Supporting information for

Injection Site-Retained Lipid Nanoparticles for Targeted Intramuscular Delivery of mRNA RSV Prefusion-F Vaccine†

Xichao Chen[a,b,‡], Honglei Zhang[b,‡], Dongyang Liu[b,‡], Jingxuan Ma[b], Lijie Jin[b], Yuqing Ma[b], Jing Li[b], Gengshen Song[b,*], Juxian Wang[a,*]

[a] Institute of Medical Biotechnology, Chinese Academy of Medical Science and Peking Union Medical College, Beijing 100021, China

[b] Beijing Youcare Kechuang Pharmaceutical Technology Co., Ltd., Beijing 100176, China

‡ These authors contributed equally to this work

* Correspondence to: J.W. wangjuxian@imb.pumc.edu.cn; G.S. songgengshen@youcareyk.com

1. Supporting information figures

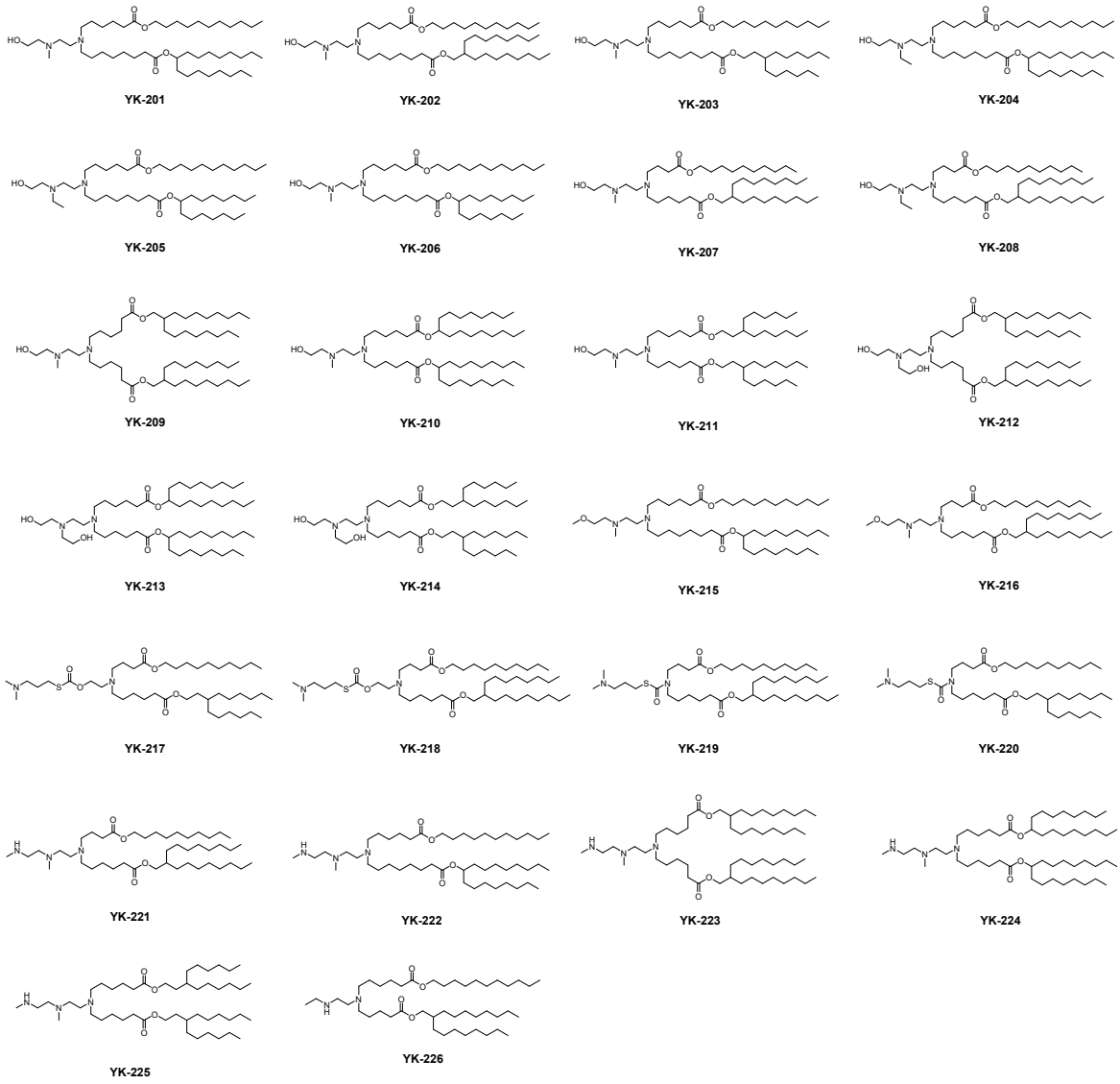


Figure S1. **Chemical structure of ionizable lipids synthesized and used in this study.**

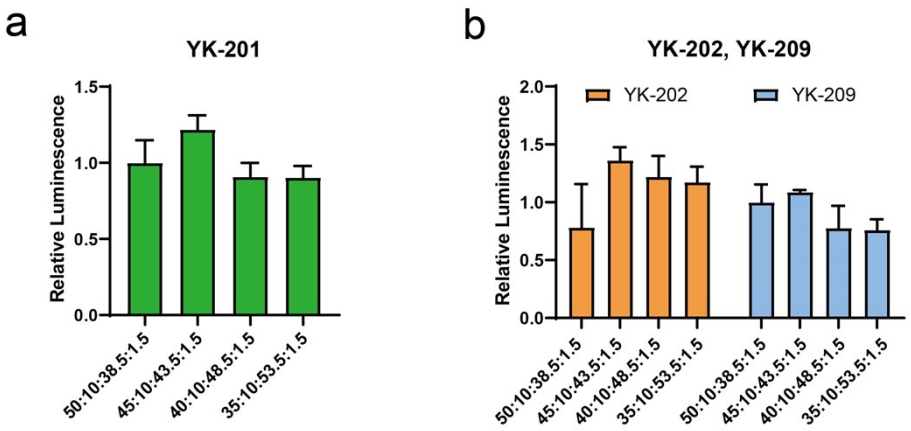


Figure S2. Optimization of the lipid ratios. Ratios are shown as ionizable lipid: DSPC: Cholesterol: DMG-PEG2000.

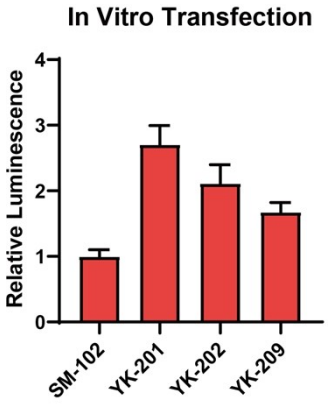


Figure S3. Comparison of the in vitro transfection efficiency between SM-102, YK-201, YK-202, and YK-209. HEK293T cells were incubated with different LNPs encapsulating Fluc mRNA for 24 h (n = 3).

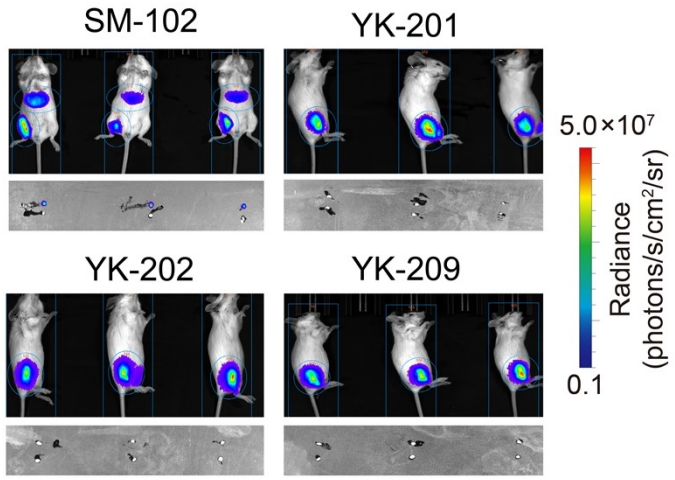


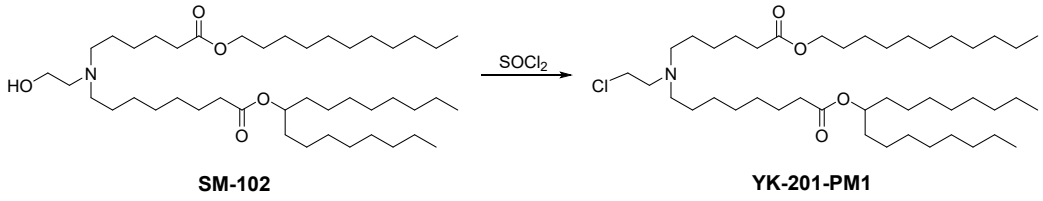
Figure S4. In vivo protein expression in inguinal lymph nodes. BALB/c mice were injected intramuscularly (i.m.) with Fluc-mRNA-LNPs (0.25 mg/kg) for 6 h, and luminescence of the whole body and inguinal lymph nodes was measured.

2. Reagents

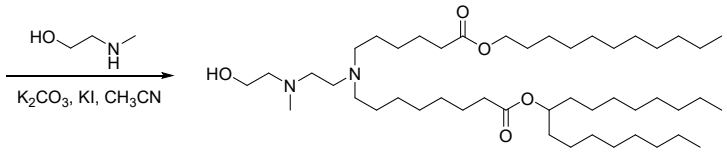
All chemicals for lipid synthesis were purchased from Sigma-Aldrich, Aladdin and Innochem. BUCHI Pure Chromatography Purification System was used to purify the crude products. The ^{1}H-NMR and LCMS were measured on a Quantum-Iplus AS400/ZA0028 NMR spectrometer and Vanquish-ISQ EM LC/MS System, respectively. CleanCap® Reagent AG (N-7113, m7GpppAmpG) was purchased from Yeasen. Fluc mRNA, eGFP mRNA and gE mRNA capped with CleanCap® Reagent AG were synthesized through an in vitro transcription process. DNA templates for encoding the Fluc and preF antigen were purchased from GenScript Biotech. The mice were purchased from Beijing Huafukang Biotech Co., Ltd. (Beijing, China). All the animal experiments were kept to the National Regulation of China for Care and Use of Laboratory Animals.

