Supplemental Information

Oxabispidine-derived ionizable lipids for effective mRNA delivery to lungs using lipid nanoparticles

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		PBS	MC3	**1**
IT dosing (0.1 mg/kg)	eGFP Protein (ng/g lung) at 24 hours	0	1.5 ± 0.2	66.2 ± 4.9
	Number of Neutrophils at 24 hours	287,500 ± 71,300	566,300 ± 61,000	400,000 ± 49,500
Inhalation (0.02 mg/kg)	eGFP Protein (ng/g lung) at 24 hours	0	-	0.377 ± 0.02
	Number of Neutrophils at 24 hours	126,250 ± 20,246	-	126,250 ± 20,246

Supplemental Table 1. Comparison of eGFP protein expression and neutrophil levels at 24 hours after intrathecal (IT) administration and Aeroneb® inhalation.

Materials and Methods

Chemicals and Materials

Most chemicals were of >95% purity and all solvents were of analytical grade and purchased from Sigma-Aldrich. Lab consumptions were purchased from Avantor and delivered by VWR. 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) was obtained from Avanti Polar Lipids; 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[carbonyl-methoxy(polyethylene glycol)-2000] (DMPE-PEG2000; also known as SUNBRIGHT PM-020CN) was obtained from NOF Corporation; and cholesterol (Chol) was obtained from Sigma-Aldrich. eGFP mRNA (996 nucleotides) Cleancap capped and modified with 5-methoxyuridine were purchased from TriLink Biotechnologies.

^{1}H NMR 500 MHz

^{1}H NMR: 500 MHz; probe: 5mm Bruker Smart probe with ATM+Z PABBO 500S1-BBF-H-D; magnet: ASCEND™ 500; Console: AVANCE Neo 500; Auto Sampler: SampleXpress™60; software: Topspin 4. Proton chemical shifts are expressed in parts per million (ppm, δ scale) and are referenced to residual protium in the NMR solvent (Chloroform-d: δ 7.26, Methanol-d4: δ 3.31, DMSO-d6: δ 2.50). Data are represented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, dt = doublet of triplets, m = multiplet, br = broad, app = apparent), integration, and coupling constant (J) in Hertz (Hz).

UPLC-MS

UPLC-MS was carried out using a Waters Acquity UPLC and Waters SQD mass spectrometer (column temp 30°C, UV detection = 210-400nm, mass spec = ESI with positive/negative switching) at a flow rate of 1 mL/min using a solvent gradient of 2 to 98% B over 1.5 mins (total runtime with equilibration back to starting conditions 2 min), where A = 0.1% formic acid in water and B = 0.1% formic acid in acetonitrile (for acid work) or A = 0.1 % ammonium hydroxide in water and B =acetonitrile (for base work). For acid analysis the column used was Waters Acquity HSS T3, 1.8 mm, 2.1 x 30 mm, for base analysis the column used was Waters Acquity BEH C18, 1.7 mm, 2.1 x 30mm.

Head group pKa analysis

The head group compounds were chemically synthesized and their pKa values were determined via the potentiometric titration (pION Inc., Billerica, MA, USA). For pH-metric pKa, the sample was prepared for the assay by adding 35 µL of a 50mM DMSO stock solution into a 5 mL glass assay vial. A triple titration was carried out under methanol-water co-solvent conditions from pH 2.0 – 12.0 at concentrations of 1.1 – 0.6 mM (the methanol mixing ratio varied from 48.2 to 29.0 % w/w). No precipitation of the sample from solution was observed and one pKa was determined from the data collected by Yasuda-Shedlovsky extrapolation of the individual results obtained.

Apparent pKa measurement

The apparent pKa of LNPs were determined using TNS assay using a modified procedure of a method previously described.[1] Several pH buffers in the range of 3.5-11 were prepared using a solution containing 10 mM HEPES, 10 mM MES, 10 mM ammonium acetate and 130 mM sodium chloride, and their pH were manually adjusted. LNPs with 10 µM of total lipid concentration were transferred to a 96-well black clear-bottom plate (Greiner). Each pH buffer solutions were transferred to the 96-well plate containing LNPs using a multichannel pipette. Amount of a 120 µM TNS solution in DMSO were added with a final TNS concentration of 6 µM. fluorescence was determined using microplate reader (PerkinElmer) with a 323 nm excitation and 435 nm emission. The data was fitted to four-parameter logistic equation (Sigmoidal,

4PL) using GraphPad Prism v8.0.1. The apparent pKa of LNPs was determined as the pH value corresponding to half of the maximum fluorescence.

LNP fabrication and characterization

The LNP formulations were prepared using a modified procedure of a method previously described.[2, 3] Stocks of the helper lipids (cholesterol, DSPC and DMPE-PEG_{2000}) were separately dissolved in ethanol at 20 mg/mL, and the stock of ionizable cationic lipid was dissolved in ethanol at 40 mg/mL. Heating in a water bath at 48°C and a vortex mixer were used to assist the dissolution of DSPC, and only clear solutions were used for lipid mixture preparation. The lipid mixtures were prepared as ionizable lipid: Cholesterol: DSPC: DMPE-PEG 2000 at 50:38.5:10:1.5 mole ratio and a total lipid concentration of 12.5 mM. The mixture was used for the LNP fabrication. eGFP mRNA stock (CleanCap® EGFP mRNA (5moU); Catalog No: L-720) was purchased from Trilink Biotechnologies (San Diego, CA, USA) and stored at -80°C until use. The frozen stock was first thawed at room temperature for 30 min, before adding nuclease free water, followed by citrate buffer (pH=3, 100 mM) with thorough mixing via gentle pipetting. The citrate concentration of the resulting RNA solution was 50 mM, while the final RNA concentration varied based on the molar ratio of ionizable lipid nitrogen (assume each ionizable lipid only has one ionizable nitrogen) and RNA phosphate (N/P). For the N/P=3 or N/P =5 formulations, the RNA concentrations were 0.244 mg/mL or 0.146 mg/mL, respectively. The RNA in citrate buffer solution was used immediately after mixing to prevent RNA degradation and/or hydrolysis. The lipid mixture and the mRNA solution were combined at a volume ratio of 3:1 (aqueous: ethanol), and at a total flow rate of 12 mL/min using a NanoAssemblr microfluidic system (Precision Nanosystems, Vancouver, BC, Canada). Crude LNPs were filtered through 0.22 µm Acrodisc® Syringe Filters with Supor® Membrane (PALL, Port Washington, NY, USA) and dialyzed against 1X PBS (pH 7.4) in Slide-A-Lyzer dialysis cassettes (ThermoFisher Scientific, Waltham, MA, USA). For LNPs to be stored at 4°C, the dialysis in PBS was for overnight. Dialyzed LNPs were concentrated via centrifugal filtration at 3000 rpm to targeted concentration using Amicon ultra-centrifugal filters (EMD Millipore, Billerica, MA, USA) and stored at 4°C until use. To prepare LNP stored at -80°C, the filtration and dialysis in PBS steps were the same except the length of dialysis was for at least 4 hours. Formulations were tested for particle size and RNA encapsulation. The size of LNPs was determined by dynamic light scattering (DLS) measurements using a Zetasizer (Malvern Panalytical Inc., Westborough, MA, USA). The concentration of mRNA was determined using the RiboGreen assay with the Quan-it kit (ThermoFisher Scientific, Waltham, MA, USA).

Animals

The in-vivo study was performed at an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited animal facility at AstraZeneca, Gothenburg, Sweden, under approval of the Animal Ethics Committee of Gothenburg (no.82-2015) and performed in accordance with the AAALAC guidelines.

Male Wistar Han rats were purchased (Charles River Germany Limited) and on arrival were caged in groups of 4 on wood shavings, with diet R70 Rat and Mouse chow (Lantmannen, Stockholm, Sweden) and were provided mains drinking water ad libitum. Animals were approximately 10 weeks of age at the start of dosing. The environment was maintained at a target temperature of 19-24°C and relative humidity of 40- 70%, with a 12-hour light/dark cycle. Animals were acclimatized to the housing conditions for at least 5 days prior to any experimental procedures. Animals that received the inhaled dose were further conditioned to the inhalation restraint procedures for up to 5 days prior to the start of dosing by gradually

increasing the duration exposure to restraint procedures up to the maximum expected duration on the respective study.

Intratracheal dosing

Rats were anaesthetized with Isoflurane mixture (air/oxygen and 3,5% isoflurane), put in a supine position with 30-40° angle and then instilled with eGFP mRNA in LNP formulation or vehicle using a modified metal cannula with bolus-bulb on the top. After the dosing, rats will be placed in cages in a supine position with the head up until regained consciousness. The animals were monitored through the experimental procedure for any signs of ill effect following the dosing with no clinical observations or abnormal change in body weight noted. At 24 hours after final dose the rats were terminated by sedation of isoflurane anesthesia and then cutting of vena cava and removal of the heart.

