Functional DNA Delivery Enabled by Lipid-Modified Charge-Altering Releasable Transporters (CARTs)

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Supporting Information

Materials and Methods.

Plasmid DNA. PKCγ-GFP (Addgene No. 21204, 2.5 μg/μL) and PKCδ-GFP (3.9 μg/μL)[1] plasmids were a generous gift of Dr. Tobias Meyer, Stanford University. Plasmid CAG-GFP (Addgene No. 11150, 1.1 μg/μL) was provided by Dr. Maria Barna, Stanford University, and plasmid CDH-GFP (BioCat CD811A-1, 1.075 μg/μL) plasmid was provided by Dr. Peter Kim, Stanford University. The transposase plasmid pCMV(CAT)T7-SB100 (Addgene No. 34879, short: pCMV-SB100)[2] was a generous gift from Dr. Zsuzsanna Izsvak, Max Delbrück Center, Berlin, Germany. The fLuc=tdTom transposon plasmid pKT2/CAG-Luc=tdTom-SP (short:

pKT2/CAGL=tTP) was generated by PCR amplification of the luciferase-tandem Tomato RFP fusion gene (fLuc=tdTom) from pcDNA3.1(+)/Luc2=tdT (Addgene No. 32904) and its insertion into the transposon expression plasmid pKT2/CAGXS (M. H. Bachmann, manuscript in preparation). Mixes of transposon and SB100 transposase plasmid were provided by Dr. Michael H. Bachmann. For more information, see Table S1.

Flow Cytometry Analysis of tdTom Expression. CHO-K1 cells were seeded at 40,000 cells/well in 24-well plates and allowed to adhere overnight at 37 °C. CART-pDNA (pDNA 1, pKT2/CAGL=tTP) complexes were prepared by mixing RNAse-free PBS, pH 5.5, (109.1 μL) and 4.17 μL pDNA mix (0.5 μg/μL stock in RNAse-free PBS) with CART **6a** (1.77 μL, from a 2 mM DMSO stock solution) to achieve specific CART:pDNA ratios (optimized to a theoretical cation:anion charge ratio of 5:1, 115 μL total volume). The complexes were incubated for 20 s at room temperature prior to treatment. The L2000 control was prepared in OptiMEM per the manufacturer's instructions with an equivalent amount of pDNA mix. The cells were washed with ~0.5 mL serum-free F-12K medium, and then 500 μL of serum-free F-12K medium was added to the wells with untreated cells, 425 μL to the wells treated with L2000-pDNA, and 462.5 μL to the wells treated with CART-pDNA complex. 75 μL of L2000-pDNA solution was added to each of three wells for a final volume of 500 μL, and 37.5 μL of the CART-pDNA complex was added to each of three separate wells for a final volume of 500 μL. The cells were incubated for 24 h at 37 °C, at which time the medium was replaced with 500 μL of serum-containing F-12K medium. The cells were incubated for an additional 24 h, washed with PBS (1.0 mL), and then trypsinized with 0.5 mL trypsin-EDTA (0.25%) for 5 min at 37 °C. F-12K medium (0.5 mL) was added and the contents of each well were transferred to a 15 mL conical tube and centrifuged (1200 rpm for 5 min). The pelleted cells were redispersed in PBS (200 μL),

transferred to FACS tubes, and analyzed by flow cytometer (LSR-II.UV, Stanford University). Results were analyzed using FlowJo software. For transfection efficiency, untreated cells were gated for no tdTom expression, and the data presented are the percentage of cells analyzed with higher tdTom expression than untreated cells.

Cell Viability Assay for Delivery of pKT2/CAGL=tTP/pCMV-SB100 Mix (pDNA mix). CHO-K1 cells were plated in a 96-well plate at a density of 10,000 cells/well in 100 μL of serum-containing F-12K medium. Cells were allowed to incubate for 24 h at 37 °C. Wells were washed with 50 μL of serum-free F-12K medium, and then serum-free F-12K medium was added to all wells to achieve a final volume of 50 μL including the treatment condition. CART-pDNA complexes were formed at a charge ratio of 5:1 and were prepared by mixing CART **6a** from a 2.0 mM stock solution (0.5 μL in DMSO) with 1.17 μL premixed pKT2/CAGL=tTP/pCMV-SB100 mix (2:1 pDNA ratio as a 0.5 μg/μL stock solution) and RNAse-free PBS, pH 5.5 (23.3 μL). The complexes were allowed to incubate for 20 s at room temperature. From these solutions either 7.3 μL, 4.87 μL, or 2.4 μL was added per well to achieve a total of 171 ng, 114 ng or 56 ng of pKT2/CAGL=tTP/pCMV-SB100 mix, respectively. CART **6a** alone was also tested by adding CART **6a** from a 2.0 mM stock solution (0.5 μL in DMSO) into RNAse-free PBS, pH 5.5 (24.47 μL). The CART was allowed to incubate for 20 s at room temperature. From these solutions 7.3 μL, 4.87 μL, or 2.4 μL was added per well. The Lipofectamine 2000 (L2000) control was prepared in serum-free OptiMEM medium per the manufacturer's instructions using either 171 ng, 114 ng or 56 ng of pKT2/CAGL=tTP/pCMV-SB100 mix per well. The treated cells then incubated at 37 °C for 24 h before medium was changed to 100 μL serum-containing F-12K medium. After incubating for another 24 h, 10 μL F-12K media containing 5 mg/mL thiazolyl blue tetrazolium bromide[3] (Fluka) was added per well

and incubated 37 °C for 2.5 hours. At this time, cells were lysed with 100 μL of a solution of 10% Triton X in 0.1 N HCl in isopropanol and allowed to incubate for another 30 min. Plates were analyzed with a VERSAmax tunable microplate reader (Molecular Devices) using SOFTmax Pro® version 3.1.1, reading at 570 nm and subtracting from 690 nm. Data from each row were normalized to untreated cells. An average of several experiments is reported as indicated; experimental error is expressed as ± SD.

Figures.

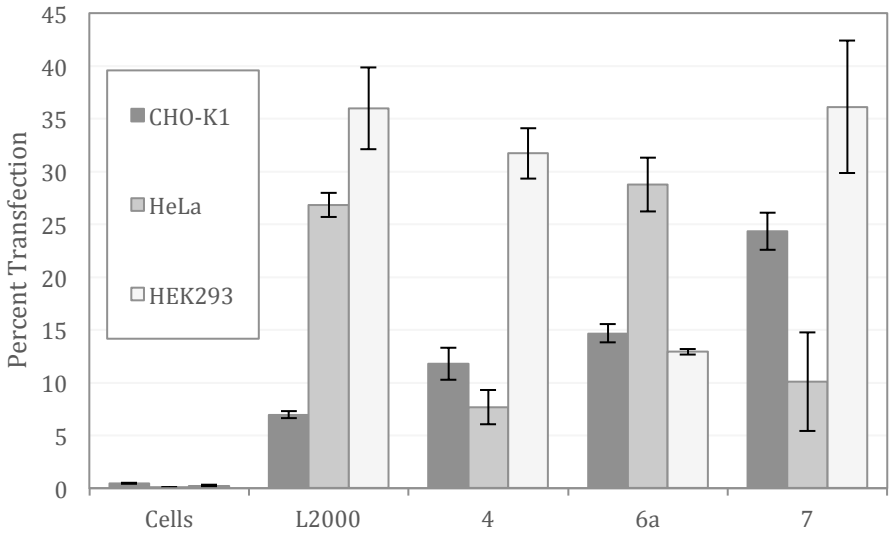

Figure S1. Transfection efficiencies of pPKCδ-GFP delivery by CARTs compared with L2000 in CHO-K1, HeLa, and HEK293 cell lines. All treatments were for 24 h and at a concentration of 1.35 ng/μL with respect to pDNA. Error is expressed as ±SD.

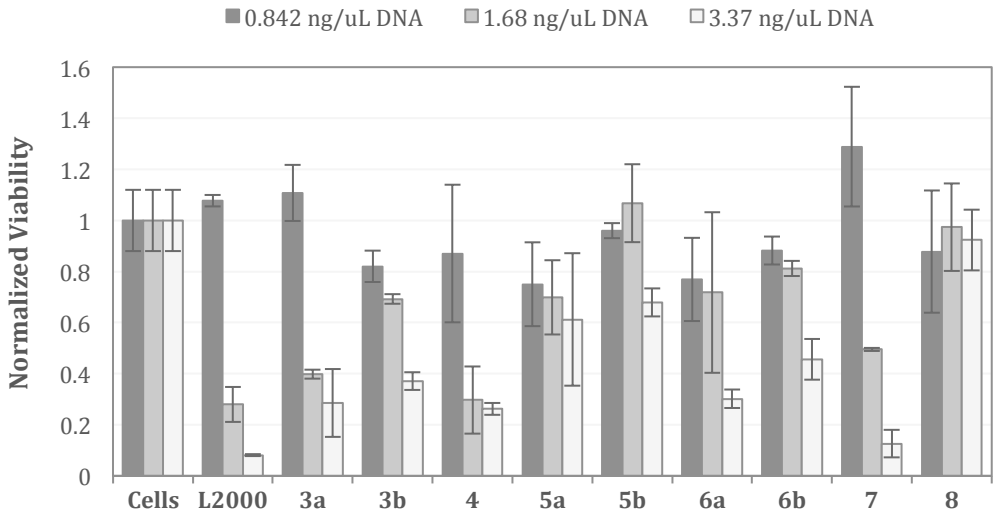

Figure S2. Normalized viability of CHO-K1 cells treated with L2000 or CART-pPKCδ-GFP complexes relative to untreated cells, as determined by MTT assay. Complexes were formulated at a charge ratio of 25:1 (+/-) and tested at a range of concentrations. Error is expressed as ± SD.

Table S1. Proteins encoded, promoters, sizes, and concentrations for plasmids used.

Proteins encoded	Promoter	Plasmid name	Size (bp)	Ratio	Concentration
Fluc, tdTom	CAG	pKT2/CAGL=tTP	9937	2	0.5 μg/μL
Transposase	CMV	pCMV(CAT)T7-SB100X	4752	1	
Fluc, tdTom	CAG	pKT2/CAGL=tTP	9937	-	1.0 μg/μL
Transposase	CMV	pCMV(CAT)T7-SB100X	4752	-	1.98 μg/μL

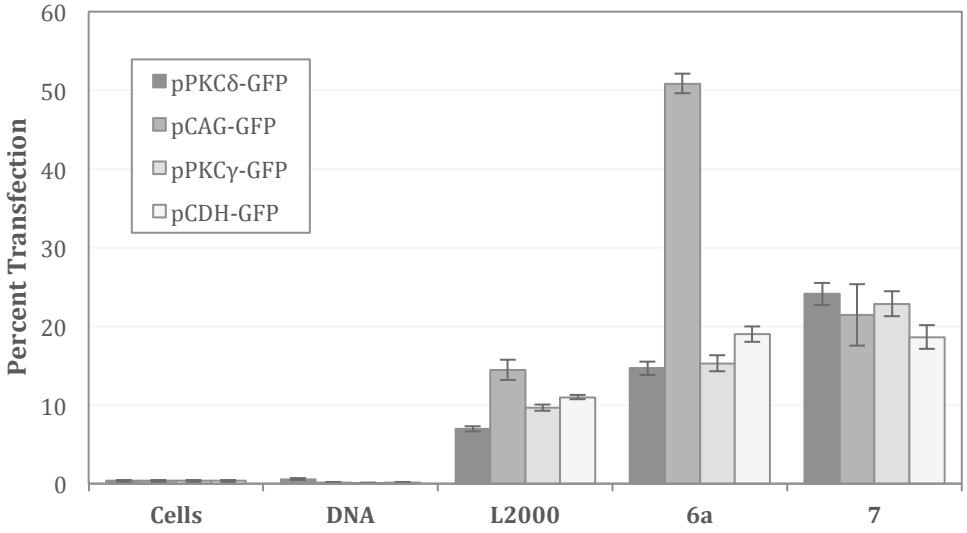

Figure S3. Percent transfection in CHO-K1 cells by complexes of L2000, $O_{11}A_9$ **6a**, or $L_{7.5}A_8$ **7** with four different plasmid DNA cargoes. All treatments were for 24 h and at a concentration of 1.35 ng/μL with respect to pDNA. Error is expressed as ± SD.

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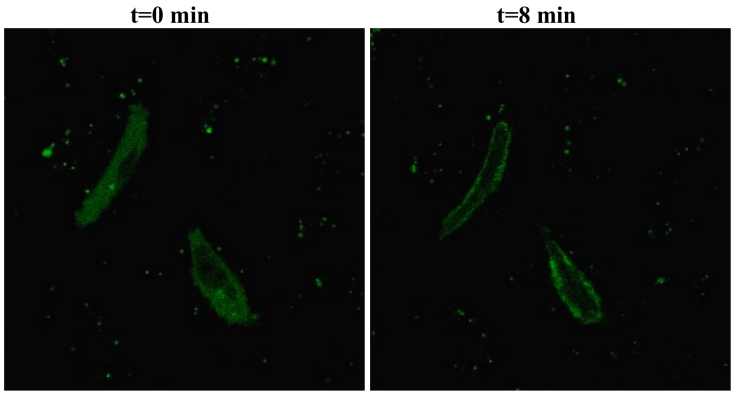


Figure S4. Confocal microscopy images of CHO-K1 cells treated with **7**-pPKCδ-GFP complexes at a 25:1 (+/-) charge ratio. (A) Confocal microscopy image of cells 48 hours after transfection. (B) Confocal microscopy image of the same CHO-K1 cells 8 minutes after treatment with 200 nM bryostatin 1.

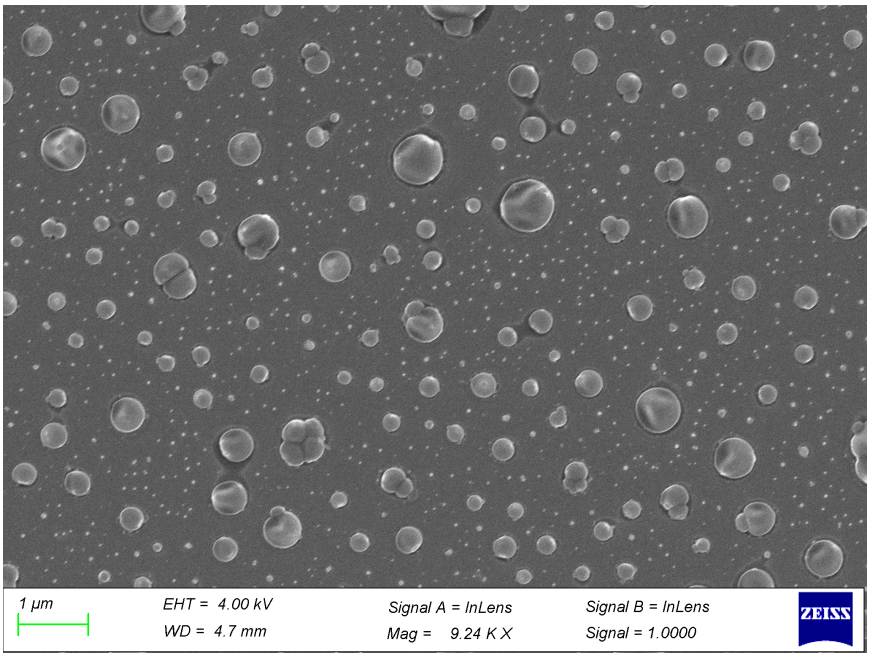


Figure S5. SEM image of CART **6a**-pPKCδ-GFP complexes on silicon wafer with sputter coating. Small particles ~30nm are hypothesized to be uncomplexed CART.

A **B**

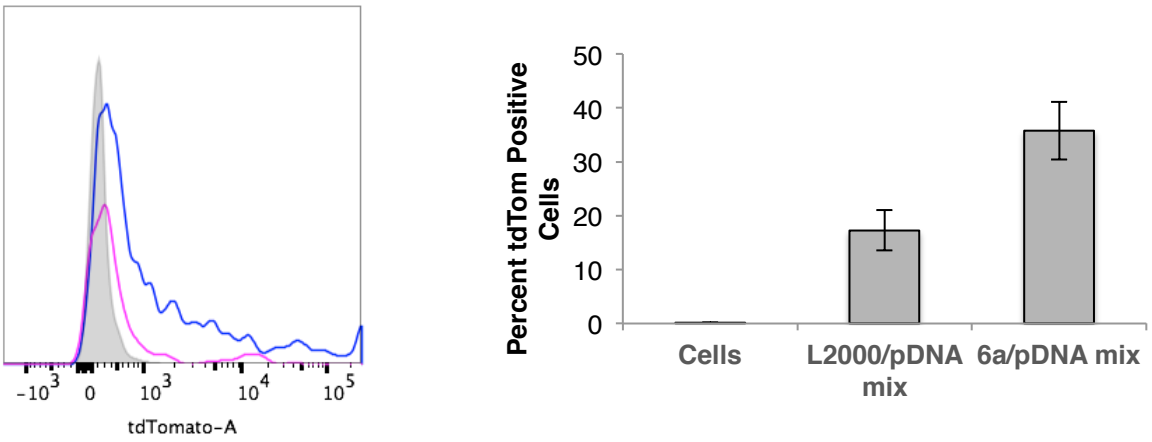


Figure S6. Flow cytometry results for CHO-K1 cells transfected with pDNA mix, using either L2000 or CART **6a** at a 5:1 (+/-) charge ratio. (A) Histogram of counts vs. tdTom fluorescence of cells alone (grey), L2000-pDNA mix (pink), and CART **6a**-pDNA mix (blue). (B) Percent

transfection based on tdTom fluorescence of cells alone, L2000-pDNA mix, and CART **6a**-pDNA mix. Error is expressed as ± SD.