3. Chemical Synthesis

heptadecan-9-yl 8-((2-((2-hydroxyethyl)(methyl)amino)ethyl)(6-oxo-6-(undecyloxy)hexyl)amino)octanoate (YK-201)



SM-102 YK-201-PM1



YK-201

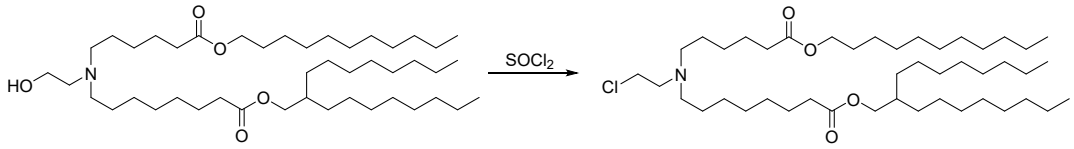
Step 1: SM-102 (10.00 g, 0.014 mol) was added to a single-neck flask, followed by the addition of 100 mL of $SOCl_2$. The reaction mixture was stirred at room temperature for 6 hours. Upon completion of the reaction, the mixture was poured into ice water to quench the reaction. The organic phase was then extracted with ethyl acetate. The organic extract was concentrated under reduced pressure to yield YK-201-PM1 as a colorless oil (9.51 g, 0.013 mol, 93.1%). $C_{44}H_{86}ClNO_4$, MS (ES): m/z ($M+H^+$) 728.5.

Step 2: To a solution of YK-201-PM1 (1.00 g, 1.37 mmol) and 2-(methylamino)ethan-1-ol (0.31 g, 4.11 mmol) in 20 mL acetonitrile were added potassium carbonate (0.61 g, 4.41 mmol) and potassium iodide (38 mg, 0.23 mmol). The mixture was heated to 70°C and stirred for 7 hours.

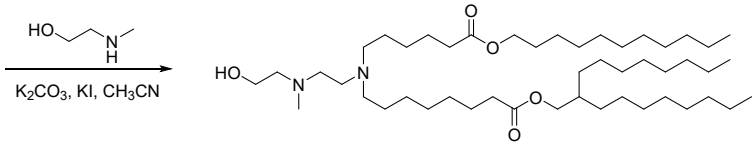
After completion, the reaction was cooled to room temperature, and the solid was removed by filtration. The filtrate was concentrated under reduced pressure, and the residue was purified by silica gel chromatography (ethyl acetate/n-hexane) to yield YK-201 (559 mg, 0.73 mmol, 53.3%). $C_{47}H_{94}N_2O_5$, MS (ES): m/z ($M+H^+$) 767.6.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 4.05 (t, *J* = 6.8 Hz, 2H), 3.57 (t, *J* = 5.1 Hz, 2H), 2.57 – 2.52 (m, 5H), 2.46 (dt, *J* = 9.8, 5.1 Hz, 4H), 2.33 – 2.25 (m, 7H), 1.68 – 1.56 (m, 6H), 1.47 (dd, *J* = 13.4, 6.0 Hz, 8H), 1.28 (d, *J* = 17.7 Hz, 50H), 0.88 (t, *J* = 6.8 Hz, 9H).

2-octyldecyl 8-((2-((2-hydroxyethyl)(methyl)amino)ethyl)(6-oxo-6-(undecyloxy)hexyl)amino)octanoate (YK-202)



YK-202-SM1 **YK-202-PM1**



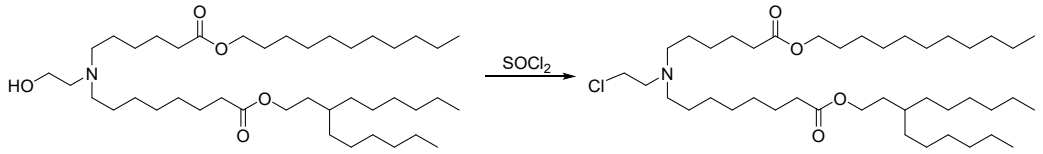
YK-202

Step 1: According to the method for preparing YK-201-PM1, YK-202-SM1 (1.50 g, 2.07 mmol) and $SOCl_2$ (10 mL) were used as starting materials to obtain YK-202-PM1 (1.63 g, 2.07 mmol, 100.0%). $C_{45}H_{88}ClNO_4$, MS (ES): m/z ($M+H^+$) 742.6.

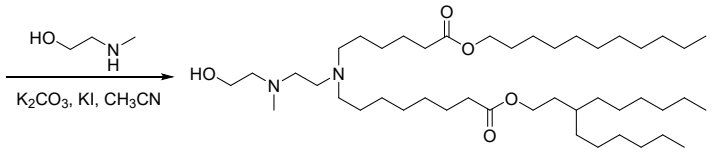
Step 2: According to the method for preparing YK-201, YK-202-PM1 (1.60 g, 2.07 mmol) and 2-(methylamino)ethan-1-ol (0.47 g, 6.21 mmol) were used as starting materials to obtain YK-202 (1.01 g, 1.29 mmol, 62.3%). $C_{48}H_{96}N_2O_5$, MS (ES): m/z ($M+H^+$) 781.3.

^{1}H NMR (400 MHz, Chloroform-*d*): δ 4.05 (t, *J* = 6.7 Hz, 2H), 3.96 (d, *J* = 5.7 Hz, 2H), 3.62 (t, *J* = 4.8 Hz, 2H), 2.72 (d, *J* = 5.4 Hz, 2H), 2.64 (dd, *J* = 14.4, 5.3 Hz, 8H), 2.36 (s, 3H), 2.30 (td, *J* = 7.4, 4.0 Hz, 4H), 1.60 (qd, *J* = 15.3, 7.7 Hz, 11H), 1.28 (d, *J* = 14.9 Hz, 52H), 0.88 (t, *J* = 6.6 Hz, 9H).

3-hexylnonyl 8-((2-((2-hydroxyethyl)(methyl)amino)ethyl)(6-oxo-6-(undecyloxy)hexyl)amino)octanoate (YK-203)



YK-203-SM1 YK-203-PM1



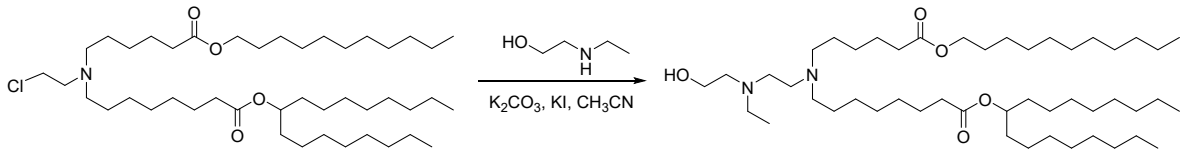
YK-203

Step 1: According to the method for preparing YK-201-PM1, YK-203-SM1 (280 mg, 0.41 mmol) and $SOCl_2$ (2 mL) were used as starting materials to obtain YK-203-PM1 (250 mg, 0.36 mmol, 87.0%). $C_{42}H_{82}ClNO_4$, MS (ES): m/z ($M+H^+$) 700.6.

Step 2: According to the method for preparing YK-201, YK-203-PM1 (150 mg, 0.22 mmol) and 2-(methylamino)ethan-1-ol (75 mg, 0.22 mmol) were used as starting materials to obtain YK-203 (120 mg, 0.16 mmol, 73.8%). $C_{45}H_{90}N_2O_5$, MS (ES): m/z ($M+H^+$) 739.7.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 4.10 (dt, *J* = 9.2, 7.0 Hz, 4H), 3.73 – 3.63 (m, 2H), 3.51 (s, 3H), 3.11 (s, 4H), 2.96 (t, *J* = 6.1 Hz, 2H), 2.88 (q, *J* = 7.8 Hz, 4H), 2.79 (t, *J* = 6.1 Hz, 2H), 2.70 (t, *J* = 4.9 Hz, 2H), 2.42 (s, 2H), 2.33 (dt, *J* = 13.2, 7.4 Hz, 4H), 1.70 – 1.59 (m, 10H), 1.37 – 1.27 (m, 41H), 0.94 – 0.88 (m, 9H).

heptadecan-9-yl 8-((2-(ethyl(2-hydroxyethyl)amino)ethyl)(6-oxo-6-(undecyloxy)hexyl)amino)octanoate (YK-204)

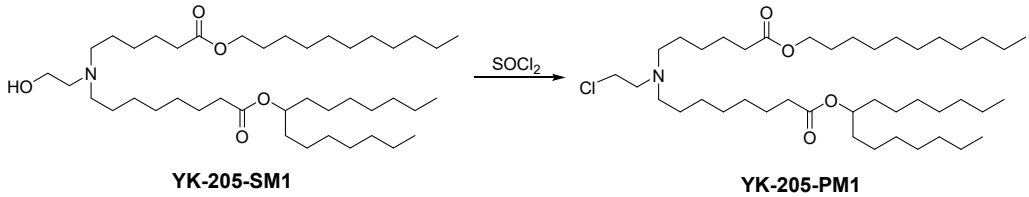


YK-201-PM1 YK-204

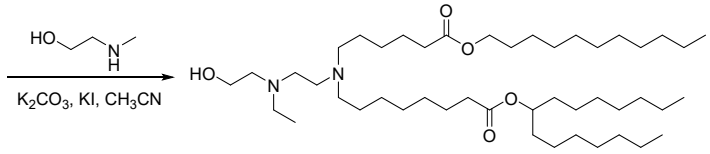
According to the method for preparing YK-201, YK-201-PM1 (88 mg, 0.12 mmol) and 2-(methylamino)ethan-1-ol (36 mg, 0.41 mmol) were used as starting materials to obtain YK-204 (52 mg, 0.07 mmol, 55.5%). $C_{48}H_{96}N_2O_5$, MS (ES): m/z ($M+H^+$) 781.7.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 5.41 – 5.24 (m, 1H), 4.85 (t, *J* = 6.2 Hz, 1H), 4.07 (q, *J* = 7.7, 6.7 Hz, 3H), 3.91 – 3.48 (m, 2H), 3.36 (d, *J* = 25.5 Hz, 2H), 3.08 (s, 2H), 2.38 – 2.15 (m, 3H), 2.11 – 1.96 (m, 1H), 1.85 (s, 3H), 1.76 – 1.46 (m, 21H), 1.26 (s, 48H), 0.94 – 0.83 (m, 9H).

pentadecan-8-yl 8-((2-(ethyl(2-hydroxyethyl)amino)ethyl)(6-oxo-6-(undecyloxy)hexyl)amino)octanoate (YK-205)



YK-205-SM1 YK-205-PM1



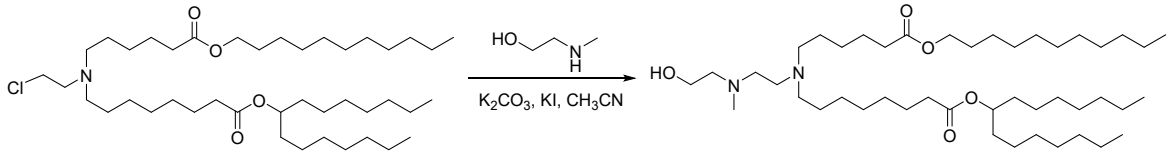
YK-205

Step 1: According to the method for preparing YK-201-PM1, YK-205-SM1 (400 mg, 0.59 mmol) and $SOCl_2$ (3 mL) were used as starting materials to obtain YK-205-PM1 (400 mg, 0.57 mmol, 96.8%). $C_{42}H_{82}ClNO_4$, MS(ES): m/z ($M+H^+$) 700.5.