Inhalation dosing

The aerosol was generated using an Aerogen Solo vibrating mesh nebulizer (Galway Ireland). The nebulizer was filled with 5.6 mL of the vehicle or the eGFP mRNA in LNP formulation and was nebulized at ca 60 pl/min for the duration of the dosing. The rats were place into a rodent restraint and then placed on an AstraZeneca designed inhalation dosing system. The animals were monitored through the experimental procedure for any signs of ill effect following the dosing with no clinical observations or abnormal change in body weight noted. At 24 hours after final dose the rats were terminated by sedation of isoflurane anesthesia and then cutting of vena cava and removal of the heart.

BAL sampling

Broncheo-alveolar lavage (BAL) was performed by manual perfusion of the whole lung. After the trachea is exposed, a polyethylene tube (PE120) was inserted and ligated with 1-0 silk suture. The tube was connected to a syringe, prefilled with 4ml of PBS at room temperature, and PBS was slowly injected into the lung. The fluid was be recollected by slow aspiration into the syringe. This procedure was be performed twice. Final BAL fluid will be transferred to a test tube (4ml, polypropylene [PP]). Tubes with BAL samples were weighed assuming that Igram is equal to 1mL. BAL was ice chilled until centrifugation (Hettich ROTANTA 46R, 1200rpm, 10min, 4°C). After centrifugation the supernatant was collected and divided onto 96-well plates (0.15 mL/well, 5 plates) and kept on dry ice (0.1 mL/well). Plates were stored at minimum of -75°C for any further analysis. The cell pellet was resuspended in 0.5 mL of PBS, kept on ice and immediately processed for cell counting. The total and differential number of cells were counted using automated SYSMEX XT-1800i Vet (Sysmex, Kobe Japan).

Organ collection

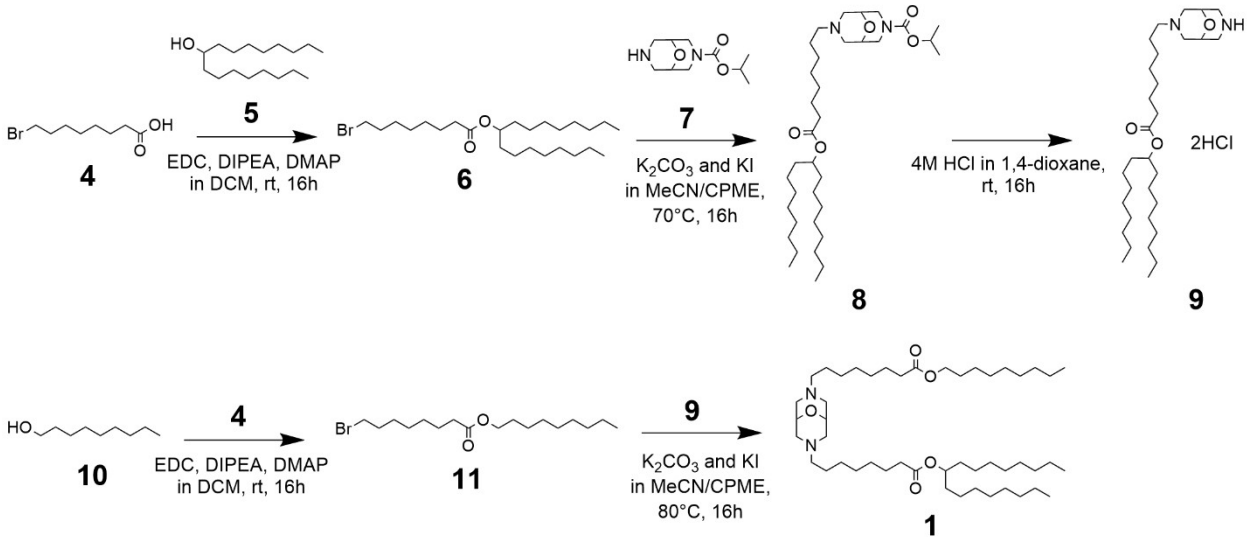
The right lung lobes were tied off and dissected out, trimmed off from non-pulmonary tissue, free from blood clots and rinsed with saline. The right lobes were abscised using a suture and cut loose and rinsed with PBS to remove any blood contamination. The superior lobe and middle lobe were weighed and collected in 7 mL precellys tubes for eGFP mRNA analysis. The inferior and post-caval lobe were weighed and collected in 7 mL precellys tubes for eGFP protein analysis. All right lobe samples are snap frozen in liquid nitrogen. Samples were saved and stored at minimum -80°C until further processing for analysis. Left lung lobe was collected for immunohistochemistry . In brief, the left lung lobe was inflated with and placed in formaldehyde (4%, VWR) for 48h fixation before being dehydrated in a series of increasing percentage of alcohol, following xylene, paraffin infiltration and embedding. The paraffin blocks with lung

tissue were sectioned in 4 µm thick sections and placed on plus slides (Menzel-Genzer) before being processed for immunohistochemistry.

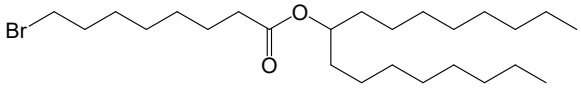
Immunohistochemistry (IHC) Staining

To detect cells expressing eGFP, glass slides were processed using the Ventana Ultra Discovery automatized staining platform and all reagents were purchased from Roche (Ventana Medical system, Inc. Roche Group, USA). Briefly, slides were deparaffinized, antigen retrieved in buffer solution (ULTRA cell conditioning (CC1)) before being treated with Discovery inhibitor (cat no 760-4840), exposed to a rabbit monoclonal antibody recognizing the amino terminus of eGFP (ab 2956S, dilution 1:100, Cell Signaling Technology) diluted in Ab diluent (cat no 760-108) for 1h at 37°C. The primary antibodies were visualized, and the background stained with the amplification system anti-rabbit cat no 760-4815), anti-HQ HRP (ref no 760-4820) and Discovery purple Kit (cat no 760-229) hematoxylin (cat no 760-2021) and bluing reagent (cat no 760-2037). Slides were view in an Olympus BX53 microscope and images confirming eGFP expressing cells were captured using XC80 Olympus camera and cellsences software.

Synthesis of **1**

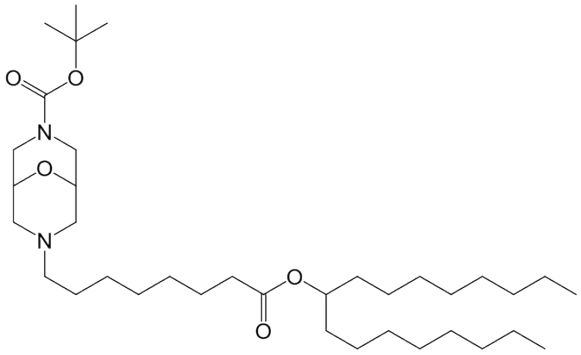


Intermediate 6: Heptadecan-9-yl 8-bromooctanoate



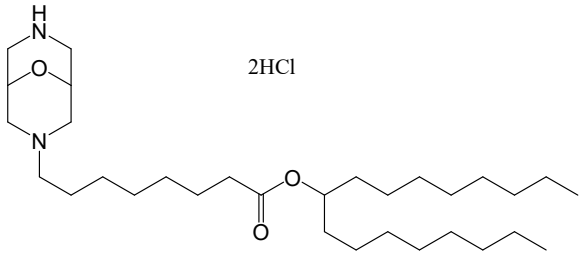
N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) (0.785 g, 4.09 mmol) was added in one portion to a stirred mixture of 8-bromooctanoic acid (intermediate 4) (0.522 g, 2.34 mmol), heptadecan-9-ol (intermediate 5) (0.5 g, 1.95 mmol), 4-dimethylaminopyridine (DMAP) (0.048 g, 0.39 mmol), and N,N-diisopropylethylamine (DIPEA) (1.396 ml, 7.99 mmol) in dichloromethane (DCM) (15 mL) under argon. The resulting mixture was stirred at room temperature for 16 hours. The reaction mixture was concentrated under reduced pressure to dryness and redissolved in ethyl acetate (EtOAc) (50 mL), and washed sequentially with saturated aqueous $NaHCO_3$ (50 mL) and saturated aqueous NaCl (50 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure to dryness to afford crude product. The resulting residue was purified by flash silica chromatography, elution gradient 0 to 20% EtOAc in hexanes. Product fractions were concentrated under reduced pressure to dryness to afford **heptadecan-9-yl 9-bromooctanoate** (0.714 g, yield - 52%) as a colorless oil.[4] ^{1}H NMR (500 MHz, Chloroform-d) δ ppm, 0.8 - 0.9 (m, 6H), 1.2 - 1.9 (m, 40H), 2.3 (t, J=7.5 Hz, 2H), 3.4 - 3.4 (m, 2H), 4.8 - 4.9 (m, 1H).

