Supplementary Information

Thiophene-based lipids for mRNA delivery to pulmonary and retinal tissues

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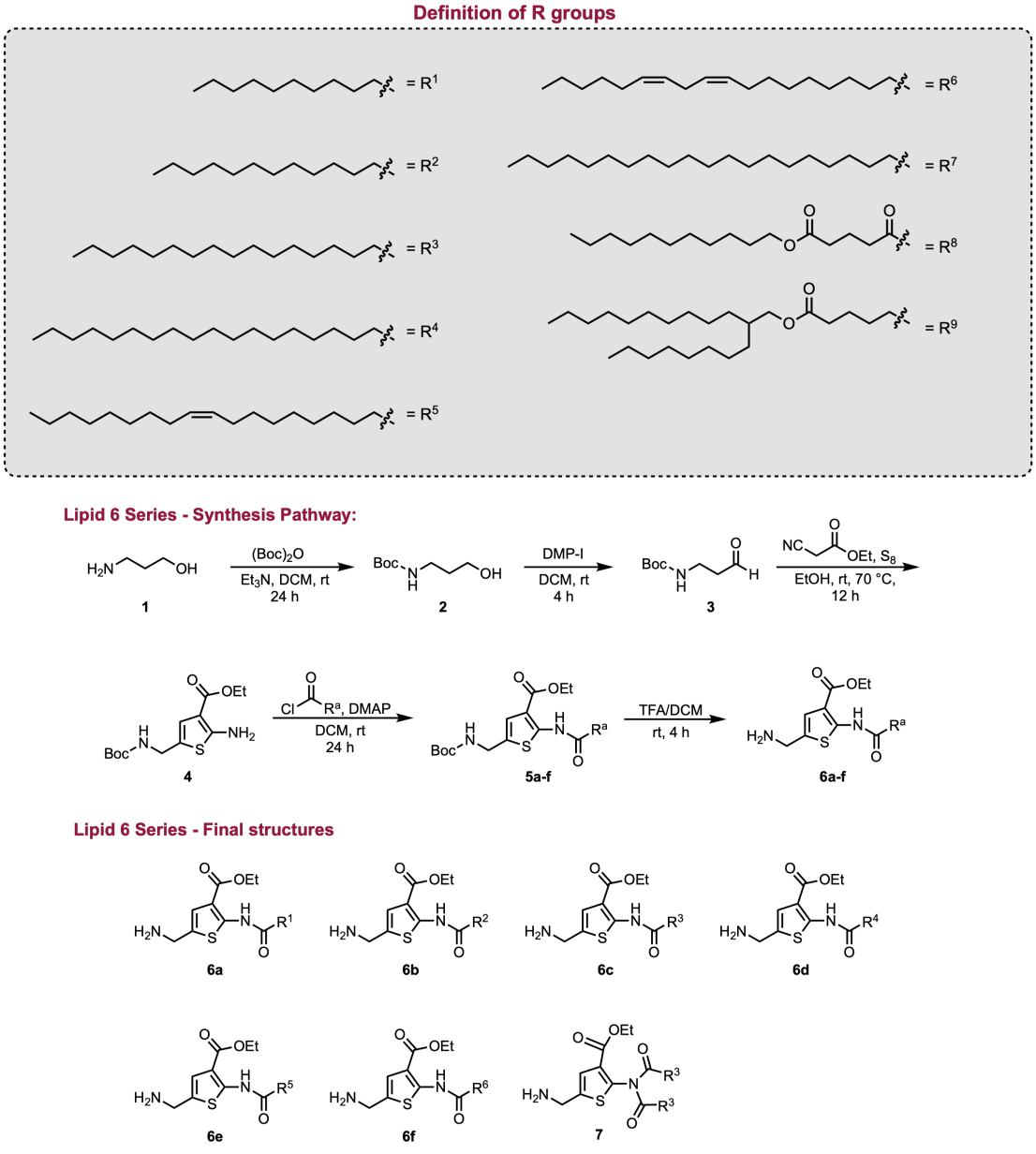
Contents: Description of synthetic scheme and detailed synthesis of key compounds; Materials and Methods; Supplementary Figures and Tables.

Synthesis



Synthesis of 2, *tert*-butyl (3-hydroxypropyl)carbamate: An overnight dried round bottom flask (RBF) was charged with 1-aminopropanol **1** (0.76 mL, 10 mmol) and anhydrous dichloromethane (10 mL). To this was added anhydrous triethylamine (1.67 mL, 12 mmol) under a nitrogen atmosphere and the reaction mixture was cooled to 0 °C using an ice bath. To this di-*tert*-butyl dicarbonate (2.18 gm, 10 mmol) was added in portions. The reaction mixture was allowed to warm to room temperature and stirred for an additional 24 hours under a nitrogen atmosphere. Subsequently, the reaction mixture was diluted with dichloromethane (50 mL), washed with 0.1 M hydrochloric acid (10 mL x 2), water (10 mL), and brine (10 mL), dried over $MgSO_4$, and concentrated under vacuum to yield a colorless oil (900 mg, yield 51%). The NMR matched the literature.

Synthesis of 3, *tert*-butyl (3-oxopropyl)carbamate: An overnight dried round bottom flask (RBF) was charged with compound **2** (0.82 gm, 4.68 mmol) and anhydrous dichloromethane (10 mL). The reaction mixture was cooled to 0 °C under a nitrogen atmosphere. To this, Dess-Martin periodinane (2.38 gm, 5.6 mmol) was added in portions over 10 minutes. The reaction mixture was stirred at 0 °C for 15 minutes, then warmed to room temperature and stirred for an additional 4 hours. Following this, the reaction mixture was diluted with diethyl ether (100 mL), 10% sodium thiosulphate solution (30 mL), and saturated sodium bicarbonate solution (30 ml). The resulting suspension was stirred vigorously until the precipitate was fully dissolved. The organic and aqueous layers were separated, and the aqueous layer was extracted with diethyl ether (2 x 60 mL). The organic layers were combined and washed with 10% sodium thiosulphate solution (2 x 30 mL), saturated sodium bicarbonate solution (2 x 30 mL), and brine (30 mL), dried over MgSO4, and concentrated in vacuo to produce a slightly yellow oil (580 mg, yield 70%) which was used without further purification.

Synthesis of 4, ethyl 2-amino-5-(((*tert*-butoxycarbonyl)amino)methyl)thiophene-3-carboxylate: To an over-night dried RBF was added **3** (580 mg, 3.35 mmol), sulfur (107.2 mg, 3.35 mmol), and ethyl cyanoacetate (0.35 mL, 3.35 mmol) in anhydrous ethanol (35 mL). The reaction mixture was stirred at room temperature for 30 minutes. Following this, morpholine (0.35 mL, 4 mmol) was added, and the reaction mixture was allowed to stir for an additional 15 minutes at room temperature. After this, the reaction mixture was heated at 70 °C for 12 hours. The reaction mixture was cooled, and the solvent was removed under reduced pressure. The crude was purified using SiO_2 chromatography to produce ethyl 2-amino-5-(((*tert*-butoxycarbonyl)amino)methyl)thiophene-3-carboxylate (**4**) as a yellow semi-solid (571 mg, yield 59%).

General protocol for the acylation reaction (Procedure A): A dry 100 mL round-bottomed flask was charged with a suitable carboxylic acid (2 mmol) and 10 mL of DCM. The reaction mixture was cooled with an ice bath to 0 °C. A 100 µL volume of anhydrous DMF was added, followed by dropwise addition of neat oxalyl chloride (2.4 mmol) over 5 minutes. The reaction was stirred at room temperature for 4 hours. The solvent was removed under reduced pressure. Excess oxalyl chloride was azeotropically removed with 2 × 5 mL portions of DCM under reduced pressure. The crude acyl chloride was used without further purification. The crude acyl chloride was dissolved into 10 mL of DCM, and the solution was cooled with an ice bath to 0 °C. To this, DMAP (5 mg, catalytic) and an amine (300 mg, 1 mmol) in 3 mL of DCM were added under nitrogen atmosphere. The reaction was stirred at 0 °C for 15 minutes, after which triethylamine (1 mL, excess) was added in portions. The ice bath was removed after 10 minutes, and the reaction was permitted to warm to room temperature. The reaction was then stirred at room temperature for 24 hours and the completion of the reaction was determined by TLC. The solvent was removed under reduced pressure. The crude product was purified using SiO_2 chromatography using hexane/ethyl acetate (EA) as a solvent.

Note: During the acylation reaction, bis-acylation compound (imide **7**, in lipid series 6 and imide **11a-d**, in lipid series 12 and 13) was isolated as minor by-product (5-12%). This by-product can be easily removed by column chromatography. The imide byproduct generation could further be minimized by reducing the reaction duration to 8-12 hours.

Synthesis of 5a-:

5a, ethyl 5-(((*tert*-butoxycarbonyl)amino)methyl)-2-octanamidothiophene-3-carboxylate was synthesized using the general procedure A from octanoyl chloride (324 mg, 2 mmol) [synthesized from octanoic acid (288 mg, 2.0 mmol), 100 µL DMF, oxalyl chloride (205 µL, 2.4 mmol), and DCM (10 mL)], analog **4** (300 mg, 1 mmol), DMAP (5 mg, catalytic), triethylamine (1 mL, excess), and DCM (13 mL). The crude product was isolated on SiO_2 using 5:1 hexane/EA to give 188 mg (44 %) of a yellow oil.

5b, ethyl 5-(((*tert*-butoxycarbonyl)amino)methyl)-2-dodecanamidothiophene-3-carboxylate was synthesized using the general procedure A from dodecanoyl chloride (436 mg, 2 mmol) [synthesized from dodecanoic acid (400 mg, 2.0 mmol), 100 µL DMF, oxalyl chloride (205 µL, 2.4 mmol), and DCM (10 mL)], analog **4** (300 mg, 1 mmol), DMAP (5 mg, catalytic), triethylamine (1 mL, excess), and DCM (13 mL). The crude product was isolated on SiO_2 using 5:1 hexane/EA to give 146 mg (30 %) of a yellow powder.

5c, ethyl 5-(((*tert*-butoxycarbonyl)amino)methyl)-2-palmitamidothiophene-3-carboxylate was synthesized using the general procedure A from palmitoyl chloride (548 mg, 2 mmol) [synthesized from palmitic acid (512 mg, 2.0 mmol), 100 µL DMF, oxalyl chloride (205 µL, 2.4 mmol), and DCM (10 mL)], analog **4** (300 mg, 1 mmol), DMAP (5 mg, catalytic), triethylamine (1 mL, excess), and DCM (13 mL). The crude product was isolated on SiO_2 using 5:1 hexane/EA to give 192 mg (35 %) of a yellow powder.

5d, ethyl 5-(((*tert*-butoxycarbonyl)amino)methyl)-2-stearamidothiophene-3-carboxylate was synthesized using the general procedure A from stearoyl chloride (606 mg, 2 mmol) [synthesized from stearic acid (569 mg, 2.0 mmol), 100 µL DMF, oxalyl chloride (205 µL, 2.4 mmol), and DCM (10 mL)], analog **4** (300 mg, 1 mmol), DMAP (5 mg, catalytic), triethylamine (1 mL, excess), and DCM (13 mL). The crude product was isolated on SiO_2 using 5:1 hexane/EA to give 155 mg (27 %) of a bright-yellow powder.

5e, ethyl 5-(((*tert*-butoxycarbonyl)amino)methyl)-2-oleamidothiophene-3-carboxylate was synthesized using the general procedure A from oleoyl chloride (602 mg, 2 mmol) [synthesized from oleic acid (565 mg, 2.0 mmol), 100 µL DMF, oxalyl chloride (205 µL, 2.4 mmol), and DCM (10 mL)], analog **4** (300 mg, 1 mmol), DMAP (5 mg, catalytic), triethylamine (1 mL, excess), and DCM (13 mL). The crude product was isolated on SiO_2 using 5:1 hexane/EA to give 169 mg (30 %) of a yellow oil.

5f, ethyl 5-(((*tert*-butoxycarbonyl)amino)methyl)-2-((9*Z*,12*Z*)-octadeca-9,12-dienamido)thiophene-3-carboxylate was synthesized using the general procedure A from linoleyl chloride (598 mg, 2 mmol) [synthesized from linoleic acid (561 mg, 2.0 mmol), 100 µL DMF, oxalyl chloride (205 µL, 2.4 mmol), and DCM (10 mL)], analog **4** (300 mg, 1 mmol), DMAP (5 mg, catalytic), triethylamine (1 mL, excess), and DCM (13 mL). The crude product was isolated on SiO_2 using 5:1 hexane/EA to give 121 mg (21 %) of a yellow oil.

5g, ethyl 5-(((*tert*-butoxycarbonyl)amino)methyl)-2-(*N*-palmitoylpalmitamido) thiophene-3-carboxylate was isolated as a by-product during the synthesis of **8c** as a yellow oil, 35 mg (4 %).

Synthesis of 6a-f and 7:

General protocol for the Boc-deprotection (Procedure B): In an overnight dried round bottom flask was added Boc-protected amines **5** and TFA:DCM (1:1, 6 mL) in a nitrogen atmosphere. The reaction was stirred at room temperature for 4 hours, after which the solvent was removed under reduced pressure to produce TFA salt of the desired amine.

6a, ethyl 5-(aminomethyl)-2-octanamidothiophene-3-carboxylate TFA salt was synthesized using the general procedure B from **5a** (50 mg, 0.11 mmol) and TFA:DCM (1:1, 6 mL). Removal of the solvent produced 46 mg (90 %) of a light-brown powder.