Step 2: According to the method for preparing YK-201, YK-205-PM1 (200 mg, 0.29 mmol) and 2-(methylamino)ethan-1-ol (127 mg, 1.43 mmol) were used as starting materials to obtain YK-205 (160 mg, 0.21 mmol, 73.2%). $C_{46}H_{92}N_2O_5$, MS (ES): m/z ($M+H^+$) 753.7.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 4.86 (p, *J* = 6.2 Hz, 1H), 4.05 (t, *J* = 6.8 Hz, 2H), 3.57 (t, *J* = 4.9 Hz, 2H), 2.69 – 2.58 (m, 7H), 2.54 (s, 4H), 2.29 (dt, *J* = 10.4, 7.5 Hz, 4H), 1.62 (dq, *J* = 14.0, 7.3 Hz, 6H), 1.49 (d, *J* = 6.3 Hz, 8H), 1.29 (d, *J* = 21.1 Hz, 46H), 1.05 (t, *J* = 7.1 Hz, 3H), 0.92 – 0.84 (m, 9H).

pentadecan-8-yl 8-((2-((2-hydroxyethyl)(methyl)amino)ethyl)(6-oxo-6-(undecyloxy)hexyl)amino)octanoate (YK-206)

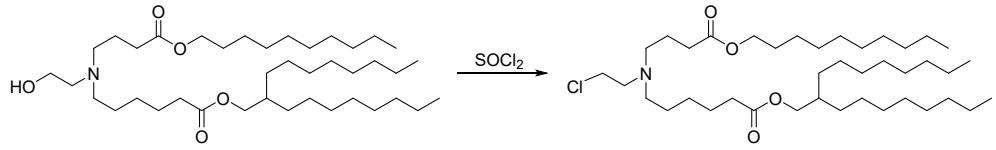


YK-205-PM1 YK-206

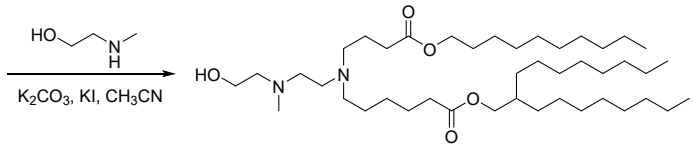
According to the method for preparing YK-201, YK-205-PM1 (200 mg, 0.29 mmol) and 2-(methylamino)ethan-1-ol (107 mg, 1.43 mmol) were used as starting materials to obtain YK-206 (127 mg, 0.17 mmol, 58.6%). $C_{45}H_{90}N_2O_5$, MS (ES): m/z ($M+H^+$) 739.7.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 4.86 (p, *J* = 6.2 Hz, 1H), 4.05 (t, *J* = 6.8 Hz, 2H), 3.56 (t, *J* = 5.1 Hz, 2H), 3.28 (s, 2H), 2.92 – 2.71 (m, 1H), 2.54 (d, *J* = 10.4 Hz, 6H), 2.44 (dt, *J* = 9.9, 4.8 Hz, 4H), 2.37 – 2.21 (m, 6H), 1.62 (dq, *J* = 14.5, 7.6 Hz, 6H), 1.46 (dt, *J* = 27.8, 9.8 Hz, 8H), 1.28 (d, *J* = 15.4 Hz, 43H), 0.88 (t, *J* = 6.7 Hz, 9H).

2-octyldecyl 6-((4-(decyloxy)-4-oxobutyl)(2-((2-hydroxyethyl)(methyl)amino)ethyl)amino)hexanoate (YK-207)



YK-207-SM1 **YK-207-PM1**



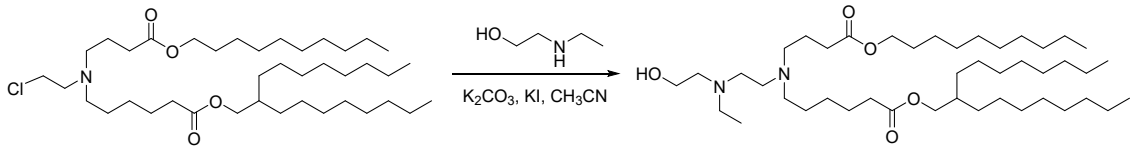
YK-207

Step 1: According to the method for preparing YK-201-PM1, YK-207-SM1 (3.0 g, 4.58 mmol) and $SOCl_2$ (5 mL) were used as starting materials to obtain YK-207-PM1 (2.9 g, 4.31 mmol, 94.2%). $C_{40}H_{78}ClNO_4$, MS (ES): m/z ($M+H^+$) 672.3.

Step 2: According to the method for preparing YK-201, YK-207-PM1 (200 mg, 0.30 mmol) and 2-(methylamino)ethan-1-ol (22 mg, 0.29 mmol) were used as starting materials to obtain YK-207 (50 mg, 0.07 mmol, 23.4%). $C_{43}H_{86}N_2O_5$, MS (ES): m/z ($M+H^+$) 711.7.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 5.43 – 5.15 (m, 1H), 4.07 (t, *J* = 6.8 Hz, 2H), 3.96 (d, *J* = 5.8 Hz, 2H), 3.87 (t, *J* = 4.6 Hz, 1H), 3.34 (d, *J* = 25.1 Hz, 3H), 3.05 (s, 3H), 2.70 (s, 2H), 2.47 (t, *J* = 6.5 Hz, 2H), 2.34 (t, *J* = 7.3 Hz, 2H), 2.01 (d, *J* = 6.9 Hz, 2H), 1.84 – 1.74 (m, 2H), 1.65 (dp, *J* = 21.2, 7.1 Hz, 5H), 1.46 – 1.37 (m, 2H), 1.37 – 1.17 (m, 44H), 0.94 – 0.85 (m, 9H).

2-octyldecyl 6-((4-(decyloxy)-4-oxobutyl)(2-(ethyl(2-hydroxyethyl)amino)ethyl)amino)hexanoate (YK-208)

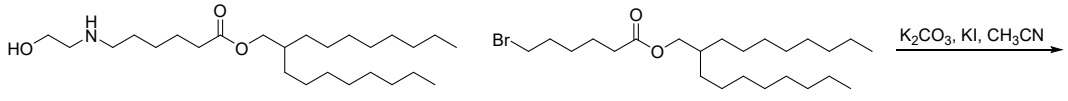


YK-207-SM1 **YK-208**

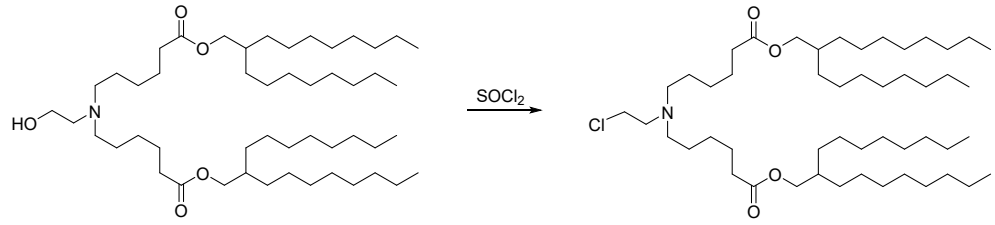
According to the method for preparing YK-201, YK-207-PM1 (150 mg, 0.22 mmol) and 2-(ethylamino)ethan-1-ol (59 mg, 0.66 mmol) were used as starting materials to obtain YK-208 (140 mg, 0.19 mmol, 87.8%). $C_{44}H_{88}N_2O_5$, MS (ES): m/z ($M+H^+$) 725.7.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 5.33 (s, 1H), 4.60 (s, 2H), 4.09 (t, *J* = 6.8 Hz, 2H), 3.99 (d, *J* = 5.8 Hz, 2H), 3.68 (t, *J* = 4.9 Hz, 2H), 2.93 – 2.54 (m, 11H), 2.36 (dt, *J* = 17.0, 7.3 Hz, 4H), 1.93 – 1.78 (m, 2H), 1.76 – 1.48 (m, 7H), 1.45 – 1.21 (m, 43H), 1.15 (t, *J* = 7.1 Hz, 3H), 1.00 – 0.78 (m, 9H).

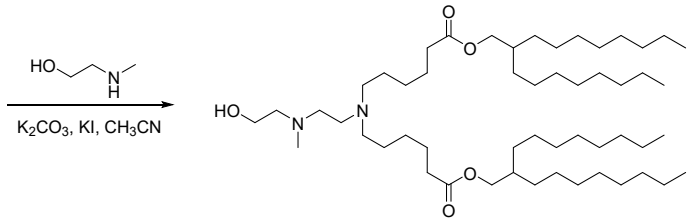
bis(2-octyldecyl) 6,6'-((2-((2-hydroxyethyl)(methyl)amino)ethyl)azanediyl)dihexanoate (YK-209)



YK-209-SM1 **YK-209-SM2**



YK-209-PM1 **YK-209-PM2**



YK-209

Step 1: To a solution of YK-209-SM1 (1.0 g, 2.34 mmol) and YK-209-SM2 (1.15 g, 2.58 mmol) in 7 mL acetonitrile were added potassium carbonate (0.97 g, 7.02 mmol) and potassium iodide (38 mg, 0.23 mmol). The mixture was heated to 70°C and stirred for 8 hours. After completion, the reaction was cooled to room temperature, and the solid was removed by filtration. The filtrate

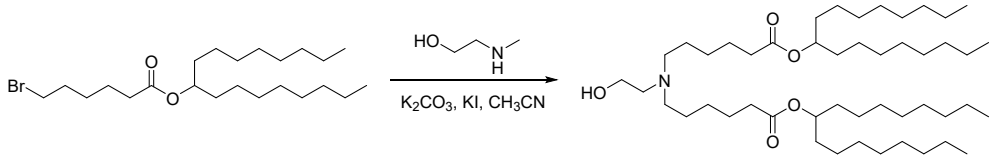
was concentrated under reduced pressure, and the residue was purified by silica gel chromatography (CH_3OH/CH_2Cl_2) to yield YK-209-PM1 (780 mg, 0.98 mmol, 41.9%). $C_{50}H_{99}NO_5$, MS (ES): m/z ($M+H^+$) 794.8.

Step 2: According to the method for preparing YK-201-PM1, YK-209-PM1 (760 mg, 0.96 mmol) and $SOCl_2$ (5 mL) were used as starting materials to obtain YK-209-PM2 (800 mg, 0.98 mmol, 102.5%). $C_{50}H_{98}ClNO_4$, MS (ES): m/z ($M+H^+$) 812.7.