Intermediate 8. Tert-butyl 7-(8-(heptadecan-9-yloxy)-8-oxooctyl)-9-oxa-3,7-diazabicyclo[3.3.1]nonane-3-carboxylate



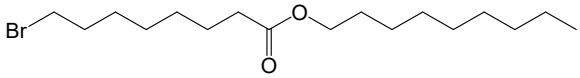
Heptadecan-9-yl 8-bromooctanoate (0.808 g, 1.75 mmol) (intermediate 6) was added dropwise to a stirred mixture of tert-butyl 9-oxa-3,7-diazabicyclo[3.3.1]nonane-3-carboxylate hydrochloride (intermediate 7) (0.386 g, 1.46 mmol), potassium carbonate (0.423 g, 3.06 mmol) and potassium iodide (0.048 g, 0.29 mmol) in acetonitrile (10 mL) under argon. The resulting mixture was stirred at 80 °C for 16 hours. The reaction mixture was concentrated under reduced pressure to dryness and redissolved in EtOAc (100 mL), and washed sequentially with saturated aqueous Na_2CO_3 (100 mL) and saturated aqueous NaCl (100 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure to dryness to afford crude product. The resulting residue was purified by flash silica chromatography, elution gradient 0 to 100% (20% MeOH and 1% NH_4OH in DCM) in DCM. Product fractions were concentrated under reduced pressure to dryness to afford **tert-butyl 7-(8-(heptadecan-9-yloxy)-8-oxooctyl)-9-oxa-3,7-diazabicyclo[3.3.1]nonane-3-carboxylate** (0.747 g, yield - 84%) as a pale yellow oil. 1H NMR (500 MHz, Chloroform-d) δ ppm, 0.8 - 0.9 (m, 6H), 1.2 - 1.7 (m, 47H), 2.0 - 2.2 (m, 2H), 2.2 - 2.3 (m, 2H), 2.4 - 2.5 (m, 2H), 2.8 - 3.0 (m, 2H), 3.2 - 3.4 (m, 2H), 3.7 - 3.8 (m, 2H), 3.9 - 4.2 (m, 2H), 4.8 - 4.9 (m, 1H).

Intermediate 9: Heptadecan-9-yl 8-(9-oxa-3,7-diazabicyclo[3.3.1]nonan-3-yl)octanoate Dihydrochloride



HCl (2.250 ml, 9.00 mmol) in dioxane was added dropwise to a stirred mixture of tert-butyl 7-(8-(heptadecan-9-yloxy)-8-oxooctyl)-9-oxa-3,7-diazabicyclo[3.3.1]nonane-3-carboxylate (intermediate 8) (0.548 g, 0.90 mmol) in dioxane (10 mL) at 0°C under argon. The resulting mixture was warmed and stirred at room temperature for 16 hours. The reaction was concentrated under reduced pressure to dryness, dissolved in DCM, and extracted with saturated sodium carbonate (3 x 50 mL). The resulting residue was purified by flash silica chromatography using DCM with 1% NH_4OH and 20% methanol in DCM with 1% NH_4OH to afford **heptadecan-9-yl 8-(9-oxa-3,7-diazabicyclo[3.3.1]nonan-3-yl)octanoate dihydrochloride** (0.472 g, yield - 96%) as a pale yellow oil. 1H NMR (500MHz, Methanol-d4) δ ppm, 0.9 (t, J=6.9 Hz, 6H), 1.3 (br s, 38H), 2.2 - 2.3 (m, 2H), 3.1 - 3.9 (m, 10H), 4.5 - 4.7 (m, 2H), 4.9 (s, 1H).

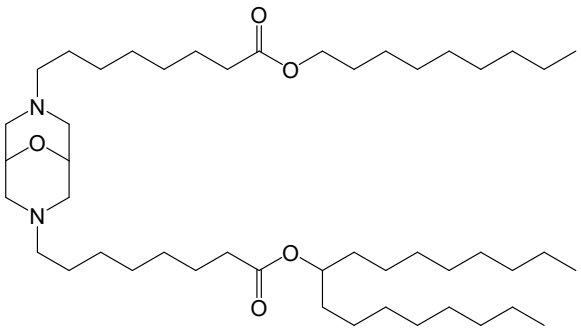
Intermediate 11: Nonyl 8-bromooctanoate



N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) (2.79 g, 14.56 mmol) was added in one portion to a stirred solution of 8-bromooctanoic acid (intermediate 4) (2.320 g, 10.40 mmol), nonan-1-ol (intermediate 10) (1.205 ml, 6.93 mmol), DMAP (0.169 g, 1.39 mmol), and N,N-diisopropylethylamine (DIPEA) (2.54 ml, 14.56 mmol) in DCM (15 mL). The resulting mixture was stirred at room temperature for 16 hours. The reaction mixture was concentrated under reduced pressure to dryness and redissolved in EtOAc (100 mL), and washed sequentially with saturated aqueous $NaHCO_3$ (100 mL) and saturated aqueous NaCl (100 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure to dryness to afford crude product. The resulting residue was purified by flash silica chromatography, elution gradient 0 to 20% EtOAc in hexanes. Product fractions were concentrated under reduced pressure to dryness to afford **nonyl 8-bromooctanoate**[4] (1.580 g, yield - 65.2%) as a colorless oil.

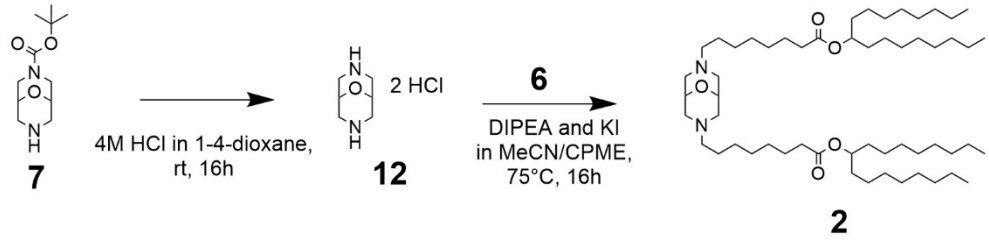
1H NMR (500MHz, Chloroform-d) δ ppm, 0.9 (m, 3H), 1.3 - 1.4 (m, 16H), 1.4 – 1.5 (m, 2H), 1.6 - 1.7 (m, 4H), 1.9 (dt, J=14.5, 7.0 Hz, 2H), 2.3 (t, J=7.5 Hz, 2H), 3.4 (t, J=6.9 Hz, 2H), 4.1 (t, J=6.7 Hz, 2H).

Compound 1: Heptadecan-9-yl 8-(7-(8-(nonyloxy)-8-oxooctyl)-9-oxa-3,7-diazabicyclo[3.3.1]nonan-3-yl)octanoate

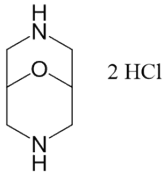


Nonyl 8-bromooctanoate (0.144 g, 0.41 mmol) (intermediate 11) was added dropwise to a stirred mixture of heptadecan-9-yl 8-(9-oxa-3,7-diazabicyclo[3.3.1]nonan-3-yl)octanoate dihydrochloride (intermediate 9) (0.2 g, 0.34 mmol) and N,N-diisopropylethylamine (DIPEA) (0.486 ml, 2.78 mmol) in acetonitrile (5 mL) under argon. The resulting mixture was stirred at 80 °C for 16 hours. The reaction mixture was concentrated under reduced pressure to dryness and redissolved in EtOAc (50 mL), and washed sequentially with saturated aqueous $NaHCO_3$ (50 mL) and saturated aqueous NaCl (50 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure to dryness to afford crude product. The resulting residue was purified twice by flash silica chromatography, elution gradient O to 100% (20% MeOH and 1% NH_4OH in DCM) in DCM. Product fractions were concentrated under reduced pressure to dryness to afford **heptadecan-9-yl 8-(7-(8-(nonyloxy)-8-oxooctyl)-9-oxa-3,7-diazabicyclo[3.3.1]nonan-3-yl)octanoate** (0.119 g, yield - 44.5%) as a pale yellow oil. **1H NMR** (500MHz, Methanol-d4) δ ppm, 0.9 (t, J=6.8 Hz, 9H), 1.3 - 1.7 (m, 63H), 2.2 - 2.3 (m, 4H), 2.3 - 2.4 (m, 4H), 2.4 -2.5 (m, 4H), 2.8 - 2.9 (m, 2H), 3.9 - 3.9 (m, 2H), 4.0 - 4.11 (m, 2H). **13C NMR** (126 MHz, CHLOROFORM-d) δ ppm, 14.1, 22.7, 25.0, 25.1, 25.3, 25.9, 26.5, 26.5, 27.3, 27.4, 28.7, 29.1, 29.2, 29.5, 29.5, 29.5, 29.6, 29.6, 31.9, 34.1, 34.4, 34.7, 56.4, 56.5, 59.7, 64.2, 64.4, 68.4, 68.4, 74.1, 173.6, 173.9. **HRMS** (ESI) m/z: $[M+H]^+$ calculated for $C_{48}H_{92}N_2O_5$ 777.7084; found 777.7091.