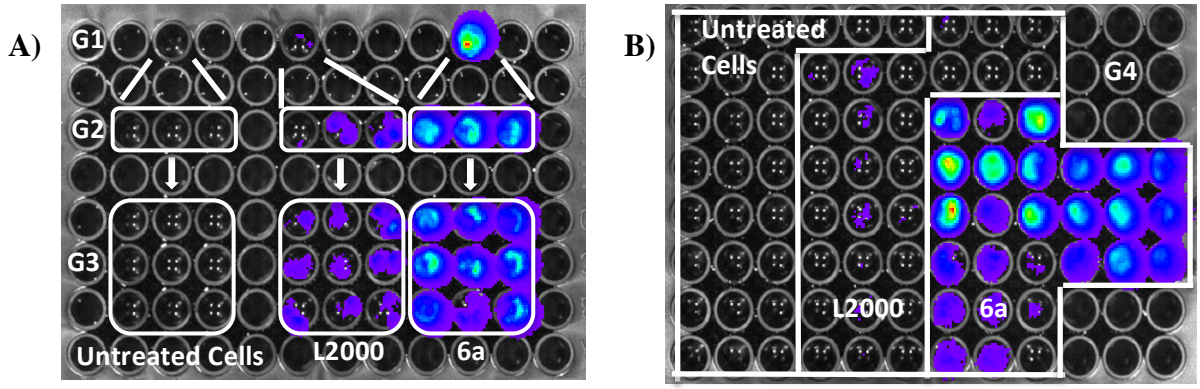


Figure S7. Plate layout for multi-generational cellular study of stable transfection of fLuc=tdTom *SB* transposon system in CHO-K1 cells by L2000 and CART **6a**. Representative image of bioluminescence on day 10. All cells started in G1 wells (treatment at 171 ng pDNA/well). (A) Plate 1 contains initial well for each condition (G1), as well as the second and third generations (G2, G3). (B) Plate 2 contains the fourth generation of cells (G4) carried forward from the G3 wells of Plate 1.

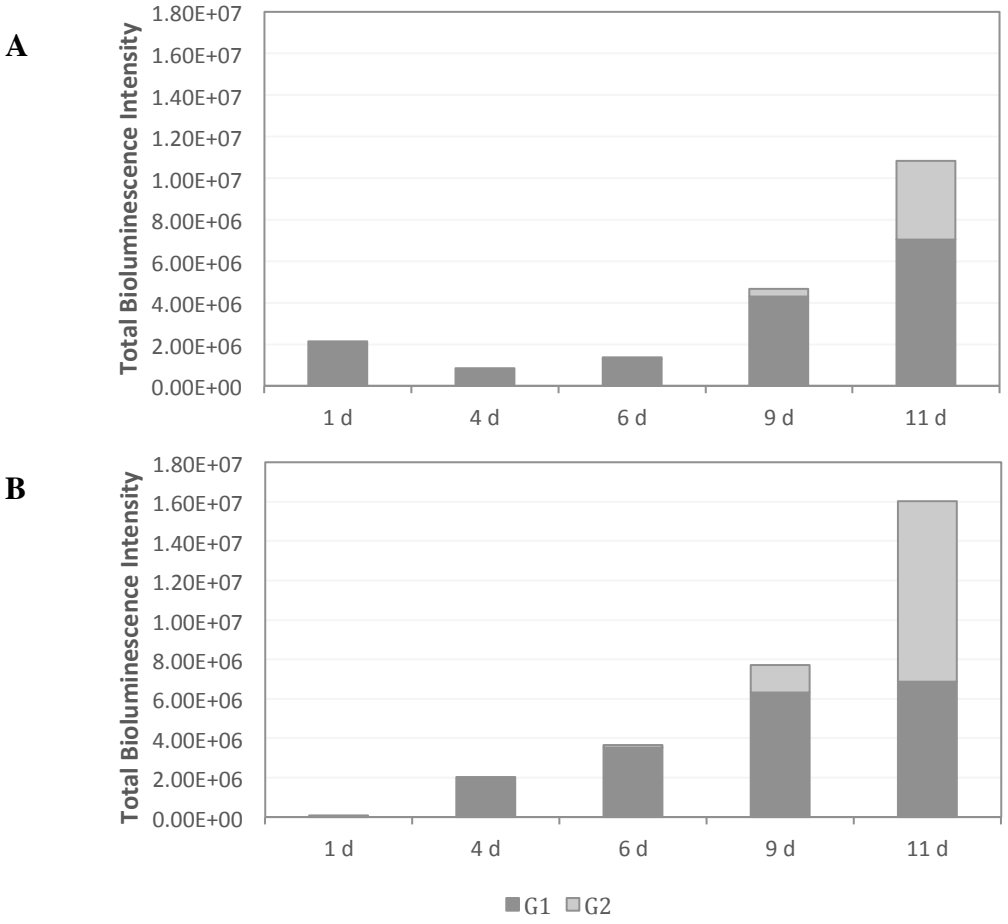


Figure S8. Bioluminescence intensity (p/s/cm^2/sr) of all CHO-K1 cells transfected with the fLuc=tdTom *SB* transposon system and then treated with puromycin over 11 days, broken down by generation (G1-G2). (A) Sum total bioluminescence resulting from transfection with L2000 for 24 h. (B) Sum total bioluminescence resulting from transfection with **6a** for 24 h. **6a**-pDNA complexes were formulated at 5:1 (+/-) charge ratio.

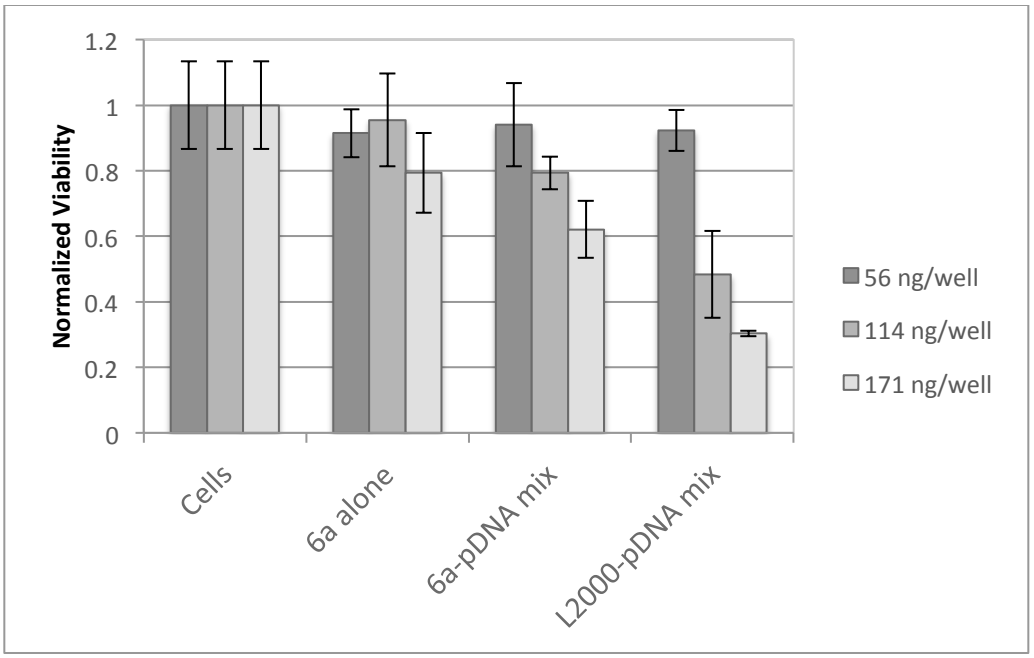


Figure S9. Cell viability assay of CART **6a** alone and **6a**-pKT2/CAGL=tTP/pCMV-SB100 mix (pDNA mix) in CHO-K1 cells, normalized to untreated cells. Cells were treated with 171 ng, 114 ng, or 56 ng of pDNA mix, with either L2000, a 5:1 (+/-) ratio of **6a**, or the corresponding amount of **6a** alone. Error is expressed as ±SD.

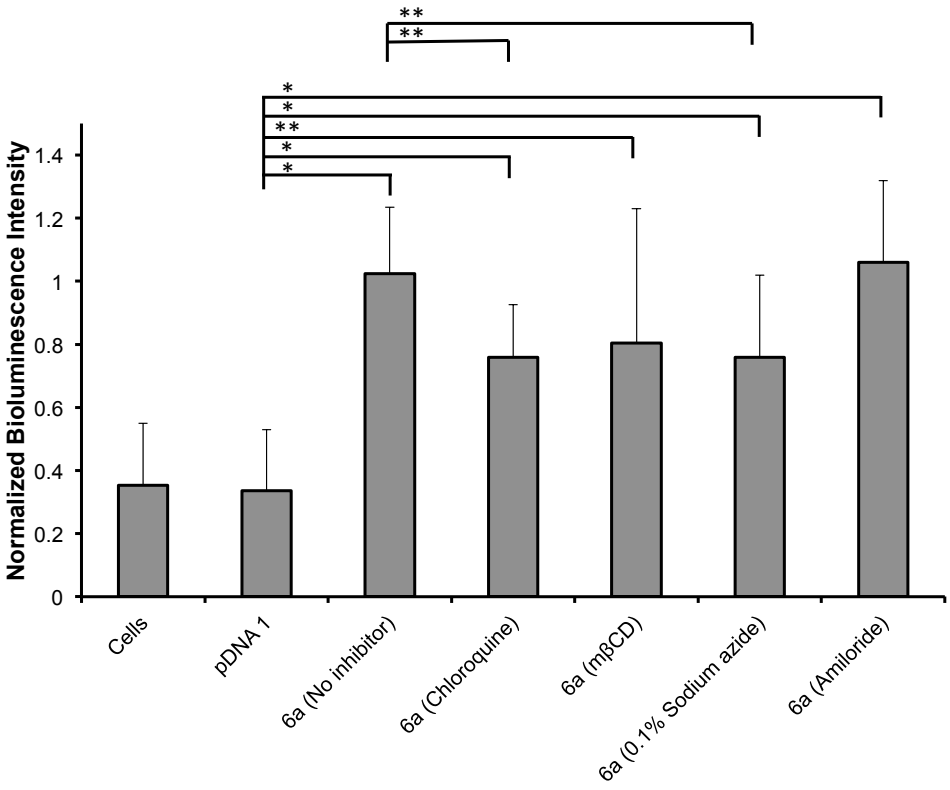
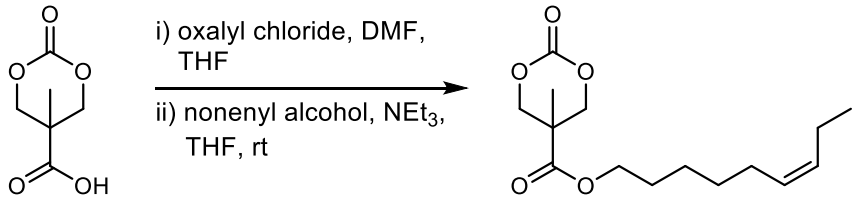


Figure S10. Normalized bioluminescence intensity of CHO-K1 cells (plated at 10,000 cells/well in black 96-well plates) treated with pKT2/CAGL=tTP alone (pDNA 1) or **6a**-pKT2/CAGL=tTP complexes at a 5:1 (+/-) charge ratio in serum-free F-12K medium using either no inhibitor, 100 μM chloroquine, 5 mM methyl-β-cyclodextrin (mβCD), 0.1% sodium azide, or 1 mM amiloride. All treatments were for 1.5 hours, after which time the media was replaced with serum-containing F-12K medium. After an additional 22.5 h, D-luciferin was added to the wells before the resultant bioluminescence was measured using an IVIS 50 charge-coupled device (CCD) camera and Living Image Software (Perkin Elmer, Waltham, MA). Error is expressed as ± SD, all samples were run in at least 10 replicates, *$p<0.0005$, **$p<0.005$.

Procedures and Characterization Data.



S1 *43%* **S2**

Procedure for nonenyl carbonate monomer S2

Catalytic dry dimethylformamide (1 drop) was added to a solution of cyclic carbonate carboxylic acid **S1**[4] (250 mg, 1.56 mmol, 1 eq.) in dry THF (8 mL) in an oven-dried 25 mL round bottom flask under N_2. To this solution was added oxalyl chloride (137 μL as a solution in 3 mL THF, 1.60 mmol, 1.02 eq.) in THF (3 mL). The reaction mixture was stirred for 1 h at rt and then the volatiles were removed *in vacuo* to yield the acid chloride as a pale yellow solid. The product was used immediately without further purification.

To a solution of cis-6-nonen-1-ol (261 μL, 1.56 mmol, 1 eq.) in THF (2 mL) was added freshly distilled triethylamine (238 μL, 1.71 mmol, 1.1 eq.). This solution was added to a solution of the crude acid chloride from above in THF (3 mL), and white precipitate immediately formed. The reaction was stirred under N_2 overnight at rt. The heterogenous reaction mixture was filtered, washed with THF (3 × 5 mL), and then concentrated *in vacuo*. Purification of the resulting residue by silica gel column chromatography (30-40% EtOAc/pentane) yielded the desired monomer as a clear oil (43%).

Characterization data for nonenyl carbonate monomer S2

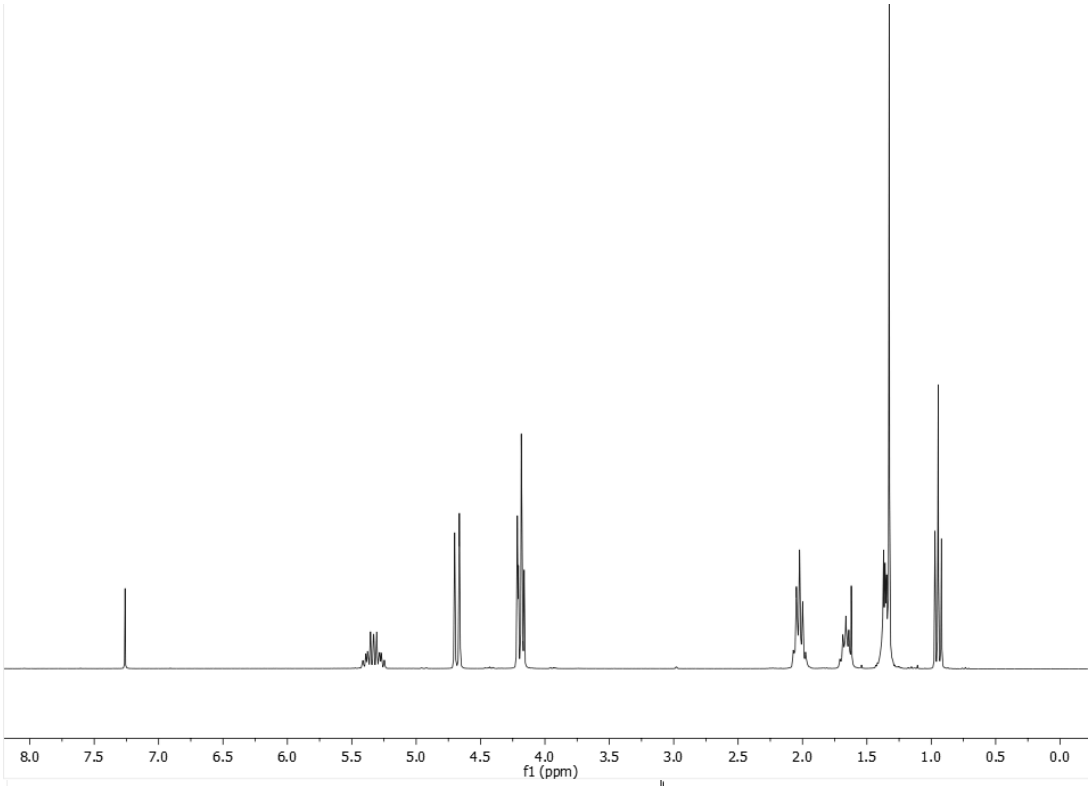
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 5.44-5.22 (m, 2H), 4.68 (d, 2H, *J* = 10.9 Hz), 4.24-4.13 (m, 4H), 2.02 (*app* p, 4H, *J* = 7.1 Hz), 1.73-1.60 (m, 2H), 1.43-1.29 (m, 4H), 1.33 (s, 3H), 0.95 (t, 3H, *J* = 7.5 Hz) ppm.

^{13}C NMR ($CDCl_3$, 75 MHz): δ = 171.2, 147.6, 132.1, 128.7, 73.1, 66.5, 40.3, 29.3, 28.4, 27.0, 25.4, 20.6, 17.8, 14.5 ppm.