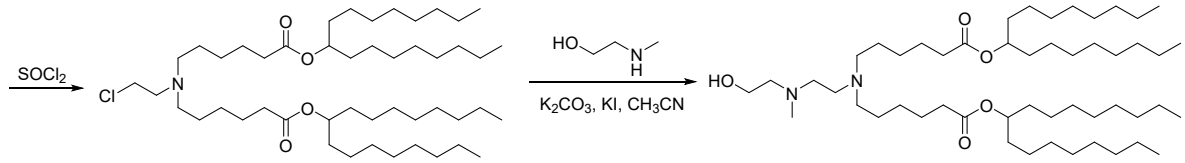
Step 3: According to the method for preparing YK-201, YK-209-PM2 (150 mg, 0.18 mmol) and 2-(methylamino)ethan-1-ol (27 mg, 0.36 mmol) were used as starting materials to obtain YK-209 (120 mg, 0.14 mmol, 78.3%). $C_{53}H_{106}N_2O_5$, MS (ES): m/z ($M+H^+$) 851.4.

^{1}H NMR (400 MHz, Chloroform-*d*): δ 3.96 (d, *J* = 5.8 Hz, 4H), 3.69 – 3.65 (m, 2H), 2.86 (s, 2H), 2.76 (s, 4H), 2.72 – 2.65 (m, 2H), 2.41 (s, 3H), 2.32 (t, *J* = 7.4 Hz, 4H), 1.66 (dt, *J* = 15.0, 7.4 Hz, 10H), 1.26 (s, 62H), 0.88 (t, *J* = 6.8 Hz, 12H).

di(heptadecan-9-yl) 6,6'-((2-((2-hydroxyethyl)(methyl)amino)ethyl)azanediyl)dihexanoate (YK-210)



YK-210-SM1 **YK-210-PM1**



YK-210-PM2 **YK-210**

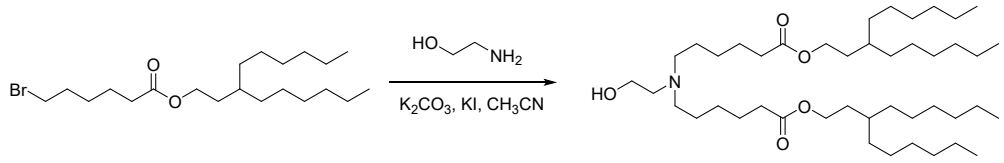
Step 1: To a solution of heptadecan-9-yl 6-bromohexanoate (700 mg, 1.62 mmol) and 2-(methylamino)ethan-1-ol (55 mg, 0.74 mmol) in 5 mL acetonitrile (CH_3CN) were added potassium carbonate (307 mg, 2.22 mmol) and potassium iodide (12 mg, 0.07 mmol). The mixture was heated to 70°C and stirred for 8 hours. After completion, the reaction was cooled to room temperature, and the solid was removed by filtration. The filtrate was concentrated under reduced pressure, and the residue was purified by silica gel chromatography (CH_3OH/CH_2Cl_2) to yield YK-210-PM1 (450 mg, 0.66 mmol, 79.4%). $C_{48}H_{95}NO_5$, MS (ES): m/z ($M+H^+$) 766.7.

Step 2: According to the method for preparing YK-201-PM1, YK-210-PM1 (450 mg, 0.59 mmol) and $SOCl_2$ (3 mL) were used as starting materials to obtain YK-210-PM2 (460 mg, 0.59 mmol, 100.0%). $C_{48}H_{94}ClNO_4$, MS (ES): m/z ($M+H^+$) 784 .6.

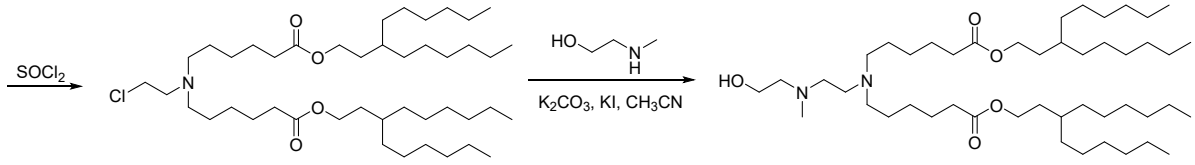
Step 3: According to the method for preparing YK-201, YK-210-PM2 (150 mg, 0.19 mmol) and 2-(methylamino)ethan-1-ol (17 mg, 0.23 mmol) were used as starting materials to obtain YK-210 (136 mg, 0.17 mmol, 86 .9%). $C_{51}H_{102}N_2O_5$, MS (ES): m/z ($M+H^+$) 823.8.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 5.30 (s, 1H), 4.85 (p, *J* = 6.2 Hz, 2H), 3.96 (s, 2H), 3.72 –3.65 (m, 2H), 2.97 (s, 1H), 2.90 (s, 3H), 2.81 (s, 2H), 2.75 – 2.67 (m, 2H), 2.42 (s, 2H), 2.30 (t, *J* = 7.3 Hz, 4H), 1.72 – 1.62 (m, 7H), 1.50 (d, *J* = 5.6 Hz, 8H), 1.26 (s, 54H), 0.88 (t, *J* = 6.8 Hz, 12H).

bis(3-hexylnonyl) 6,6'-((2-((2-hydroxyethyl)(methyl)amino)ethyl)azanediyl)dihexanoate (YK-211)



YK-211-SM1 **YK-211-PM1**



YK-211-PM2 **YK-211**

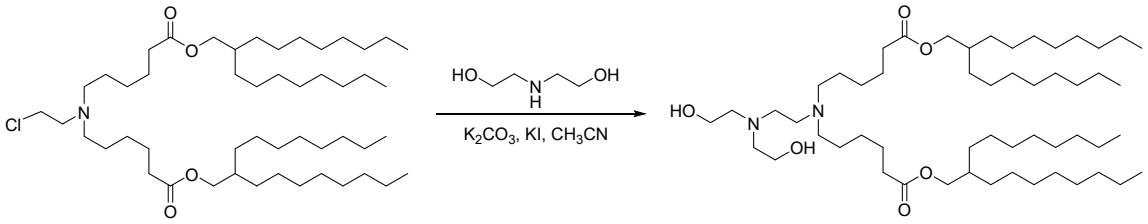
Step 1: According to the method for preparing YK-210-PM1, 3-hexylnonyl 6-bromohexanoate (828 mg, 2.05 mmol) and 2-aminoethan-1-ol (50 mg, 0.82 mmol) were used as starting materials to obtain YK-211-PM1 (470 mg, 0.66 mmol, 80.8%). $C_{44}H_{87}NO_5$, MS (ES): m/z ($M+H^+$) 710.5.

Step 2: According to the method for preparing YK-201-PM1, YK-211-PM1 (470 mg, 0.66 mmol) and $SOCl_2$ (3 mL) were used as starting materials to obtain YK-211-PM2 (420 mg, 0.58 mmol, 87.5%). $C_{44}H_{86}ClNO_4$, MS (ES): m/z ($M+H^+$) 728.6.

Step 3: According to the method for preparing YK-201, YK-211-PM2 (100 mg, 0.14 mmol) and 2-(methylamino)ethan-1-ol (12 mg, 0.16 mmol) were used as starting materials to obtain YK-211 (57 mg, 0.07 mmol, 53.1%). $C_{47}H_{94}N_2O_5$, MS (ES): m/z ($M+H^+$) 767.7.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 4.08 (t, *J* = 7.2 Hz, 4H), 3.82 – 3.75 (m, 2H), 3.31 (d, *J* = 5.6 Hz, 4H), 3.13 (dd, *J* = 18.9, 10.6 Hz, 5H), 2.87 (s, 2H), 2.56 (s, 2H), 2.33 (t, *J* = 7.2 Hz, 4H), 1.78 (p, *J* = 7.9 Hz, 3H), 1.73 – 1.62 (m, 4H), 1.57 (q, *J* = 7.0 Hz, 4H), 1.43 (dt, *J* = 14.4, 7.5 Hz, 6H), 1.25 (s, 42H), 0.88 (t, *J* = 6.7 Hz, 12H).

bis(2-octyldecyl) 6,6'-((2-(bis(2-hydroxyethyl)amino)ethyl)azanediyl)dihexanoate (YK-212)

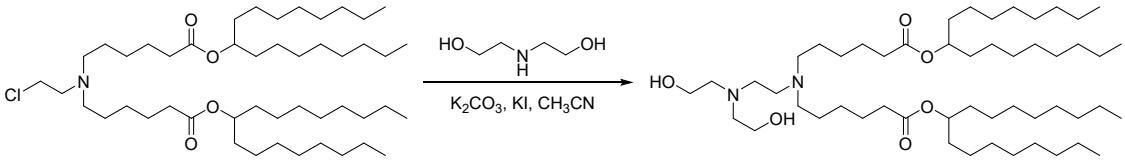


YK-209-PM2 **YK-212**

According to the method for preparing YK-201, YK-209-PM2 (100 mg, 0.12 mmol) and 2,2'-azanediylbis(ethan-1-ol) (26 mg, 0.24 mmol) were used as starting materials to obtain YK-212 (80 mg, 0.09 mmol, 75.6%). $C_{54}H_{108}N_2O_6$, MS (ES): m/z ($M+H^+$) 881.8.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 4.00 (d, *J* = 5.8 Hz, 4H), 3.69 (dd, *J* = 5.6, 4.0 Hz, 4H), 3.04 – 2.93 (m, 5H), 2.85 (t, *J* = 5.4 Hz, 2H), 2.75 (t, *J* = 4.8 Hz, 4H), 2.36 (t, *J* = 7.3 Hz, 4H), 1.70 (tq, *J* = 11.0, 6.7, 5.6 Hz, 10H), 1.30 (s, 63H), 0.95 – 0.89 (m, 12H).

di(heptadecan-9-yl) 6,6'-((2-(bis(2-hydroxyethyl)amino)ethyl)azanediyl)dihexanoate (YK-213)

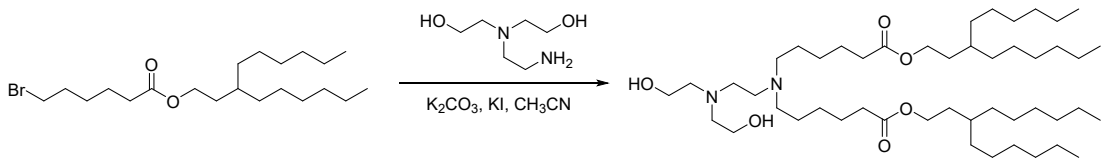


YK-210-PM2 **YK-213**

According to the method for preparing YK-201, YK-210-PM2 (150 mg, 0.19 mmol) and 2,2'-azanediylbis(ethan-1-ol) (21 mg, 0.20 mmol) were used as starting materials to obtain YK-213 (50 mg, 0.06 mmol, 30.8%). $C_{52}H_{104}N_2O_6$, MS (ES): m/z ($M+H^+$) 853.8.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 4.00 (d, *J* = 5.8 Hz, 4H), 3.69 (dd, *J* = 5.6, 4.0 Hz, 4H), 3.04 – 2.93 (m, 5H), 2.85 (t, *J* = 5.4 Hz, 2H), 2.75 (t, *J* = 4.8 Hz, 4H), 2.36 (t, *J* = 7.3 Hz, 4H), 1.70 (tq, *J* = 11.0, 6.7, 5.6 Hz, 10H), 1.30 (s, 63H), 0.95 – 0.89 (m, 12H).