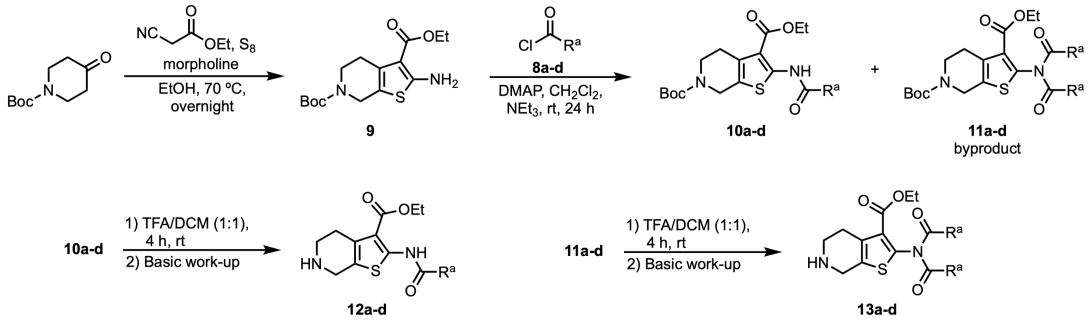
6b, ethyl 5-(aminomethyl)-2-dodecanamidothiophene-3-carboxylate TFA salt was synthesized using the general procedure B from **5b** (50 mg, 0.10 mmol) and TFA:DCM (1:1, 6 mL). Removal of the solvent produced 47 mg (91 %) of a light-yellow powder.

6c, ethyl 5-(aminomethyl)-2-palmitamidothiophene-3-carboxylate TFA salt was synthesized using the general procedure B from **5c** (50 mg, 0.09 mmol) and TFA:DCM (1:1, 6 mL). Removal of the solvent produced 44 mg (86 %) of a white powder.

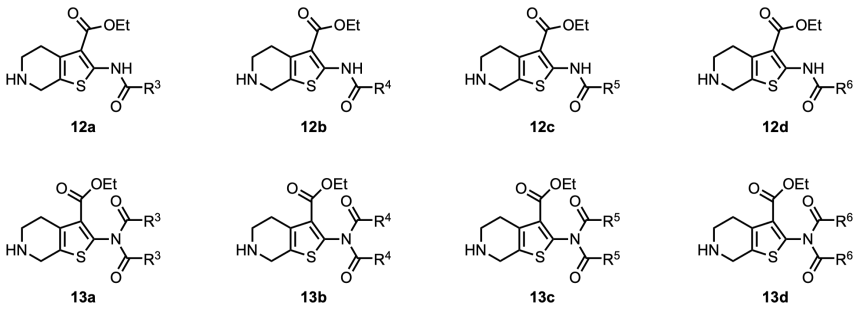
6d, ethyl 5-(aminomethyl)-2-stearamidothiophene-3-carboxylate TFA salt was synthesized using the general procedure B from **5d** (50 mg, 0.09 mmol) and TFA:DCM (1:1, 6 mL). Removal of the solvent produced 49 mg (95 %) of a yellow powder.

6e, ethyl 5-(aminomethyl)-2-oleamidothiophene-3-carboxylate TFA salt was synthesized using the general procedure B from **5e** (50 mg, 0.09 mmol) and TFA:DCM (1:1, 6 mL). Removal of the solvent produced 50 mg (98 %) of a yellow powder.
6f, ethyl 5-(aminomethyl)-2-((9*Z*,12*Z*)-octadeca-9,12-dienamido)thiophene-3-carboxylate TFA salt was synthesized using the general procedure B from **5f** (50 mg, 0.09 mmol) and TFA:DCM (1:1, 6 mL). Removal of the solvent produced 50 mg (98 %) of a yellow powder.
7, ethyl 5-(aminomethyl)-2-(*N*-palmitoylpalmitamido)thiophene-3-carboxylate TFA salt was synthesized using the general procedure B from **5g** (50 mg, 0.06 mmol) and TFA:DCM (1:1, 6 mL). Removal of the solvent produced 48 mg (94 %) of a yellow powder.

Lipid Series 12 and 13 - Synthesis Pathway:



Lipid Series 12 and 13 - Final Structures:



Synthesis of 9, 6-(*tert*-butyl)-3-ethyl 2-amino-4,7-dihydrothieno[*2,3-c*] pyridine-3,6(*5H*)-dicarboxylate: A 250 mL one-neck round bottom flask was charged with ethyl cyanoacetate (3.2 mL, 24 mmol), *tert*-butyl 4-oxopiperidine-1-carboxylate (3.7 g, 20 mmol), sulfur (768 mg, 24 mmol), morpholine (5 mL, excess), and ethanol (50 mL). The mixture was stirred at 70 °C for 12 hours. The reaction mixture was cooled, filtered, and washed with cold EtOH followed by water. The solid collected was dried to yield 5.35 g (82 %) of a light yellow crystalline solid. ^{1}H NMR (400 MHz, CDCl3): δ 6.06 (s, 2H), 4.36 (s, 2H), 4.29 (q, *J* = 7.2 Hz, 2H), 3.63 (t, 2H), 2.82 (s, 2H), 1.49 (s, 9H), 1.35 (t, *J* = 7.2 Hz, 3H), matching the literature.

Synthesis of 10a-d:
10a, 6-(*tert*-butyl) 3-ethyl 2-palmitamido-4,7-dihydrothieno[2,3-*c*]pyridine-3,6(5*H*)-dicarboxylate was synthesized using the general procedure A from palmitoyl chloride (**8a**, 548 mg, 2 mmol) [synthesized from palmitic acid (512 mg, 2.0 mmol), 100 µL DMF, oxalyl chloride (205 µL, 2.4 mmol), and DCM (10 mL)], analog **9** (326 mg, 1 mmol), DMAP (5 mg, catalytic), triethylamine (1 mL, excess), and DCM (13 mL). The crude product was isolated on SiO_2 using 5:1 hexane/EA to give 212 mg (37 %) of a light-yellow oil.

10b, 6-(*tert*-butyl) 3-ethyl 2-stearamido-4,7-dihydrothieno[2,3-*c*]pyridine-3,6(5*H*)-dicarboxylate was synthesized using the general procedure A from stearoyl chloride (**8b**, 606 mg, 2 mmol) [synthesized from stearic acid (569 mg, 2.0 mmol), 100 µL DMF, oxalyl chloride (205 µL, 2.4 mmol), and DCM (10 mL)], analog **9** (326 mg, 1 mmol), DMAP (5 mg, catalytic), triethylamine (1 mL, excess), and DCM (13 mL). The crude product was isolated on SiO_2 using 5:1 hexane/EA to give 208 mg (35 %) of a yellow film.
10c, 6-(*tert*-butyl) 3-ethyl 2-oleamido-4,7-dihydrothieno[2,3-*c*]pyridine-3,6(5*H*)-dicarboxylate was synthesized using the general procedure A from oleoyl chloride (**8c**, 602 mg, 2 mmol) [synthesized from oleic acid (565 mg, 2.0 mmol), 100 µL DMF, oxalyl chloride (205 µL, 2.4 mmol), and DCM (10 mL)], analog **9** (326 mg, 1 mmol), DMAP (5 mg, catalytic), triethylamine (1 mL, excess), and DCM (13 mL). The crude product was isolated on SiO_2 using 5:1 hexane/EA to give 200 mg (34 %) of a yellow oil.
10d, 6-(*tert*-butyl) 3-ethyl 2-((9*Z*,12*Z*)-octadeca-9,12-dienamido)-4,7-dihydrothieno[2,3-*c*]pyridine-3,6(5*H*)-dicarboxylate was synthesized using the general procedure A from linoleyl chloride (**8d**, 598 mg, 2 mmol) [synthesized from linoleic acid (561 mg, 2.0 mmol), 100 µL DMF, oxalyl chloride (205 µL, 2.4 mmol), and DCM (10 mL)], analog **9** (326 mg, 1 mmol), DMAP (5 mg, catalytic), triethylamine (1 mL, excess), and DCM (13 mL). The crude product was isolated on SiO_2 using 5:1 hexane/EA to give 194 mg (33 %) of a yellow oil.

Synthesis of 11a-d:
11a, 6-(*tert*-butyl) 3-ethyl 2-(*N*-palmitoylpalmitamido)-4,7-dihydrothieno[2,3-*c*]pyridine-3,6(5*H*)-dicarboxylate was isolated as a by-product during the synthesis of **10a** as a yellow film, 102 mg (12 %).
11b, 6-(*tert*-butyl) 3-ethyl 2-(*N*-stearoylstearamido)-4,7-dihydrothieno[2,3-*c*]pyridine-3,6(5*H*)-dicarboxylate was isolated as a by-product during the synthesis of **10b** as a yellow powder, 68 mg (8 %).
11c, 6-(*tert*-butyl) 3-ethyl 2-(*N*-oleoyloleamido)-4,7-dihydrothieno[2,3-*c*]pyridine-3,6(5*H*)-dicarboxylate was isolated as a by-product during the synthesis of **10c** as a yellow oil, 96 mg (11 %).
11d, 6-(*tert*-butyl) 3-ethyl 2-((9*Z*,12*Z*)-*N*-((9*Z*,12*Z*)-octadeca-9,12-dienoyl)octadeca-9,12-dienamido)-4,7-dihydrothieno[2,3-*c*]pyridine-3,6(5*H*)-dicarboxylate was isolated as a by-product during the synthesis of **10d** as a yellow oil, 90 mg (10 %).

Synthesis of 12a-d:
12a, ethyl 2-palmitamido-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylate was synthesized using the general procedure B from **10a** (100 mg, 0.17 mmol) and TFA:DCM (1:1, 6 mL). The resulting solution was diluted with DCM (20 mL), washed with 0.5 N NaOH (pH ~ 12) and brine (10 mL), dried over $MgSO_4$, and concentrated under vacuum to yield 62 mg (75 %) of a white powder. **MS** (ESI^+) m/z: $[M + H]^+$ Calculated for $C_{26}H_{45}N_2O_3S^+$ 465.3; Found 465.6.
12b, ethyl 2-stearamido-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylate was synthesized using the general procedure B from **10b** (100 mg, 0.17 mmol) and TFA:DCM (1:1, 6 mL). The resulting solution was diluted with DCM (20 mL), washed with 0.5 N NaOH (pH ~ 12), and brine (10 mL), dried over $MgSO_4$, and concentrated under vacuum to yield 56 mg (67 %) of a yellow powder.
12c, ethyl 2-oleamido-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylate was synthesized using the general procedure B from **10c** (100 mg, 0.17 mmol) and TFA:DCM (1:1, 6 mL). The resulting solution was diluted with DCM (20 mL), washed with 0.5 N NaOH (pH ~ 12) and brine (10 mL), dried over $MgSO_4$, and concentrated under vacuum to yield 65 mg (78 %) of a yellow film.
12d, ethyl 2-((9*Z*,12*Z*)-octadeca-9,12-dienamido)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylate was synthesized using the general procedure B from **10d** (100 mg, 0.17 mmol) and

TFA:DCM (1:1, 6 mL). The resulting solution was diluted with DCM (20 mL), washed with 0.5 N NaOH (pH ~ 12), and brine (10 mL), dried over $MgSO_4$, and concentrated under vacuum to yield 48 mg (58 %) of a yellow oil. **MS** (ESI^+) m/z: $[M + H]^+$ Calculated for $C_{46}H_{83}N_2O_4S^+$ 489.3; Found 489.6.

Synthesis of 13a-d:

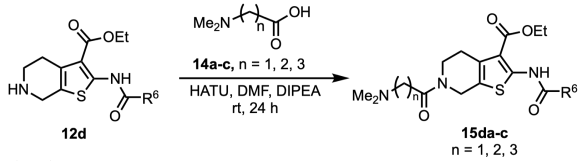
13a, ethyl 2-(*N*-palmitoylpalmitamido)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylate was synthesized using the general procedure B from **11a** (100 mg, 0.12 mmol) and TFA:DCM (1:1, 6 mL). The resulting solution was diluted with DCM (20 mL), washed with 0.5 N NaOH (pH ~ 12) and brine (10 mL), dried over $MgSO_4$, and concentrated under vacuum to yield 68 mg (78 %) of a yellow powder. **MS** (ESI^+) m/z: $[M + H]^+$ Calculated for $C_{42}H_{75}N_2O_4S^+$ 703.5; Found 704.0.

13b, ethyl 2-(*N*-stearoylstearamido)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylate was synthesized using the general procedure B from **11b** (100 mg, 0.11 mmol) and TFA:DCM (1:1, 6 mL). The resulting solution was diluted with DCM (20 mL), washed with 0.5 N NaOH (pH ~ 12), and brine (10 mL), dried over $MgSO_4$, and concentrated under vacuum to yield 62 mg (70 %) of a yellow powder. **MS** (ESI^+) m/z: $[M + H]^+$ Calculated for $C_{46}H_{83}N_2O_4S^+$ 759.6; Found 760.0.

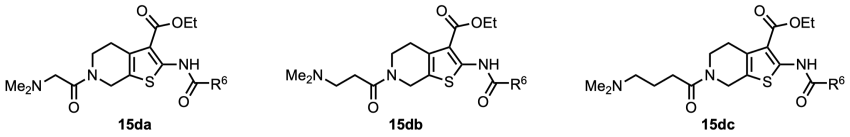
13c, ethyl 2-(*N*-oleoyloleamido)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylate was synthesized using the general procedure B from **11c** (100 mg, 0.11 mmol) and TFA:DCM (1:1, 6 mL). The resulting solution was diluted with DCM (20 mL), washed with 0.5 N NaOH (pH ~ 12) and brine (10 mL), dried over $MgSO_4$, and concentrated under vacuum to yield 66 mg (75 %) of a yellow powder.

13d, ethyl 2-((9*Z*,12*Z*)-*N*-((9*Z*,12*Z*)-octadeca-9,12-dienoyl)octadeca-9,12-dienamido)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylate was synthesized using the general procedure B from **11d** (100 mg, 0.11 mmol) and TFA:DCM (1:1, 6 mL). The resulting solution was diluted with DCM (20 mL), washed with 0.5 N NaOH (pH ~ 12), and brine (10 mL), dried over $MgSO_4$, and concentrated under vacuum to yield 70 mg (79 %) of a yellow oil.