IR (thin film): ν = 3003, 2962, 2934, 2858, 1764, 1735, 1465, 1404. 1332, 1239, 1177, 1138, 1103, 765 cm^{-1}.

HRMS (ES+, *m/z*) calculated for $C_{15}H_{24}O_5Na^+$: 307.1498, Found 307.1519.

R_f = 0.40 (35% EtOAc/pentane), one blue spot, *p*-anisaldehyde + UV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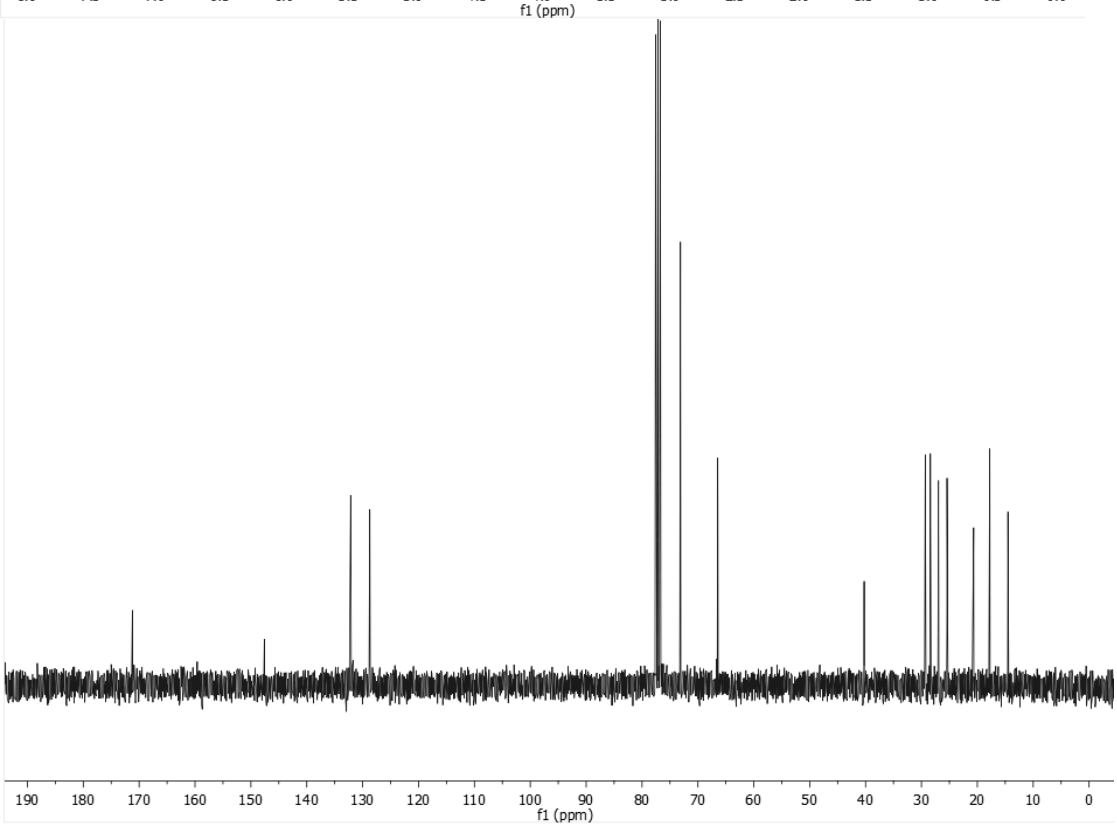
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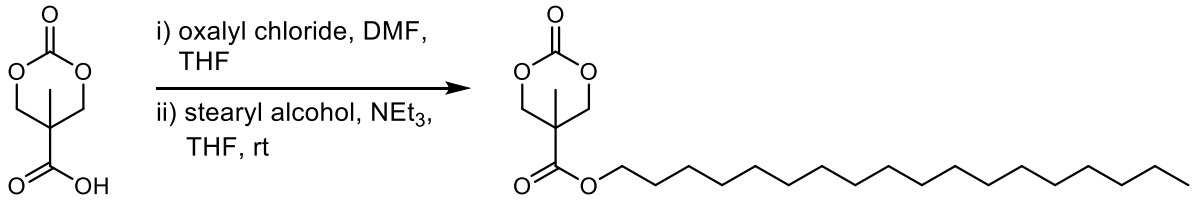


S1 ***52%*** **S3**

Procedure for stearyl carbonate monomer S3

Catalytic dry dimethylformamide (2 drops) was added to a solution of cyclic carbonate carboxylic acid **S1**[4] (250 mg, 1.56 mmol, 1 eq.) in dry THF (8 mL) in an oven-dried 25 mL round bottom flask under N_2. To this solution was added oxalyl chloride (137 μL as a solution in 3 mL THF, 1.59 mmol, 1.02 eq.). The reaction mixture was stirred for 1 h at rt and then the volatiles were removed *in vacuo* to yield the acid chloride as a pale yellow solid. The product was used immediately without further purification.

To a solution of stearyl alcohol (422 mg, 1.56 mmol, 1 eq.) in THF (2 mL) was added freshly distilled triethylamine (238 μL, 1.71 mmol, 1.1 eq.). This solution was added to a solution of the crude acid chloride from above in THF (3 mL), and white precipitate immediately formed. The reaction mixture was stirred overnight at rt. The heterogeneous reaction mixture was filtered, washed with THF (3× 5 mL) and then concentrated *in vacuo*. Purification of the resulting residue by silica gel column chromatography (30→40% EtOAc/pentane) yielded the desired monomer as a waxy white solid (52%).

Characterization data for stearyl carbonate monomer S3

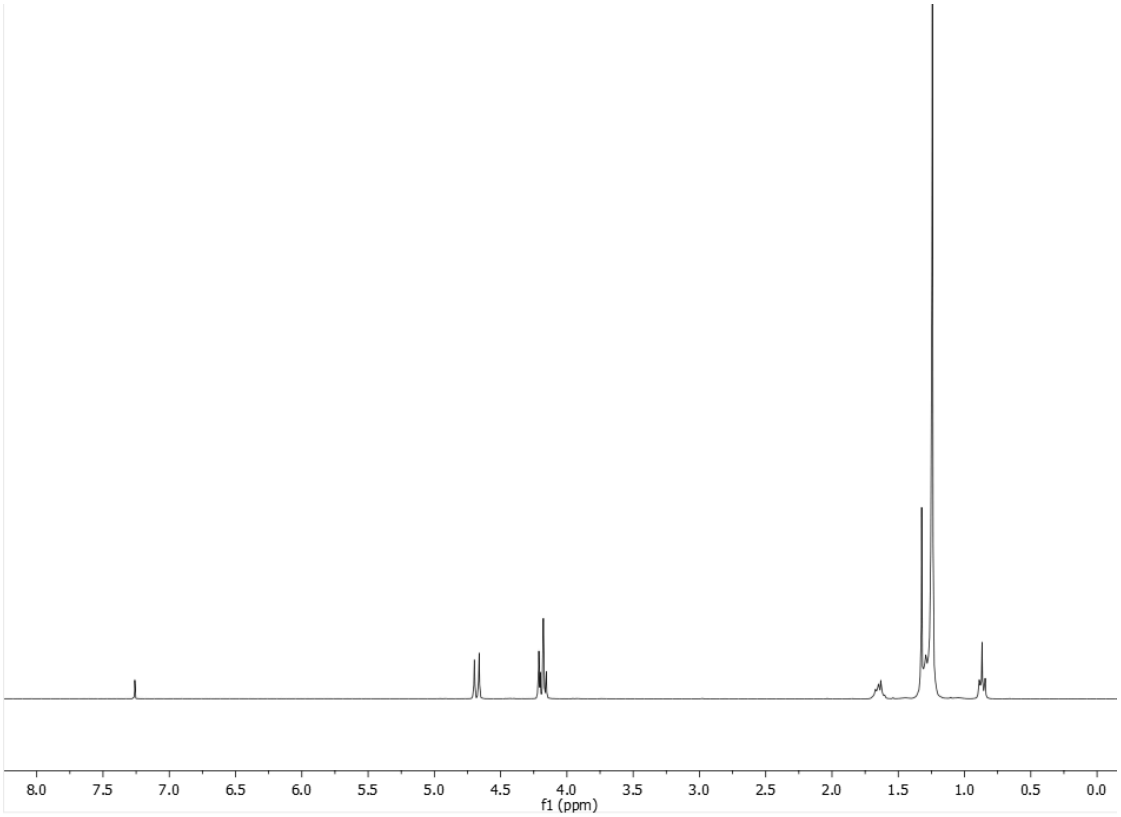
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 4.68 (d, 2H, *J* = 10.9 Hz), 4.23-4.14 (m, 4H), 1.70-1.58 (m, 2H), 1.37-1.17 (m, 33H), 0.92-0.82 (m, 3H) ppm.

^{13}C NMR ($CDCl_3$, 125 MHz): δ = 171.3, 147.7, 73.6, 66.7, 40.4, 32.2, 30.0 (broad), 29,8, 29.7, 29.6, 29.4, 28.9, 28.6, 28.4, 25.9, 23.0, 18.0, 17.8, 14.3 ppm.

IR (thin film) ν = 2955, 2914, 2848, 1737, 1470, 1239, 1184, 1133, 1104 cm^{-1}.

HRMS = (ES+, *m/z*) calculated for $C_{24}H_{44}O_5Na^+$: 435.3098, Found 435.3081.

R_f = 0.2 (20% EtOAc/pentane), one blue spot, *p*-anisaldehyde + UV.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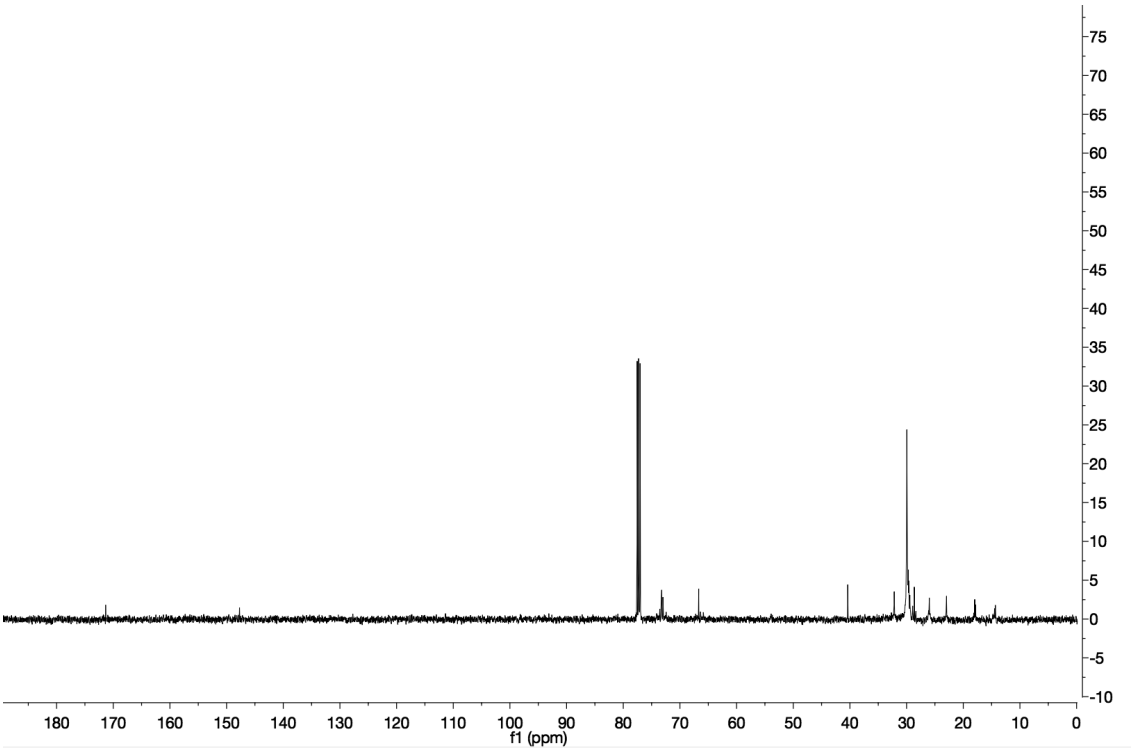
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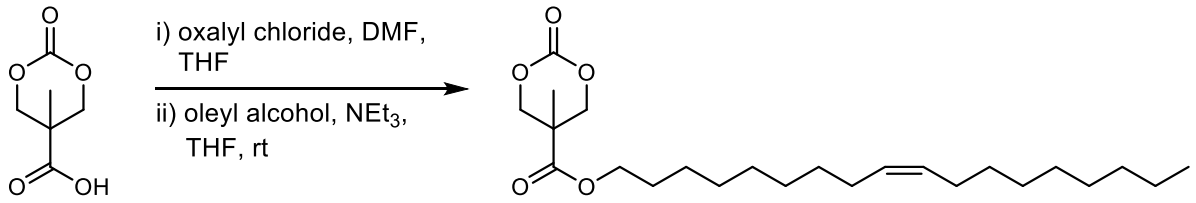


S1 ***46%*** **S4**

Procedure for oleyl carbonate monomer S4

Catalytic dry dimethylformamide (2 drops) was added to a solution of cyclic carbonate carboxylic acid **S1**[4] (250 mg, 1.56 mmol, 1 eq.) in dry THF (8 mL) in an oven-dried 25 mL round bottom flask under N_2. To this solution was added oxalyl chloride (137 μL as a solution in 3 mL THF, 1.59 mmol, 1.02 eq.). The reaction mixture was stirred for 1 h at rt and then the volatiles were removed *in vacuo* to yield the acid chloride as a pale yellow solid. The product was used immediately without further purification.

To a solution of oleyl alcohol (493 μL, 1.56 mmol, 1 eq.) in THF (2 mL) was added freshly distilled triethylamine (238 μL, 1.71 mmol, 1.1 eq.). This solution was added to a solution of the crude acid chloride from above in THF (3 mL), and pale yellow precipitate immediately formed. The reaction mixture was stirred under N_2 overnight at rt. The heterogeneous reaction mixture was filtered, washed with THF (3 × 5 mL) and then concentrated *in vacuo*. Purification of the resulting residue by silica gel column chromatography (30→40% EtOAc/pentane) yielded the desired monomer as a clear oil (46%).

Characterization data for oleyl carbonate monomer S4

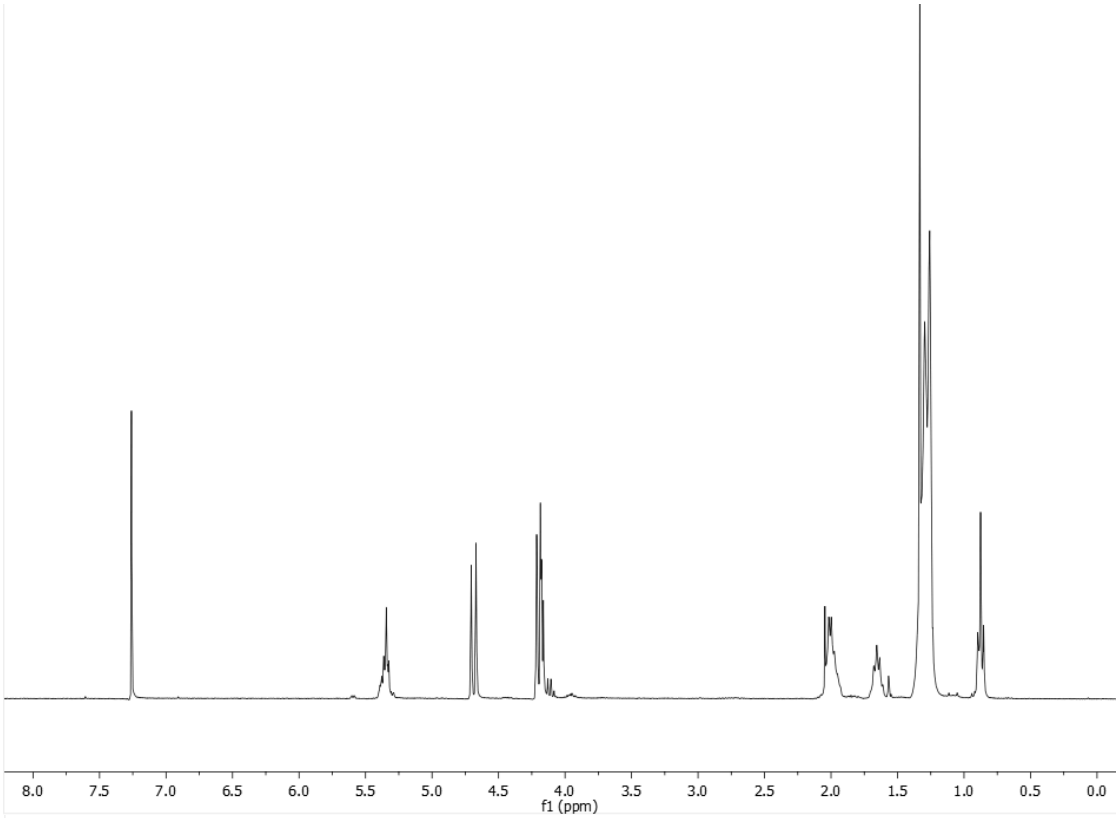
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 5.41-5.28 (m, 2H), 4.69 (d, 2H, *J* = 10.8 Hz), 4.24-4.13 (m, 4H), 2.07-1.91 (m, 4H), 1.66 (*app* p, 2H, *J* = 6.7 Hz), 1.39-1.18 (m, 25H), 0.88 (t, 3H, *J* = 6.6 Hz) ppm.