bis(3-hexylnonyl) 6,6'-((2-(bis(2-hydroxyethyl)amino)ethyl)azanediyl)dihexanoate (YK-214)

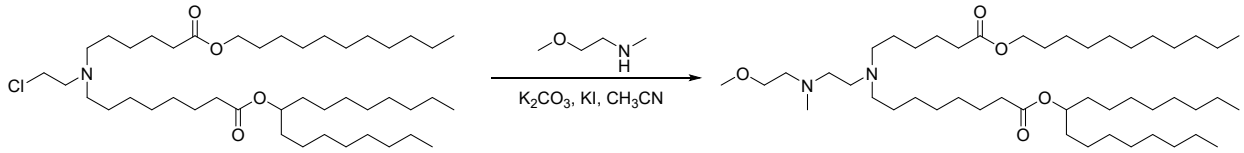


YK-211-SM1 **YK-214**

To a solution of 3-hexylnonyl 6-bromohexanoate (303 mg, 0.75 mmol) and 2,2'-((2-aminoethyl)azanediyl)bis(ethan-1-ol) (50 mg, 0.34 mmol) in 2 mL acetonitrile (CH_3CN) were added potassium carbonate (140 mg, 1.02 mmol) and potassium iodide (5.6 mg, 0.034 mmol). The mixture was heated to 70°C and stirred for 24 hours. After completion, the reaction was cooled to room temperature, and the solid was removed by filtration. The filtrate was concentrated under reduced pressure, and the residue was purified by silica gel chromatography (CH_3OH/CH_2Cl_2) to yield YK-214 (120 mg, 0.15 mmol, 44.3%), $C_{48}H_{96}N_2O_6$, MS (ES): m/z ($M+H^+$) 797.8.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 4.11 (t, *J* = 7.2 Hz, 4H), 4.02 – 3.74 (m, 1H), 3.65 (t, *J* = 4.9 Hz, 4H), 2.74 (dd, *J* = 12.4, 7.4 Hz, 11H), 2.34 (t, *J* = 7.4 Hz, 4H), 1.64 (dq, *J* = 29.0, 7.4 Hz, 11H), 1.50 – 1.20 (m, 49H), 0.94 – 0.89 (m, 12H).

heptadecan-9-yl 8-((2-((2-methoxyethyl)(methyl)amino)ethyl)(6-oxo-6-(undecyloxy)hexyl)amino)octanoate (YK-215)

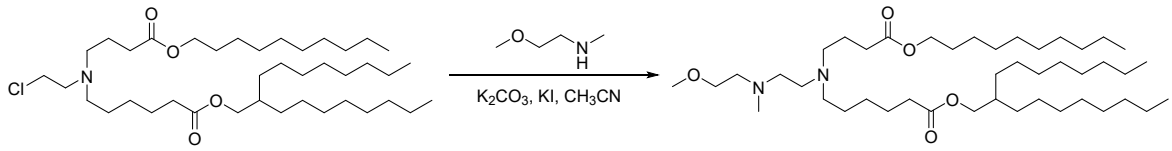


YK-201-PM1 **YK-215**

According to the method for preparing YK-201, YK-201-PM1 (95 mg, 0.13 mmol) and 2-methoxy-N-methylethan-1-amine (14 mg, 0.16 mmol) were used as starting materials to obtain YK-215 (98 mg, 0.12 mmol, 96.5%). $C_{48}H_{96}N_2O_5$, MS (ES): m/z ($M+H^+$) 781.2.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 4.86 (t, *J* = 6.2 Hz, 1H), 4.05 (t, *J* = 6.8 Hz, 2H), 3.36 (s, 3H), 3.15 (t, *J* = 6.3 Hz, 3H), 3.01 (dq, *J* = 11.7, 7.2, 5.6 Hz, 6H), 2.77 (t, *J* = 5.0 Hz, 2H), 2.45 (s, 2H), 2.30 (dt, *J* = 17.9, 7.4 Hz, 4H), 1.85 – 1.57 (m, 10H), 1.50 (d, *J* = 6.3 Hz, 4H), 1.41 – 1.18 (m, 50H), 0.88 (t, *J* = 6.6 Hz, 9H).

2-octyldecyl 6-((4-(decyloxy)-4-oxobutyl)(2-((2-methoxyethyl)(methyl)amino)ethyl)amino)hexanoate (YK-216)

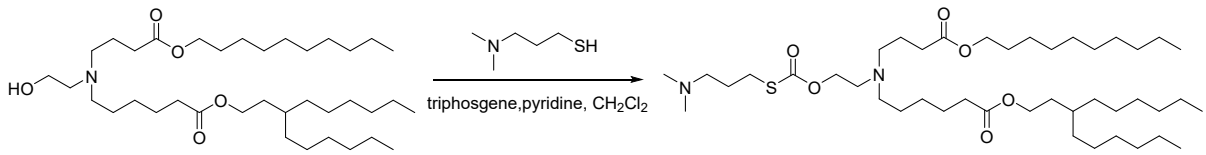


YK-207-PM1 **YK-216**

According to the method for preparing YK-201, YK-207-PM1 (170 mg, 0.25 mmol) and 2-methoxy-N-methylethan-1-amine (89 mg, 0.45mmol) were used as starting materials to obtain YK-216 (140 mg, 0.19 mmol, 77 .2%). $C_{44}H_{88}N_2O_5$, MS (ES): m/z ($M+H^+$) 725.6.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 4.09 (t, *J* = 6.7 Hz, 2H), 4.00 (d, *J* = 5.8 Hz, 2H), 3.58 (t, *J* = 5.3 Hz, 2H), 3.39 (s, 3H), 2.94 – 2.50 (m, 11H), 2.51 – 2.25 (m, 7H), 1.88 (d, *J* = 8.2 Hz, 2H), 1.75 – 1.48 (m, 7H), 1.50 – 1.20 (m, 43H), 0.98 – 0.83 (m, 9H).

3-hexylnonyl 11-(4-(decyloxy)-4-oxobutyl)-2-methyl-7-oxo-8-oxa-6-thia-2,11-diazaheptadecan-17-oate (YK-217)

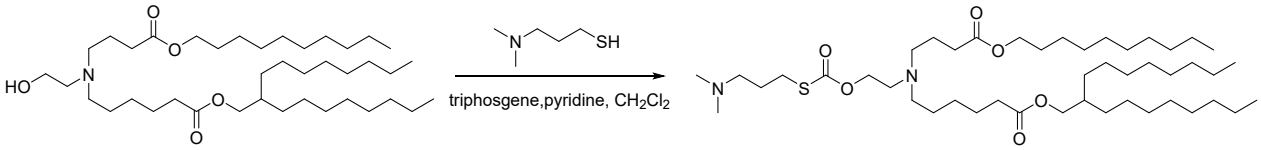


YK-217-SM1 **YK-217**

YK-317-SM1 (100 mg, 0.16 mmol), 3-(dimethylamino)propane-1-thiol (20 mg, 0.32 mmol), and pyridine (100 mg, 0.16 mmol) were dissolved in dichloromethane (5 mL) under a nitrogen atmosphere. The mixture was cooled in an ice bath, and triphosgene (77 mg, 0.26 mmol) was added dropwise. After the addition, the reaction mixture was allowed to warm to room temperature and stirred for 8 hours. Following the reaction, a saturated aqueous solution of sodium bicarbonate (5 mL) was added dropwise, followed by 20 mL of dichloromethane. The organic phase was separated, washed with saturated sodium chloride solution, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to yield YK-217 (60 mg, 0.03 mmol, 16.5%), $C_{43}H_{84}N_2O_6S$, MS (ES): m/z ($M+H^+$) 757.2.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 4.14 – 4.00 (m, 2H), 3.96 (t, *J* = 4.7 Hz, 2H), 3.34 (s, 4H), 2.91 (td, *J* = 7.2, 3.3 Hz, 2H), 2.47 (d, *J* = 7.9 Hz, 2H), 2.31 (t, *J* = 7.0 Hz, 8H), 1.95 – 1.78 (m, 4H), 1.62 (d, *J* = 11.7 Hz, 6H), 1.31 – 1.23 (m, 45H), 0.87 (dt, *J* = 7.0, 3.4 Hz, 9H).

2-octyldecyl 11-(4-(decyloxy)-4-oxobutyl)-2-methyl-7-oxo-8-oxa-6-thia-2,11-diazaheptadecan-17-oate (YK-218)

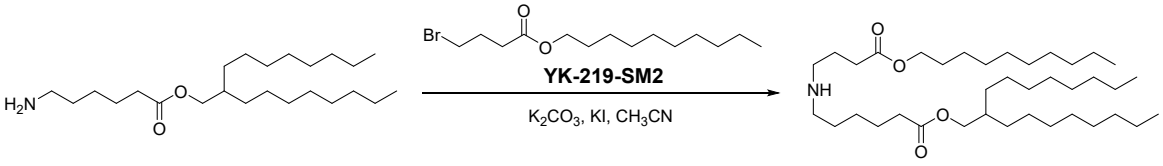


YK-207-SM1 **YK-218**

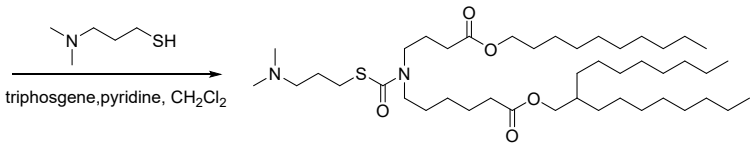
According to the method for preparing YK-217, YK-207-SM1 (100 mg, 0.15 mmol) and 3-(dimethylamino)propane-1-thiol (36.5 mg, 0.31 mmol) were used as starting materials to obtain YK-218 (60 mg , 0.08 mmol, 50.0%). $C_{46}H_{90}N_2O_6S$, MS (ES): m/z ($M+H^+$) 799.7.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 4.23 (t, *J* = 6.2 Hz, 2H), 4.05 (t, *J* = 6.8 Hz, 2H), 3.96 (d, *J* = 5.8 Hz, 2H), 2.89 (t, *J* = 7.2 Hz, 2H), 2.70 (t, *J* = 6.2 Hz, 2H), 2.46 (ddd, *J* = 12.4, 8.0, 6.1 Hz, 5H), 2.31 (d, *J* = 7.4 Hz, 9H), 1.91 – 1.83 (m, 2H), 1.77 – 1.68 (m, 2H), 1.62 (tt, *J* = 7.8, 4.5 Hz, 5H), 1.46 – 1.38 (m, 2H), 1.28 (d, *J* = 14.4 Hz, 46H), 0.88 (t, *J* = 6.7 Hz, 9H).

