1. Abbreviations

Oligomer naming system: "F253" refers to fluorous-tagged ("F") trimer with side chains 2, 5, and 3, with 2 being nearest the fluorous tag. The structure of F253 is shown in Figure 2. Structures of monomer side chains corresponding to each number are shown in Figure 3a. "253" refers to the same trimer after removal of the fluorous tag.

2. Materials and Methods

2.1 List of Materials

Materials

All Fluorous materials (Fluorous CbZ-OSu, Fluorous silica, Fluorous 96-well SPE plates): Fluorous Technologies

Multiarm PEGs (4 arm PEG NHS ester, 4 arm PEG azide): Creative PEGWorks

RNA sequences were generously provided by Alnylam Pharmaceuticals

Cellular assays: Promega Dual Glo, Promega CellTiter-Glo

Transfection reagents: Lipofectamine 2000, Lipofectamine RNAiMax (Life Technologies)
Cell culture materials: Life Technologies
All other reagents were purchased from Sigma Aldrich

Instrumentation

Flash chromatography: Teledyne ISCO CombiFlash
LCMS : Waters Acquity UPLC with a Xevo QToF LC/MS
NMR - VARIAN Inova-500 NMR Spectrometer with an Oxford Instruments Ltd. superconducting magnet
HPLC: Agilent HPLC with a Waters OST C18 column
Liquid Handling robot, chemical synthesis: Freeslate CM3
Liquid Handling robot, cellular assays: Tecan Freedom Evo
Microplate reader: Tecan Infinite

2.2 Methods

I. Synthesis and Purification of monomers

a. **Solid phase extraction through fluorous silica (FSPE).** Materials containing the Fluorous purification tag were routinely purified by solid phase extraction through Fluorous silica. In a typical bench scale purification for 40 g silica (or 2-3 g reaction mixture), the silica was first washed with 50 mL DMF and then preconditioned with 120 mL 85:15 methanol:water. The reaction mixture was loaded into the silica in up to 10 mL DMF. Non-Fluorous impurities were removed by washing with 150-200 mL 85:15 methanol:water. The fluorous product was then collected by washing the silica with 200-250 mL THF or acetone. The columns were then washed with 200 - 300 mL of 1:1:0.01 methanol:THF:TFA; this fraction often contained additional product that could be recovered. This general procedure was scaled to accommodate the amount of material to be purified.

b. Fluorous-CbZ-protected monomers

i. **Fluorous-CbZ-protected hydroxyproline.** N-[4-(1H, 1H, 2H, 2H-perfluorodecyl) benzyloxy-carbonyloxy] succinimide (16 mmol, 11.12 g) was combined with 1.5 eq. (24 mmol, 3.14 g) trans-4-hydroxy-L-proline in 65 mL anhydrous DMF. Triethylamine (2 eq, 3.9 mL) was added and the mixture was stirred at room temperature for 2 days or until LCMS analysis of the mixture showed that the NHS ester starting material was completely reacted. The product was purified by solid phase extraction on Fluorous silica. The reaction mixture was split into two batches and loaded on to 60 g of fluorous silica as described. Batches that required further purification after

solid phase extraction were re-purified by normal phase flash chromatography on a silica column using a gradient of hexane to ethyl acetate. 90-95% yield.

ii. **Fluorous-CbZ-protected monomer 1.** Fluorous-CbZ-protected hydroxyproline (2 mmol, 1.42 g) was dissolved in 4 mL DMF. Amine side chain (N,N,N' trimethylethylenediamine, 1.5 eq, 3 mmol, 389 microliters) and DIPEA (2 eq, 4 mmol, 695 μL) were added to the mixture. PyBOP (1.2 eq, 2.4 mmol, 1.25 g) was dissolved in 4 mL DMF and added to the reaction. The mixture was stirred at 50°C overnight. Product was purified by solid phase extraction through 40g of fluorous silica. If further purification was required, the product was purified by normal phase flash chromatography on a 25-gram silica column using a gradient of dichloromethane to Ultra solvent (88% dichloromethane, 20% methanol, 2% ammonium hydroxide). 60% yield.

iii. **Fluorous-CbZ-protected monomer 2.** The same procedure was followed as for Fluorous-CbZ-protected monomer 1, using 2-methylaminomethyl-1,3-dioxolane (1.5 eq, 3 mmol, 341 μL) as the amine side chain. Batches that required further purification after solid phase extraction were re-purified by reverse-phase HPLC (Agilent column info here) using a gradient of 5-95% acetonitrile. 60% yield.