^{13}C NMR ($CDCl_3$, 75 MHz): δ = 171.2, 147.6, 130.1, 129.9, 73.1, 66.6, 40.3, 32.8, 32.1, 29.9, 29.8, 29.7, 29.5, 29.5, 29.3, 29.3, 28.5, 27.4, 27.3, 25.9, 22.8, 17.8, 14.3 ppm.

IR (thin film) ν = 3508, 2925, 2855, 1760, 1466, 1404, 1332, 1241, 1179, 1139, 1104, 968, 930, 806, 765, 737, 704 cm^{-1}.

HRMS = (ES+, *m/z*) calculated for $C_{24}H_{42}O_5Na^+$: 433.2898, Found 433.2924.

R_f = 0.26 (20% EtOAc/pentane), one blue spot, *p*-anisaldehyde + UV.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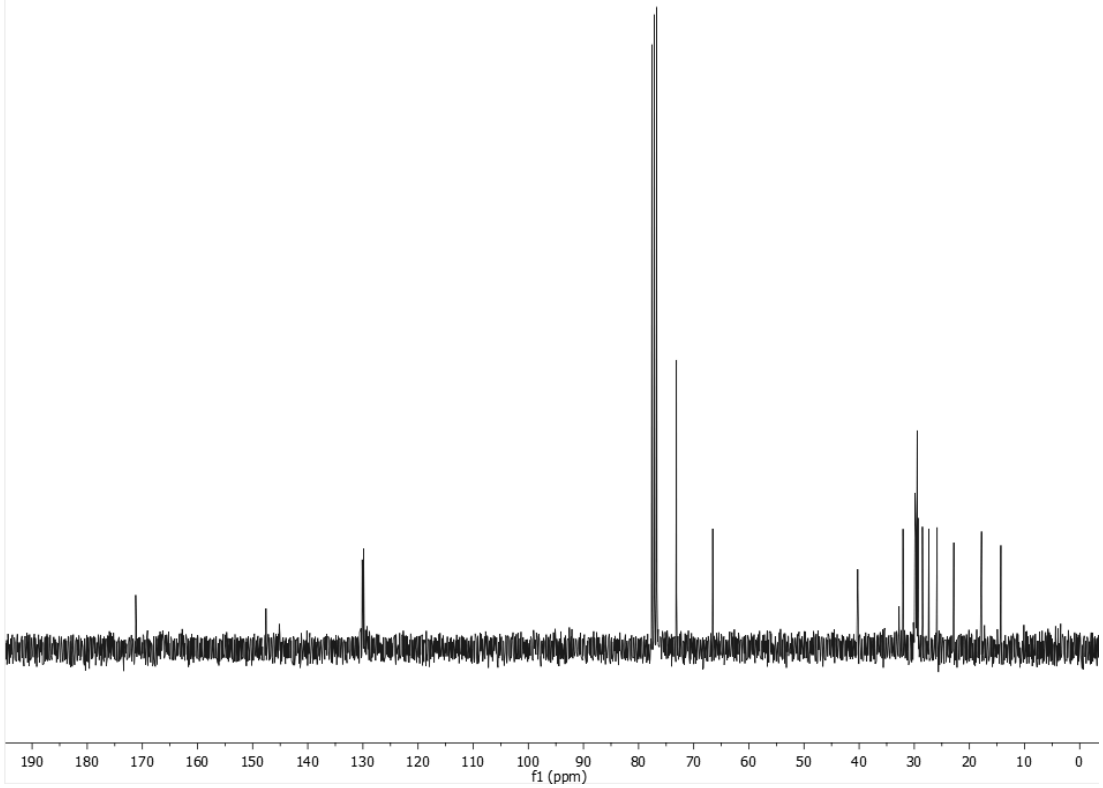
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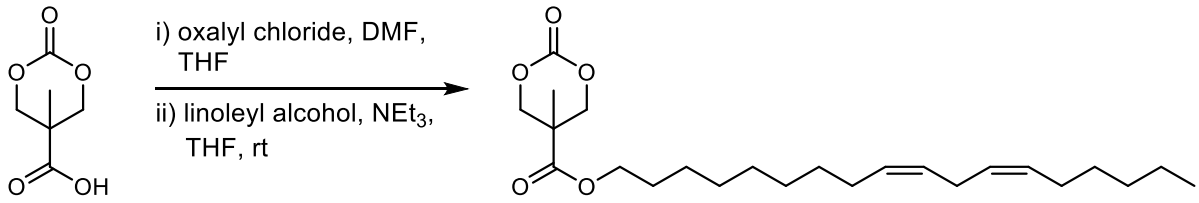


S1 ***40%*** **S5**

Procedure for linoleyl carbonate monomer S5

Catalytic dry dimethylformamide (1 drop) was added to a solution of cyclic carbonate carboxylic acid **S1**[4] (48 mg, 0.30 mmol, 1 eq.) in dry THF (1.5 mL) in an oven-dried 10 mL round bottom flask under N_2. To this solution was added oxalyl chloride (26 μL as a solution in 0.6 mL THF, 0.306 mmol, 1.02 eq.). The reaction mixture was stirred for 1 h at rt and then the volatiles were removed *in vacuo* to yield the acid chloride as a pale yellow solid. The product was used immediately without further purification.

To a solution of linoleyl alcohol (80 mg, 0.30 mmol, 1 eq.) in THF (0.6 mL) was added freshly distilled triethylamine (46 μL, 0.33 mmol, 1.1 eq.). This solution was added to a solution of the crude acid chloride from above in THF (1 mL), and white precipitate immediately formed. The reaction mixture was stirred under N_2 overnight at rt. The heterogeneous reaction mixture was filtered, washed with THF (3 × 5 mL) and then concentrated *in vacuo*. Purification of the resulting residue by silica gel column chromatography (30→40% EtOAc/pentane) yielded the desired monomer as a clear oil (40%).

Characterization data for linoleyl carbonate monomer S5

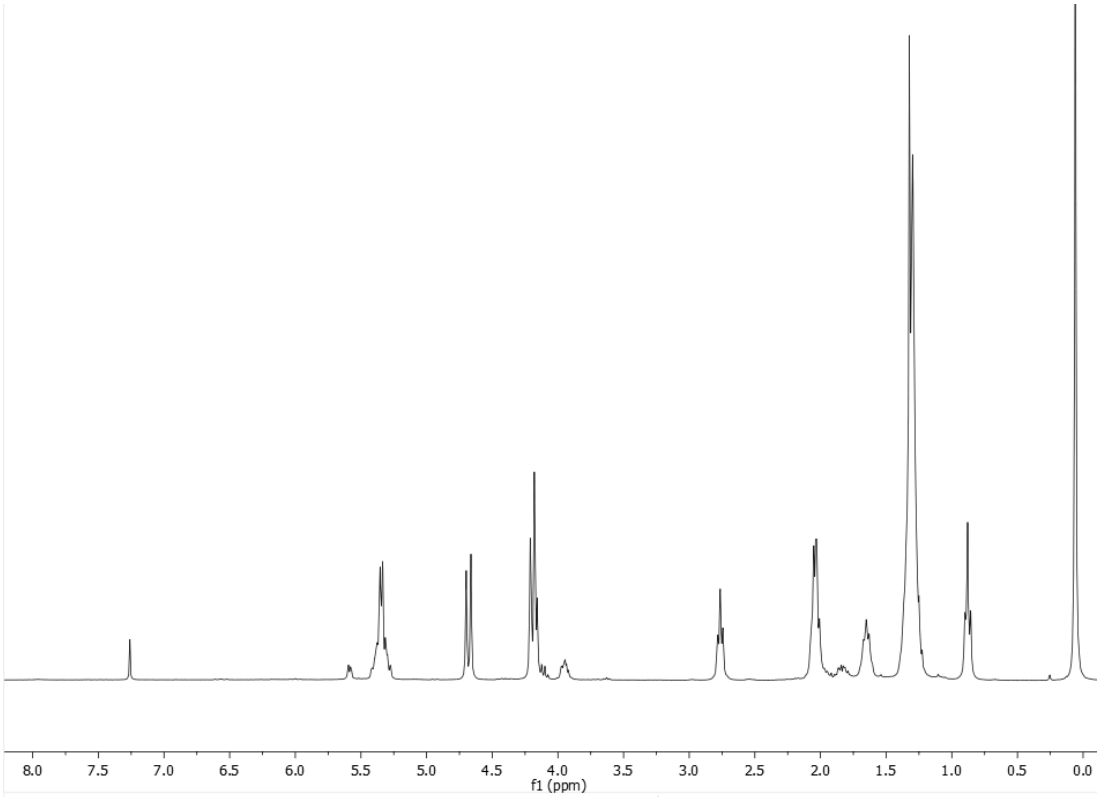
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 5.45-5.23 (m, 4H), 4.68 (d, 2H, *J* = 10.8 Hz), 4.24-4.13 (m, 4H), 2.76 (t, 2H, *J* = 6.1 Hz), 2.12-1.97 (m, 4H), 1.65 (*app* p, 2H, *J* = 7.0 Hz), 1.40-1.19 (m, 19H), 0.88 (t, 3H, *J* = 6.5 Hz) ppm.

^{13}C NMR ($CDCl_3$, 75 MHz): δ = 171.2, 147.6, 130.3, 130.2, 128.1, 128.0, 73.1, 66.5, 40.2, 32.7, 31.6, 30.0, 29.7, 29.5, 29.3, 29.2, 28.5, 27.3, 25.8, 25.7, 22.7, 17.8, 14.2 ppm.

IR (thin film) ν = 3451, 2931, 2857, 1736, 1467, 1407, 1260, 1180, 1137, 1102, 798 cm^{-1}.

HRMS = (ES+, *m/z*) calculated for $C_{24}H_{40}O_5Na^+$: 431.2798, Found 431.2763.

R_f = 0.5 (40% EtOAc/pentane), one blue spot, *p*-anisaldehyde + UV.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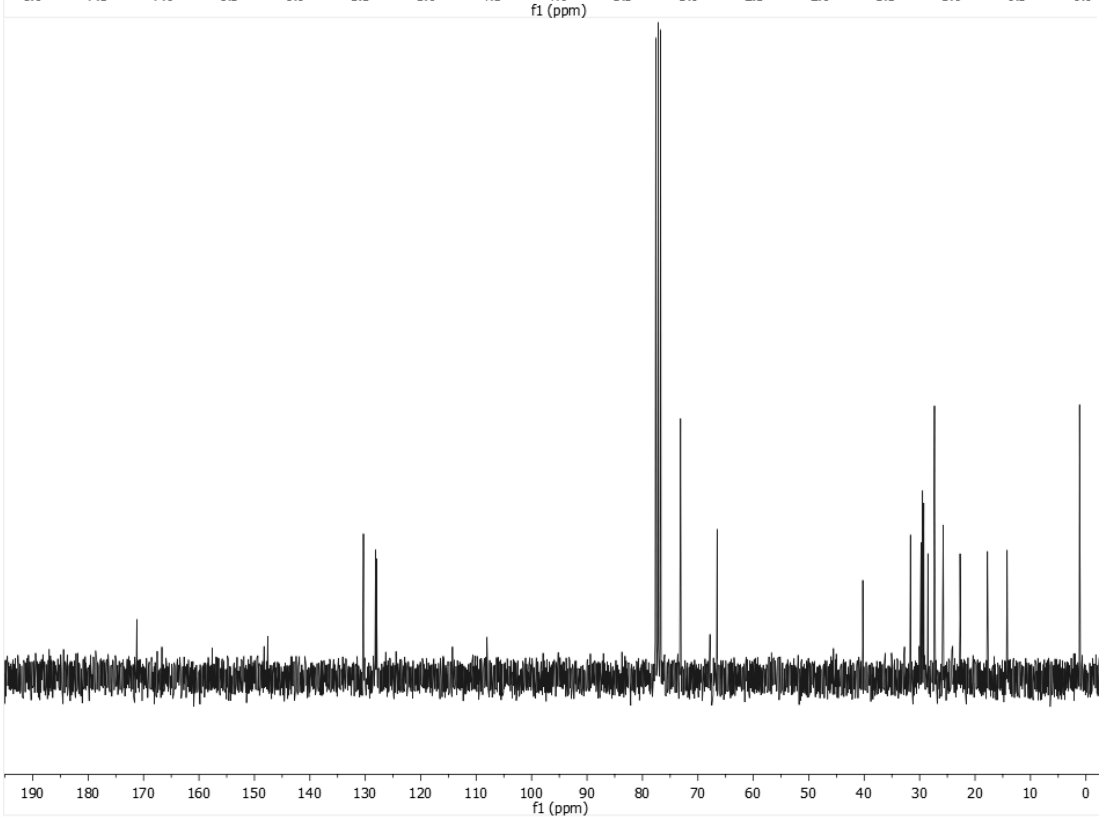
f1 (ppm)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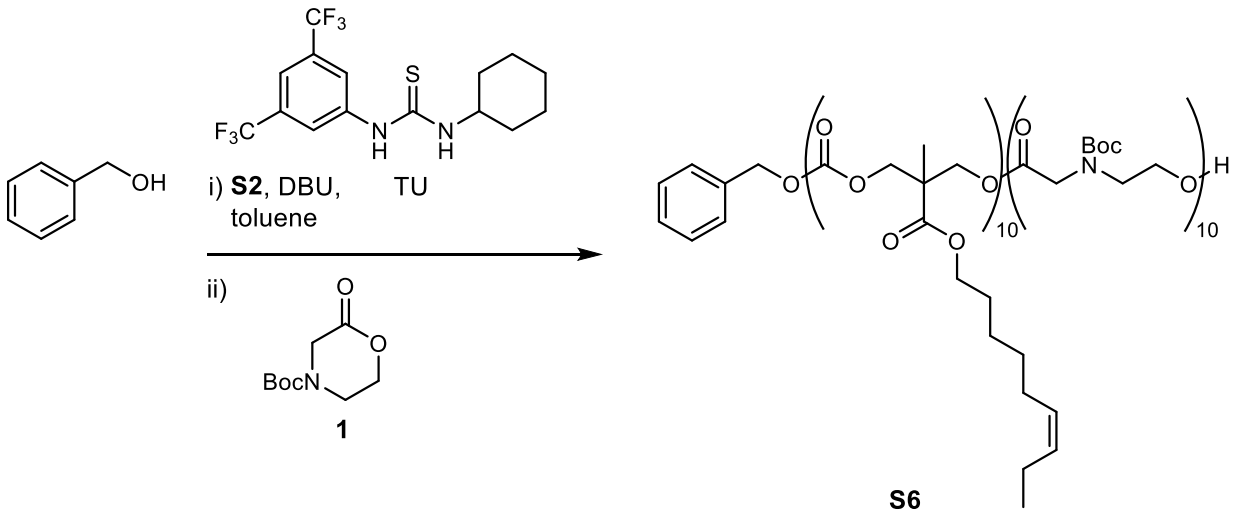


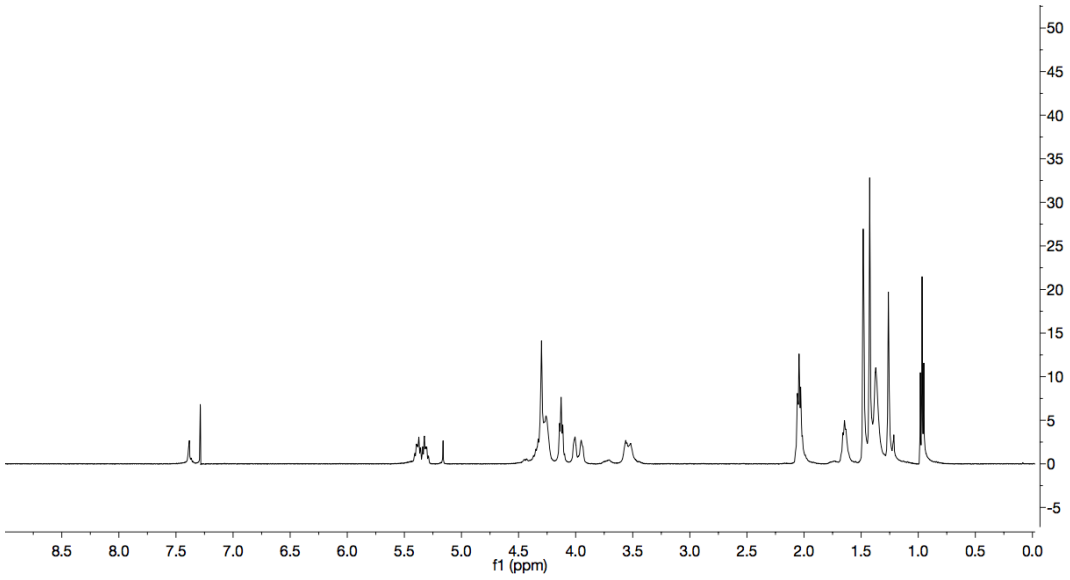
Procedure for Boc-protected CART co-oligomer N_{10}:A_{10}, S6

In a glovebox under N_2, methyl trimethylene carbonate (MTC)-nonenyl monomer **S2** (33.8 mg, 0.199 mmol, 12 eq), thiourea catalyst (TU, 5 mol% with respect to monomer, 2.2 mg, 6 μmol, 0.63 eq.), and benzyl alcohol (9.9 μL as a 1M solution in toluene, 0.01 mmol, 1 eq.) were added to a 1-dram flame-dried vial. This was then further diluted with toluene (109 μL, for a final concentration of 1M with respect to monomer). One drop of DBU (5 mol% with respect to monomer, 6 μmol, 0.63 eq.) was added to the reaction vial and the reaction was stirred in the glovebox at rt for 2 h. before N-Boc morpholinone monomer **1**[5] (26.5 mg, 0.132 mmol, 13.3 eq.) was added to the reaction vial as a solid. After the reaction stirred at rt for an additional 3.5 h, benzoic acid was added to quench the catalyst. The crude reaction solution was dialyzed against MeOH (1.0 kDa dialysis bag) overnight. Concentration afforded protected nonenyl co-oligomer **S6** as a clear oil. Degree of polymerization was determined by ^{1}H NMR end group analysis, and the molecular weight distribution (M_w/M_n; also polydispersity index, PDI) was determined by gel permeation chromatography (GPC).