2-octyldecyl 6-((4-(decyloxy)-4-oxobutyl)(((3-(dimethylamino)propyl)thio)carbonyl)amino)hexanoate (YK-219)



YK-219-SM1 **YK-219-PM1**



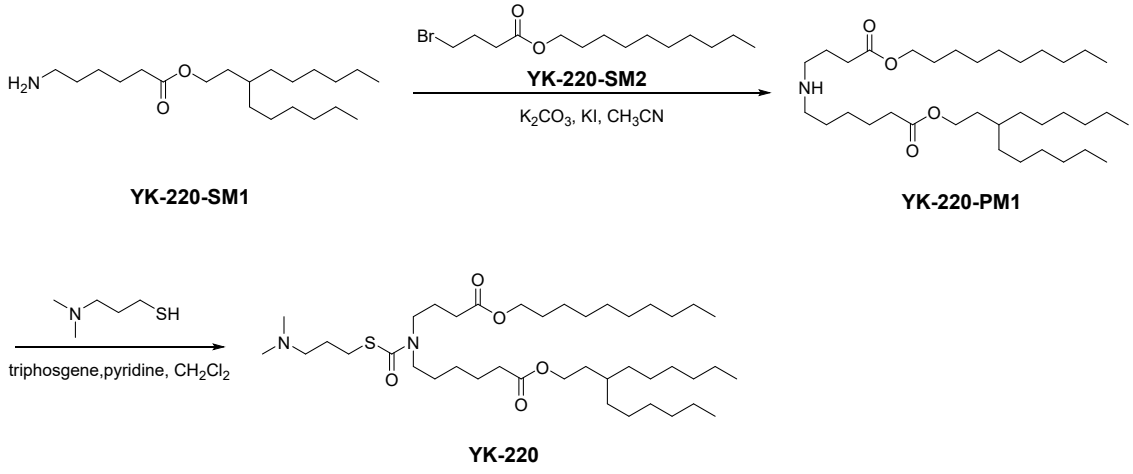
YK-219

Step 1: According to the method for preparing YK-207-PM1, YK-219-SM1 (459 mg, 1.19 mmol) and YK-219-SM2 (304 mg, 0.99 mmol) were used as starting materials to obtain YK-219-PM1 (310 mg, 0.51 mmol, 51.3%), $C_{38}H_{75}NO_4$, MS (ES): m/z ($M+H^+$) 610.5.

Step 2: According to the method for preparing YK-217, YK-219-PM1 (310 mg, 0.51 mmol) and 3-(dimethylamino)propane-1-thiol (303 mg, 2.54 mmol) were used as starting materials to obtain YK-219 (82 mg, 0.11 mmol, 21.3%). $C_{44}H_{86}N_2O_5S$, MS (ES): m/z ($M+H^+$) 755.5.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 4.10 (q, *J* = 4.9, 3.5 Hz, 2H), 3.99 (t, *J* = 4.6 Hz, 2H), 3.37 (s, 3H), 2.95 (td, *J* = 7.2, 3.4 Hz, 2H), 2.50 (d, *J* = 7.8 Hz, 2H), 2.36 (d, *J* = 3.6 Hz, 9H), 2.07 – 1.84 (m, 4H), 1.70 – 1.59 (m, 6H), 1.43 – 1.26 (m, 47H), 0.91 (dt, *J* = 7.0, 3.4 Hz, 9H).

3-hexylnonyl 6-((4-(decyloxy)-4-oxobutyl)(((3-(dimethylamino)propyl)thio)carbonyl)amino)hexanoate (YK-220)

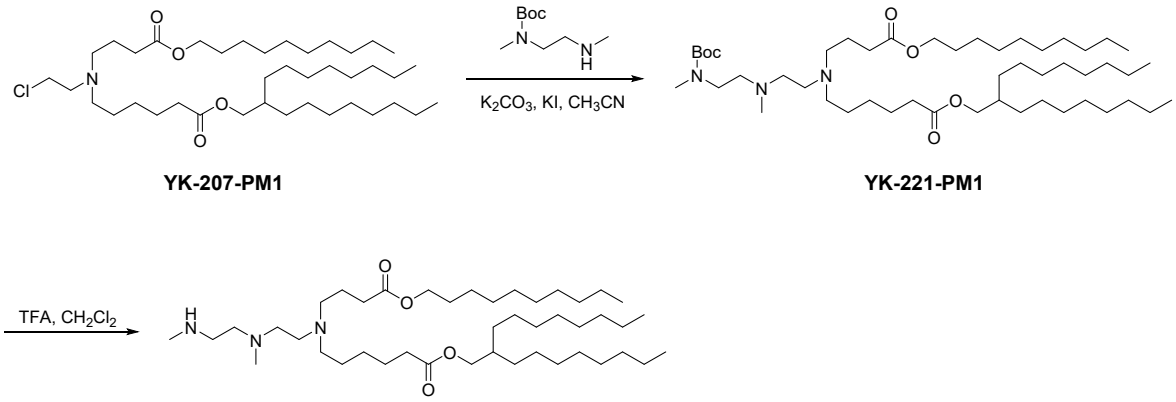


Step 1: According to the method for preparing YK-207-PM1, YK-220-SM1 (391 mg, 1.15 mmol) and YK-220-SM2 (292 mg, 0.95 mmol) were used as starting materials to obtain YK-220-PM1 (249 mg, 0.44 mmol, 46.2%), $C_{35}H_{69}NO_4$, MS (ES): m/z ($M+H^+$) 568.5.

Step 2: According to the method for preparing YK-217, YK-220-PM1 (200 mg, 0.35 mmol) and 3-(dimethylamino)propane-1-thiol (209 mg, 1.75 mmol) were used as starting materials to obtain YK-220 (76 mg , 0.11 mmol, 30.4%). $C_{41}H_{80}N_2O_5S$, MS (ES): m/z ($M+H^+$) 713.6.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 4.12 – 4.02 (m, 4H), 3.35 (s, 4H), 2.92 (t, *J* = 7.2 Hz, 2H), 2.49 – 2.40 (m, 2H), 2.30 (s, 8H), 1.60 (dp, *J* = 20.8, 7.2 Hz, 8H), 1.44 – 1.18 (m, 40H), 0.88 (t, *J* = 6.8 Hz, 9H).

2-octyldecyl 6-((4-(decyloxy)-4-oxobutyl)(2-(methyl(2-(methylamino)ethyl)amino)ethyl)amino)hexanoate (YK-221)



YK-207-PM1 YK-221-PM1

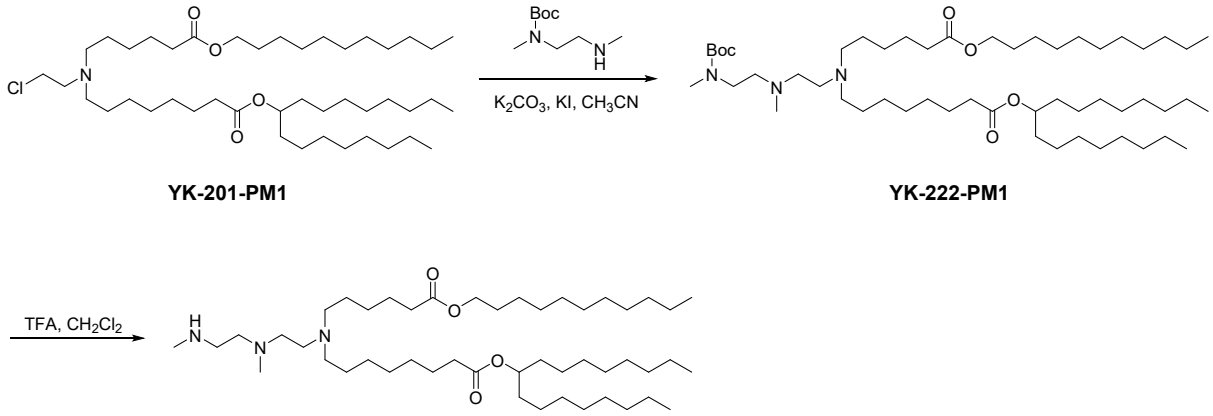
YK-221

Step 1: According to the method for preparing YK-201, YK-207-PM1 (150 mg, 0.22 mmol) and *tert*-butyl methyl(2-(methylamino)ethyl)carbamate (45 mg, 0.22 mmol) were used as starting materials to obtain YK-221-PM1 (120 mg, 0.15 mmol, 66.2%). $C_{49}H_{97}N_3O_6$, MS (ES): m/z ($M+H^+$) 824.7.

Step 2: To a solution of YK-221-PM1 (120 mg, 0.15 mmol) in dicholoromethane was added trifluoroacetic acid (0.5 mL). The mixture was stirred at room temperature for 2 h. After completion of the reaction, the solvent was removed under reduced pressure, and the residue was dissolved in 10 mL of ethyl acetate. The organic phase was washed with a saturated aqueous sodium bicarbonate solution, then concentrated under reduced pressure. The residue was purified by silica gel column chromatography to yield YK-221 (80 mg, 0.11 mmol, 73.6%). $C_{44}H_{89}N_3O_4$, MS (ES): m/z ($M+H^+$) 724.6.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 4.06 (t, *J* = 6.8 Hz, 2H), 3.96 (d, *J* = 5.8 Hz, 1H), 3.21 (s, 3H), 3.05 (s, 2H), 2.91 (s, 1H), 2.73 (s, 2H), 2.54 (s, 2H), 2.49 – 2.17 (m, 11H), 2.03 (d, *J* = 11.0 Hz, 3H), 1.82 (s, 2H), 1.61 (d, *J* = 7.3 Hz, 4H), 1.53 – 1.40 (m, 2H), 1.26 (d, *J* = 3.9 Hz, 42H), 0.88 (t, *J* = 6.7 Hz, 9H).

heptadecan-9-yl 8-((2-(methyl(2-(methylamino)ethyl)amino)ethyl)(6-oxo-6-(undecyloxy)hexyl)amino)octanoate (YK-222)