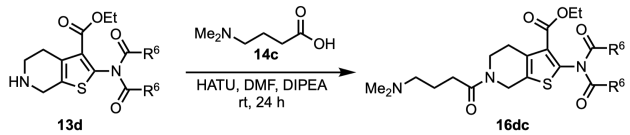
Lipid Series 15 - Synthesis Pathway:



Lipid Series 15 - Final structures:



Lipid 16dc Preparation:



General protocol for the amide formation using HATU coupling (Procedure C): A solution of the amine (0.2 mmol), acid (0.6 mmol), DIPEA (105 µl, 0.6 mmol) in DMF (under argon) was stirred for 15 minutes at room temperature in a 25 mL RBF (overnight dried) under nitrogen

atmosphere. HATU (228 mg, 0.6 mmol) was then added, and the reaction was allowed to stir for 24 hours (reaction progress monitored by TLC). The reaction mixture was diluted with water (30 mL) and extracted with DCM (15 mL × 5). The organic layers were combined, washed with brine (30 mL), and dried over $MgSO_4$. The solvent was removed, and the crude was then purified by SiO_2 flash chromatography (Biotage® Isolera™) to obtain the desired product. For basic amines, a solvent system comprising of 1-9% methanol/DCM (1 % of 7N NH_3 additive) was used. For neutral analogs, a solvent system comprising of hexanes/ethyl acetate (6-50%) was used.

Synthesis of 15da-c:

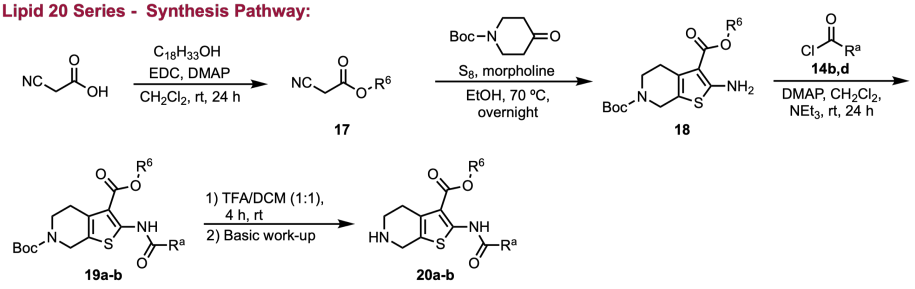
15da, ethyl 6-(dimethylglycyl)-2-((9*Z*,12*Z*)-octadeca-9,12-dienamido)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylate: Using the general procedure C, with **15d** (98 mg, 0.2 mmol), *N*,*N*-Dimethylglycine (**14a**, 62 mg, 0.6 mmol), DIPEA (105 µl, 0.6 mmol), HATU (228 mg, 0.6 mmol), and DMF (4mL), **17da** was prepared as a yellow solid, 28 mg (24 %).

15db, ethyl 6-(3-(dimethylamino)propanoyl)-2-((9*Z*,12*Z*)-octadeca-9,12-dienamido)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylate: Using the general procedure C, with **15d** (98 mg, 0.2 mmol), 3-(Dimethylamino)propionic acid hydrochloride (**14b**, 92 mg, 0.6 mmol), DIPEA (105 µl, 0.6 mmol), HATU (228 mg, 0.6 mmol), and DMF (4mL), **17db** was prepared as a yellow oil, 32 mg (27 %). **MS** (ESI^+) m/z: $[M + H]^+$ Calculated for $C_{33}H_{54}N_3O_4S^+$ 588.4; Found 588.6.

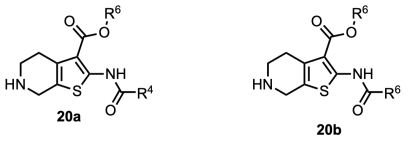
15dc, ethyl 6-(4-(dimethylamino)butanoyl)-2-((9*Z*,12*Z*)-octadeca-9,12-dienamido)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylate: Using the general procedure C, with **15d** (98 mg, 0.2 mmol), 4-(dimethylamino)butanoic acid hydrochloride (**14c,** 100 mg, 0.6 mmol), DIPEA (105 µl, 0.6 mmol), HATU (228 mg, 0.6 mmol), and DMF (4mL), **17dc** was prepared as a yellow oil, 41 mg (34 %). **MS** (ESI^+) m/z: $[M + H]^+$ Calculated for $C_{34}H_{56}N_3O_4S^+$ 602.4; Found 602.7.

Synthesis of 16dc, ethyl 6-(4-(dimethylamino)butanoyl)-2-((9*Z*,12*Z*)-*N*-((9*Z*,12*Z*)-octadeca-9,12-dienoyl)octadeca-9,12-dienamido)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylate: Using the general procedure C, with **13d** (150 mg, 0.2 mmol), 4-(dimethylamino)butanoic acid hydrochloride (100 mg, 0.6 mmol), DIPEA (105 µl, 0.6 mmol), HATU (228 mg, 0.6 mmol), and DMF (4mL), **16dc** was prepared as a yellow oil, 74 mg (43 %).

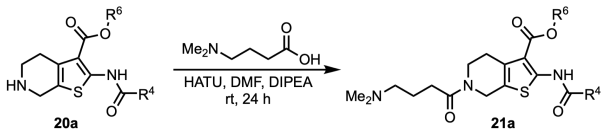
Lipid 20 Series - Synthesis Pathway:



Lipid 20 Series - Final Structures:



Lipid 21 Series - Preparation:



General protocol for EDC coupling (Procedure D): An oven-dried round-bottomed flask was charged with a suitable acid (1 eq.), amine (1.2 eq.), DMAP (catalytic), and DCM at room temperature under nitrogen atmosphere. The reaction was allowed to stir for 15 minutes, then EDC (*N*-(3- Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride, 1.2 eq.) was added. The reaction mixture was stirred for an additional 24 hours at room temperature. After this, the crude was dissolved in 50 mL of DCM and washed with 3 × 20 mL of 0.5 N HCl, 2 × 20 mL of water, followed by brine wash. The crude was dried over sodium sulfate and purified using SiO_2 flash chromatography (Biotage® Isolera™) with 10-50 % of hexane/ethyl acetate to give rise to the final compound.

Synthesis of 17, (9*Z*,12*Z*)-octadeca-9,12-dien-1-yl 2-cyanoacetate: Using the general procedure D, with cyanoacetic acid (2.9 gm, 35 mmol), linoleyl alcohol (14 mL, 45.5 mmol), DMAP (280 mg, 2.3 mmol), EDC (8 gm, 42 mmol), and DCM (100 mL), **17** was prepared as a yellow powder, 9.3 gm (80 %).

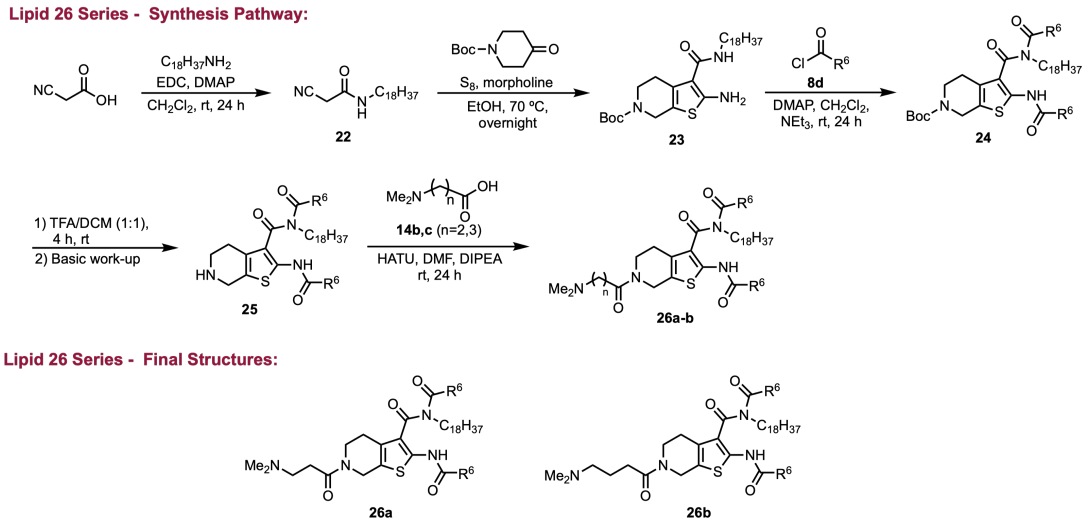
Synthesis of 18, 6-(*tert*-butyl) 3-((9*Z*,12*Z*)-octadeca-9,12-dien-1-yl) 2-amino-4,7-dihydrothieno[2,3-*c*]pyridine-3,6(5*H*)-dicarboxylate: A 250 mL one-neck round bottom flask was charged with **17** (1.6 gm, 4.8 mmol), *tert*-butyl 4-oxopiperidine-1-carboxylate (960 g, 4.8 mmol), sulfur (154 mg, 4.8 mmol), morpholine (1 mL, excess), and ethanol (25 mL). The mixture was stirred at 70 ^{o}C for 12 hours. The reaction mixture was cooled, and the solvent was evaporated under reduced pressure to give rise to the crude, which was purified using SiO_2 flash chromatography (Biotage® Isolera™) with 6-25 % of hexane/ethyl acetate to give rise to **18**, (2.2 gm, 84 %) as a yellow oil.

Synthesis of 19a-b:
19a, 6-(*tert*-butyl) 3-((9*Z*,12*Z*)-octadeca-9,12-dien-1-yl) 2-stearamido-4,7-dihydrothieno[2,3-*c*]pyridine-3,6(5*H*)-dicarboxylate was synthesized using the general procedure A from stearoyl chloride (606 mg, 2 mmol) [synthesized from stearic acid (569 mg, 2.0 mmol), 100 µL DMF, oxalyl chloride (205 µL, 2.4 mmol), and DCM (10 mL)], analog **18** (547 mg, 1 mmol), DMAP (5 mg, catalytic), triethylamine (1 mL, excess), and DCM (13 mL). The crude product was purified using SiO_2 flash chromatography (Biotage® Isolera™) with 6-25 % of hexane/ethyl acetate to give rise to **19a**, (340 mg, 42 %) as a yellow powder.
19b, 6-(*tert*-butyl) 3-((9*Z*,12*Z*)-octadeca-9,12-dien-1-yl) 2-((9*Z*,12*Z*)-octadeca-9,12-dienamido)-4,7-dihydrothieno[2,3-*c*]pyridine-3,6(5*H*)-dicarboxylate was synthesized using the general procedure A from linoleyl chloride (**14d**, 598 mg, 2 mmol) [synthesized from linoleic acid (561 mg, 2.0 mmol), 100 µL DMF, oxalyl chloride (205 µL, 2.4 mmol), and DCM (10 mL)], analog **18** (547 mg, 1 mmol), DMAP (5 mg, catalytic), triethylamine (1 mL, excess), and DCM (13 mL). The crude product was purified using SiO_2 flash chromatography (Biotage® Isolera™) with 6-25 % of hexane/ethyl acetate to give rise to **19b**, (356 mg, 44 %) as a yellow oil.

Synthesis of 20a-b:
20a, (9*Z*,12*Z*)-octadeca-9,12-dien-1-yl 2-stearamido-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylate was synthesized using the general procedure B from **19a** (713 mg, 1 mmol) and TFA:DCM (1:1, 6 mL). The resulting solution was diluted with DCM (20 mL), washed with 0.5 N NaOH (pH ~ 12), and brine (50 mL), dried over $MgSO_4$, and concentrated under vacuum to yield 600 mg (84 %) of a yellow powder.
20b, (9*Z*,12*Z*)-octadeca-9,12-dien-1-yl 2-((9*Z*,12*Z*)-octadeca-9,12-dienamido)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylate was synthesized using the general procedure B from **19b** (709 mg, 1 mmol) and TFA:DCM (1:1, 6 mL). The resulting solution was diluted with

DCM (20 mL), washed with 0.5 N NaOH (pH ~ 12), and brine (50 mL), dried over $MgSO_4$, and concentrated under vacuum to yield 612 mg (86 %) of a yellow oil. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 11.26 (s, 1H), 5.41 – 5.27 (m, 8H), 4.25 (t, *J* = 6.6 Hz, 2H), 3.90 (t, *J* = 1.8 Hz, 2H), 3.09 (s, 2H), 2.81 – 2.71 (m, 6H), 2.45 (t, *J* = 7.6 Hz, 2H), 2.24 (s, 1H), 2.03 (q, *J* = 6.9 Hz, 8H), 1.78 – 1.66 (m, 4H), 1.44 – 1.22 (m, 30H), 0.91 – 0.83 (m, 6H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 170.2, 166.6, 148.2, 130.3, 130.3, 130.1, 130.1, 129.4, 128.1, 128.1, 128.0, 127.9, 125.4, 111.3, 64.8, 44.3, 43.5, 36.9, 31.6, 29.7, 29.7, 29.5, 29.4, 29.4, 29.3, 29.3, 29.2, 29.2, 28.8, 27.5, 27.3, 27.3, 26.2, 25.7, 25.3, 22.7, 14.1. **MS** (ESI^+) m/z: $[M + H]^+$ Calculated for $C_{44}H_{73}N_2O_3S^+$ 709. 5; Found 709.9.

Synthesis of 21a, (9*Z*,12*Z*)-octadeca-9,12-dien-1-yl 6-(4-(dimethylamino)butanoyl)-2-((9*Z*,12*Z*)-octadeca-9,12-dienamido)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylate: Using the general procedure C, with **20a** (214 mg, 0.3 mmol), 4-(dimethylamino)butanoic acid hydrochloride (167 mg, 1 mmol), DIPEA (175 µl, 1 mmol), HATU (380 mg, 1 mmol), and DMF (8 mL), **21a** was prepared as a yellow powder, 106 mg (43 %).



Synthesis of 22, 2-cyano-*N*-octadecylacetamide: Using the general procedure D, with cyanoacetic acid (2.98 gm, 35 mmol), stearyl amine (11.1g, 35 mmol), DMAP (280 mg, 2.3 mmol), EDC (8 gm, 42 mmol), and DCM (100 mL), **22** was prepared as a yellow crystalline powder, 4.8 gm (41 %).