Characterization data for Boc-protected CART co-oligomer N_{10}:A_{10}, S6

^{1}H NMR ($CDCl_3$, 500 MHz) δ = 7.39-7.30 (m, 5H), 5.41-5.23 (m, 21H), 5.17-5.12 (s, 2H), 4.46-4.16 (m, 58H), 4.15-4.05 (m, 20H), 4.02-3.80 (m, 20H), 3.6-3.32 (m, 19H), 2.09-1.92 (m, 40H), 1.79-1.55 (m, 24H) 1.50-1.28 (m, 130H), 1.25-1.18 (m, 30H), 1.02-0.82 (m, 27H) ppm.

GPC (THF): M_n: 4098 g/mol, M_w: 5424 g/mol; Đ: 1.32.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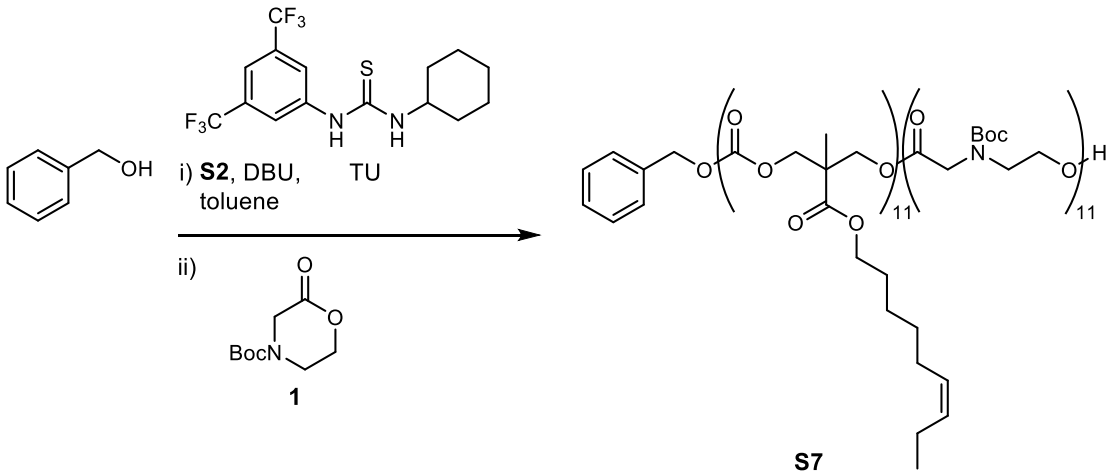


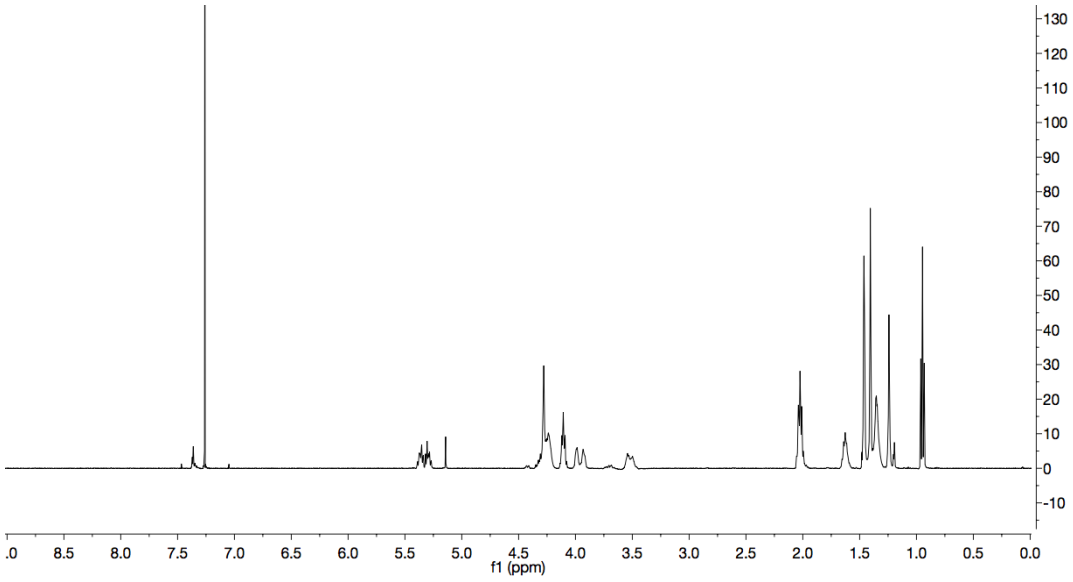
Procedure for Boc-protected CART co-oligomer: N_{11}:A_{11}, S7

In a glovebox under N_2, methyl trimethylene carbonate (MTC)-nonenyl monomer **S2** (25.3 mg, 89 μmol, 9 eq), thiourea catalyst (TU, 5 mol% with respect to monomer, 1.7 mg, 4 μmol, 0.45 eq.), and benzyl alcohol (9.9 μL as a 1M solution in toluene, 0.01 mmol, 1 eq.) were added to a 1-dram flame-dried vial. This was then further diluted with toluene (79 μL, for a final concentration of 1M with respect to monomer). One drop of DBU (5 mol% with respect to monomer, 4 μmol, 0.45 eq.) was added to the reaction vial and the reaction was stirred in the glovebox at rt for 2 h. before N-Boc morpholinone monomer **1**[5] (21.9 mg, 0.109 mmol, 11 eq.) was added to the reaction vial as a solid. After the reaction stirred at rt for an additional 3.5 h, benzoic acid was added to quench the catalyst. The crude reaction solution was dialyzed against MeOH (1.0 kDa dialysis bag) overnight. Concentration afforded protected nonenyl co-oligomer **S7** as a clear oil. Degree of polymerization was determined by ^{1}H NMR end group analysis, and the molecular weight distribution (M_w/M_n; also polydispersity index, PDI) was determined by gel permeation chromatography (GPC).

Characterization data for Boc-protected CART co-oligomer: N_{11}:A_{11}, S7

^{1}H NMR ($CDCl_3$, 500 MHz) δ = 7.40-7.30 (m, 5H), 5.44-5.23 (m, 23H), 5.17-5.12 (s, 2H), 4.46-4.16 (m, 66H), 4.15-4.05 (m, 22H), 4.02-3.80 (m, 23H), 3.6-3.32 (m, 17H), 2.09-1.92 (m, 47H), 1.79-1.55 (m, 22H) 1.50-1.40 (m, 110H), 1.38-1.28 (m, 51 H), 1.25-1.18 (m, 33H), 1.02-0.82 (m, 34H) ppm.

GPC (THF): M_n: 4107 g/mol, M_w: 5027 g/mol; Đ: 1.22.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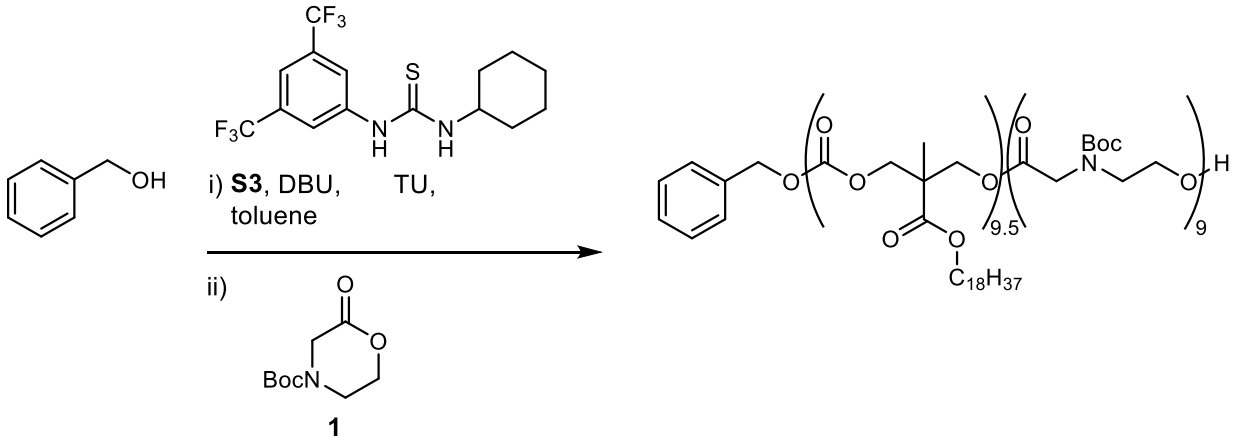


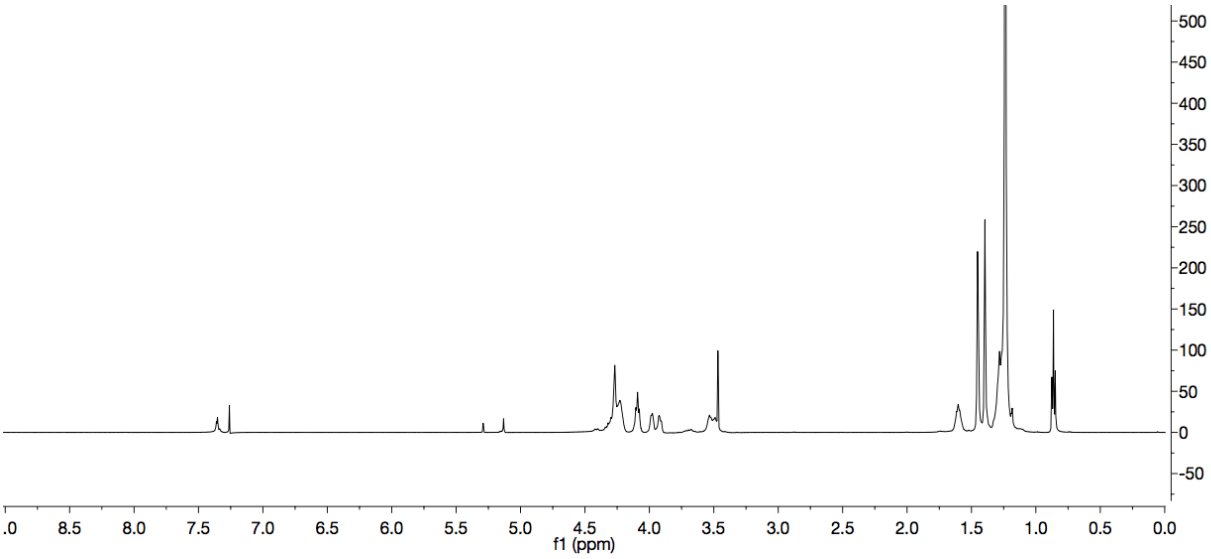
Procedure for Boc-protected CART co-oligomer: $S_{9.5}$:A_9, S8

In a glovebox under N_2, methyl trimethylene carbonate (MTC)-stearyl monomer **S3** (38 mg, 92 μmol, 12.5 eq), thiourea catalyst (TU, 5 mol% with respect to monomer, 1.7 mg, 5 μmol, 0.62 eq.), and benzyl alcohol (7.4 μL as a 1M solution in toluene, 7 μmol, 1 eq.) were added to a 1-dram flame-dried vial. This was then further diluted with toluene (85.5 μL, for a final concentration of 1M with respect to monomer). One drop of DBU (5 mol% with respect to monomer, 5 μmol, 0.62 eq.) was added to the reaction vial and the reaction was stirred in the glovebox at rt for 2 h. before N-Boc morpholinone monomer **1**[5] (21.6 mg, 0.11 mmol, 14.5 eq.) was added to the reaction vial as a solid. After the reaction stirred at rt for an additional 3.5 h, benzoic acid was added to quench the catalyst. The crude reaction solution was dialyzed against MeOH (1.0 kDa dialysis bag) overnight. Concentration afforded protected stearyl co-oligomer **S8** as a clear oil. Degree of polymerization was determined by ^{1}H NMR end group analysis, and the molecular weight distribution (M_w/M_n; also polydispersity index, PDI) was determined by gel permeation chromatography (GPC).

Characterization data for Boc-protected CART co-oligomer: $S_{9.5}$:A_9, S8

^{1}H NMR ($CDCl_3$, 500 MHz) δ = 7.40-7.30 (m, 5H), 5.17-5.12 (s, 2H), 4.46-4.16 (m, 56H), 4.15-4.05 (m, 18H), 4.02-3.80 (m, 18H), 3.6-3.32 (m, 18H), 1.68-1.53 (m, 20H) 1.51-1.35 (m, 89H), 1.34-1.08 (m, 310H), 0.93-0.80 (m, 27H) ppm.

GPC (THF): M_n*:* 5683 g/mol, M_w*:* 7400 g/mol; Đ: 1.31.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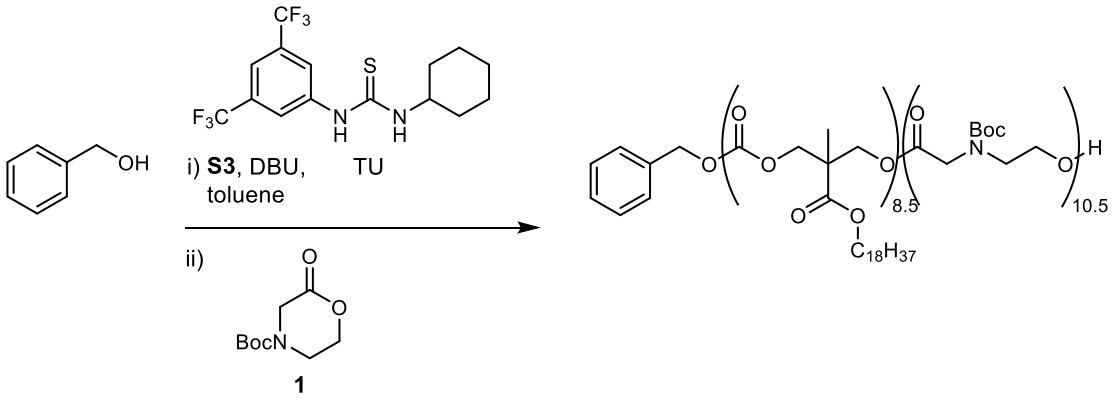


S9

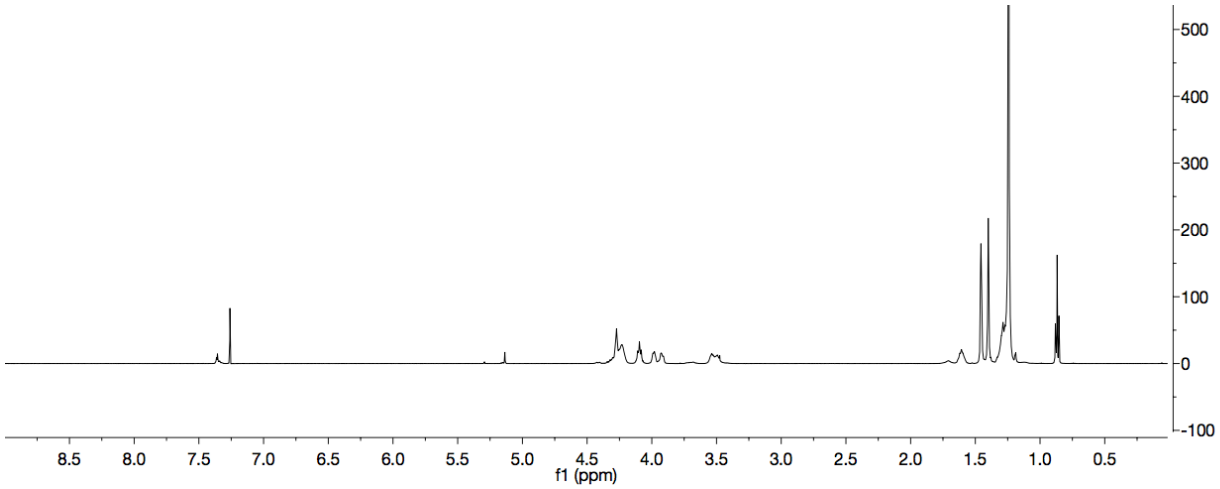
Procedure for Boc-protected CART co-oligomer: $S_{8.5}$:$A_{10.5}$, S9

In a glovebox under N_2, methyl trimethylene carbonate (MTC)-stearyl monomer **S3** (51.1 mg, 0.124 mmol, 12.5 eq), thiourea catalyst (TU, 5 mol% with respect to monomer, 2.3 mg, 6 μmol, 0.62 eq.), and benzyl alcohol (9.9 μL as a 1M solution in toluene, 0.01 mmol, 1 eq.) were added to a 1-dram flame-dried vial. This was then further diluted with toluene (113 μL, for a final concentration of 1M with respect to monomer). One drop of DBU (5 mol% with respect to monomer, 6 μmol, 0.62 eq.) was added to the reaction vial and the reaction was stirred in the glovebox at rt for 2 h. before N-Boc morpholinone monomer **1**[5] (28.9 mg, 0.143 mmol, 14.5 eq.) was added to the reaction vial as a solid. After the reaction stirred at rt for an additional 3.5 h, benzoic acid was added to quench the catalyst. The crude reaction solution was dialyzed against MeOH (1.0 kDa dialysis bag) overnight. Concentration afforded protected stearyl co-oligomer **S9** as a clear oil. Degree of polymerization was determined by ^{1}H NMR end group analysis, and the molecular weight distribution (M_w/M_n; also polydispersity index, PDI) was determined by gel permeation chromatography (GPC).

