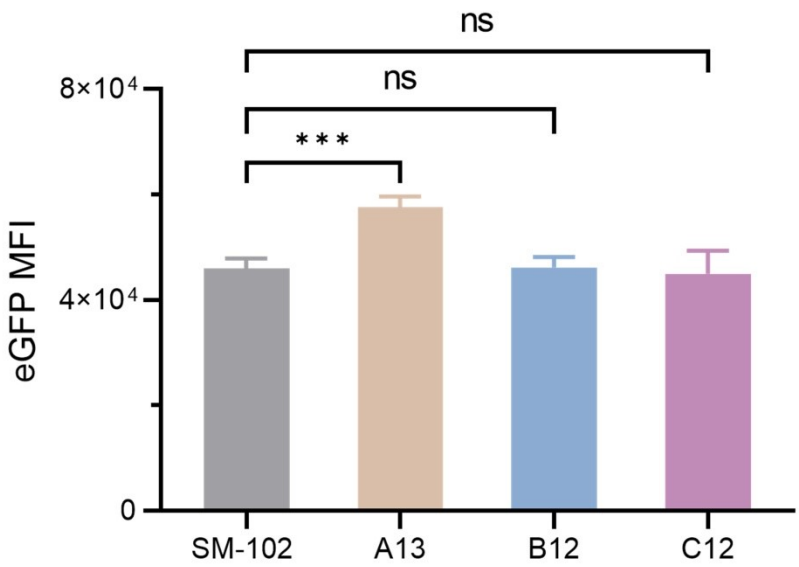


Fig. S28. Confocal images of endosomal escape. HePG2 cells were treated with Cy5-labelled Ova mRNA@A13 LNPs, Cy5-labelled Ova mRNA@B12 LNPs, Cy5-labelled Ova mRNA@C12 LNPs or Cy5-labelled Ova mRNA@SM-102 LNPs respectively for 1 or 2 h before staining with LysoTracker Green and Hoechst 33342. The Pearson's correlation coefficients (r_P) were determined using ImageJ software.



(A)



(B)

Fig. S29. (A) Histograms and percentages of eGFP-positive HepG2 cells at 0, 1, 4 and 6 h of incubation for our LNPs and the benchmark SM-102 LNP as determined by flow cytometry analysis. (B) Histograms of mean fluorescence intensity (MFI) (a.u.) reflecting eGFP expression for four LNP formulations at 6 h post-incubation with HepG2 cells.