Synthesis of 23, *tert*-butyl 2-amino-3-(octadecylcarbamoyl)-4,7-dihydrothieno[2,3-*c*]pyridine-6(5*H*)-carboxylate : A 250 mL one-neck round bottom flask was charged with **22** (2.6 gm, 7.7 mmol), *tert*-butyl 4-oxopiperidine-1-carboxylate (1.4 g, 7 mmol), sulfur (246 mg, 7.7 mmol), morpholine (1.5 mL, excess), and ethanol (20 mL). The mixture was stirred at 70 °C for 12 hours. The reaction mixture was cooled, and the solvent was evaporated under reduced pressure to give rise to the crude, which was purified using SiO2 flash chromatography (Biotage® Isolera™) with 10-50 % of hexane/ethyl acetate to give rise to **23**, (2.8 gm, 72 %) as a yellow powder.

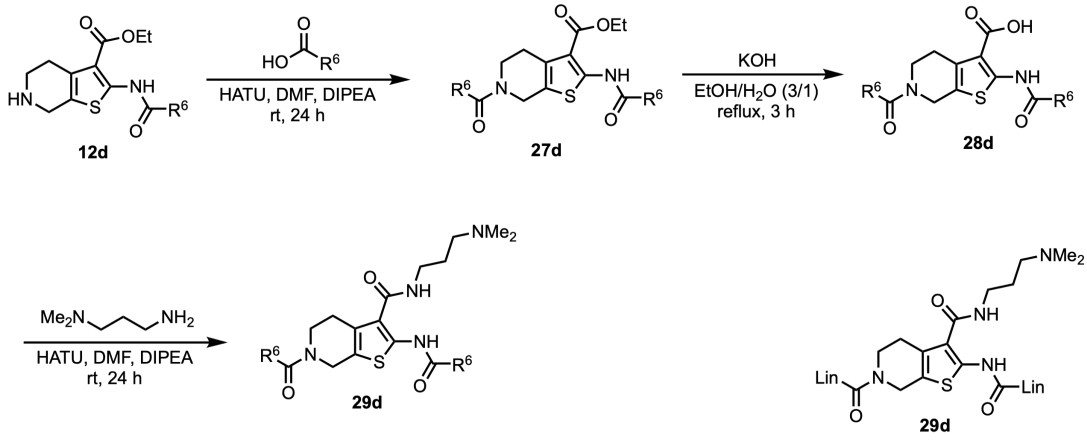
Synthesis of 24, *tert*-butyl 2-((9*Z*,12*Z*)-octadeca-9,12-dienamido)-3-(((9*Z*,12*Z*)-octadeca-9,12-dienoyl)(octadecyl)carbamoyl)-4,7-dihydrothieno[2,3-*c*]pyridine-6(5*H*)-carboxylate was synthesized using the general procedure A from linoleyl chloride (**14d**, 1495 mg, 5 mmol) [synthesized from linoleic acid (1402 mg, 5 mmol), 100 µL DMF, oxalyl chloride (513 µL, 6 mmol),

and DCM (50 mL)], analog **23** (550 mg, 1 mmol), DMAP (5 mg, catalytic), triethylamine (1 mL, excess), and DCM (25 mL). The crude product was isolated on SiO_2 using 10-50 % hexane/EA to give 648 mg (60 %) of a yellow semi-solid.

Synthesis of 25, 2-((9*Z*,12*Z*)-octadeca-9,12-dienamido)-*N*-((9*Z*,12*Z*)-octadeca-9,12-dienoyl)-*N*-octadecyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxamide was synthesized using the general procedure B from **24** (600 mg, 0.56 mmol) and TFA:DCM (1:1, 6 mL). The resulting solution was diluted with DCM (40 mL), washed with 0.5 N NaOH (pH ~ 12), and brine (30 mL), dried over $MgSO_4$, and concentrated under vacuum to yield 512 mg (94 %) of a yellow oil.

Synthesis of 26a-b:
26a, 6-(3-(dimethylamino)propanoyl)-2-((9*Z*,12*Z*)-octadeca-9,12-dienamido)-*N*-((9*Z*,12*Z*)-octadeca-9,12-dienoyl)-*N*-octadecyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-: Using the general procedure C, with **25** (500 mg, 0.51 mmol), 3-(Dimethylamino)propionic acid hydrochloride (153 mg, 1 mmol), DIPEA (175 µl, 1 mmol), HATU (380 mg, 1 mmol), and DMF (8 mL), **26a** was prepared as a yellow film, 212 mg (38 %).
26b, 6-(4-(dimethylamino)butanoyl)-2-((9*Z*,12*Z*)-octadeca-9,12-dienamido)-*N*-((9*Z*,12*Z*)-octadeca-9,12-dienoyl)-*N*-octadecyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxamide: Using the general procedure C, with **25** (500 mg, 0.51 mmol), 4-(dimethylamino)butanoic acid hydrochloride (167 mg, 1 mmol), DIPEA (175 µl, 1 mmol), HATU (380 mg, 1 mmol), and DMF (8 mL), **26b** was prepared as a yellow film, 188 mg (34 %).

Lipid Series 29 - Synthesis Pathway A:



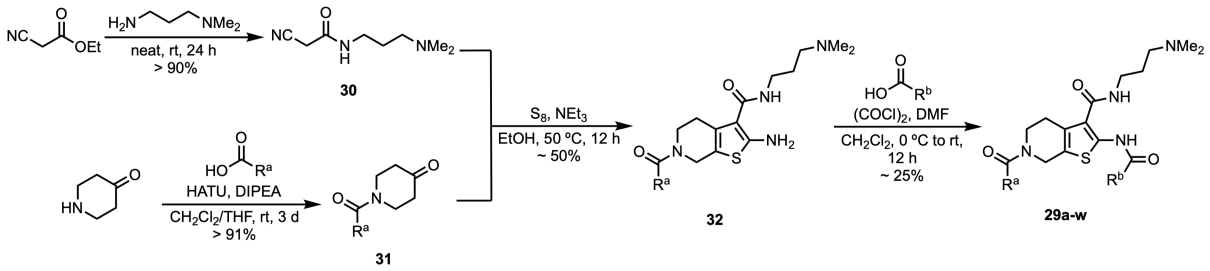
Synthesis of 27d, ethyl 2-((9*Z*,12*Z*)-octadeca-9,12-dienamido)-6-((9*Z*,12*Z*)-octadeca-9,12-dienoyl)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylate: Using the general procedure C, with **12d** (976 mg, 2 mmol), linoleic acid (1.24 mL, 4 mmol), DIPEA (700 µl, 4 mmol), HATU (1.52 gm, 4 mmol), and DMF (12 mL), **27d** was prepared as a white semi-solid, 1.1 gm (73 %).

Synthesis of 28d, 2-((9*Z*,12*Z*)-octadeca-9,12-dienamido)-6-((9*Z*,12*Z*)-octadeca-9,12-dienoyl)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylic acid: An oven-dried 100 mL round bottom flask was charged with **27d** (1.1 gm, 1.46 mmol) and ethanol (12 mL). Then a 10 % solution of potassium hydroxide in water (4.5 mL, 8 mmol) was added, and the reaction was allowed to stir for an additional 15 minutes at room temperature. The reaction mixture was then refluxed for 3 hours. The progress was monitored using TLC. After completion, the reaction was allowed to be cooled, and 1 N HCl (pH ~ 3-4), DCM (25 mL), and water (25 mL) were added. The organic phase was separated and washed with 3 × 20 mL water, followed by brine. The crude

was dried over sodium sulfate and purified using SiO_2 flash chromatography (Biotage® Isolera™) with 10-50 % of hexane/ethyl acetate to give rise to **28d** (308 mg, 29 %) as a light-yellow powder.

Synthesis of 29d, *N*-(3-(dimethylamino)propyl)-2-((9*Z*,12*Z*)-octadeca-9,12-dienamido)-6-((9*Z*,12*Z*)-octadeca-9,12-dienoyl)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxamide: Using the general procedure C, with **28d** (140 mg, 0.2 mmol), N^1,N^1-dimethylpropane-1,3-diamine (60 µl, 0.5 mmol), DIPEA (70 µl, 0.4 mmol), HATU (152 mg, 0.4 mmol), and DMF (4 mL), **29d** was prepared as a colorless oil, 28 mg (17 %).**^{1}H NMR** (400 MHz, $CDCl_3$) δ 12.08 (d, *J* = 61.1 Hz, 1H), 7.55 (d, *J* = 69.0 Hz, 1H), 5.42 – 5.26 (m, 8H), 4.62 (d, *J* = 48.8 Hz, 2H), 3.84 (t, *J* = 5.7 Hz, 1H), 3.69 (t, *J* = 5.7 Hz, 1H), 3.51 (p, *J* = 5.1 Hz, 2H), 2.83 (d, *J* = 5.5 Hz, 1H), 2.76 (dd, *J* = 13.3, 6.6 Hz, 5H), 2.44 (m, 6H), 2.24 (d, *J* = 11.5 Hz, 4H), 2.03 (q, *J* = 7.0 Hz, 8H), 1.68 (m, 6H), 1.41 – 1.21 (m, 30H), 0.87 (t, *J* = 6.7 Hz, 6H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 172.3, 171.8, 170.5, 170.4, 166.0, 165.9, 146.1, 146.1, 130.3, 130.2, 128.2, 128.1, 128.0, 127.7, 125.8, 123.9, 122.3, 113.9, 113.6, 59.3, 59.0, 45.6, 44.6, 43.2, 41.2, 40.4, 40.1, 39.5, 38.7, 36.9, 33.9, 33.7, 31.6, 29.8, 29.7, 29.7, 29.5, 29.5, 29.3, 29.3, 29.2, 27.3, 27.1, 25.9, 25.7, 25.5, 25.4, 25.3, 22.7, 14.2. **HRMS** (ESI$^+$) m/z: $[M + H]^+$ Calculated for $C_{49}H_{83}N_4O_3S^+$ 807.6180; Found 807.6164.

Lipid Series 29 - Synthesis Pathway B:



Synthesis of 30: *N*-(3-(dimethylamino)propyl)-2-isocyanoacetamide **30** was synthesized according to literature procedure (1).

Synthesis of piperidinones 31: A Schlenk was charged with a solution of the desired acid (1.1 equiv.) in anhydrous THF:CH_2Cl_2 (1:1) under N_2-atmosphere. DIPEA (2.5 equiv.) and HATU or HBTU (2.2 equiv.) were added successively and the reaction mixture was stirred for 15 min before the addition of 4-piperidone monohydrate hydrochloride (1.0 equiv.). The reaction mixture was left to stir for 72 h at room temperature. The reaction was quenched with saturated $NaHCO_3$ (aq.) and extracted with CH_2Cl_2 (3x). The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography.

Synthesis of 2-aminothiophenes 32: Gewald Reaction: A Schlenk flask was charged with the 2-isocyanoacetamide **30** (1.0 equiv.), the desired piperidin-4-one **31** (1 equiv.), sulfur (1.0 equiv.), EtOH and NEt_3 (1.0 equiv.) under N_2-atmohphere. The reaction mixture was stirred overnight at 50 ºC. After allowing the reaction mixture to cool down to room temperature the solvent was removed under reduced pressure and the crude residue was purified by column chromatography. To remove the NEt_3-HCl salt the isolated mixture was dissolved in anhydrous THF and solids were filtered off, and the solvent removed under reduced pressure to yield the desired thiophene.

Synthesis of 29a-w: A Schlenk flask was charged with acid (1.0 equiv.) and DMF (0.1 equiv.) in anhydrous CH_2Cl_2 under N_2-atmohphere. Oxalyl chloride (1.5 equiv.) was added and the reaction mixture stirred for 3 h at room temperature. The solvent was removed under reduced pressure

and the crude material was dissolved in CH_2Cl_2. After addition of thiophene (1.0 equiv.) the reaction mixture was stirred overnight at room temperature. The solvent was removed under reduced pressure and the crude residue purified by column chromatography. To remove the NEt_3-HCl salt the isolated mixture was dissolved in anhydrous THF and solids were filtered off, and the solvent removed under reduced pressure to yield the desired thiophene lipid.

29a ($R^a = R^2$, $R^b = R^2$): ***N*-(3-(dimethylamino)propyl)-2-dodecanamido-6-dodecanoyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxamide**. **MS** (ESI^+) m/z: $[M + H]^+$ Calculated for $C_{37}H_{67}N_4O_3S^+$ 647.5; Found 647.4.

29b ($R^a = R^2$, $R^b = R^3$): ***N*-(3-(dimethylamino)propyl)-6-dodecanoyl-2-palmitamido-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxamide**. **MS** (ESI^+) m/z: $[M + H]^+$ Calculated for $C_{41}H_{75}N_4O_3S^+$ 703.5; Found 703.5.

29c ($R^a = R^2$, $R^b = R^4$): ***N*-(3-(dimethylamino)propyl)-6-dodecanoyl-2-stearamido-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxamide**. **MS** (ESI^+) m/z: $[M + H]^+$ Calculated for $C_{43}H_{79}N_4O_3S^+$ 731.5; Found 731.5.

29e ($R^a = R^2$, $R^b = R^8$): **undecyl 5-((3-((3-(dimethylamino)propyl)carbamoyl)-6-dodecanoyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridin-2-yl)amino)-5-oxopentanoate**. **MS** (ESI^+) m/z: $[M + H]^+$ Calculated for $C_{41}H_{73}N_4O_5S^+$ 733.5; Found 733.5.

29f ($R^2 = R^a$, $R^b = R^9$): **2-octyldodecyl 5-((3-((3-(dimethylamino)propyl)carbamoyl)-6-dodecanoyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridin-2-yl)amino)-5-oxopentanoate**. **MS** (ESI^+) m/z: $[M + H]^+$ Calculated for $C_{50}H_{91}N_4O_5S^+$ 859.7; Found 859.7.

29g ($R^a = R^2$, $R^b = R^5$) ***N*-(3-(dimethylamino)propyl)-6-dodecanoyl-2-oleamido-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxamide**. **MS** (ESI^+) m/z: $[M + H]^+$ Calculated for $C_{43}H_{77}N_4O_3S^+$ 729.5; Found 729.5.