Characterization data for Boc-protected CART co-oligomer: $S_{8.5}$:$A_{10.5}$, S9

^{1}H NMR ($CDCl_3$, 500 MHz) δ = 7.40-7.30 (m, 5H), 5.17-5.12 (s, 2H), 4.38-4.16 (m, 50H), 4.15-4.05 (m, 18H), 4.02-3.80 (m, 21H), 3.6-3.32 (m, 22H), 1.68-1.53 (m, 18H) 1.52-1.37 (m, 94H), 1.37-1.13 (m, 276H), 0.93-0.80 (m, 25H) ppm.

GPC (THF): M_n: 5445 g/mol, M_w: 7402 g/mol; Đ: 1.36.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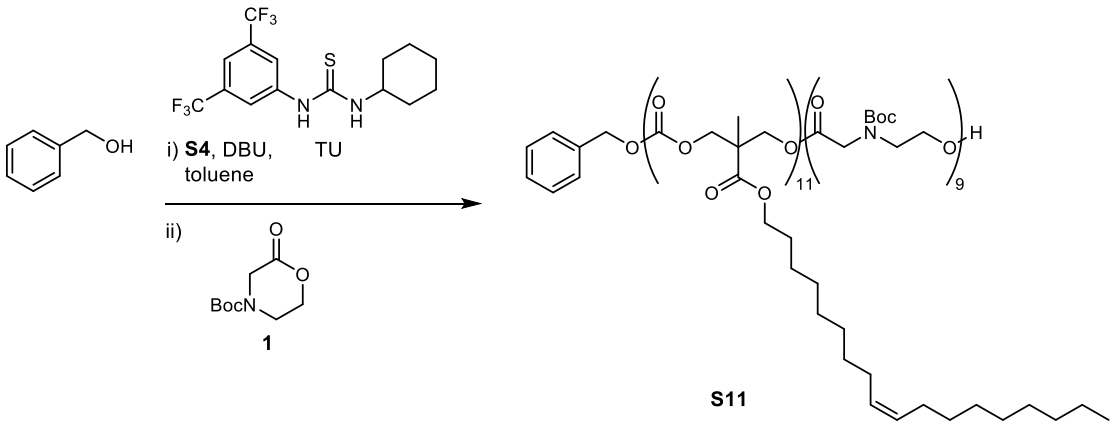
f1 (ppm)



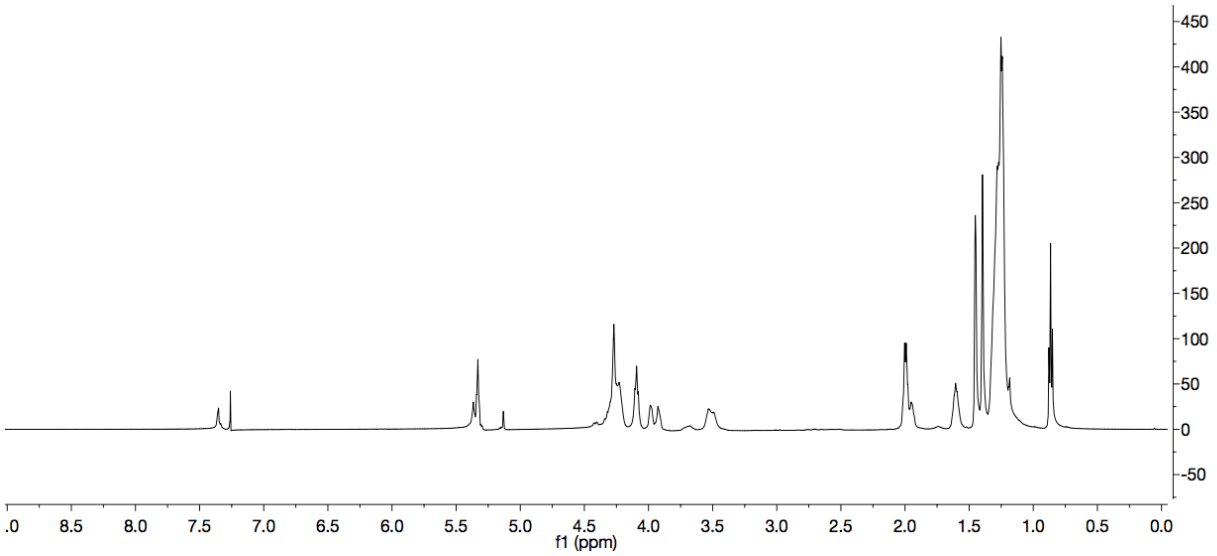
Procedure for Boc-protected CART co-oligomer: O_{11}:A_9, S11

In a glovebox under N_2, methyl trimethylene carbonate (MTC)-oleyl monomer **S4** (48.8 mg, 0.119 mmol, 12 eq), thiourea catalyst (TU, 5 mol% with respect to monomer, 2.2 mg, 6 μmol, 0.66 eq.), and benzyl alcohol (9.9 μL as a 1M solution in toluene, 0.01 mmol, 1 eq.) were added to a 1-dram flame-dried vial. This was then further diluted with toluene (117 μL, for a final concentration of 1M with respect to monomer). One drop of DBU (5 mol% with respect to monomer, 6 μmol, 0.66 eq.) was added to the reaction vial and the reaction was stirred in the glovebox at rt for 2 h. before N-Boc morpholinone monomer **1**[5] (24.5 mg, 0.123 mmol, 12.3 eq.) was added to the reaction vial as a solid. After the reaction stirred at rt for an additional 3.5 h, benzoic acid was added to quench the catalyst. The crude reaction solution was dialyzed against MeOH (1.0 kDa dialysis bag) overnight. Concentration afforded protected oleyl co-oligomer **S11** as a clear oil. Degree of polymerization was determined by ^{1}H NMR end group analysis, and the molecular weight distribution (M_w/M_n; also polydispersity index, PDI) was determined by gel permeation chromatography (GPC).

Characterization data for Boc-protected CART co-oligomer: O_{11}:A_9, S11

^{1}H NMR ($CDCl_3$, 500 MHz) δ = 7.40-7.30 (m, 5H), 5.55-5.27 (m, 23H), 5.17-5.12 (s, 2H), 4.60-4.16 (m, 66H), 4.15-4.05 (m, 22H), 4.02-3.80 (m, 16H), 3.6-3.32 (m, 18H), 2.14-1.88 (m, 39H), 1.69-1.52 (m, 21H), 1.52-1.37 (m, 83H), 1.37-1.01 (m, 296H), 0.95-0.79 (m, 34H) ppm.

GPC (THF): M_n: 5080 g/mol, M_w: 7038 g/mol; Đ: 1.39.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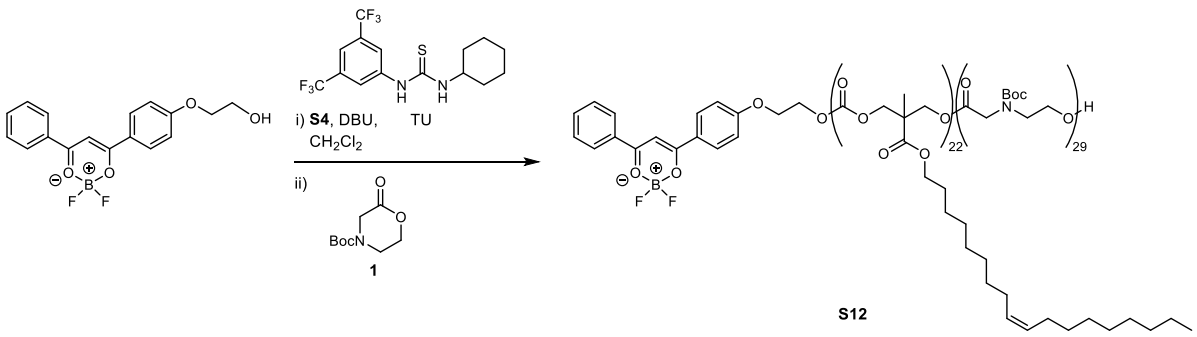


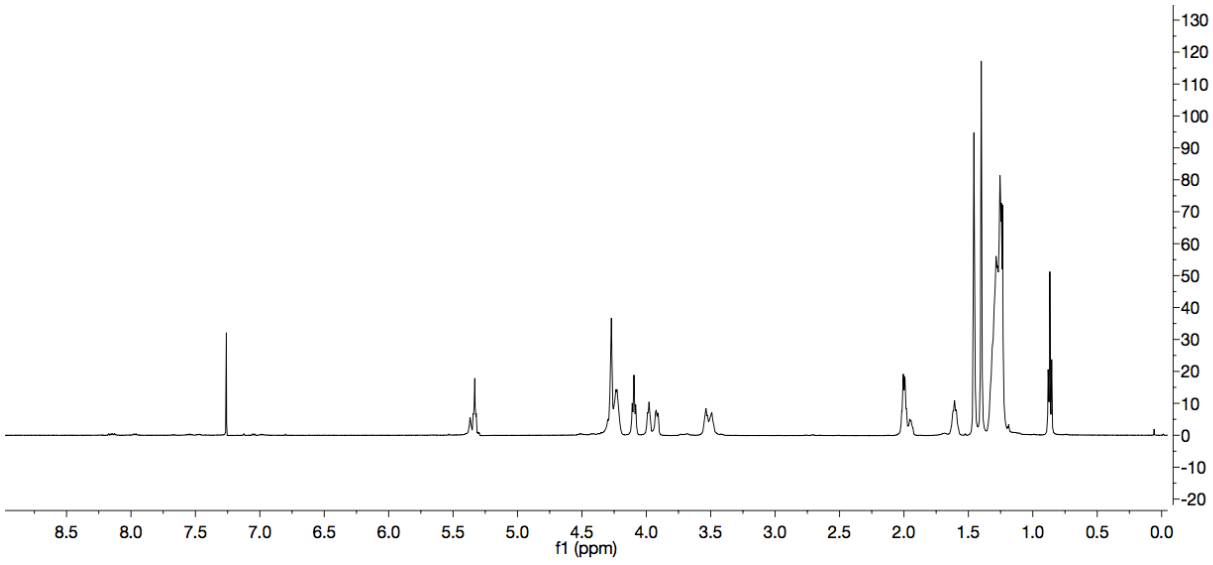
Procedure for Boc-protected CART co-oligomer: O_{22}:A_{29}, S12

In a glovebox under N_2, methyl trimethylene carbonate (MTC)-oleyl monomer **S4** (50 mg, 0.122 mmol, 122 eq), thiourea catalyst (TU, 5 mol% with respect to monomer, 2.2 mg, 6 μmol, 0.66 eq.), and BDK initiator (3.3 mg, 3 μmol, 1 eq.) were added to a 1-dram flame-dried vial. This was then diluted with CH_2Cl_2 (122 μL, for a final concentration of 1M with respect to monomer). One drop of DBU (5 mol% with respect to monomer, 6 μmol, 0.6 eq.) was added to the reaction vial and the reaction was stirred in the glovebox at rt for 2 h. before N-Boc morpholinone monomer **1**[5] (30 mg, 0.149 mmol, 15 eq.) was added to the reaction vial as a solid. After the reaction stirred at rt for an additional 3.5 h, benzoic acid was added to quench the catalyst. The crude reaction solution was dialyzed against MeOH (1.0 kDa dialysis bag) overnight. Concentration afforded protected oleyl co-oligomer **S12** as a clear oil. Degree of polymerization was determined by ^{1}H NMR end group analysis, and the molecular weight distribution (M_w/M_n; also polydispersity index, PDI) was determined by gel permeation chromatography (GPC).

Characterization data for Boc-protected CART co-oligomer: O_{22}:A_{29}, S12

^{1}H NMR ($CDCl_3$, 300 MHz) δ = 8.27-7.92 (m, 4H), 7.71-7.36 (m, 3H), 7.17-6.88 (m, 3H), 5.46-5.22(m, 44H), 4.35-4.19 (m, 148H), 4.15-4.05 (m, 47H), 4.03-3.80 (m, 58H), 3.60-3.32 (m, 58.5H), 2.07-1.89 (m, 84H), 1.68-1.54 (m, 45H), 1.50-1.00 (m, 868H), 0.91-0.79 (m, 72H) ppm.

GPC (THF): M_n: 12219 g/mol, M_w: 16051 g/mol; Đ: 1.31.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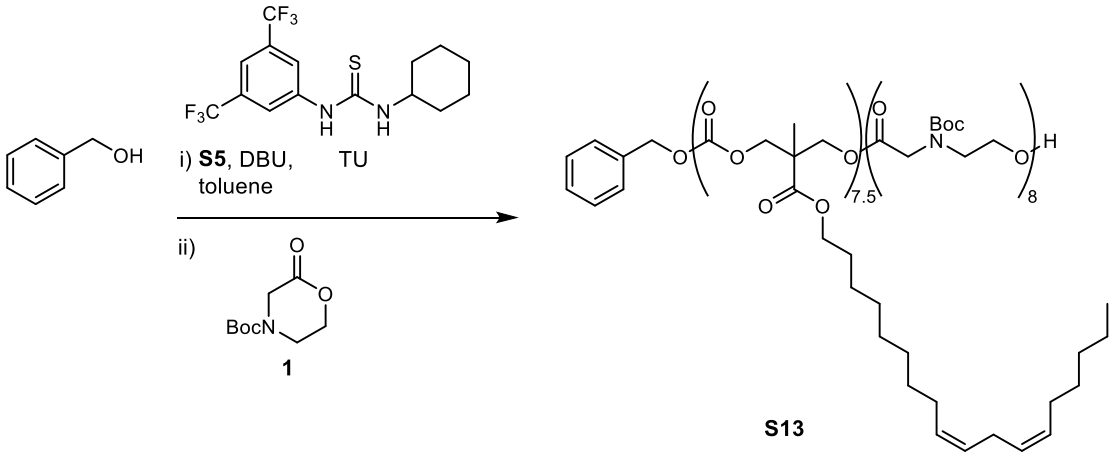


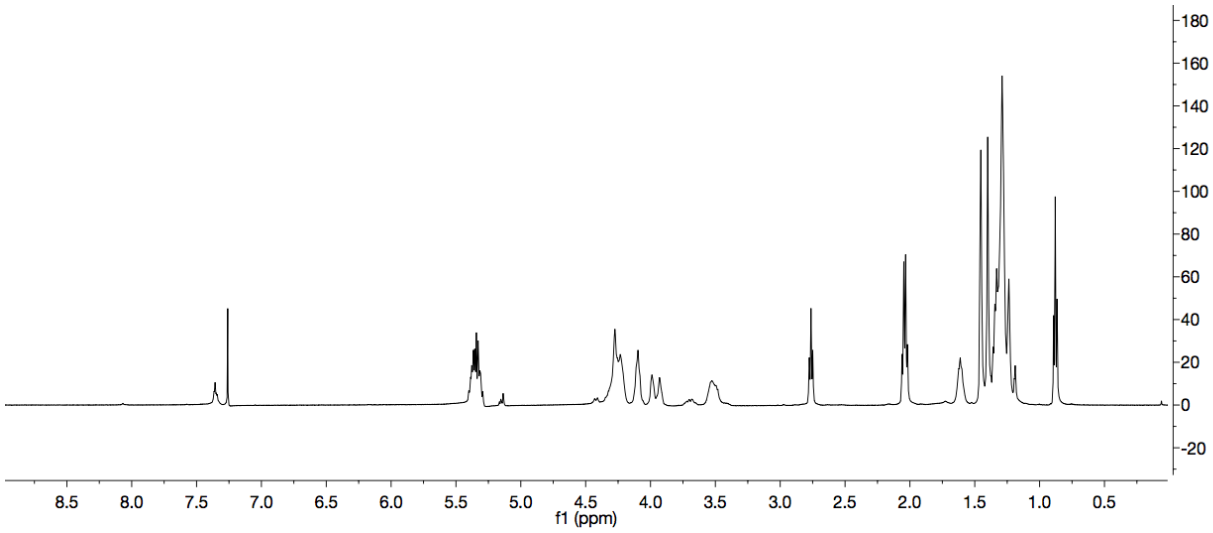
Procedure for Boc-protected CART co-oligomer: $L_{7.5}$:A_8, S13

In a glovebox under N_2, methyl trimethylene carbonate (MTC)-linoleyl monomer **S5** (20.0 mg, 49 μmol, 12.5 eq), thiourea catalyst (TU, 5 mol% with respect to monomer, 0.9 mg, 2 μmol, 0.61 eq.), and benzyl alcohol (3.9 μL as a 1M solution in toluene, 4 μmol, 1 eq.) were added to a 1-dram flame-dried vial. This was then further diluted with toluene (45 μL, for a final concentration of 1M with respect to monomer). One drop of DBU (5 mol% with respect to monomer, 6 μmol, 0.61 eq.) was added to the reaction vial and the reaction was stirred in the glovebox at rt for 2 h. before N-Boc morpholinone monomer **1**[5] (11.4 mg, 57 μmol, 14.5 eq.) was added to the reaction vial as a solid. After the reaction stirred at rt for an additional 3.5 h, benzoic acid was added to quench the catalyst. The crude reaction solution was dialyzed against MeOH (1.0 kDa dialysis bag) overnight. Concentration afforded protected linoleyl co-oligomer **S13** as a clear oil. Degree of polymerization was determined by ^{1}H NMR end group analysis, and the molecular weight distribution (M_w/M_n; also polydispersity index, PDI) was determined by gel permeation chromatography (GPC).