iv. **Fluorous-CbZ-protected monomer 3.** The same procedure was followed as for Fluorous-CbZ-protected monomer 1, using N-benzylmethylamine (1.5 eq, 3 mmol, 385 μL) as the amine side chain. Batches that required further purification after solid phase extraction were re-purified by normal phase flash chromatography on a 25-gram silica column using a gradient of dichloromethane to 80:20 dichloromethane:methanol. 80% yield.

v. **Fluorous-CbZ-protected monomer 4.** The same procedure was followed as for Fluorous-CbZ-protected monomer 1, using N-methyldodecylamine (1.5 eq, 3 mmol, 751 μL) as the amine side chain. Batches that required further purification after solid phase extraction were re-purified by normal phase flash chromatography on a 25-gram silica column using a gradient of hexane to ethyl acetate. 80% yield.

vi. **Fluorous-CbZ-protected monomer 5.** The same procedure was followed as for Fluorous-CbZ-protected monomer 1, using 4-morpholinopiperidine (1.5 eq, 3 mmol, 504 mg) as the amine side chain. Batches that required further purification after solid phase extraction were re-purified by normal phase flash chromatography on a 25-gram silica column using a gradient of dichloromethane to

Ultra solvent (88% dichloromethane, 20% methanol, 2% ammonium hydroxide). 60% yield.

vii. **Fluorous-CbZ-protected monomer 6.** The same procedure was followed as for Fluorous-CbZ-protected monomer 1, using 2-[2-(1H-Imidazol-1-yl) ethyl] piperidine dihydrochloride (1.2 eq, 2.4 mmol, 605 mg) as the amine side chain, with an additional 2.4 eq of DIPEA to solubilize the amine in a portion of the DMF prior to addition to the reaction. This reaction was run at room temperature overnight. Batches that required further purification after solid phase extraction were re-purified by reverse phase HPLC using a gradient of 20 – 95% acetonitrile. 60% yield.

viii. **Fluorous-CbZ-protected monomer 7.** The same procedure was followed as for Fluorous-CbZ-protected monomer 1, using PEG500-amine (1.5 eq, 3 mmol, 1500 mg) as the amine side chain. Batches that required further purification after solid phase extraction were re-purified by reversed phase flash chromatography. 60% yield.

c. CbZ-protected monomers

i. **CbZ-protected monomer 1.** Z-Hyp-OH (Aldrich) (13.2 mmol, 3.5 g) was dissolved in 20 mL DMF. Amine side chain (N,N,N' trimethylethylenediamine, 1.5 eq, 19.8 mmol, 2.57 mL) and DIPEA (2 eq, 26.4 mmol, 4.59 mL) were added to the mixture. PyBOP (1.2 eq, 15.8 mmol, 8.24 g) was dissolved in 30 mL DMF and added to the reaction. The mixture was stirred at 50°C overnight. Product was purified by reverse phase flash chromatography on a C18 column using a slow gradient from 100% water to 95% acetonitrile. 90% yield.

ii. **CbZ-protected monomer 2.** The same procedure was followed as for CbZ-protected monomer 1, using 2-methylaminomethyl-1,3-dioxolane (1.5 eq, 19.8 mmol, 2.25 mL) as the amine side chain. Product was purified by reverse phase flash chromatography on a C18 column using a slow gradient from 100% water to 95% acetonitrile. 88% yield.

iii. **CbZ-protected monomer 3.** The same procedure was followed as for CbZ-protected monomer 1, using N-benzylmethylamine (1.5 eq, 19.8 mmol, 4.96 mL) as the amine side chain. Product was purified by normal phase flash chromatography on a silca column using a gradient of hexane to ethyl acetate (5-95%). 95% yield.

iv. **CbZ-protected monomer 4.** The same procedure was followed as for CbZ-protected monomer 1, using N-methyldodecylamine (1.5 eq, 19.8 mmol, 2.56 mL) as the amine side chain. Product was purified by normal phase flash chromatography on a silca column using a gradient of hexane to ethyl acetate (5-95%). 95% yield.

v. **CbZ-protected monomer 5.** The same procedure was followed as for CbZ-protected monomer 1, using 4-morpholinopiperidine (1.5 eq, 19.8 mmol, 3.33 g) as the amine side chain. Product was purified by normal phase flash chromatography on a silica column using a gradient dichloromethane to Ultra solvent (88% dichloromethane, 20% methanol, 2% ammonium hydroxide). 80% yield.