YK-201-PM1 **YK-222-PM1**

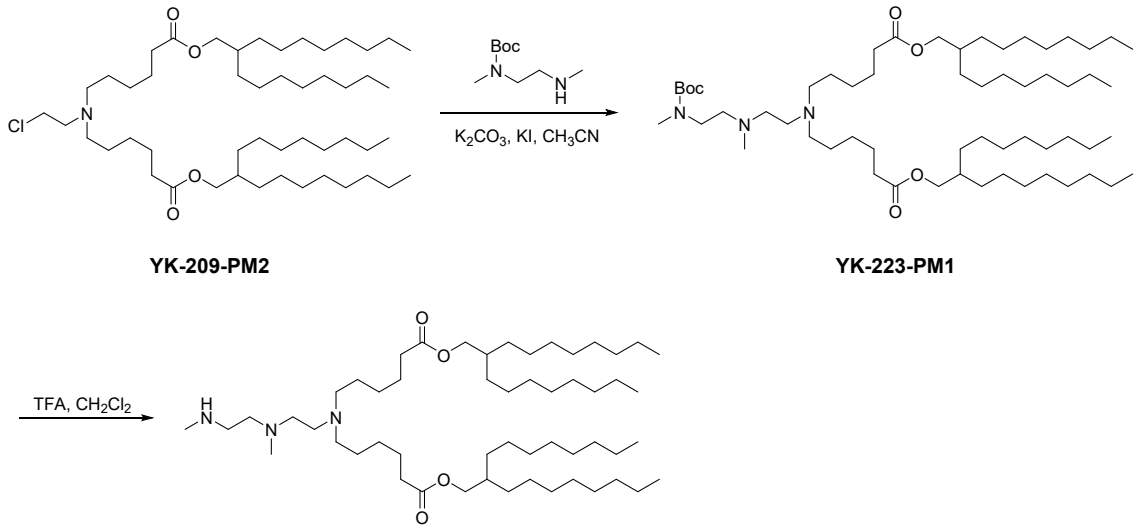
YK-222

Step 1: According to the method for preparing YK-201, YK-201-PM1 (100 mg, 0.14 mmol) and *tert*-butyl methyl(2-(methylamino)ethyl)carbamate (28 mg, 0.15 mmol) were used as starting materials to obtain YK-222-PM1 (120 mg, 0.14 mmol , 97.4%). $C_{49}H_{97}N_3O_6$, MS (ES): m/z ($M+H^+$) 880.3.

Step 2: According to the method for preparing YK-221, YK-222-PM1 (120 mg, 0.14 mmol) was used as starting material to obtain YK-222 (67 mg, 0.09 mmol, 61.3%). $C_{48}H_{97}N_3O_4$, MS (ES): m/z ($M+H^+$) 780.4.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 4.93 – 4.80 (m, 1H), 4.05 (t, *J* = 6.8 Hz, 2H), 3.15 (t, *J* = 5.4 Hz, 4H), 3.02 (s, 3H), 2.83 (s, 1H), 2.73 (s, 2H), 2.37 – 2.21 (m, 7H), 2.01 (d, *J* = 5.5 Hz, 6H), 1.63 (tt, *J* = 13.9, 7.4 Hz, 10H), 1.50 (d, *J* = 6.4 Hz, 4H), 1.46 – 1.15 (m, 49H), 0.93 – 0.85 (m, 9H).

bis(2-octyldecyl) 6,6'-((2-(methyl(2-(methylamino)ethyl)amino)ethyl)azanediyl)dihexanoate (YK-223)



YK-209-PM2 **YK-223-PM1**

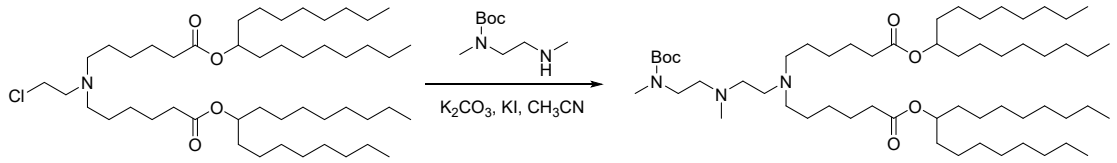
YK-223

Step 1: According to the method for preparing YK-201, YK-209-PM2 (150 mg, 0.18 mmol) and *tert*-butyl methyl(2-(methylamino)ethyl)carbamate (45 mg, 0.24 mmol) were used as starting materials to obtain YK-223-PM1 (160 mg, 0.17 mmol, 92.2%). $C_{59}H_{117}N_3O_6$, MS (ES): m/z ($M+H^+$) 964.9.

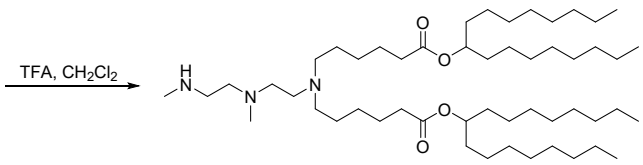
Step 2: According to the method for preparing YK-221, YK-223-PM1 (160 mg, 0.17 mmol) was used as starting material to obtain YK-223 (120 mg, 0.14 mmol, 81.7%). $C_{54}H_{109}N_3O_4$, MS (ES): m/z ($M+H^+$) 864.9.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 4.12 (q, *J* = 7.1 Hz, 1H), 3.97 (d, *J* = 5.8 Hz, 3H), 2.95 (d, *J* = 4.8 Hz, 1H), 2.88 – 2.40 (m, 10H), 2.38 – 2.25 (m, 6H), 2.05 (s, 2H), 1.74 – 1.12 (m, 74H), 0.88 (d, *J* = 6.9 Hz, 12H).

di(heptadecan-9-yl) 6,6'-((2-(methyl(2-(methylamino)ethyl)amino)ethyl)azanediyl)dihexanoate (YK-224)



YK-210-PM2 **YK-224-PM1**



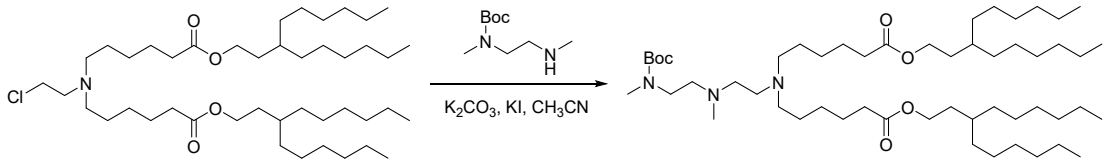
YK-224

Step 1: According to the method for preparing YK-201, YK-210-PM2 (150 mg, 0.19mmol) and *tert*-butyl methyl(2-(methylamino)ethyl)carbamate (43 mg, 0.23 mmol) were used as starting materials to obtain YK-224-PM1 (120 mg, 0.13 mmol, 55.7%). $C_{57}H_{113}N_3O_6$, MS (ES): m/z ($M+H^+$) 936.8.

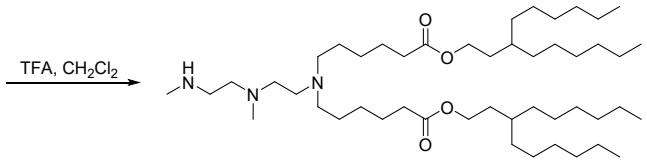
Step 2: According to the method for preparing YK-221, YK-224-PM1 (100 mg, 0.11 mmol) was used as starting material to obtain YK-224 (70 mg, 0.08 mmol, 76.1%). $C_{52}H_{105}N_3O_4$, MS (ES): m/z ($M+H^+$) 836.7.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 4.86 (q, *J* = 6.1 Hz, 2H), 4.12 (q, *J* = 7.1 Hz, 1H), 2.90 – 2.82 (m, 2H), 2.76 – 2.41 (m, 9H), 2.30 (q, *J* = 8.3, 7.4 Hz, 6H), 2.04 (s, 1H), 1.63 (q, *J* = 7.4 Hz, 4H), 1.57 – 1.39 (m, 12H), 1.28 (d, *J* = 15.2 Hz, 56H), 0.88 (t, *J* = 6.8 Hz, 12H).

bis(3-hexylnonyl) 6,6'-((2-(methyl(2-(methylamino)ethyl)amino)ethyl)azanediyl)dihexanoate (YK-225)



YK-211-PM2 **YK-225-PM1**



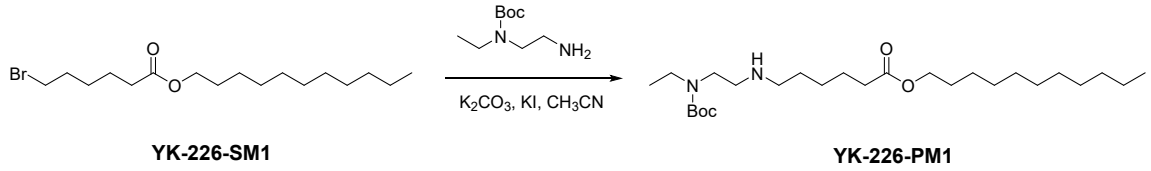
YK-225

Step 1: According to the method for preparing YK-201, YK-211-PM2 (170 mg, 0.21 mmol) and *tert*-butyl methyl(2-(methylamino)ethyl)carbamate (43 mg, 0.23 mmol) were used as starting materials to obtain YK-225-PM1 (120 mg, 0.14 mmol, 64.9%). $C_{53}H_{105}N_3O_6$, MS (ES): m/z ($M+H^+$) 880.8.

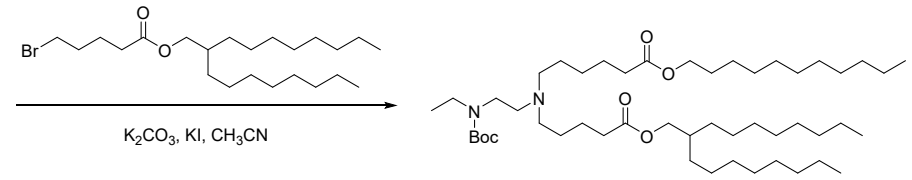
Step 2: According to the method for preparing YK-221, YK-225-PM1 (120 mg, 0.14 mmol) was used as starting material to obtain YK-225 (80 mg, 0.10 mmol, 73.2%). $C_{48}H_{97}N_3O_4$, MS (ES): m/z ($M+H^+$) 780.7.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 4.08 (t, *J* = 7.1 Hz, 4H), 3.48 (s, 3H), 3.04 – 2.86 (m, 5H), 2.29 (dd, *J* = 17.2, 9.6 Hz, 5H), 1.61 (ddd, *J* = 26.8, 14.3, 7.3 Hz, 10H), 1.47 – 1.35 (m, 6H), 1.25 (s, 45H), 0.88 (t, *J* = 6.1 Hz, 12H).