Characterization data for Boc-protected CART co-oligomer: $L_{7.5}$:A_8, S13

^{1}H NMR ($CDCl_3$, 500 MHz) δ = 7.45-7.30 (m, 5H), 5.55-5.21 (m, 31H), 5.17-5.12 (s, 2H), 4.50-4.16 (m, 45H), 4.16-4.04 (m, 16H), 4.02-3.80 (m, 14H), 3.68-3.32 (m, 17H), 2.87-2.67 (m, 15H), 2.11-1.93 (m, 30H), 1.69-1.52 (m, 16H), 1.49-1.37 (m, 71H), 1.36-1.11 (m, 152H), 0.94-0.81 (m, 23H) ppm.

GPC (THF): M_n: 3884 g/mol, M_w: 5977 g/mol; Đ: 1.53.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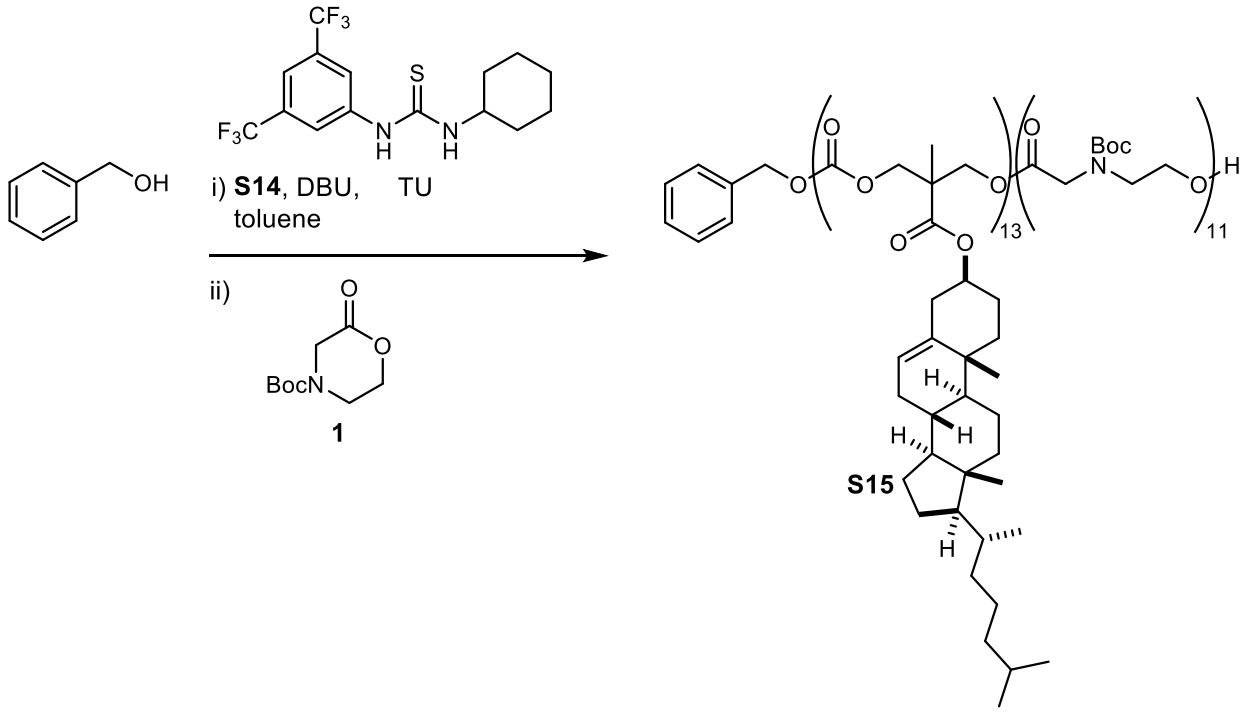
0.5
f1 (ppm)



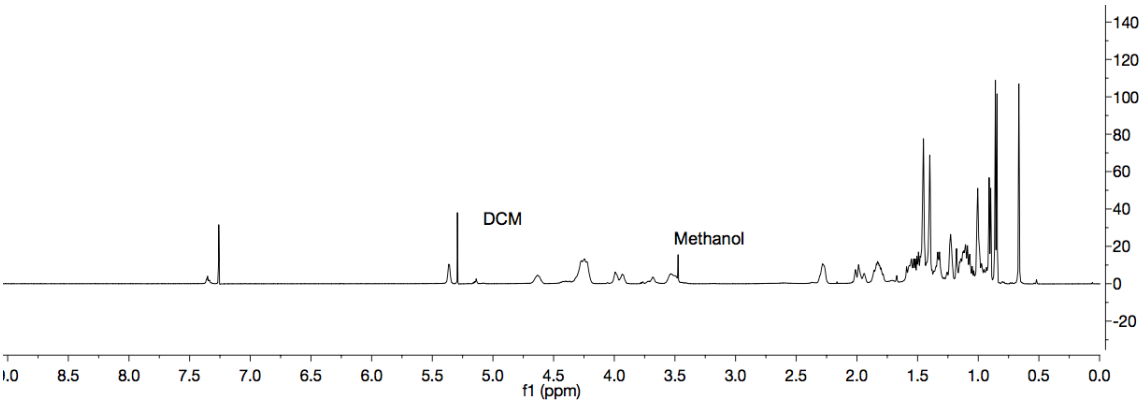
Procedure for Boc-protected CART co-oligomer: $Chol_{13}$:A_{11}, S15

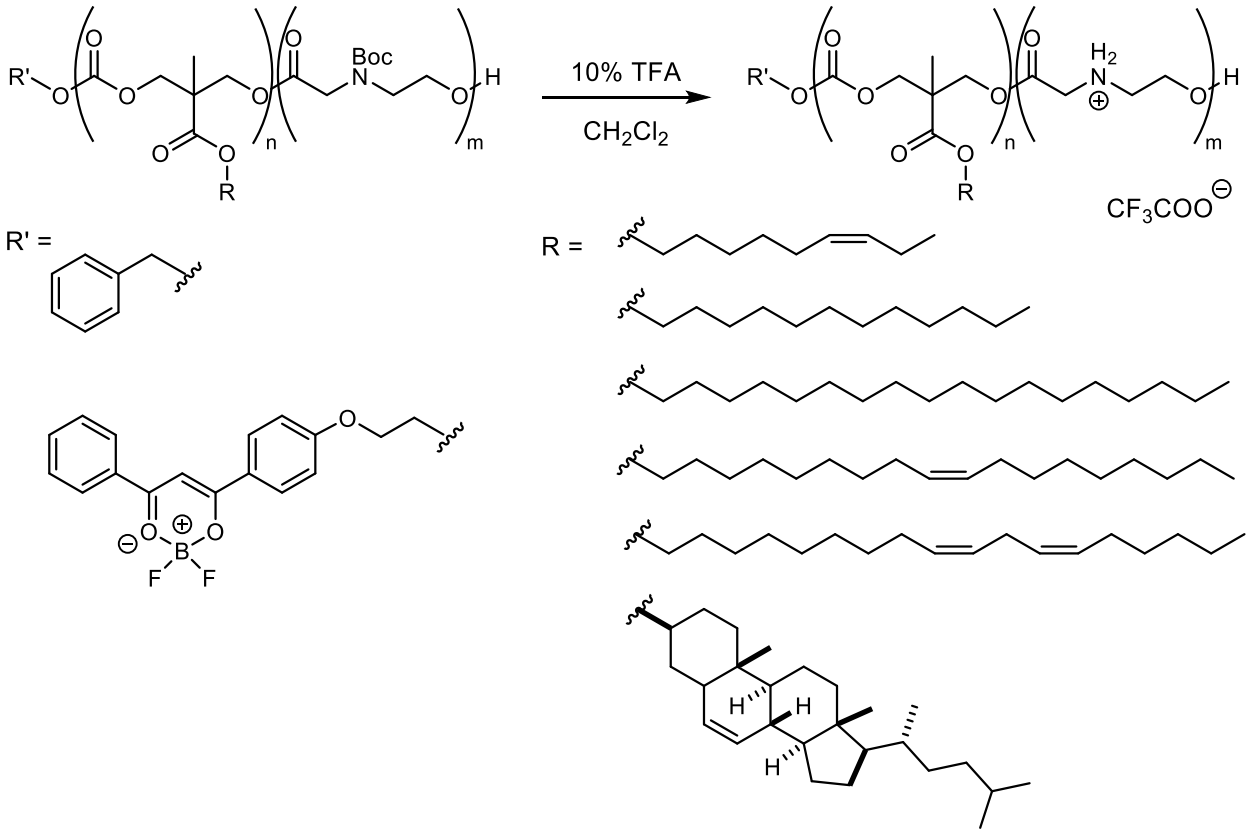
In a glovebox under N_2, methyl trimethylene carbonate (MTC)-cholesteryl monomer **S14**[6] (55.0 mg, 0.104 mmol, 10.4 eq), thiourea catalyst (TU, 5 mol% with respect to monomer, 1.7 mg, 5 μmol, 0.51 eq.), and benzyl alcohol (9.9 μL as a 1M solution in toluene, 1 mmol, 1 eq.) were added to a 1-dram flame-dried vial. This was then further diluted with toluene (90 μL, for a final concentration of 0.96M with respect to monomer). One drop of DBU (4 mol% with respect to monomer, 5 μmol, 0.51 eq.) was added to the reaction vial and the reaction was stirred in the glovebox at rt for 2 h. before N-Boc morpholinone monomer **1**[5] (23.0 mg, 0.114 mmol, 11.5 eq.) was added to the reaction vial as a solid. After the reaction stirred at rt for an additional 3.5 h, benzoic acid was added to quench the catalyst. The crude reaction solution was dialyzed against MeOH (1.0 kDa dialysis bag) overnight. Concentration afforded protected cholesteryl co-oligomer **S15** as a clear oil. Degree of polymerization was determined by ^{1}H NMR end group analysis.

Characterization of Boc-protected CART co-oligomer: $Chol_{13}$:A_{11}, S15

^{1}H NMR ($CDCl_3$, 500 MHz) δ = 7.45-7.30 (m, 5H), 5.43-5.21 (m, 13H), 5.17-5.11 (s, 2H), 4.69-4.57 (m, 13H), 4.45-4.12 (m, 68H), 4.11—3.90 (m, 22H), 3.59-3.47 (m, 22H), 2.35-2.19 (m, 26H), 2.05-1.90 (m, 29H), 1.89-1.75 (m, 43H), 1.63-1.28 (m, 242H), 1.28-0.89 (m, 263H), 0.88-0.81 (m,76H), 0.71 0.64 (m, 38H) ppm.

GPC (THF): M_n: 3376 g/mol, M_w: 4287 g/mol; Đ: 1.27.


DCM
Methanol
140
120
100
80
60
40
20
0
-20
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)

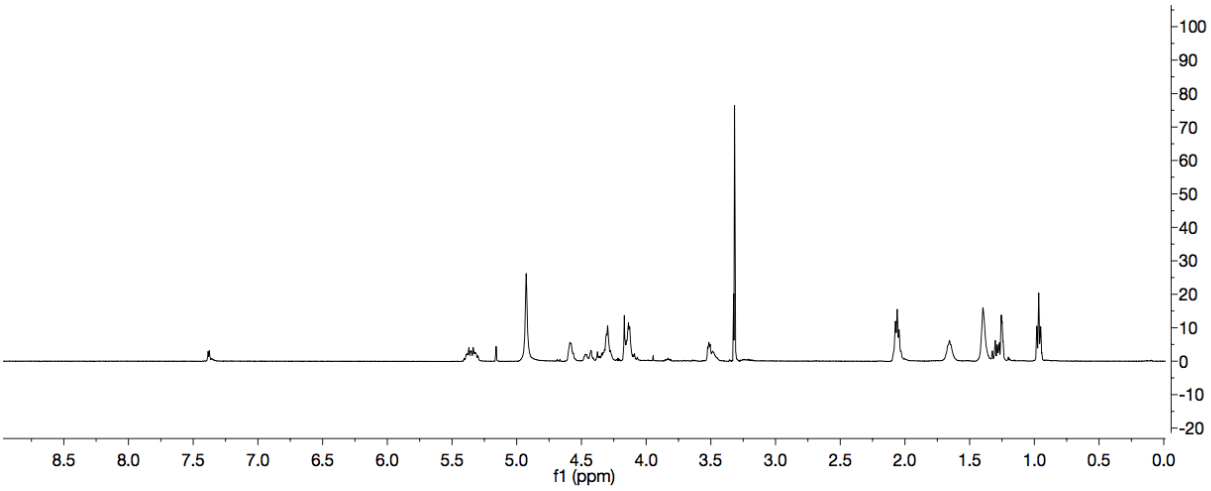


General procedure for Boc deprotection of CART co-oligomers 3a-8

Trifluoroacetic acid (TFA, 0.5 mL) was added to a vial containing Boc-protected co-oligomer (7.5-45 mg) dissolved in dry CH_2Cl_2 (4.5 mL). The reaction was sealed under N_2 and stirred at rt overnight. The solvent was concentrated *in vacuo* to afford the desired co-oligomer as a light brown oil. Complete deprotection was confirmed by ^{1}H NMR.

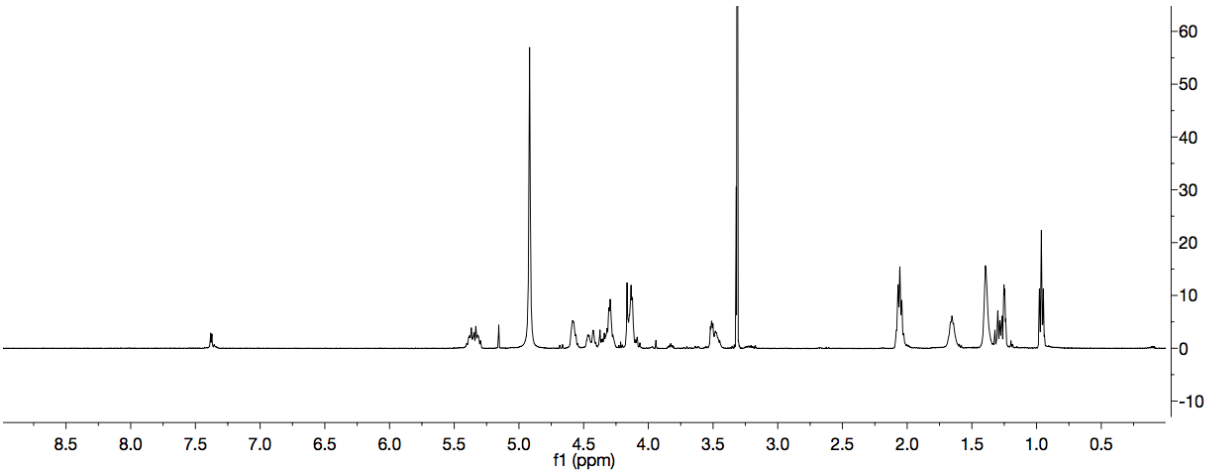
Characterization data for CART co-oligomer N_{10}:A_{10} 3a

^{1}H NMR (CD_3OD, 500 MHz) δ = 7.48-7.27 (m, 5H), 5.44-5.26 (m, 20H), 5.17-5.12 (s, 2H), 4.62-4.52 (m, 16.5H), 4.51-4.23 (m, 47H), 4.19-4.05 (m, 45H), 3.56-3.41 (m, 20H), 2.14-1.98 (m, 43H), 1.72-1.57 (m, 24H), 1.46-1.08 (m, 81H), 0.99-0.93 (m, 32H) ppm.