29h ($R^a = R^2$, $R^b = R^6$): ***N*-(3-(dimethylamino)propyl)-6-dodecanoyl-2-((9*Z*,12*Z*)-octadeca-9,12-dienamido)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxamide**. **MS** (ESI^+) m/z: $[M + H]^+$ Calculated for $C_{43}H_{75}N_4O_3S^+$ 727.5; Found 727.5.

29i ($R^a = R^2$, $R^b = R^7$): ***N*-(3-(dimethylamino)propyl)-6-dodecanoyl-2-icosanamido-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxamide**. **MS** (ESI^+) m/z: $[M + H]^+$ Calculated for $C_{45}H_{83}N_4O_3S^+$ 759.6; Found 759.5.

29j ($R^a = R^4$, $R^b = R^4$): ***N*-(3-(dimethylamino)propyl)-2-stearamido-6-stearoyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxamide**. **MS** (ESI^+) m/z: $[M + H]^+$ Calculated for $C_{49}H_{91}N_4O_3S^+$ 815.7; Found 815.7.

29k ($R^a = R^4$, $R^b = R^8$): **undecyl 5-((3-((3-(dimethylamino)propyl)carbamoyl)-6-stearoyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridin-2-yl)amino)-5-oxopentanoate**. **MS** (ESI^+) m/z: $[M + H]^+$ Calculated for $C_{47}H_{85}N_4O_5S^+$ 817.6; Found 817.7.

29l ($R^a = R^3$, $R^b = R^2$): ***N*-(3-(dimethylamino)propyl)-2-dodecanamido-6-palmitoyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxamide**. **MS** (ESI^+) m/z: $[M + H]^+$ Calculated for $C_{41}H_{75}N_4O_3S^+$ 703.6; Found 703.6.

29m ($R^a = R^3$, $R^b = R^3$): ***N*-(3-(dimethylamino)propyl)-2-palmitamido-6-palmitoyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxamide**. **MS** (ESI^+) m/z: $[M + H]^+$ Calculated for $C_{45}H_{83}N_4O_3S^+$ 759.6; Found 759.7.

29n ($R^a = R^7$, $R^b = R^5$): ***N*-(3-(dimethylamino)propyl)-6-icosanoyl-2-oleamido-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxamide**. **MS** (ESI^+) m/z: $[M + H]^+$ Calculated for $C_{51}H_{93}N_4O_3S^+$ 841.7; Found 841.7.

29o ($R^a = R^7$, $R^b = R^6$): ***N*-(3-(dimethylamino)propyl)-6-icosanoyl-2-((9*Z*,12*Z*)-octadeca-9,12-dienamido)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxamide**. **MS** (ESI^+) m/z: $[M + H]^+$ Calculated for $C_{51}H_{91}N_4O_3S^+$ 839.7; Found 839.7.

29p ($R^a = R^7$, $R^b = R^7$): ***N*-(3-(dimethylamino)propyl)-2-icosanamido-6-icosanoyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxamide**. **MS** (ESI^+) m/z: $[M + H]^+$ Calculated for $C_{53}H_{99}N_4O_3S^+$ 871.7; Found 871.7.

29q ($R^a = R^7$, $R^b = R^8$): **undecyl 5-((3-((3-(dimethylamino)propyl)carbamoyl)-6-icosanoyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridin-2-yl)amino)-5-oxopentanoate**. **MS** (ESI^+) m/z: $[M + H]^+$ Calculated for $C_{49}H_{89}N_4O_5S^+$ 845.6; Found 845.6.
29r ($R^a = R^7$, $R^b = R^2$): ***N*-(3-(dimethylamino)propyl)-2-dodecanamido-6-icosanoyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxamide**. **MS** (ESI^+) m/z: $[M + H]^+$ Calculated for $C_{45}H_{83}N_4O_3S^+$ 759.6; Found 760.0.
29s ($R^a = R^7$, $R^b = R^3$): ***N*-(3-(dimethylamino)propyl)-6-icosanoyl-2-palmitamido-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxamide**. **MS** (ESI^+) m/z: $[M + H]^+$ Calculated for $C_{49}H_{91}N_4O_3S^+$ 816.1; Found 815.7.
29t ($R^a = R^9$, $R^b = R^9$): **2-octyldodecyl 5-(3-((3-(dimethylamino)propyl)carbamoyl)-2-(5-((2-octyldodecyl)oxy)-5-oxopentanamido)-4,7-dihydrothieno[2,3-*c*]pyridin-6(5*H*)-yl)-5-oxopentanoate**. **MS** (ESI^+) m/z: $[M + H]^+$ Calculated for $C_{63}H_{115}N_4O_7S^+$ 1071.8; Found 1072.0.
29u ($R^a = R^6$, $R^b = R^9$): **2-octyldodecyl 5-((3-((3-(dimethylamino)propyl)carbamoyl)-6-((9*Z*,12*Z*)-octadeca-9,12-dienoyl)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridin-2-yl)amino)-5-oxopentanoate**. **MS** (ESI^+) m/z: $[M + H]^+$ Calculated for $C_{56}H_{99}N_4O_5S^+$ 939.7; Found 940.1.
29v ($R^a = R^6$, $R^b = R^4$): ***N*-(3-(dimethylamino)propyl)-6-((9*Z*,12*Z*)-octadeca-9,12-dienoyl)-2-stearamido-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxamide**. **MS** (ESI^+) m/z: $[M + H]^+$ Calculated for $C_{49}H_{87}N_4O_3S^+$ 811.6; Found 812.0.
29w ($R^a = R^6$, $R^b = R^5$): ***N*-(3-(dimethylamino)propyl)-6-((9*Z*,12*Z*)-octadeca-9,12-dienoyl)-2-oleamido-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxamide**. **MS** (ESI^+) m/z: $[M + H]^+$ Calculated for $C_{49}H_{85}N_4O_3S^+$ 809.6; Found 809.1.

Materials and Methods

Synthesis, purification, and analysis

All solvents and reagents were used as received unless noted otherwise. All reactions were conducted in dry glassware and under an atmosphere of nitrogen unless otherwise noted. All TLCs were obtained on Sorbent Technologies polyester backed Silica G TLC Plates of thickness 200 µm. All compounds were purified by silica gel chromatography either via manual flash chromatography or by Biotage Isolera™ flash chromatography unless noted otherwise. The compound's identity and purity were confirmed via NMR (^{1}H and/or ^{13}C) and/or Mass Spectrometry. All proton and carbon NMR spectra were obtained with a 500 MHz or a 400 MHz Oxford spectrospin cryostat, controlled by a Bruker Avance system, and were acquired using Bruker TOPSPIN 2.0 acquisition software. Acquired FIDs were analyzed using MestReC 3.2 or MestReNova 9.0. Mass spectrometry analysis was conducted with Advion Expression® CMS. HRMS analysis was conducted either at Oregon State University or Oregon Health & Science University and are ± 10 PPM or ± 0.003 dalton of theoretical. All ^{1}H and ^{13}C NMR spectra were taken in CDCl3 (Chloroform-*d*) unless otherwise noted and are reported as ppm relative to TMS as an internal standard.

Formulation and characterization of the nanoparticles

Formulation of mRNA LNPs and characterization

LNPs were prepared via microfluidic mixing of ethanol (lipid) and aqueous (nucleic acid) solutions in the NanoAssemblr Benchtop mixer (Precision NanoSystems). Ethanol solution comprised of ionizable lipid/phospholipid/cholesterol/DMG-PEG2k mixture at the molar ratio of 50/10/38.5/1.5 and a total lipid concentration of 5.5 mM. mRNA (Trilink Biotech, San Diego CA) was mixed with water and 50 mM citrate buffer at pH 4.0, keeping the N/P (ionizable lipid: mRNA) ratio constant at 5.67. Mixing was performed at 9-12 ml/min and 3:1 volume ratio of aqueous:ethanol, respectively. Following formulation, LNPs were diluted in sterile DPBS (pH 7.4) and dialyzed against 3 L of 1x PBS for 4 hours at room temperature using a 10KDa Slide-A-Lyzer dialysis cassette, before being transferred to fresh PBS solution overnight. LNPs were concentrated after dialysis using a MWCO, 100 kDa Amicon Ultra-4 mL centrifugal filter unit for in-vitro and in-vivo studies (Millipore, Burlington, MA). LNP size and PDI were determined using Dynamic Light Scattering (DLS) in the Zetasizer Nano ZS (Malvern Panalytical Inc., Westborough MA) by diluting 1000-fold into a 1x PBS solution at 25 ºC. For high-throughput formulation work, a Hamilton liquid handling system was used. Similar to the microfluidic process, lipid stock in ethanol and mRNA stock in citric buffer were rapidly mixed at 1:3 volume ratio, respectively. The LNP mix was then diluted 1:5 with PBS. Size characterization for high-throughput formulation work was performed on Stunner (Unchained Labs). Quant-iT RiboGreen assay kit was used to estimate mRNA encapsulation efficiency and concentration.

TNS assay

TNS assay was performed as described elsewhere (2). A master buffer stock consisting of 10 mM sodium phosphate, 10 mM sodium borate, 10 mM sodium citrate and 150 mM sodium chloride. Buffers were titrated with HCl or NaOH to achieved desired pH in 0.5 pH increments and preheated to 37°C for 30 minutes prior to the assay. Lipoplexes were prepared by rapid mixing of lipid components (in ethanol) with mRNA to yield 40 ng/µl final mRNA concentration; no significant differences were observed between lipoplexes and dialyzed LNPs. In a black 96 well plate, 90 µl of each buffer was mixed with 3.26 µl of lipoplex/LNP sample and 2 µl of 300 µM 6-(*p*-Toluidino)-2-naphthalenesulfonic acid sodium salt (TNS reagent) in DMSO solution. Each well was then carefully mixed for at least three cycles; each pH point was repeated in triplicate. Fluorescence

was then recorded using a Tecan M200 Pro microplate reader at excitation/emission wavelengths of 325/435nm, respectively. Data was fitted by a sigmoidal curve and the fit was reported.

Cryo-TEM image acquisition and processing

Falcon III and K3 Summit cameras with DED at 300kV were used to capture cryo-TEM images. The Vitrobot Mark IV system (FEI) was used to plunge-freeze a copper lacey carbon film-coated Cryo-EM grid (Quantifoil, R1.2/1.3 300 Cu mesh). 2 µL of LNP was dispensed onto the glow discharged grids in the Vitrobot chamber maintained at a temperature of 23 ºC and a relative humidity of 100% to freeze the samples. The sample was incubated for 30 seconds before being blotted with filter paper for 3 seconds before being submerged in liquid ethane cooled by liquid nitrogen. The frozen grids were examined for any defects, clipped, and assembled into cassettes. The images were taken at an electron dose of 15-20 e−/Å^2 and processed and analyzed using Fiji.

In vitro studies

Cell Culture

All cell culture media and supplies were obtained from Thermo Fisher Scientific (Waltham, MA). Commercially available mammalian cell lines used to measure cytotoxicity and transfection efficiency include HeLa (human cervical epithelial cells), HEK293T/17 (human embryonic kidney cells), HepG2 (human liver epithelial-like cells), and Jurkat E6-1 (human T-lymphocytes). 661W cone cells were generously provided by Prof. Muayyad Al-Ubaidi, University of Houston, Houston, TX. 661w cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone Laboratories Inc., Logan, UT), 1× penicillin/streptomycin (Thermo Fisher, Federal Way, WA), 23 mg/l Putrescine, 40 µl of β-Mercaptoethanol, 300 mg/l glutamine, and 40 µg of hydrocortisone 21-hemisuccinate and progesterone. Human Embryonic Kidney 293T/17 cells (CRL-11268; ATCC, Manassas, VA) and HeLa cells were also cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. All the cells were maintained in an atmosphere of 5% CO_2 at 37°C.

In vitro transfection

A cell density of 4000/well was plated in white, clear-bottom 96-well plates and allowed to grow to 60-70% confluency for 24 hours at 37 ºC. 661w, HeLa, and HEK293T cells were treated with LNPs encapsulating Fluc mRNA at concentrations of 100 and 200 nM in the medium in all transfection studies. All the cells were incubated for an additional 24 and 48 hours. Cell viability (Promega CellTiter FluorTM Cell Viability Assay) and luciferase expression (Promega One-GloTM Luciferase Assay) were assessed. Cell viability signal was used to normalize luciferase expression.

In vivo studies

Animals

Balb/c, Ai9, C57BL6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Ai9 is a Cre reporter tool designed to have a *lox*P-flanked STOP cassette preventing transcription of tdTomato under the control of a ubiquitous promoter. Following Cre-mediated recombination Ai9, mice express robust tdTomato. All mice used in the experiments were 1-6 months old and were either bred in-house or used directly from the supplier for the studies. All mice were handled in according with the animal welfare guidelines outlined by OHSU Institutional Animal Care and Use Committee (IACUC) (Protocols TR01_IP00001707 and TR02_IP00000610). Two male rhesus macaques, aged 10 years old, were used for this study. All protocols involving NHPs were approved by OHSU IACUC (Protocol TR02_IP00000768). All

animal work was conducted in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals and Animal Research.

Intravenous and intramuscular injections

For intravenous and intramuscular injections, LNP were diluted with sterile 1x PBS to a desired dose and mice were sedated with isoflurane. The intravenous injection was performed by tail-vein or retro-orbital injection, intramuscular injection was performed in the hind flanks of the mouse. Mice that received a dose of LNPs were housed until bioluminescence imaging, typically for 24 hours unless stated otherwise. Prior to bioluminescence imaging, the mice were sedated by inhalation of 2.5% isoflurane. The sedated mice then received intraperitoneal injection of D-luciferin at 150 mg/kg and rested for 10 minutes. Bioluminescence imaging was performed on the mice with various exposure times ranging from 1 to 300 seconds using IVIS Lumina XRMS from PerkinElmer. To evaluate the protein expression at an organ level, internal organs of a mouse, such as the liver, lungs, kidneys, spleen, lymph nodes, and heart, and external tissues of mouse, such as muscle tissues close to a site of injection, were excised.