Synthesis of **2**

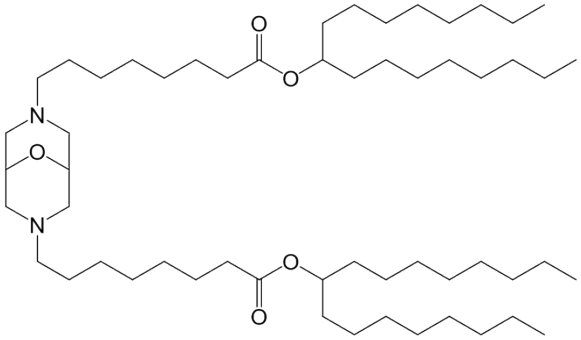


Intermediate 12: 9-oxa-3,7-diazabicyclo[3.3.1]nonane dihydrochloride



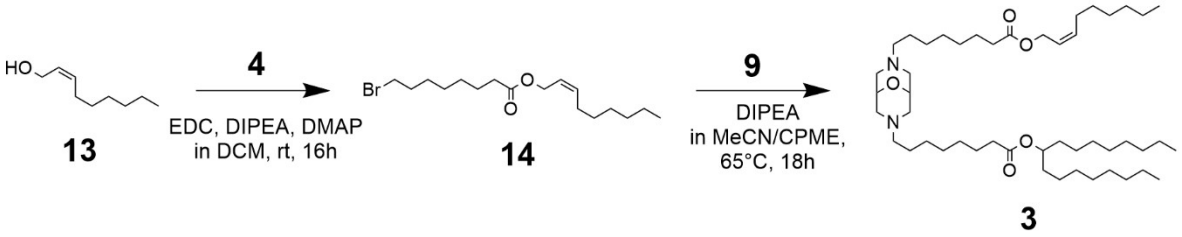
HCl (2 ml, 65.83 mmol) was added dropwise to a stirred mixture of tert-butyl 9-oxa-3,7-diazabicyclo[3.3.1]nonane-3-carboxylate hydrochloride (intermediate 7) (0.3 g, 1.13 mmol) in 1,4-dioxane (8 mL) under argon. The reaction was stirred overnight at room temperature until precipitate formed. The reaction mixture was concentrated under reduced pressure to dryness to afford to afford **9-oxa-3,7-diazabicyclo[3.3.1]nonane dihydrochloride** (0.220 g, yield - 97%) as a white powder. 1H NMR (500 MHz, Methanol-d4) δ ppm, 3.5 (s, 8H), 4.4 (br s, 2H).

Compound 2: Di(heptadecan-9-yl) 8,8'-(9-oxa-3,7-diazabicyclo[3.3.1]nonane-3,7-diyl)dioctanoate

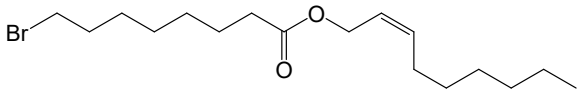


Heptadecan-9-yl 8-bromooctanoate (intermediate 6) (0.285 g, 0.62 mmol) was added dropwise to a stirred mixture of 9-oxa-3,7-diazabicyclo[3.3.1]nonane dihydrochloride (intermediate 12) (0.04 g, 0.20 mmol), N,N-diisopropylethylamine (DIPEA) (0.142 ml, 0.82 mmol), and potassium iodide (6.60 mg, 0.04 mmol) in acetonitrile (5 mL) at 25°C under argon. The resulting mixture was stirred at 80°C for 16 hours. The reaction mixture was concentrated under reduced pressure to dryness and redissolved in EtOAc (50 mL), and washed sequentially with saturated aqueous Na_2CO_3 (50 mL) and saturated aqueous NaCl (50 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure to dryness to afford crude product. The resulting residue was purified by flash silica chromatography, elution gradient 0 to 100% (20% MeOH and 1% NH_4OH in DCM) in DCM. Product fractions were concentrated under reduced pressure to dryness to afford **di(heptadecan-9-yl) 8,8'-(9-oxa-3,7-diazabicyclo[3.3.1]nonane-3,7-diyl)dioctanoate** (0.062 g, yield - 34.9%) as a pale yellow oil. **1H NMR** (500 MHz, Chloroform-d) δ ppm, 0.9 (t, J=6.8 Hz, 12H), 1.2 - 1.7 (m, 76H), 1.9 - 2.3 (m, 8H), 2.4 - 2.6 (m, 4H), 2.7 - 3.0 (m, 4H), 3.8 - 4.0 (m, 2H), 4.8 - 4.9 (m, 2H). **13C NMR** (126 MHz, CHLOROFORM-d) δ ppm, 14.1, 22.7, 25.0, 25.1, 25.3, 26.5, 26.5, 27.4, 29.2, 29.2, 29.5, 29.5, 31.9, 34.1, 34.7, 53.4, 56.5, 59.7, 59.8, 68.3, 68.3, 68.4, 68.5, 74.1, 173.6. **HRMS** (ESI) m/z: $[M+H]^+$ calculated for $C_{56}H_{108}N_2O_5$ 889.8336; found 889.8344.

Synthesis of **3**

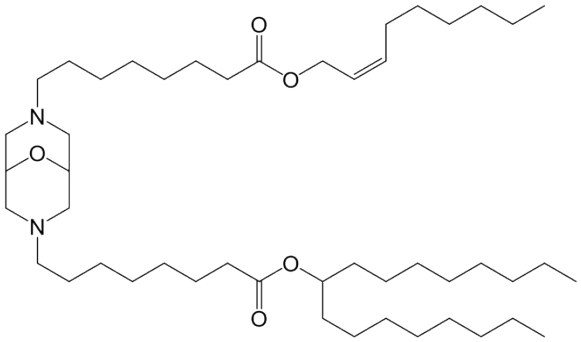


Intermediate 14: (Z)-non-2-en-1-yl 8-bromooctanoate



N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) (2.83 g, 14.76 mmol) was added in one portion to a stirred mixture of 8-bromooctanoic acid (intermediate 4) (2.353 g, 10.55 mmol), N,N-diisopropylethylamine (DIPEA) (3.68 ml, 21.09 mmol), (Z)-non-2-en-1-ol (intermediate 13) (1 g, 7.03 mmol), and DMAP (0.172 g, 1.41 mmol) in DCM (15 mL) under argon. The resulting mixture was stirred at room temperature for 16 hours. The reaction mixture was concentrated under reduced pressure to dryness and redissolved in EtOAc (100 mL), and washed sequentially with saturated aqueous $NaHCO_3$ (100 mL) and saturated aqueous NaCI (100 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure to dryness to afford crude product. The resulting residue was purified by flash silica chromatography, elution gradient O to 100% EtOAc in hexanes. Product fractions were concentrated under reduced pressure to dryness to afford **(Z)-non-2-en-1-yl 8-bromooctanoate**[4] (1.930 g, yield - 79%) as a pale yellow oil. 1H NMR (500MHz, Chloroform-d) δ ppm, 0.8 – 1.0 (m, 3H), 1.2 - 1.5 (m, 14H), 1.6 - 1.7 (m, 2H), 1.7 - 1.9 (m, 2H), 2.1 (br d, J=7.5 Hz, 2H), 2.3 (t, J=7.6 Hz, 2H), 3.3 - 3.7 (m, 2H), 4.6 (d, 2H), 5.5 - 5.7 (m, 2H).

Compound 3: Heptadecan-9-yl (Z)-8-(7-(8-(non-2-en-1-yloxy)-8-oxooctyl)-9-oxa-3,7-diazabicyclo[3.3.1]nonan-3-yl)octanoate