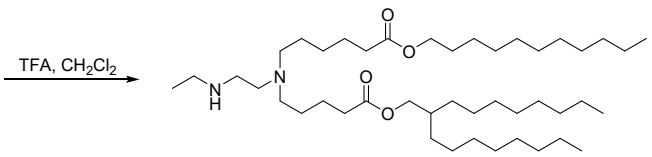
undecyl 6-((2-(ethylamino)ethyl)(5-((2-octyldecyl)oxy)-5-oxopentyl)amino)hexanoate (YK-226)



YK-226-SM1 **YK-226-PM1**



YK-226-PM2



YK-226

Step 1: To a solution of YK-226-SM1 (278 mg, 0.80 mmol) and tert-butyl (2-aminoethyl)(ethyl)carbamate (150 mg, 0.80 mmol) in 3 mL acetonitrile were added potassium carbonate (330 mg, 2.38 mmol) and potassium iodide (13 mg, 0.08 mmol). The mixture was heated to 70°C and stirred for 4 hours. After completion, the reaction was cooled to room temperature, and the solid was removed by filtration. The filtrate was concentrated under reduced pressure, and the residue was purified by silica gel chromatography (CH_3OH/CH_2Cl_2) to yield YK-226-PM1 (190 mg, 0.46 mmol, 57.3%), $C_{23}H_{46}N_2O_4$, MS (ES): m/z ($M+H^+$) 415.4.

Step 2: To a solution of YK-226-PM1 (190 mg, 0.42 mmol) and 2-octyldecyl 5-bromopentanoate (180 mg, 0.46 mmol) in 3 mL acetonitrile were added potassium carbonate (173 mg, 1.26 mmol) and potassium iodide (7 mg, 0.04 mmol). The mixture was heated to 70°C and stirred for 24 hours. After completion, the reaction was cooled to room temperature, and the solid was removed by filtration. The filtrate was concentrated under reduced pressure, and the residue was purified by silica gel chromatography (PE/EA) to yield YK-226-PM2 (281 mg, 0.35 mmol, 82.7%). $C_{49}H_{96}N_2O_6$, MS (ES): m/z ($M+H^+$) 809.7.

Step 3: According to the method for preparing YK-221, YK-226-PM2 (281 mg, 0.35 mmol) was used as starting material to obtain YK-226 (210 mg, 0.30 mmol, 84.6%). $C_{44}H_{88}N_2O_4$, MS (ES): m/z ($M+H^+$) 709.6.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 4.09 (t, *J* = 6.8 Hz, 2H), 4.00 (d, *J* = 5.8 Hz, 2H), 3.40 – 3.18 (m, 1H), 2.86 – 2.73 (m, 4H), 2.64 (t, *J* = 6.0 Hz, 2H), 2.46 (q, *J* = 7.1 Hz, 4H), 2.34 (q, *J* = 7.7 Hz, 4H), 1.73 – 1.58 (m, 7H), 1.54 – 1.42 (m, 4H), 1.37 – 1.20 (m, 49H), 0.97 – 0.89 (m, 9H).

4. Experimental Sections

4.1 Cells and viruses. Human embryonic kidney-293T (HEK293T) cell lines were maintained in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific) supplemented with 10% (vol/vol) dialyzed fetal bovine serum (FBS, Thermo Fisher Scientific), 100 U/mL penicillin, and 100 mg/mL streptomycin in a humidified atmosphere at 37 °C with 5% CO_2. The parental RSV A2 (ATCC VR-1540) and B18537 (VR-1580) were obtained from ATCC (American Type Culture Collection).

4.2 mRNA synthesis by in vitro transcription. DNase/RNase-free water was added in a 1.5 mL centrifuge tube, followed by 10× transcription buffer, NTPs, and CleanCap® Reagent AG. After each addition, ensure thorough mixing, then gently centrifuge to collect the contents at the bottom of the tube. Next, murine RNase inhibitor, inorganic pyrophosphatase, T7 RNA polymerase, and linearized DNA template were added and mixed. After 2 h incubation at 37 °C, DNase I (2 U/μL) was added and samples were incubated at 37 °C for another 30 minutes to digest the DNA template. Finally, RNA was purified using chromatographic purification method.

4.3 Preparation of mRNA-LNPs. The cationic lipids were dissolved in ethanol with DSPC (AVT (Shanghai) Pharmaceutical Technology Co., Ltd.), cholesterol (AVT (Shanghai) Pharmaceutical Technology Co., Ltd.) and DMG-PEG2000 according to a molar ratio of 45:10:43.5:1.5, respectively, to prepare ethanol lipid solution. For SM-102, a molar ratio of 50:38.5:10:1.5 was used. The ethanol lipid solution was quickly added to citrate buffer (pH=4~5), and vortexed for 30s for later use. mRNA was diluted in citrate buffer (pH=4~5) to give an aqueous mRNA solution. Next, the lipid mixture was mixed with mRNA solution (with a volume ratio of 3:1) via microfluidics at a total flow rate of 12 mL/min. The resulting formulations were diluted to 10 times volume with PBS (Phosphate-Buffered Saline) buffer (pH=7.4, Life Science), and then ultrafiltered with a 300 KDa ultrafiltration tube to remove ethanol. Finally, the LNPs encapsulated mRNA was filtrated through a 0.22 μm filter after diluting the mixtures by PBS to a certain volume.

4.4 Physiochemical characterization of LNPs. The particle size and polydispersity index (PDI) were determined by dynamic light scattering using Malvern laser particle size analyzer. 25 μL of the liposome solution was weighed, diluted to a final volume of 125 μL with normal saline, and added to the sample pool. Each sample was measured in triplicate. The measurement conditions were: 90° scattering angle, and the temperature is 25 °C. The encapsulation efficiency was determined by Quant-iT™ RiboGreen RNA Quantitation Kit (ThermoFisher).

4.5 Luminescent detection of Fluc-mRNA. HEK-293T cells, cultured and passaged as described above, were seeded into a 96-well plate at a density of 1.0×10^4 cells per well and incubated for 24 hours. An LNP formulation containing 50 ng of Fluc-mRNA was added to the cell culture medium of a 96-well plate, and further incubated for another 24 h. The corresponding reagent was added according to the instructions of the Gaussia Luciferase Assay Kit, and the fluorescence expression intensity of each well was detected by microplate reader (BioTek Synergy H1).

4.6 Cell survival rate determination. An LNP formulation containing 1.5 μg of Fluc-mRNA or the formulation of Lipofectamine 3000 were added to the cell culture medium of a 96-well plate, and further incubated for 24 hours. 10 μL of CCK-8 solution was then added to each well, and the culture plate was incubated in an incubator for 1 hour. The absorbance at 450 nm was measured by microplate reader (BioTek Synergy H1).

4.7 In vivo validation of the delivery efficiency. The LNP formulation containing Fluc-mRNA was injected intramuscularly into female BALB/C mice aged 4-6 weeks old and weighed 17-19 g, and the mice were intraperitoneally injected with fluorescent imaging substrate at specific time points after administration, where the mice were free to move for 5 minutes, and then the average radiation intensity (corresponding to fluorescence expression intensity) of the protein expression of the mRNA carried by the LNP in the mice was detected by the IVIS Spectrum instrument (AniView 600).

4.8 Enzyme-linked immunosorbent assay (ELISA). The high-binding 96-well plates (Corning) were coated with 50 μL of RSV prefusion-F (preF) protein (Acro Biosystems, RSF-V52H7) per well with a concentration of 2.0 μg/mL at 4 °C overnight. The plates were then washed three times with Washing Buffer (Biolegend, 421601) and incubated with Blocking Buffer (BovoGen, BSAS). The serum collected from immunized and control mice were initially diluted at 1:500. Then, a four-fold

serial dilution was performed on all serum samples. 50 µL of samples per well were added to the plates for 2 h at 37 °C. Then, the plates were washed and then incubated with goat anti-mouse IgG (H+L) secondary antibody with HRP at 1:5,000 (Abcam, ab97023) for 45 min at 37 °C. Next, the plates were washed and 50 µL of TMB substrate solution (Biolegend, 421101) was added for 10 min incubation at 37 °C. Then, 50 µL of stop solution (Biolegend, 423001) was added. SpectraMax iD3 Reader (Molecular Devices) was used to measure the Optical Density (OD450 and OD630).

4.9 Vaccination and neutralization studies. Mice received intramuscular injections with preF-mRNA-LNPs (0.5 µg/animal) at weeks 0 and 3. Serum was collected 4 weeks after the second immunization and anti-RSV preF IgG titer was measured by ELISA as described above. To measure neutralizing titers, sera were heat inactivated and serially diluted into a 96-well plate.200 $TCID_{50}$ of RSV/A2 or RSV/B18537 virus were mixed with serial diluted sera at 1:1 ratio and incubated at 37°C for 2h. A 8-point, 3-fold dilution curve was generated for each sample with starting concentration at 1:30. After incubation, Hep2 cells (ATCC/CCL-23) were added to each well (2.5×10^4 cells/well) and the plates were incubated at 37 °C for 5 days. Cells were then washed and fixed with acetone. Each well was then blocked by 5% BSA for 1h at room temperature. After washing with TBST three times, each well were incubated with Human respiratory syncytial virus (RSV) Fusion glycoprotein/RSV-F Neutralizing Antibody (Sino Biological, 11049-R338), followed by HRP-labeled Goat Anti-Rabbit IgG(H+L) (Beyotime Biotechnology, A0208). Next, the wells were washed and 100 µL of TMB substrate solution (Biolegend, 421101) was added for 10 min incubation at room temperature. Then, 100 µL of stop solution (hydrochloric acid) was added. SpectraMax iD3 Reader (Molecular Devices) was used to measure the Optical Density (OD450). Titers were calculated by four-parameter curve fit using GraphPad Prism® 7 software.

Mice were then challenged by RSV/A2 virus on day 43, and euthanized on day 47 for tissue harvesting. Viral titers in lungs were analyzed via plaque assay.

4.10 Histological analysis. Immediately after euthanasia, the left lung lobe was harvested and fixed by submersion in 4% paraformaldehyde for more than 24 h. Following fixation, the lungs were embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E). The blinded pathologist evaluated the H&E stained slides for evidence of peribronchiolitis (inflammatory cell infiltration around the bronchioles), perivasculitis (inflammatory cell infiltration around the small

blood vessels), interstitial pneumonia (inflammatory cell infiltration and thickening of alveolar walls), and alveolitis (cells within the alveolar spaces). Slides were scored on a 0–4 severity scale.

4.11 Toxicity evaluation. To assess the in vivo toxicity of preF-mRNA-LNPs, mice were intramuscularly injected with preF-mRNA-LNPs (0.5 mg/kg of mRNA), with DPBS used as a control. Serum samples were collected 6 h post-injection to measure cytokine levels on ELISA plate reader of IL-1β, TNF-α, IL-6, and IFN-γ, according to the manufacturer's instructions (Elabscience). Sera were obtained at Day 12 after three injections at Day 0, 3, 7 to evaluate the liver and kidney function (Automated Biochemical Analyzer).