Barcode study

Barcode design: DNA barcodes were single stranded oligonucleotides, 91 bases long with three phosphorothioate bonds at each end (Integrated DNA Technologies). Oligonucleotide sequences and primers are listed in Table S1. The central unique barcode was 8 nucleotides, flanked by semi-randomized bases (5'-NWNH, 3'-NWH, Fig. S4). Each oligonucleotide contained forward and reverse universal primer sites for amplification.

Administration and tissue isolation: BALB/c mice (N=5) were administered IV the injection mix containing **20b** and MC3 LNPs encapsulating barcode DNA with or without FLuc mRNA, and a naked barcode in PBS. Total barcode dose was 1.8 μg (200-400 ng per barcode); total nucleic acid dose was 31.5 μg. 6 hours after the administration, mice were imaged in IVIS to confirm mRNA transfection and euthanized immediately after the imaging. Then, tissues were isolated and snap-frozen in liquid nitrogen.

PCR amplification and Illumina sequencing: Tissues were homogenized in 1.5 mL tubes using disposable pestles. Tissue was lysed and DNA isolated using the DNeasy Blood & Tissue kit (Qiagen) and DNA concentration measured. A nested two step PCR approach was used to amplify barcodes and prepare for Illumina sequencing. Barcode DNA was amplified from each crude DNA preparation using the HiFi HotStart ReadyMix kit (KAPA) with the following recipe: 12.5 μL Master Mix, 1 μL F Universal Primer (5 μM), 1 μL R Universal Primer (5 μM), 200 ng DNA template, H2O up to 25 μL. Cycling conditions were: 22x: 20s at 98°C, 15s at 69°, 15s at 72°, 1x: 30s at 72°. PCR products were purified using Wizard SV PCR and Gel Clean-up System (Promega) and eluting in 35 μL water. Purified PCR products were amplified and Illumina adapter and index sequences were added through a second PCR using HiFi HotStart and the following recipe: 12.5 μL Master Mix, 1 μL F p5 Index Primer (5 μM), 1μL R p7 Index Primer, 1 μL DNA template for PCR reaction 1, 9.5 μL H2O. Cycling conditions: 1x: 3m at 95°C, 12x: 20s 95°, 20s at 62°, 30s at 72°, 1x: 2m at 72°. PCR products were run out on 1.5% Tris-acetate-EDTA agarose and bands were excised, pooled, and purified with Wizard SV PCR and Gel Clean-up System. Concentration of these purified PCR products, the barcode library, was quantified by KAPA Library Quantification Kit for sequencing. The library was loaded onto flow cells at 2 nM concentration. Multiplexed, next generation sequencing runs were performed on an Illumina MiSeq machine with a v2 150 chip. Primers were designed based on Nextera XT adapter and index sequences.

Barcode count analysis: A custom Python script (see link in Main text) was used to extract raw barcode counts from sequencing data for each sample. These counts were normalized to the

barcoded LNP mixture injected into mice, producing a “fold-change” value for each barcode per sample.

Subretinal injections

Subretinal injections, tissue preparation, and analysis were performed as reported elsewhere (3). Before the subretinal injection, the eyes were dilated by topical administration of 0.5% proparacaine, 1% tropicamide, and 2.5% phenylephrine and anesthetized with ketamine (100 mg/kg)/xylazine (10 mg/kg) cocktail administered intraperitoneally. To initiate the injection, 2.5% hypromellose was placed over the eye and a 30-gauge needle was used to make an incision in the limbus. After that, a glass coverslip was placed over the eye to allow visualization of the retina. Using a Hamilton syringe with a 33-gauge blunt needle, 1 µL of Cre-LNPs (300 ng mRNA/injection) were delivered to the subretinal space. To observe the retinal detachment, 2% fluorescein was added to the injection solution. Scleral incisions in the limbus were made nasally for most injections, therefore LNPs were delivered temporally.

Fundus imaging

In vivo retinal imaging was performed with the Micron IV (Phoenix Research Laboratories, Pleasanton, CA). To observe general retinal morphology, bright-field images were acquired. To capture tdTomato, we used a 534/42-nm Bright Line single-band bandpass filter (Semrock,Rochester, NY). Light intensity, exposure, and gain were kept consistent across all tdTomato images.

Tissue preparation

Cre LNP injected mice were sacrificed immediately after fundus imaging. The limbus of PBS and formulation injected eyes were marked at the 12 o'clock position with a hot needle to aid orientation. Whole globes were enucleated and immediately placed in 4% paraformaldehyde overnight at 4 ºC. The next day, whole globes were incubated in 30% sucrose in PBS for 2 hours at 4 °C before embedding in cryostat compound (Tissue-Tek O.C.T. Compound, code # 4583). The embedded eyes were snap-frozen in a dry ice bath. Eyes were sectioned at 12 microns with a cryostat (Microtome HM550; Walldorf, Germany) and stored at -20 °C.

Immunofluorescence (IF) preparation and confocal imaging

Retinal cryosections were dried for at least 30 minutes, washed three times in 1x PBS, and counter-stained with DAPI for 5 min at room temperature. After a final rinse, retinal sections were mounted in a Fluoromount-G, and cover-slipped. Retinal sections were analyzed by the TCS SP8 X (Leica Microsystems, Buffalo Grove, IL) confocal microscope. All the images were captured with identical exposure settings at 40x magnification using Z-stacks.

NHP in vivo delivery and imaging

Prior to surgery, one animal received daily intramuscular injections of 1 mg/kg prednisone for 2 weeks. On the day of surgery, pupils were dilated to a minimum of 8 mm using phenylephrine (2.5%; Bausch and Lomb, Rochester, NY, USA) and tropicamide (1% tropicacyl; Akorn, Lake Forest, IL, USA) eye drops. LNPs were administered into the subretinal space through a 27G/38G subretinal cannula (#5194, Microvision, Redmond, WA, USA) using an Alcon Constellation vitrectomy machine and a pars plana transvitreal approach. Two 50 µl subretinal blebs, one superior and one inferior to the fovea, were generated with either saline as control or LNPs at low (50 ng/ul) and high dose (500 ng/ul). After the injection, dexamethasone (0.5 ml and 10 mg/ml) and cefazolin (0.5 ml and 125 mg/ml) were administered subconjunctivally. The immune suppressed animal also received a subtenon injection of triamcinolone (40mg). There were no

complications noted during surgery. The animals received comprehensive multimodal retinal imaging before injection (baseline) and at 48 hours after injection. For each imaging session, the animal was anesthetized by an intramuscular injection of Telazol (1:1 mixture of tiletamine hydrochloride and zolazepam hydrochloride, 3.5 to 5.0 mg/kg) and maintained with ketamine (1 to 2 mg/kg) as required. Heart rate and peripheral blood oxygen saturation were monitored by pulse oximetry. Rectal temperature was maintained between 37.0° and 38.0°C by water-circulating heated pads. For image acquisition, animals were positioned prone with the head supported by a chinrest; the pupils were dilated to a minimum of 8 mm using phenylephrine (2.5%; Bausch and Lomb, Rochester, NY, USA) and tropicamide (1% tropicacyl; Akorn, Lake Forest, IL, USA) eye drops. A speculum was inserted to hold the lids open, and custom contact lenses were placed on the cornea to maintain hydration and improve image quality. Imaging included fundus autofluorescence and SD-OCT (Spectralis, Heidelberg, Franklin, MA). Following imaging, contact lenses and eyelid specula were removed, and erythromycin ointment was applied to each eye.

NHP immunofluorescence imaging

Following humane euthanasia by a veterinary pathologist, eyes were collected and immersion fixed in 4% paraformaldehyde in PBS for 24 hours. Fixed eye cups were cryoprotected in 10%, 20% and 30% sucrose solutions, embedded in OCT compound, and sectioned using a Leica cryostat (CM1850, Leica, Wetzlar, Germany) at 16µm. Every slide was visualized for native GFP expression to identify the borders of each bleb. For quantification of LNP expression, primary antibodies consisted of anti-GFP (ab290, Abcam, Cambridge, UK), anti-cone arrestin, and anti-rod arrestin (generously provided by W.C. Smith at UF). Secondaries included a combination of Alexa Fluor 488 (GFP), Alex Fluor 568 (rods) and Alexa Fluor 633 (cones), which were used at 1:300 dilution. All slides were counterstained with DAPI.

Photoreceptor quantification

For each bleb, 10 representative 20x confocal microscope images were collected with uniform laser intensity settings for EGFP, cone arrestin, and rod arrestin fluorescence. For each image, total cone, total rod, GFP+ cone and GFP+ rod counts were generated. The GFP+ cone and GFP+ rod counts were divided by the total cone and total rod counts to generate a % transfected cones and % transfected rods, respectively. To compare transfected photoreceptors across groups, we ran a balanced two-way ANOVA along with Tukey's honest significance test using a confidence interval of 95%. A $p < 0.05$ was considered significant.

Supplementary Figures

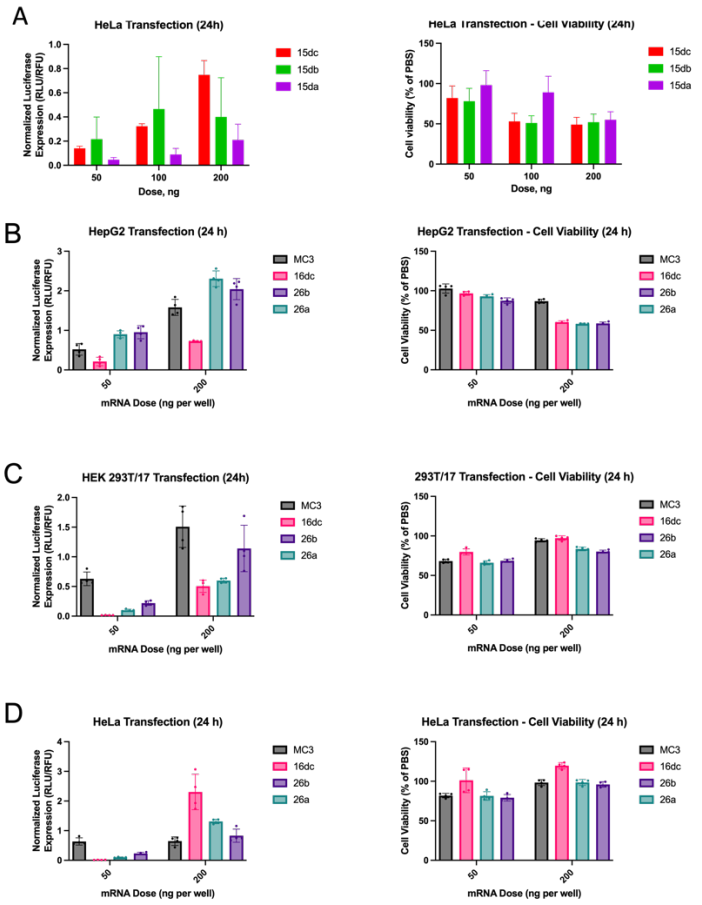


Figure S1. (A) Results of in vitro screening (HeLa cells) of 15d lipid derivatives incorporating various ionizable head modifiers. Transfection of HepG2 (B), HEK293T/17 (C), and HeLa (D) cells with Fluc LNPs containing 16dc, 26a, and 26b suggest variable cell tropism depending on the ionizable lipid structure.

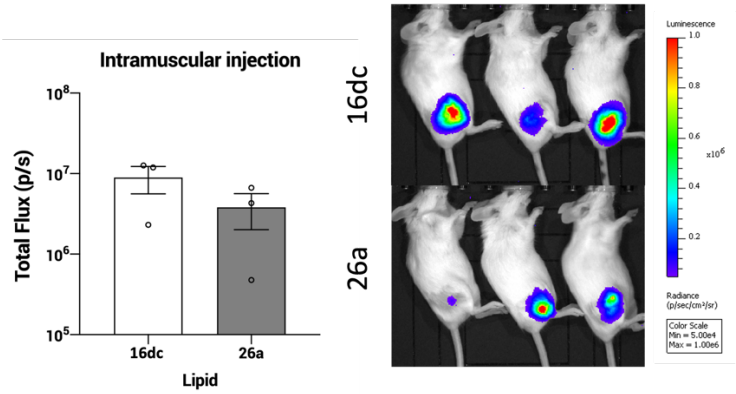


Figure S2. Results of intramuscular injection for LNPs containing lipid 19da and 26a.

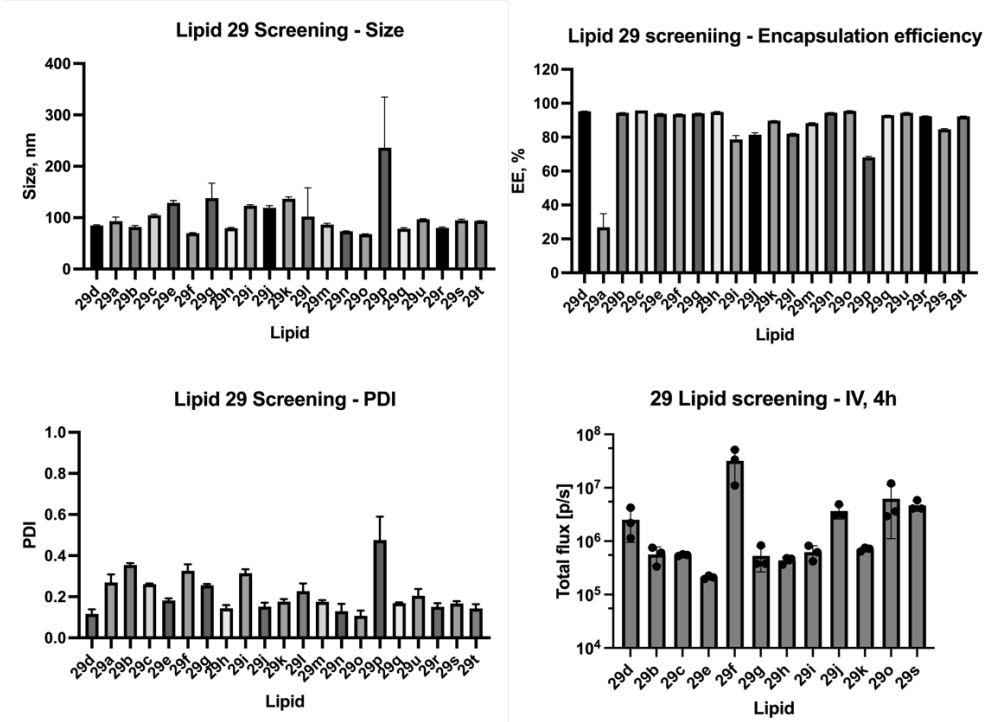


Figure S3. Summary of LNP properties with lipids **29a-w**.