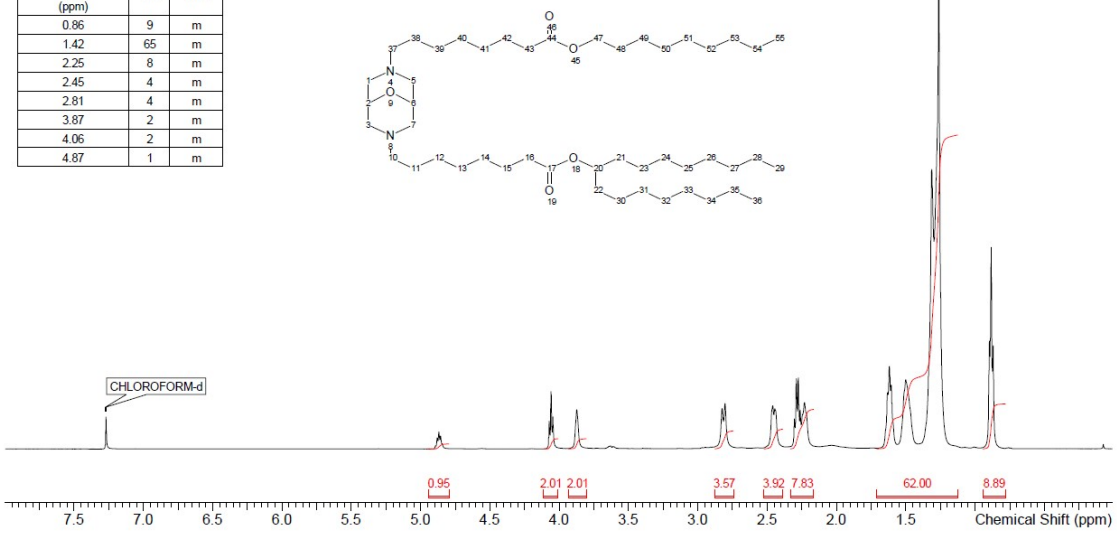
The heptadecan-9-yl 8-(9-oxa-3,7-diazabicyclo[3.3.1]nonan-3-yl)octanoate dihydrochloride (intermediate 9) dissolved in 1:1 acetonitrile/cyclopentyl methyl ether (10 mL) and N,N-diisopropylethylamine (DIPEA) (0.212 mL, 1.21 mmol) was added at 20°C under nitrogen. The resulting mixture was stirred at 20 °C for 30 minutes. The (Z)-non-2-en-1-yl 8-bromooctanoate (intermediate 14) (0.123 g, 0.35 mmol) was added dropwise to the stirred solution under nitrogen. The resulting mixture was heated to 80 °C and stirred over a period of 18 hours under nitrogen. The reaction mixture was concentrated under reduced pressure to dryness and redissolved in EtOAc (50 mL), and washed sequentially with saturated aqueous $NaHCO_3$ (50 mL) and saturated aqueous sodium chloride (50 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure to dryness to afford crude product. The resulting residue was purified by flash silica chromatography, elution gradient 0 to 100% (20% MeOH and 1% NH_4OH in DCM) in DCM. Product fractions were concentrated under reduced pressure to afford **heptadecan-9-yl (Z)-8-(7-(8-(non-2-en-1-yloxy)-8-oxooctyl)-9-oxa-3,7-diazabicyclo[3.3.1]nonan-3-yl)octanoate** (0.214 g, yield - 93%) as a pale yellow oil. **1H NMR** (500MHz, Methanol-d4) δ ppm, 0.8 – 0.9 (m, 9H), 1.2 - 1.7 (m, 56H), 2.1 (br d, J=7.2 Hz, 2H), 2.3 (dt, J=14.4, 7.4 Hz, 4H), 2.8 - 3.0 (m, 8H), 3.6 (br d, J=11.7 Hz, 4H), 4.1 -4.1 (m, 2H), 4.6 (d, J=6.9 Hz, 2H), 4.9 (br t, J=6.1 Hz, 1H), 5.5 - 5.7 (m, 2H). **13C NMR** (126 MHz, CHLOROFORM-d) δ ppm, 14.3, 14.4, 18.7, 18.7, 22.9, 22.9, 25.3, 25.6, 25.6, 27.8, 29.1, 29.4, 29.5, 29.6, 29.8, 29.8, 29.9, 29.9, 31.9, 32.1, 34.4, 34.6, 34.9, 35.1, 56.7, 58.7, 60.5, 74.4, 91.4, 123.6, 135.6, 173.9, 174.0. **HRMS** (ESI) m/z: $[M+H]^+$ calculated for $C_{48}H_{90}N_2O_5$ 775.6928; found 775.6951.

^{1}H NMR spectrum of **1** in chloroform-d

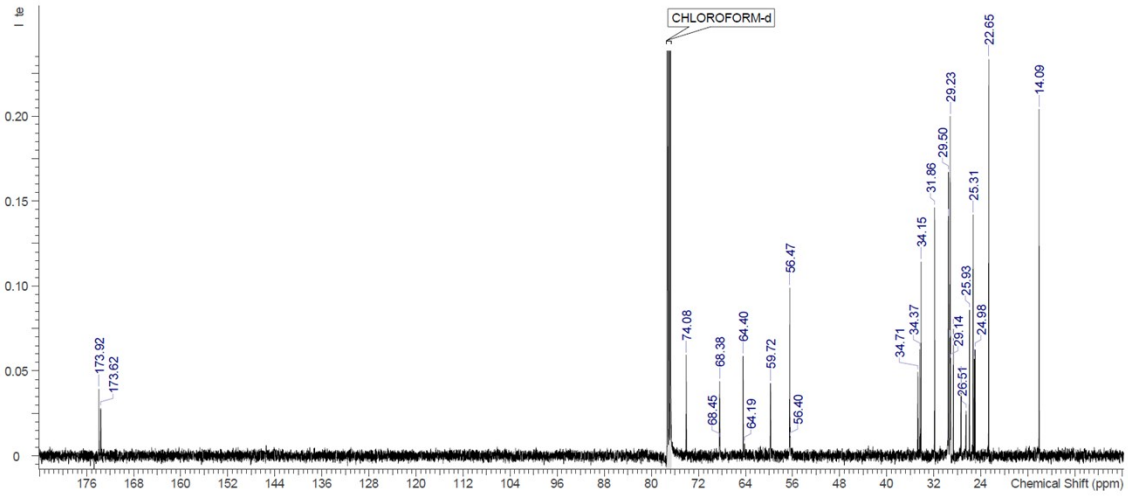
^{1}H NMR (500 MHz, CHLOROFORM-*d*) δ ppm 0.8 - 0.9 (m, 9 H) 1.1 - 1.7 (m, 65 H) 2.2 - 2.3 (m, 8 H) 2.4 - 2.5 (m, 4 H) 2.7 - 2.9 (m, 4 H) 3.8 - 3.9 (m, 2 H) 4.0 - 4.1 (m, 2 H) 4.8 - 4.9 (m, 1 H)

Number of Nuclei: 95 H's / 92 H's (spectrum / structure)

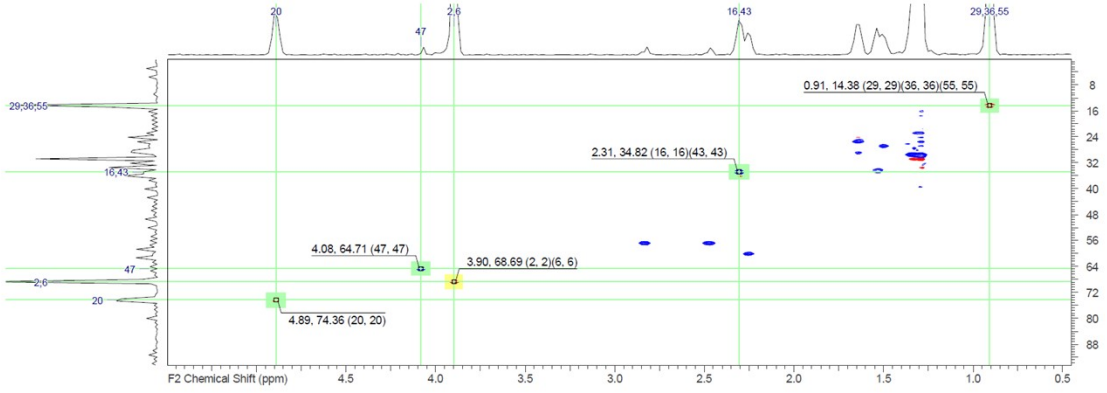
Shift1 (ppm)	H's	Type
0.86	9	m
1.42	65	m
2.25	8	m
2.45	4	m
2.81	4	m
3.87	2	m
4.06	2	m
4.87	1	m