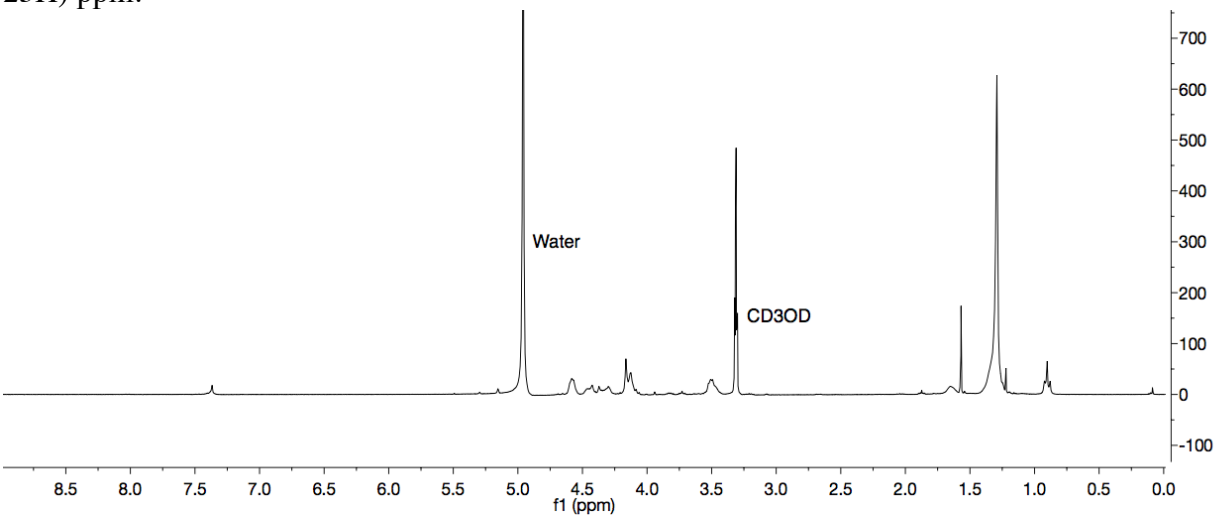
Characterization data for CART co-oligomer N_{11}:A_{11} 3b

^{1}H NMR (CD_3OD, 500 MHz) δ = 7.48-7.27 (m, 5H), 5.48-5.26 (m, 22H), 5.17 (s, 2H), 4.67-4.52 (m, 18H), 4.53-4.23 (m, 52H), 4.19-4.05 (m, 46H), 3.59-3.40 (m, 22H), 2.14-1.96 (m, 45H), 1.72-1.54 (m, 25H), 1.46-1.17 (m, 84H), 1.02-0.89 (m, 34H) ppm.



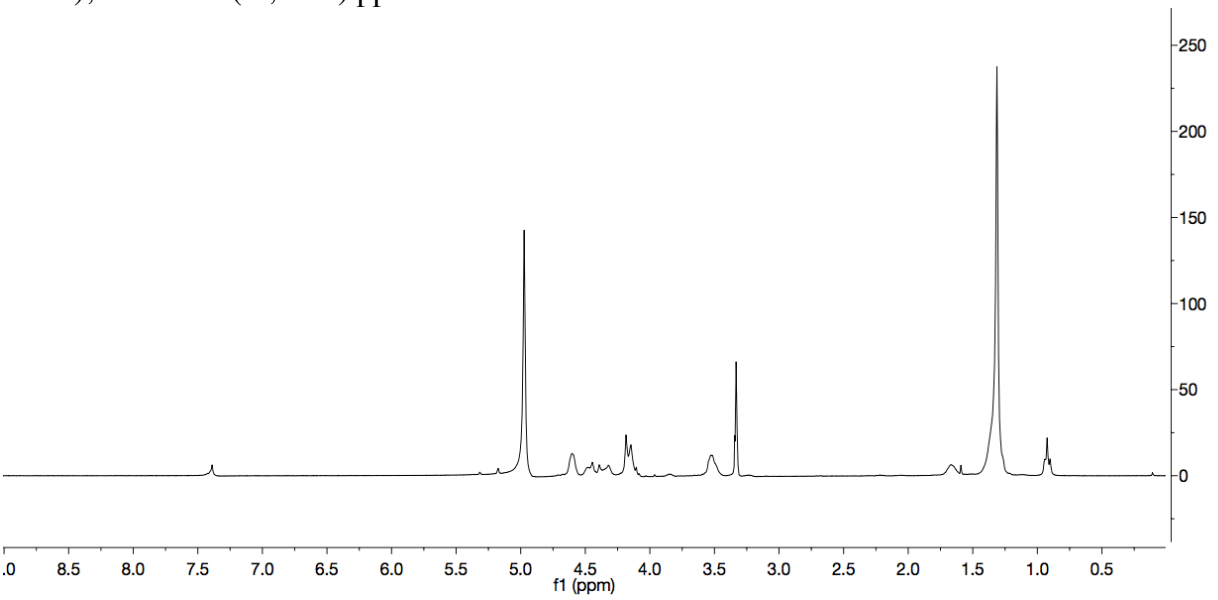
Characterization data for S_9:$A_{9.5}$ 5a

^{1}H NMR (CD_3OD, 300 MHz) δ = 7.48-7.27 (m, 5H), 5.17 (s, 2H), 4.68-4.51 (m, 20H), 4.53-4.01 (m, 68H), 3.56-3.40 (m, 26H), 1.80-1.59 (m, 17H), 1.45-1.18 (m, 236H), 1.01-0.79 (m, 23H) ppm.



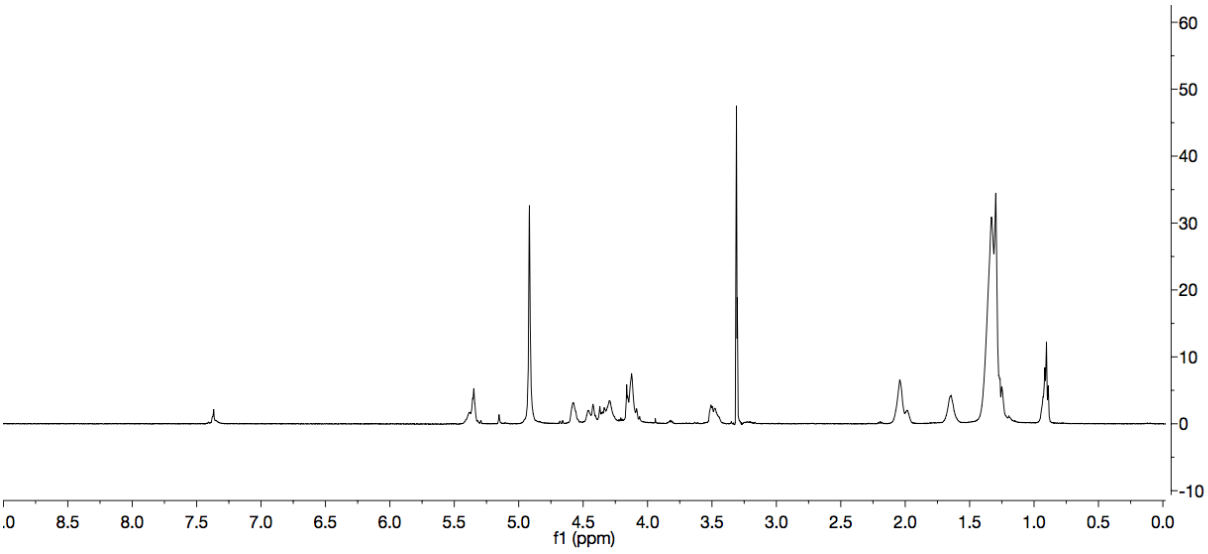
Characterization data for $S_{8.5}$:A_{10} 5b

^{1}H NMR (CD_3OD, 500 MHz) δ = 7.48-7.27 (m, 5H), 5.17 (s, 2H), 4.68-4.51 (m, 20H), 4.53-4.24 (m, 28H), 4.24-4.01 (m, 36H), 3.59-3.40 (m, 25H), 1.76-1.53 (m, 18H), 1.45-1.18 (m, 221H), 1.00-0.79 (m, 20H) ppm.



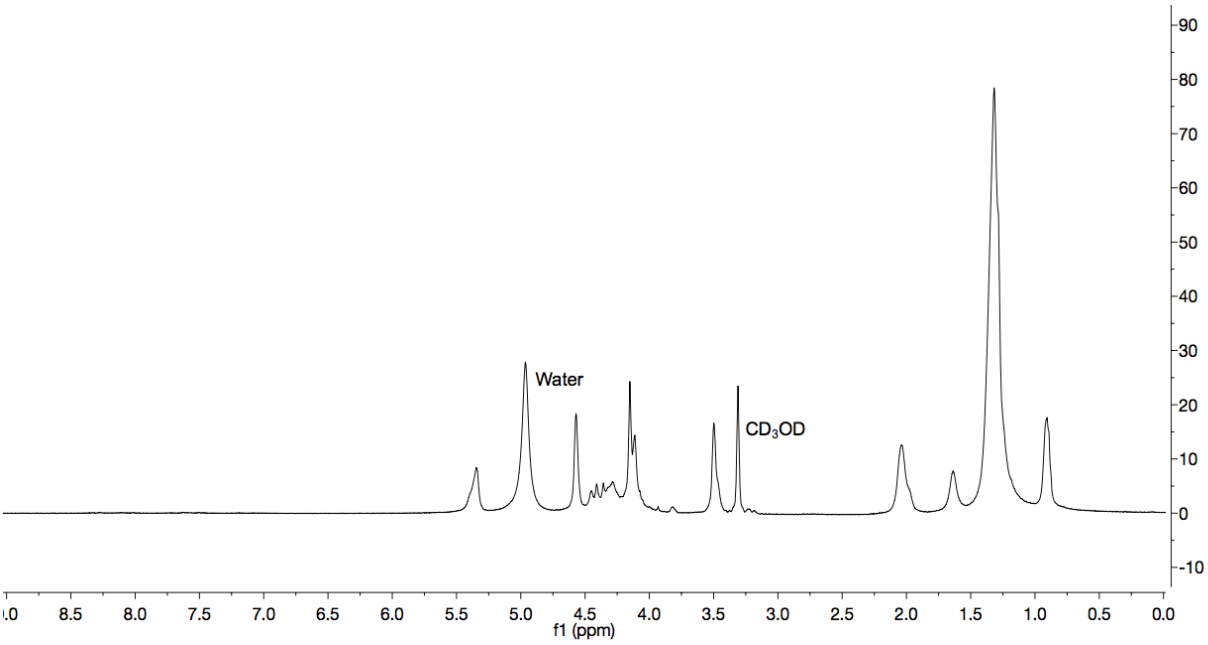
Characterization data for O_{11}:A_9 6a

^{1}H NMR (CD_3OD, 500 MHz) δ = 7.48-7.20 (m, 5H), 5.49-5.27 (m, 20H), 5.17 (s, 2H), 4.68-4.51 (m, 14H), 4.53-4.24 (m, 44H), 4.24-4.01 (m, 33H), 3.59-3.40 (m, 18H), 2.15-1.85 (m, 39H), 1.80-1.51 (m, 26H), 1.51-1.03 (m, 289H), 1.03-0.72 (m, 37H) ppm.



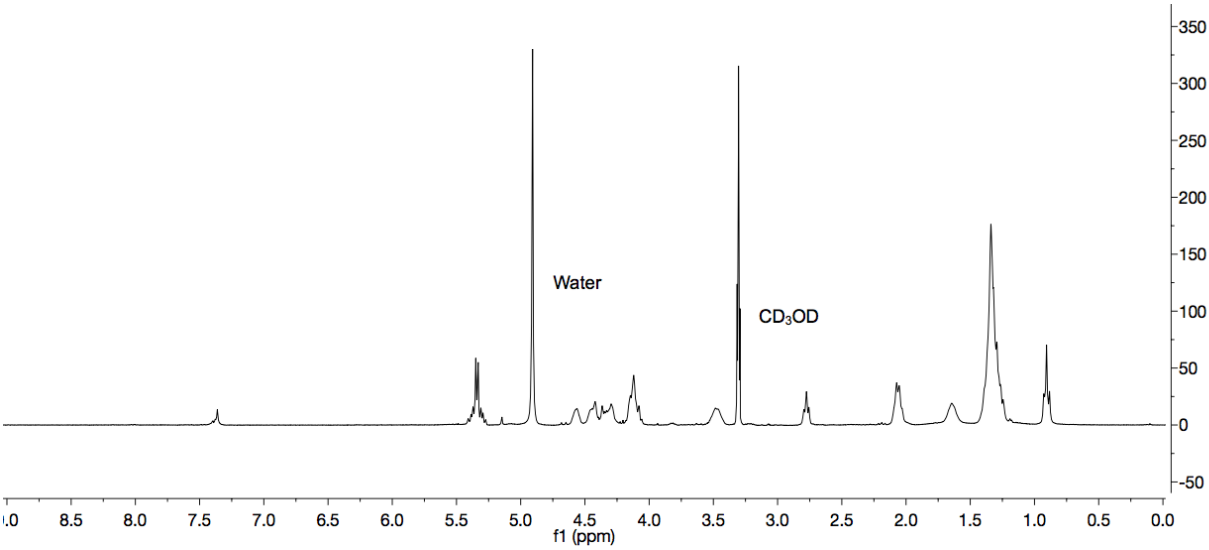
Characterization data for O_{22}:A_{29} 6b

^{1}H NMR (CD_3OD, 300 MHz) δ = 8.63-7.00 (m, 7H), 5.53-5.19 (m, 44H), 4.75-4.51 (m, 51H), 4.49-4.42 (m, 82H), 4.42-4.05 (m, 87H), 3.90-3.77 (m, 2H), 3.65-3.41 (m, 49H), 2.30-1.86 (m, 70H), 1.73-1.59 (m, 37H), 1.47-1.31 (m, 78H), 1.31-1.16 (m, 61H), 1.08-0.71 (m, 65H) ppm.



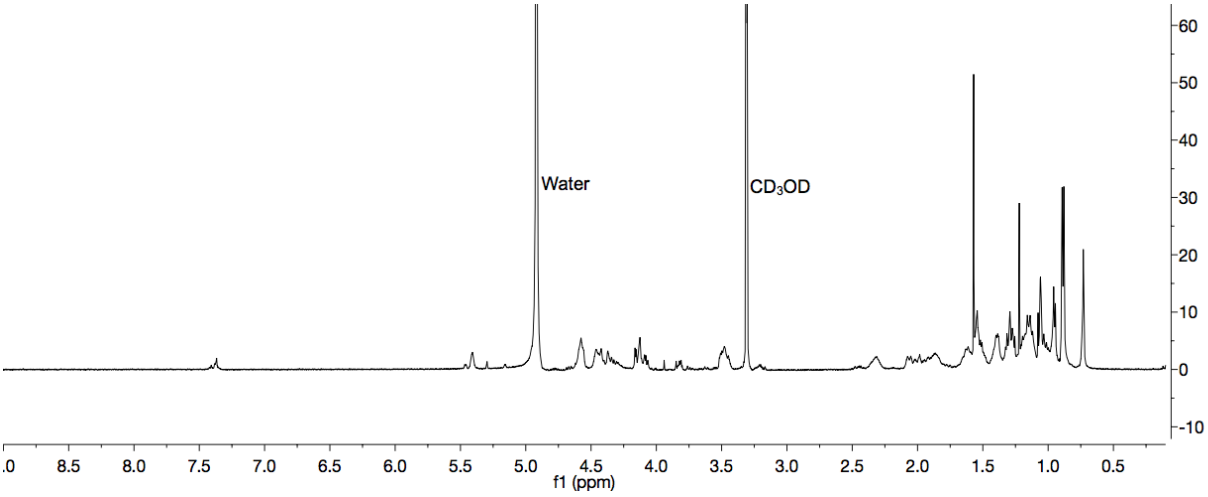
Characterization data for $L_{7.5}$:A_8 7

^{1}H NMR (CD_3OD, 300 MHz) δ = 7.48-7.20 (m, 5H), 5.57-5.24 (m, 32H), 5.15 (s, 2H), 4.64-4.51 (m, 12H), 4.53-4.24 (m, 42H), 4.24-4.01 (m, 37H), 3.59-3.40 (m, 18H), 2.86-2.70 (m, 14H), 2.15-1.85 (m, 32H), 1.80-1.51 (m, 24H), 1.51-1.03 (m, 187H), 0.98-0.81 (m, 30H) ppm.



Characterization of $Chol_{13}$:A_{11} 8

^{1}H NMR (CD_3OD, 500 MHz) δ = 7.48-7.20 (m, 5H), 5.53-5.38 (m, 11H), 5.15 (s, 2H), 4.72-4.51 (m, 26H), 4.50-4.22 (m, 44H), 4.22-3.99 (m, 26H), 3.60-3.36 (m, 26H), 2.49-2.19 (m, 20H), 2.19-1.72 (m, 58H), 1.71-1.24 (m, 177H), 1.21-0.91 (m, 168H), 0.91-0.81 (m, 72H), 0.79-0.67 (m, 33H) ppm.



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