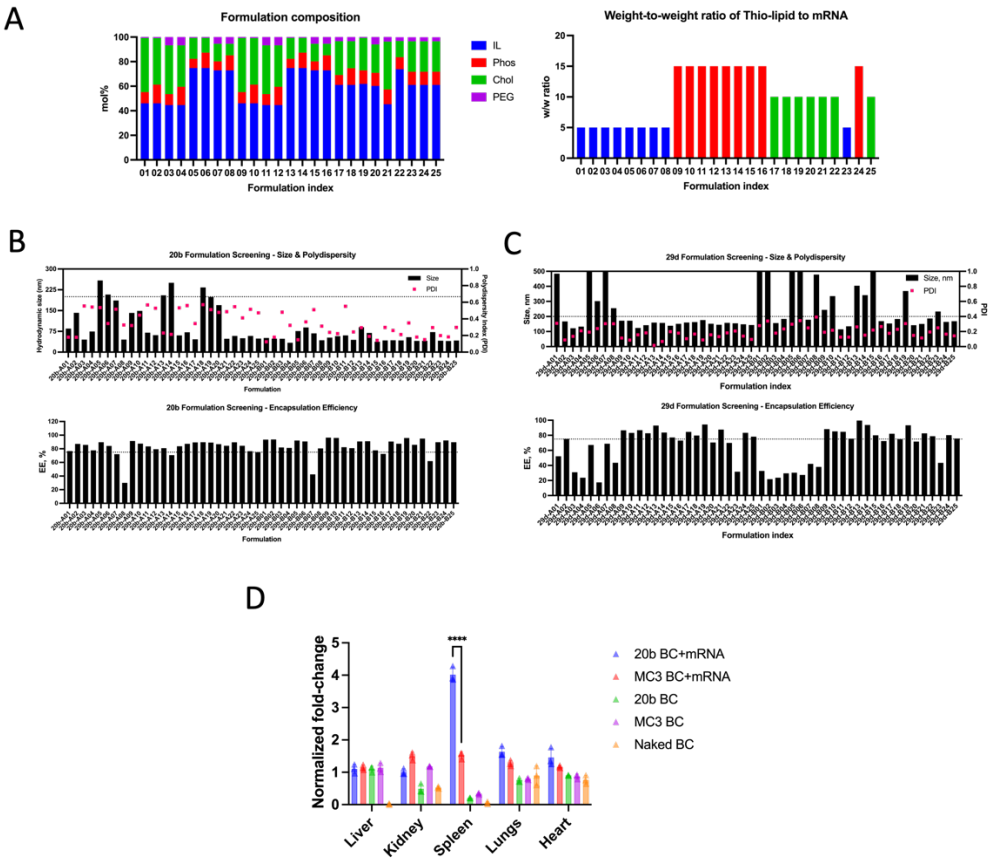


Figure S4. (A) Initial formulation library design includes varying both molar % (left) and w/w ratio between IL and mRNA (right), and two structural lipids – DOPE (formulations A) and DSPC (formulations B). Results of high-throughput formulation prescreening for (B) **20b** and (C) **29d**. LNP QA cutoffs (size > 200nm, PDI > 0.2 and EE < 75%) are shown in the graphs. (C) results of NGS sequencing for **20b**-barcode LNP intravenous screening. DNA barcode counts were normalized to the injection solution.

G*A*T*GCTCTCATACGAACTCGTCCNHNWCCTGCTAGTCCACGTCCATGTCCACCNWNH[8nt Seq]NWHGTGGTTAGTCGAGCAGAGAC*T*A*G
Universal primer site — Barcode — Universal primer site

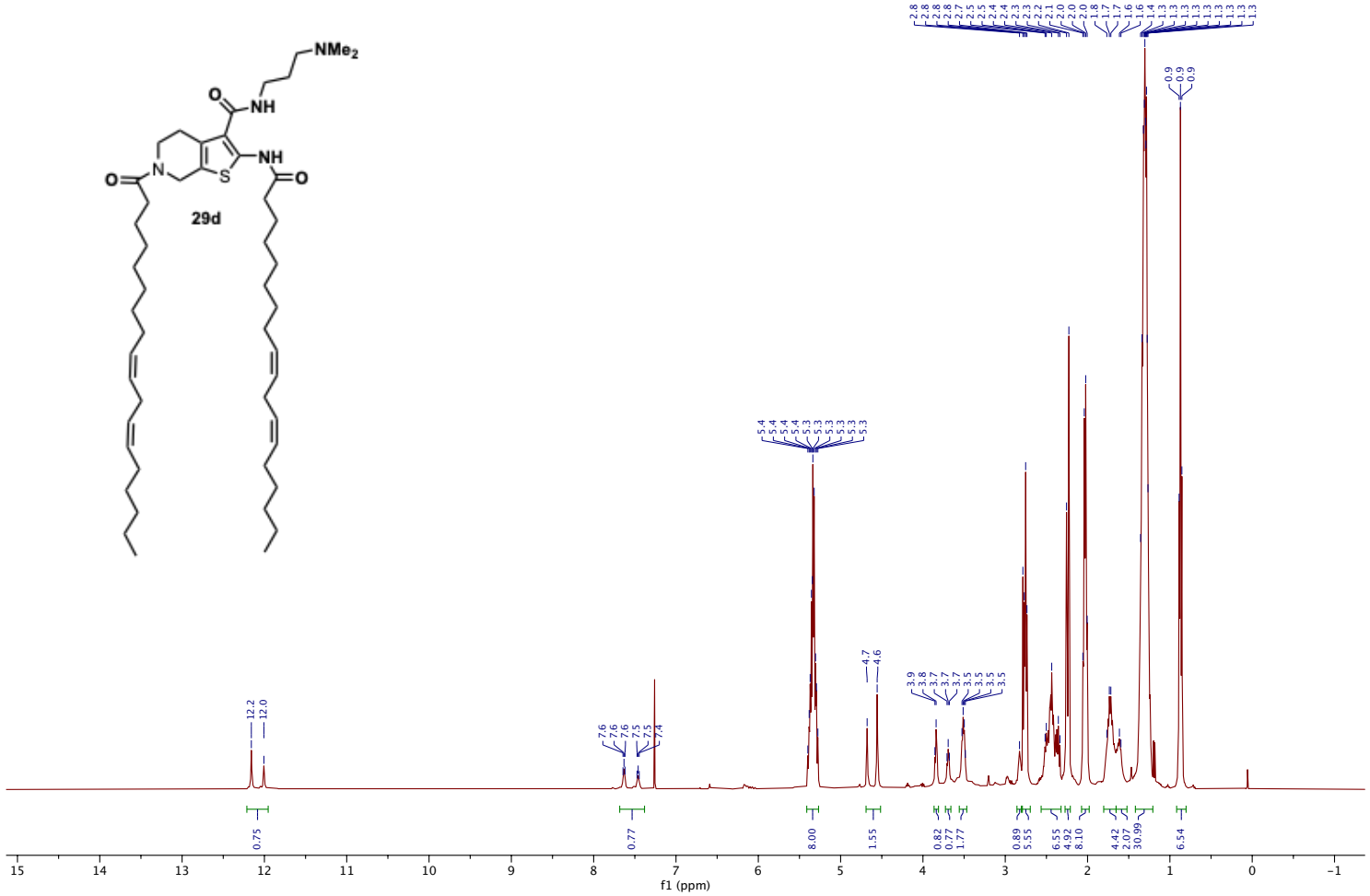
Fig. S5: Barcode template sequence

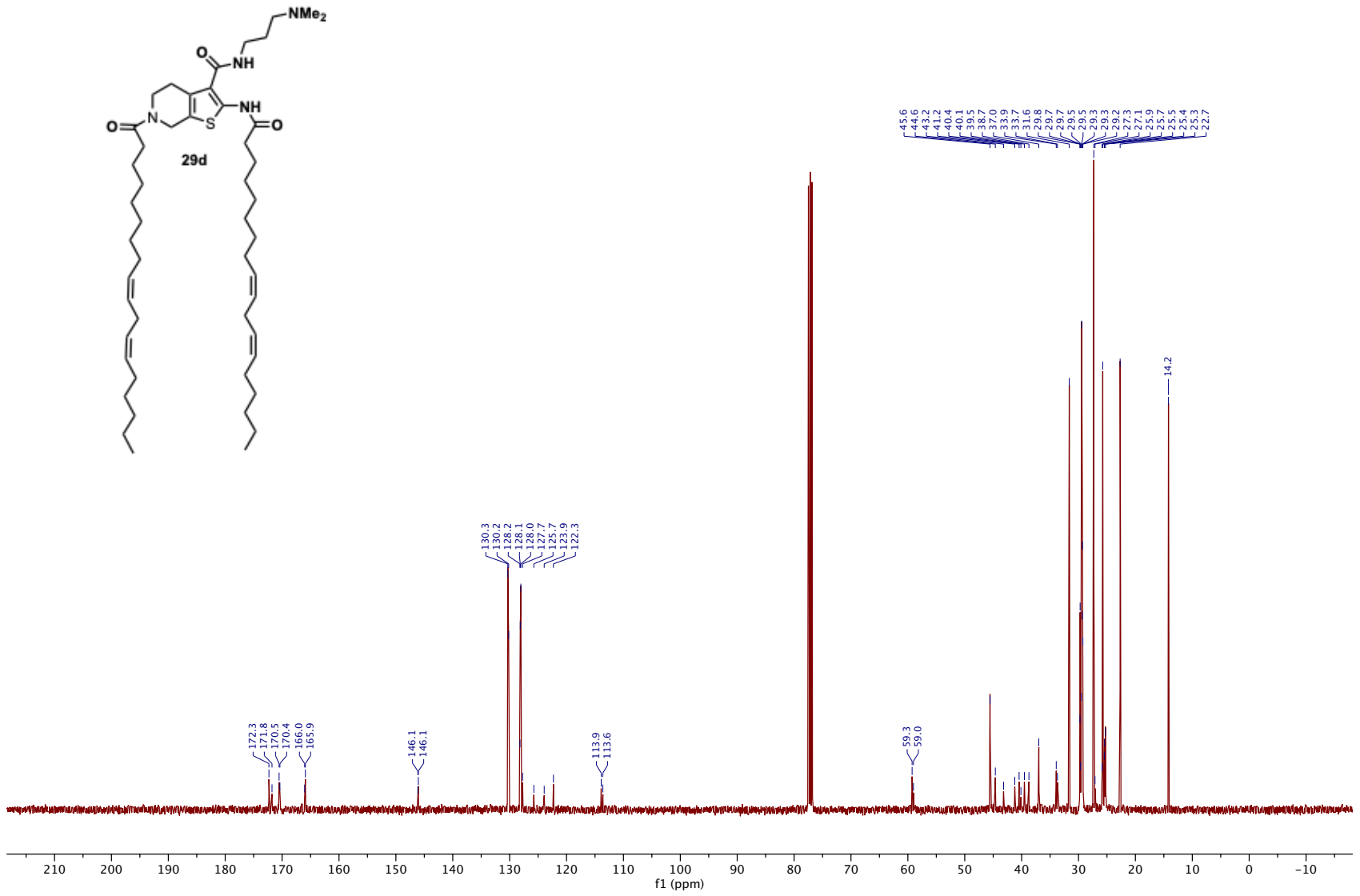
Table S1: Oligonucleotide and primer sequences

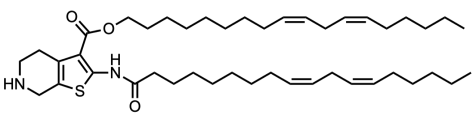
F universal primer	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCTCTCATACGAACTCGTCC
R universal primer	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGTCTCTGCTCGACTAACCAC
F p5 index S502 primer	AATGATACGGCGACCACCGAGATCTACAC CTCTCTAT TCGTCGGCAGCGTC
F p5 index S503 primer	AATGATACGGCGACCACCGAGATCTACAC TATCCTCT TCGTCGGCAGCGTC
F p5 index S505 primer	AATGATACGGCGACCACCGAGATCTACAC GTAAGGAG TCGTCGGCAGCGTC
F p5 index S506 primer	AATGATACGGCGACCACCGAGATCTACAC ACTGCATA TCGTCGGCAGCGTC
F p5 index S507 primer	AATGATACGGCGACCACCGAGATCTACAC AAGGAGTA TCGTCGGCAGCGTC

R p7 index N701 primer	CAAGCAGAAGACGGCATACGAGAT TAAGGCGA GTCTCGTGGGCTCGG
R p7 index N702 primer	CAAGCAGAAGACGGCATACGAGAT CGTACTAG GTCTCGTGGGCTCGG
R p7 index N703 primer	CAAGCAGAAGACGGCATACGAGAT AGGCAGAA GTCTCGTGGGCTCGG
R p7 index N704 primer	CAAGCAGAAGACGGCATACGAGAT TCCTGAGC GTCTCGTGGGCTCGG
R p7 index N705 primer	CAAGCAGAAGACGGCATACGAGAT GGACTCCT GTCTCGTGGGCTCGG
Barcode 1 8nt seq	GACACAGT
Barcode 2 8nt seq	GCATAACG
Barcode 3 8nt seq	ACAGAGGT
Barcode 4 8nt seq	CCACTAAG
Barcode 5 8nt seq	TGTTCCGT

Figure S6. NMR spectroscopy data for key compounds.