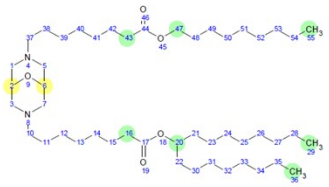


^{13}C NMR spectrum of **1** in chloroform-d

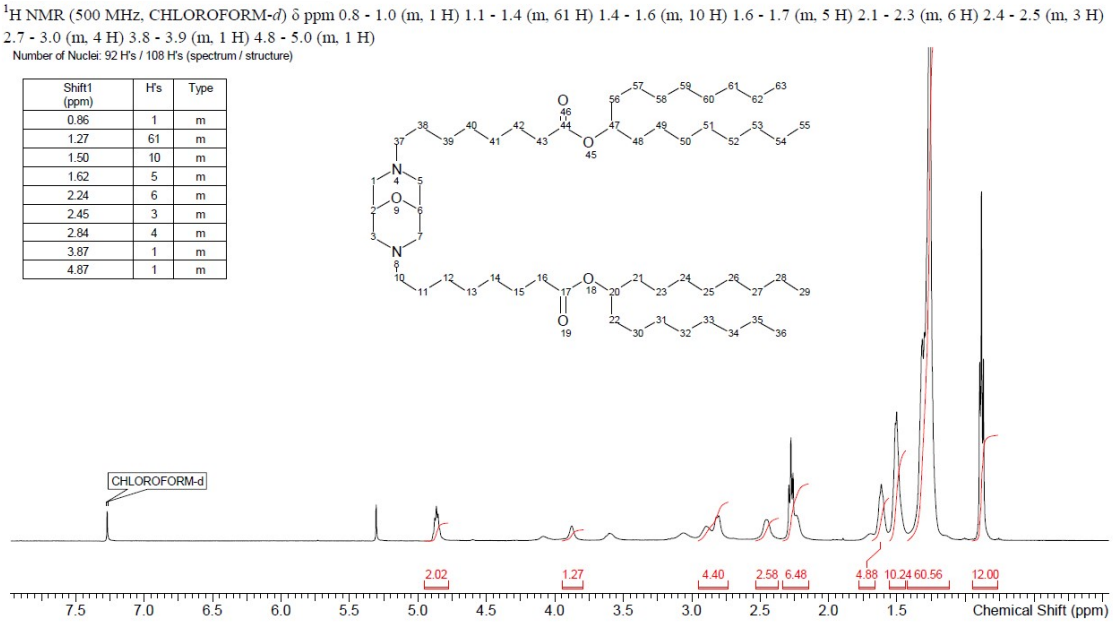


HSQC NMR spectrum of **1** in chloroform-d

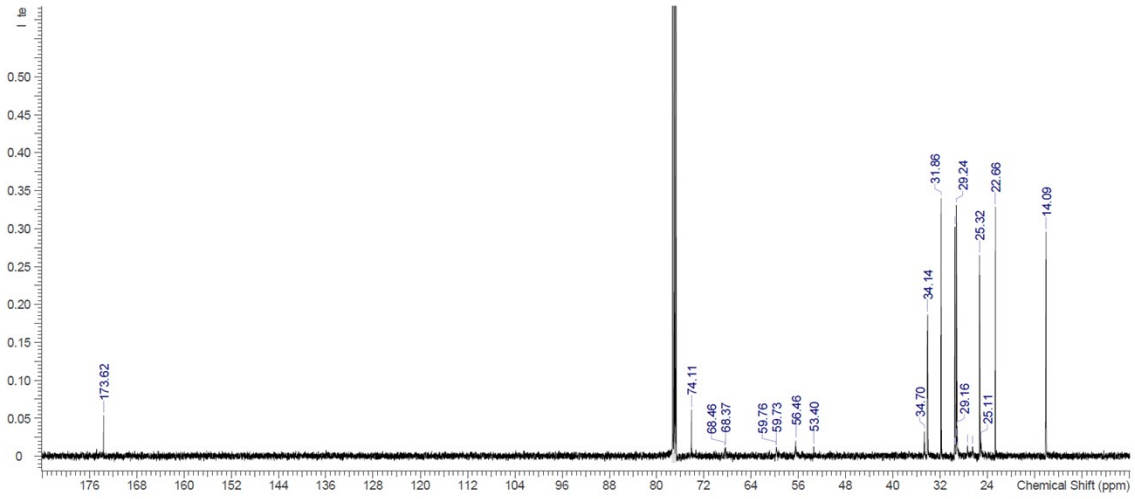




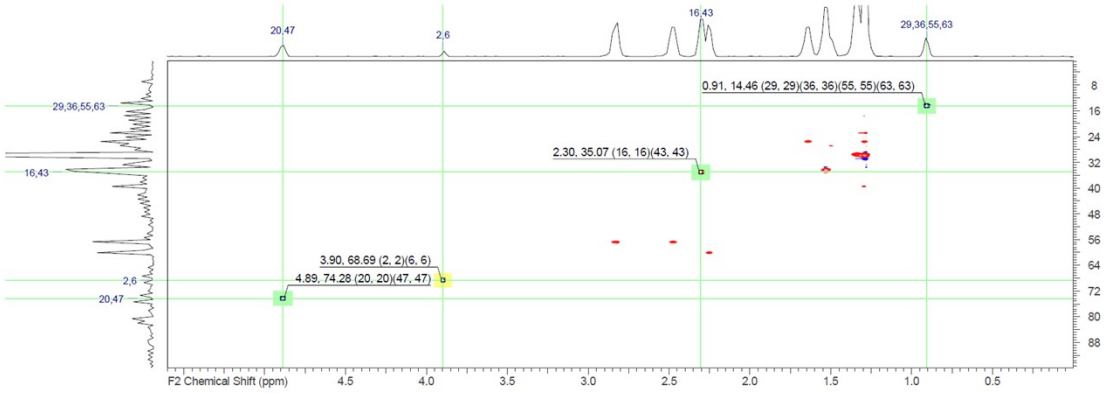
[1]H NMR spectrum of **2** in chloroform-d

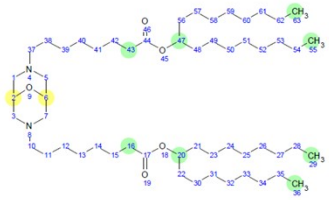


[13]C NMR spectrum of **2** in chloroform-d



HSQC NMR spectrum of **2** in chloroform-d



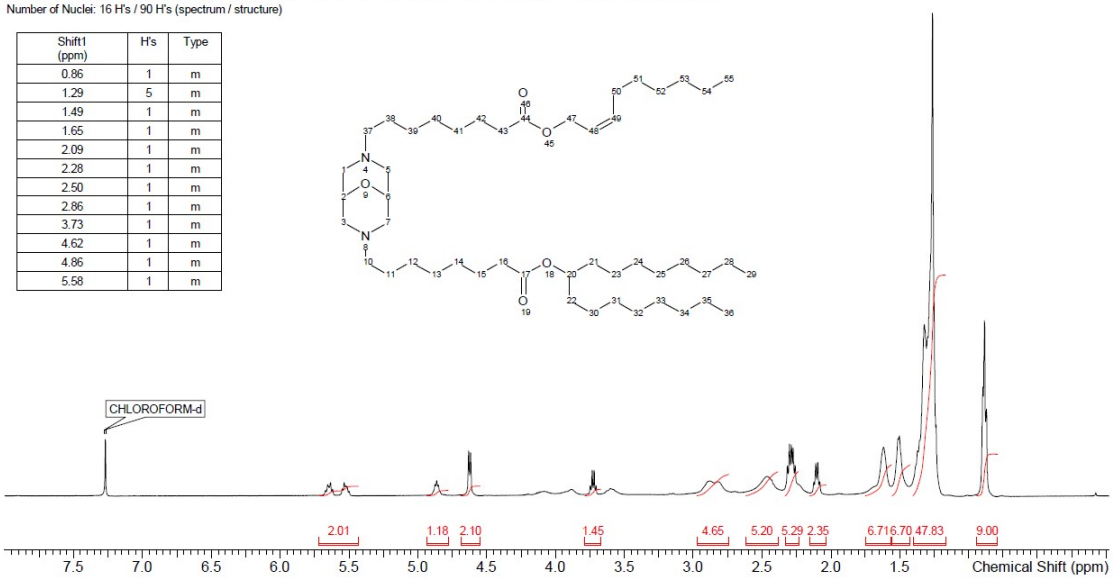


^{1}H NMR spectrum of **3** in chloroform-d

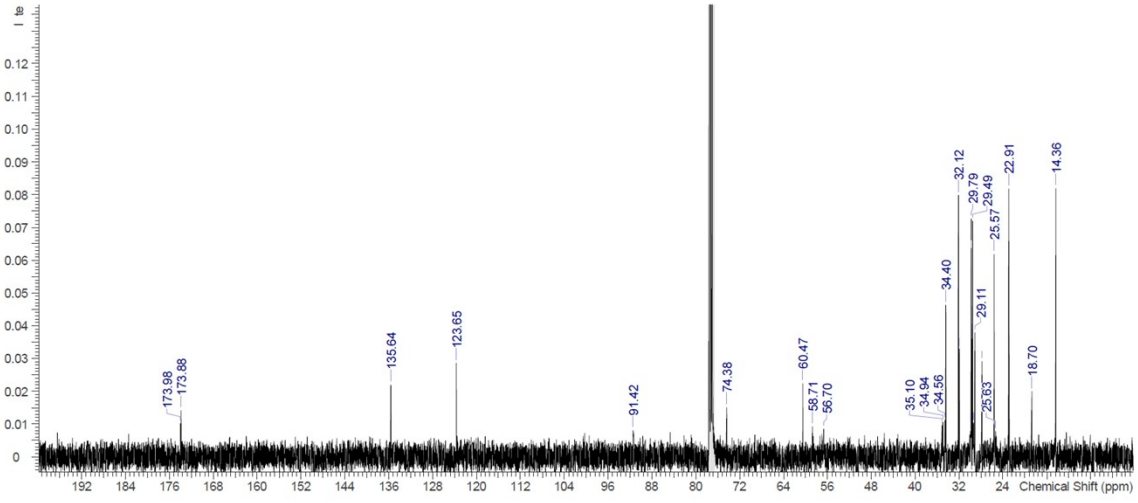
^{1}H NMR (500 MHz, CHLOROFORM-*d*) δ ppm 0.8 - 0.9 (m, 1 H) 1.2 - 1.4 (m, 5 H) 1.4 - 1.6 (m, 1 H) 1.6 - 1.7 (m, 1 H) 2.0 - 2.2 (m, 1 H) 2.2 - 2.3 (m, 1 H) 2.4 - 2.6 (m, 1 H) 2.7 - 3.0 (m, 1 H) 3.7 - 3.8 (m, 1 H) 4.5 - 4.7 (m, 1 H) 4.8 - 4.9 (m, 1 H) 5.4 - 5.7 (m, 1 H)

Number of Nuclei: 16 H's / 90 H's (spectrum / structure)

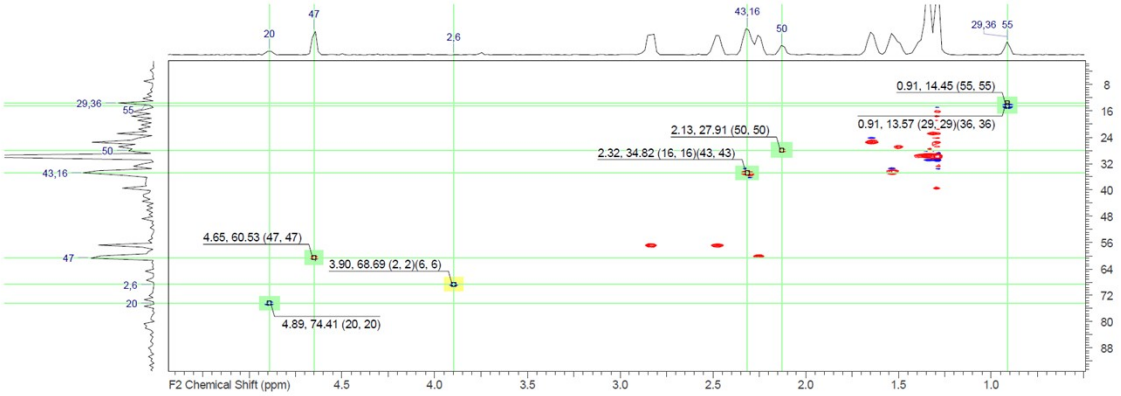
Shift1 (ppm)	H's	Type
0.86	1	m
1.29	5	m
1.49	1	m
1.65	1	m
2.09	1	m
2.28	1	m
2.50	1	m
2.86	1	m
3.73	1	m
4.62	1	m
4.86	1	m
5.58	1	m

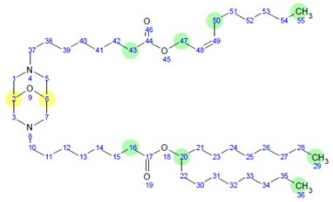


^{13}C NMR spectrum of **3** in chloroform-d



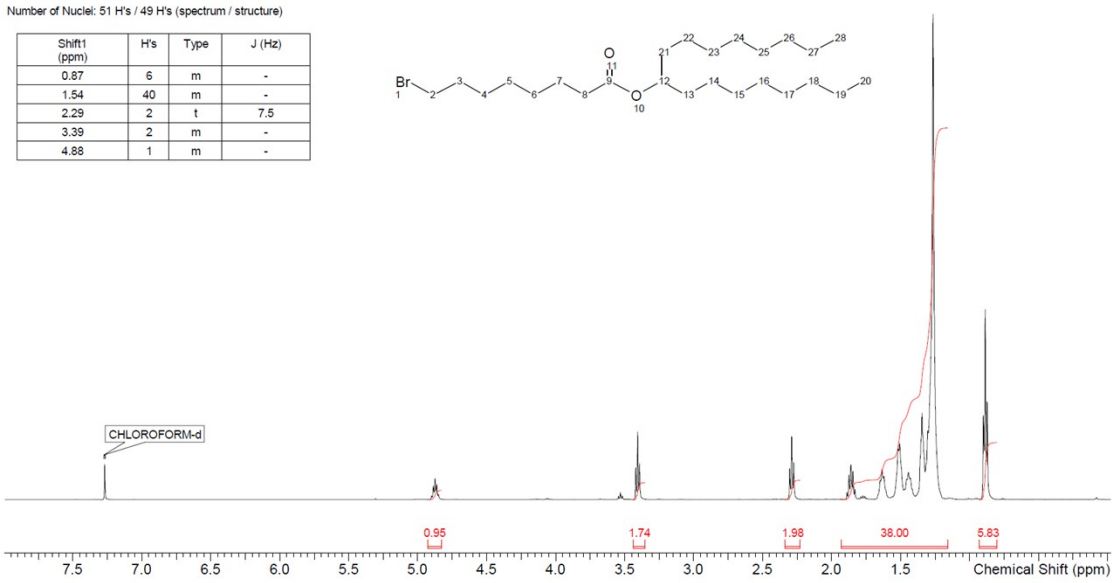
HSQC NMR spectrum of **3** in chloroform-d





^{1}H NMR (500 MHz, chloroform-d) of intermediate **6**

^{1}H NMR (500 MHz, CHLOROFORM-*d*) δ ppm 0.8 - 0.9 (m, 6 H) 1.2 - 1.9 (m, 40 H) 2.3 (t, *J*=7.5 Hz, 2 H) 3.4 - 3.4 (m, 2 H) 4.8 - 4.9 (m, 1 H)

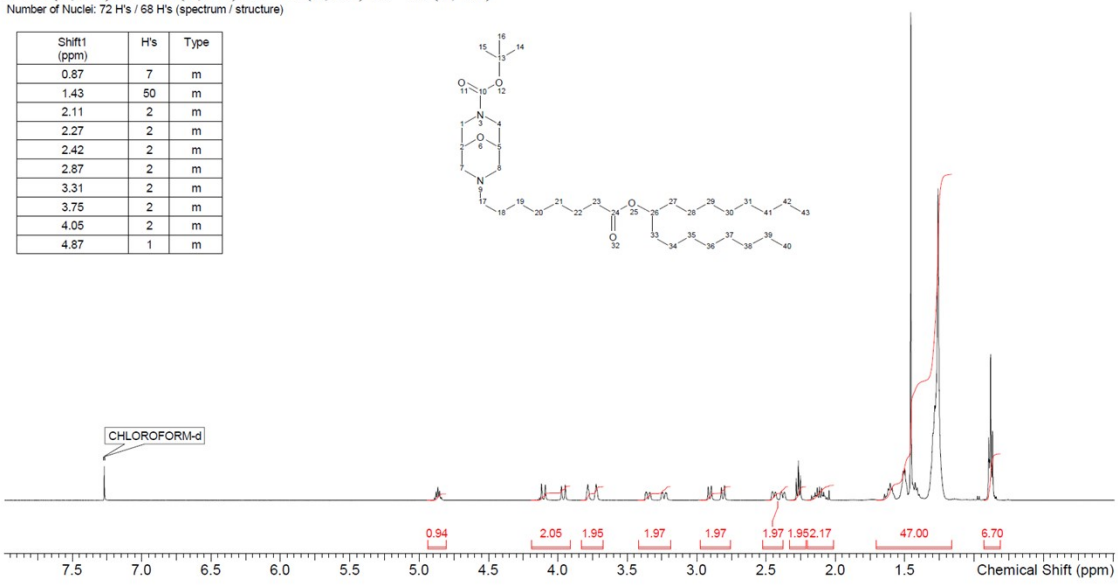


^{1}H NMR (500 MHz, chloroform-d) of intermediate **8**

^{1}H NMR (500 MHz, CHLOROFORM-*d*) δ ppm 0.8 - 0.9 (m, 7 H) 1.2 - 1.7 (m, 50 H) 2.0 - 2.2 (m, 2 H) 2.2 - 2.3 (m, 2 H) 2.3 - 2.5 (m, 2 H) 2.8 - 3.0 (m, 2 H) 3.2 - 3.4 (m, 2 H) 3.7 - 3.8 (m, 2 H) 3.9 - 4.2 (m, 2 H) 4.8 - 4.9 (m, 1 H)

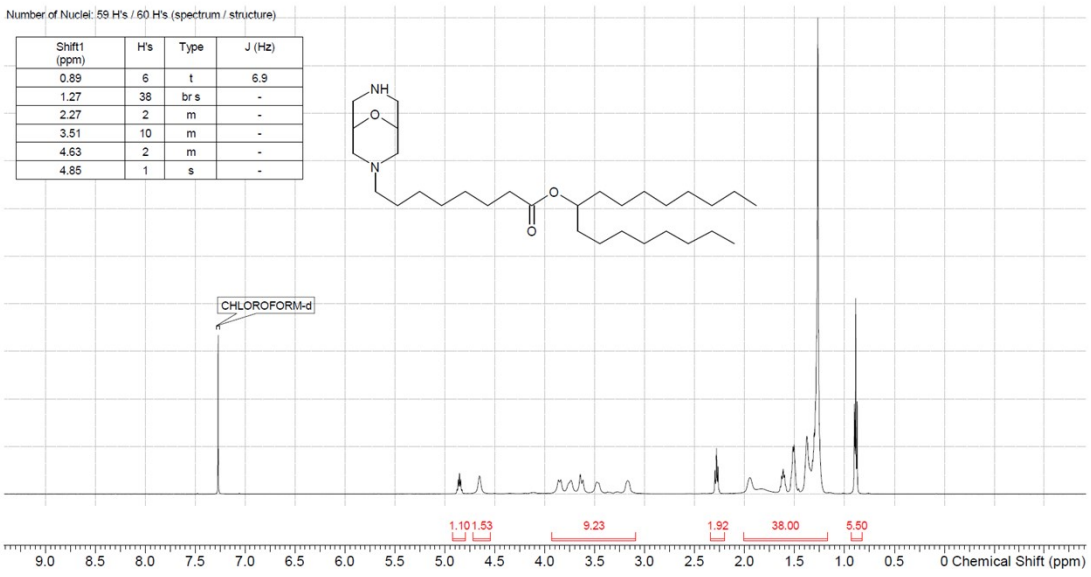
Number of Nuclei: 72 H's / 68 H's (spectrum / structure)