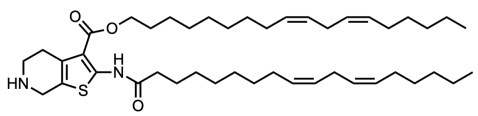



NMe2
O
NH
NH
O
N
S
O
29d
45.6
44.6
43.2
41.2
40.4
40.1
39.5
38.7
37.0
33.9
33.7
31.6
29.8
29.7
29.7
29.5
29.5
29.3
29.3
29.2
27.3
27.1
25.9
25.7
25.5
25.4
25.3
22.7
14.2
130.3
130.2
128.2
128.1
128.0
127.7
125.7
123.9
122.3
172.3
171.8
170.5
170.4
166.0
165.9
146.1
146.1
113.9
113.6
59.3
59.0
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
−10
f1 (ppm)



20b

11.3
5.3
4.3
4.2
3.9
3.4
3.1
2.8
2.7
2.5
2.4
2.2
2.1
2.0
1.7
1.3
0.9
0.98
8.00
1.99
1.86
1.87
5.90
2.00
1.23
8.11
4.05
31.42
6.13
f1 (ppm)



20b

31.6
29.7
29.7
29.7
29.5
29.4
29.4
29.3
29.3
29.2
29.2
28.8
27.5
27.3
27.3
26.2
25.7
25.3
22.7
14.2
130.3
130.3
130.1
130.1
129.4
128.2
128.1
128.0
128.0
125.4
170.2
166.6
148.2
111.3
64.8
44.3
43.5
37.0
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)

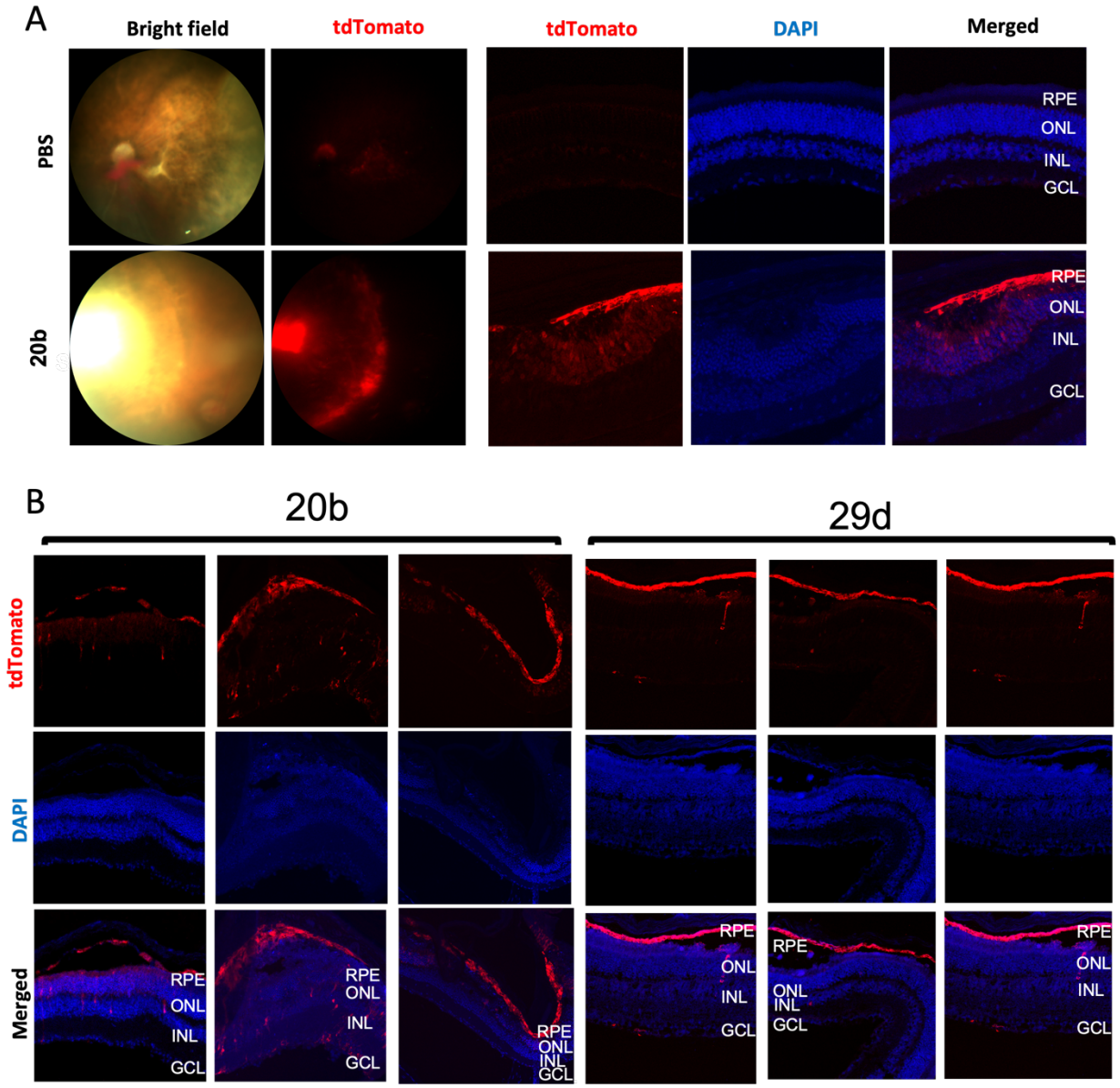


Figure S7. (A) Representative fundus images of Ai9 mice retina 96 hours after a subretinal injection of 300ng Cre mRNA delivered in **20b** LNPs or a PBS control. (B) Immunofluorescence images of retinal sections after a subretinal administration of LNPs with **20b** or **29d** LNPs in Ai9 mice (300ng mRNA dose).

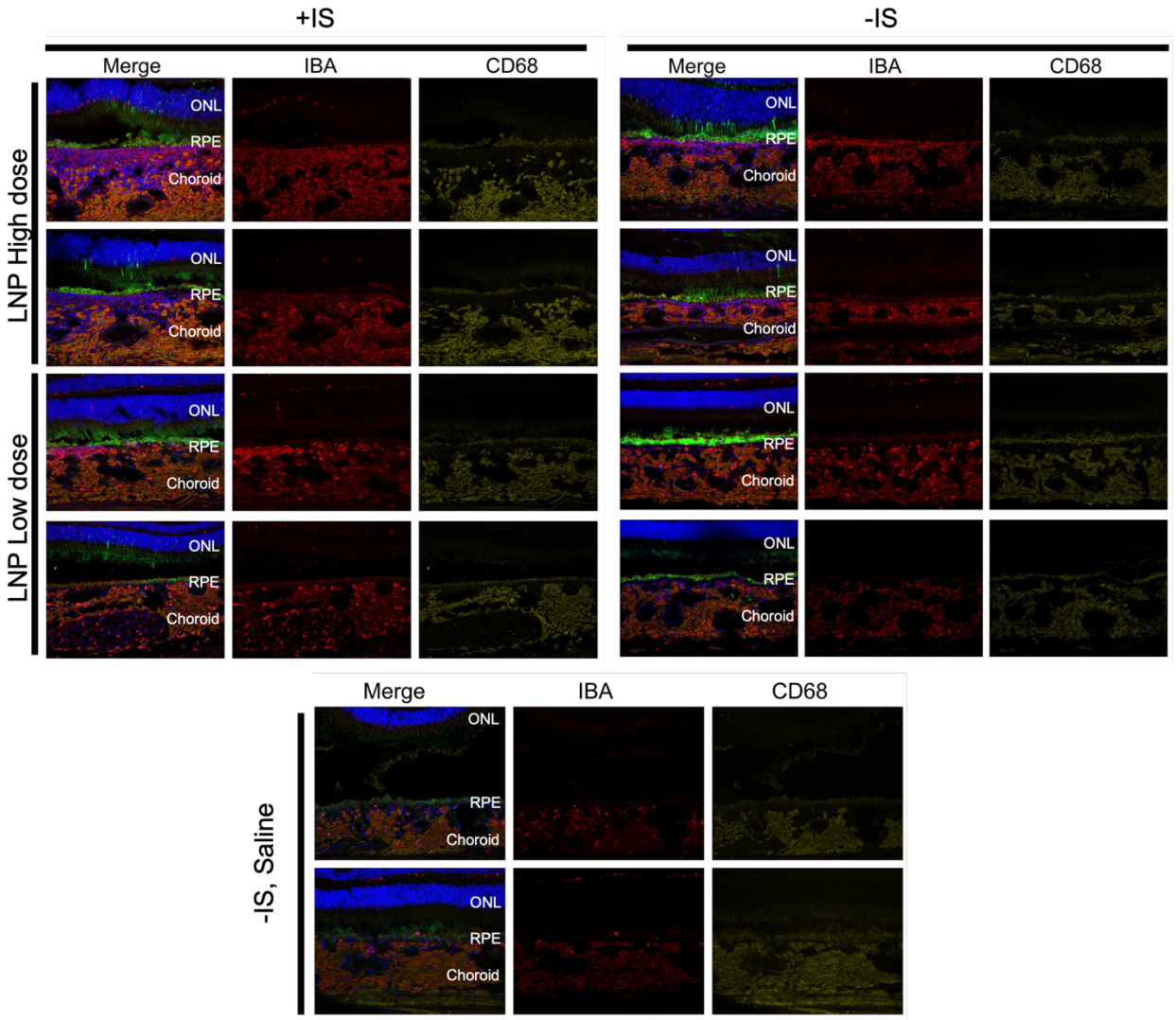


Figure S8. Representative immunofluorescence images of NHP retinal sections after a subretinal administration of LNPs with **20b** lipid (high dose – 25µg EGFP mRNA, low dose - 2.5µg EGFP mRNA). IBA and CD68 antibodies were used to evaluate the extent of immune cell infiltration.

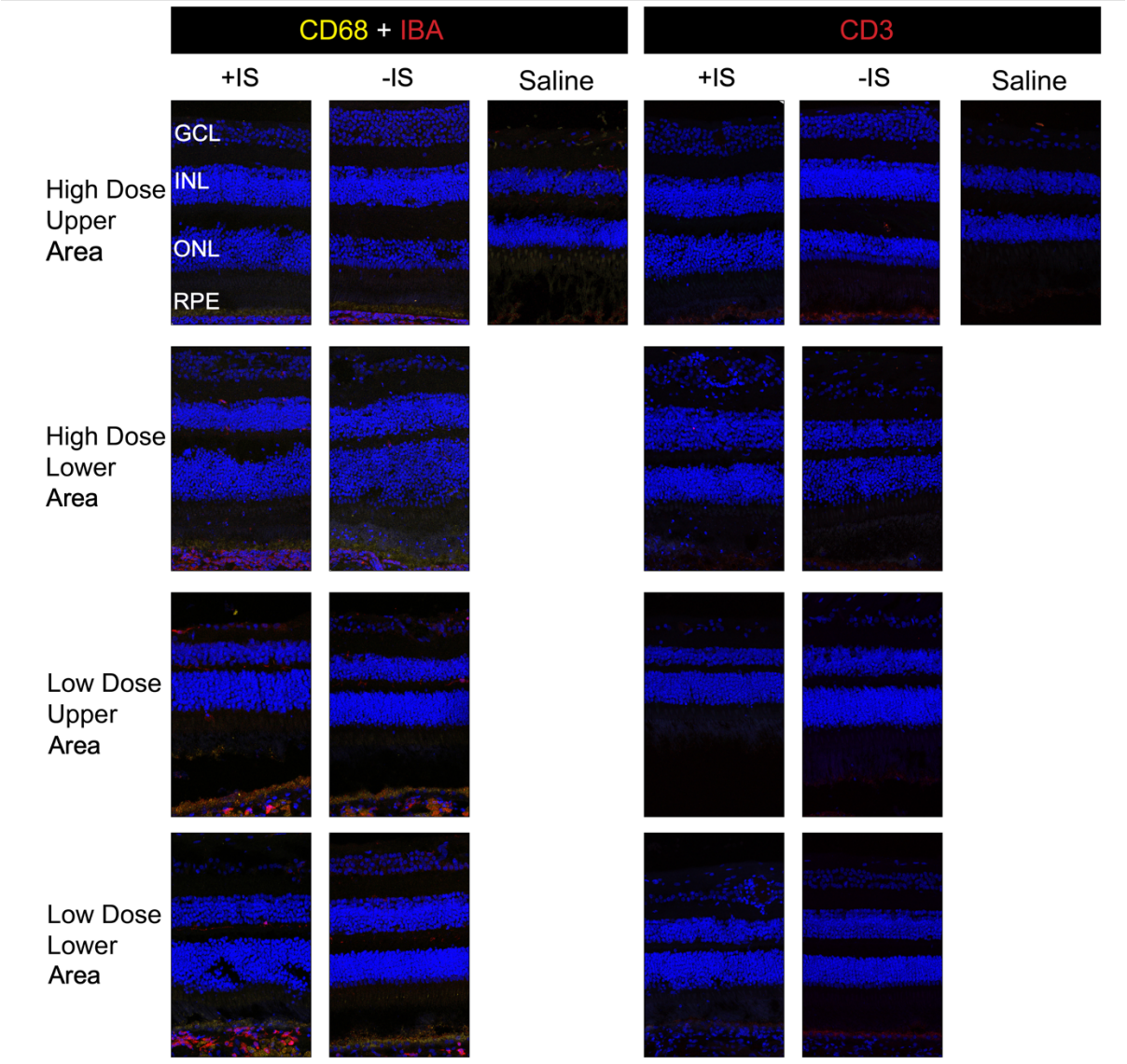


Figure S9. Immunofluorescence images after DAPI, Iba1, CD68, and CD3 staining to investigate the immune response in the neural retina following the subretinal injection of EGFP mRNA - **20b** LNP in non-human primate.

References cited

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