Shift1 (ppm)	H's	Type
0.87	7	m
1.43	50	m
2.11	2	m
2.27	2	m
2.42	2	m
2.87	2	m
3.31	2	m
3.75	2	m
4.05	2	m
4.87	1	m

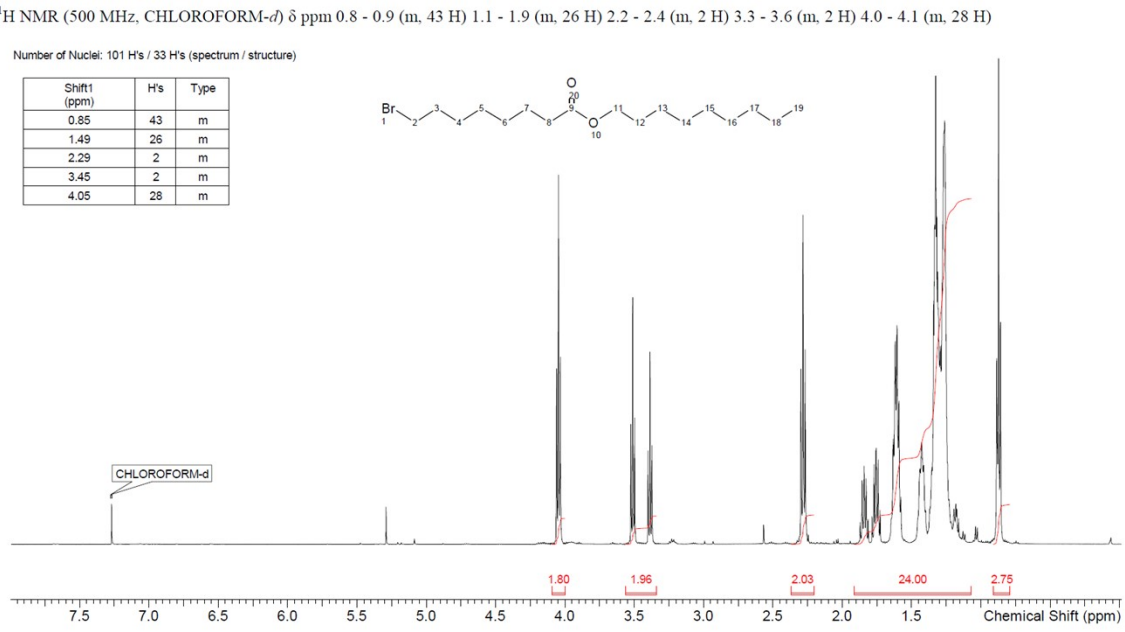


^{1}H NMR (500 MHz, chloroform-d) of intermediate **9**

^{1}H NMR (500 MHz, CHLOROFORM-*d*) δ ppm 0.9 (t, *J*=6.9 Hz, 6 H) 1.3 (br s, 38 H) 2.2 - 2.3 (m, 2 H) 3.1 - 3.9 (m, 10 H) 4.5 - 4.7 (m, 2 H) 4.9 (s, 1 H)

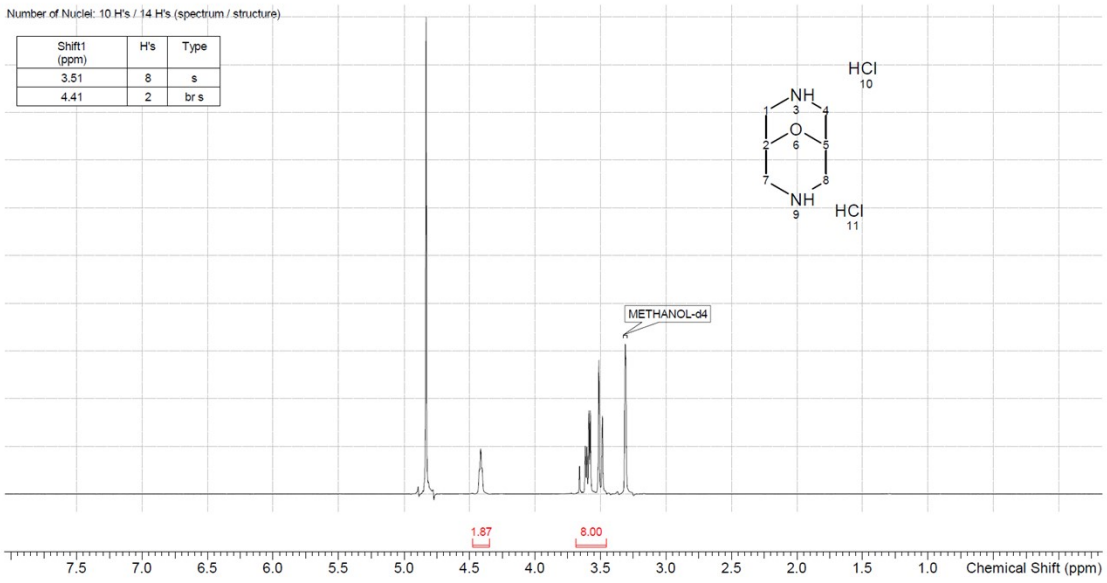


^{1}H NMR (500 MHz, chloroform-d) of intermediate **11**



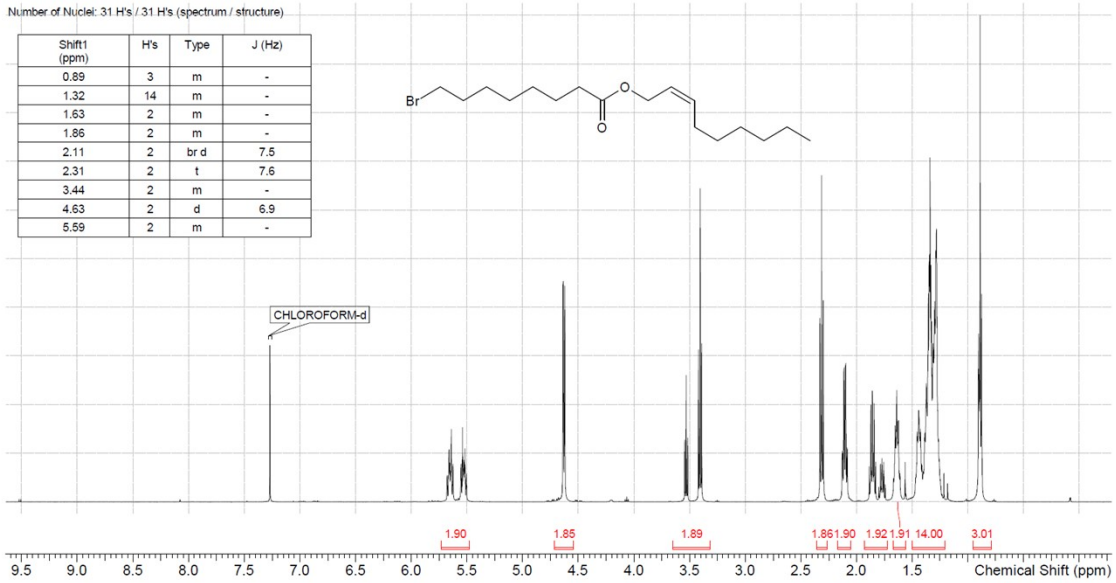
^{1}H NMR (500 MHz, methanol-d_4) of intermediate **12**

^{1}H NMR (500 MHz, METHANOL-d_4) δ ppm 3.5 (s, 8 H) 4.4 (br s, 2 H)



^{1}H NMR (500 MHz, chloroform-d) of intermediate **14**

^{1}H NMR (500 MHz, CHLOROFORM-*d*) δ ppm 0.8 - 1.0 (m, 3 H) 1.2 - 1.5 (m, 14 H) 1.6 - 1.7 (m, 2 H) 1.7 - 1.9 (m, 2 H) 2.1 (br d, *J*=7.5 Hz, 2 H) 2.3 (t, *J*=7.6 Hz, 2 H) 3.3 - 3.7 (m, 2 H) 4.6 (d, *J*=6.9 Hz, 2 H) 5.5 - 5.7 (m, 2 H)



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