vi. **CbZ-protected monomer 6.** Z-Hyp-OH (Aldrich) (15.1 mmol, 4 g) was dissolved in 10 mL DMF. Amine side chain (2-[2-(1H-Imidazol-1-yl) ethyl] piperidine dihydrochloride, 1.2 eq, 18.1 mmol, 4.56 g) was combined with DIPEA (3.5 eq, 52.8 mmol,9.2 mL) in 15 mL DMF and added to the reaction mixture. PyBOP (1.2 eq, 18.1 mmol, 9.42 g) was dissolved in 35 mL DMF and added to the reaction. The mixture was stirred at room temperature overnight. Product was purified by reverse phase flash chromatography on a C18 column using a slow gradient from 100% water to 95% acetonitrile. 55% yield.

d. **Removal of CbZ protecting group to yield monomers 1-6.**

i. **Monomer 1.** CbZ-protected monomer 1 was dissolved in DMF to a concentration of 0.25 M and added to palladium catalyst (30 wt% on activated carbon) at a ratio of roughly 3:1 CbZ-protected monomer: palladium on carbon. A hydrogen balloon was attached to the reaction flask, which was stirred for at least 3 h at room temperature. After reaction, the hydrogen balloon was removed and ~5 mL of triethylamine was added to the mixture, which was allowed to stir for another 10 min. The catalyst was removed by filtration and rinsed thoroughly with methanol and water. The eluent was concentrated and purified by reverse phase flash chromatography on a C18 column using several column volumes of 100% water followed by a slow gradient up to 20% acetonitrile. 53% yield.

ii. **Monomer 2.** The same reaction and purification methods were used as for monomer 1. 74% yield.

iii. **Monomer 3.** The same reaction procedure used as for monomer 1. Product was purified by reverse phase flash chromatography on a C18 column using a gradient of 5-95% acetonitrile. 90% yield.

iv. **Monomer 4.** The same reaction procedure used as for monomer 1. Product was purified by reverse phase flash chromatography on a C18 column using a gradient of 5-95% acetonitrile. 80% yield

v. **Monomer 5.** The same reaction procedure used as for monomer 1. Product was purified by reverse phase flash chromatography on a C18 column using several column volumes of 100% water followed by a slow gradient up to 50% acetonitrile. 70% yield.

vi. **Monomer 6.** The same reaction procedure used as for monomer 1. Product was purified by reverse phase flash chromatography on a C18 column using several column volumes of 100% water with 1% triethylamine, followed by a slow gradient up to 20% acetonitrile with 1% triethylamine. 65% yield.

II. **Oligomer synthesis and purification**

a. **Bench top synthesis and purification for individual batches**. Fluorous CbZ-protected monomer was weighed in an oven-dried glass vial which was fitted with a rubber septum and fitted with a line flowing dry nitrogen. Ten to twelve molar equivalents of carbonyldiimidazole were added in DMF and the mixture was stirred to dissolve the starting monomer to a final concentration of 0.1 M. After stirring for one hour at room temperature, the mixture was cooled to 0° C before quenching. Excess CDI was quenched with a 1:1 mixture of water and acetonitrile such that a minimum amount of water was added to quench excess CDI (1 molar equivalent of water: excess CDI). The mixture was then evaporated to dryness, then resuspended in benzene and evaporated twice to remove residual water. The material was placed back under nitrogen flow and the second monomer (6 equivalents, deprotected) was added in DMF to a final concentration of 0.1 M starting monomer. The mixture was stirred at room temperature overnight, then purified by solid phase extraction through fluorous silica, as described previously. Yields ranged from 85 – 100%

b. **High-throughput synthesis and purification.** Synthesis steps were carried out by a Freeslate CM3 robot, using 1-mL glass vials in 96-well plate format. All vials, stir bars, and reaction blocks were dried overnight at 150° C before use. The interior of the robot was purged with nitrogen such that moisture levels were below 150 ppm for the duration of the synthesis. Reagent addition scripts were written in Automation Studio and executed using the Library Studio software.

i. **Stock solutions**. Monomers were dissolved in anhydrous DMF to a concentration of 0.24M for fluorous CbZ-protected monomers and 0.58M for deprotected monomers, with the exception of Monomer 3, which was dissolved in 1:1 DMF:THF to 0.12M, and Monomer 5, which was dissolved in 1:1 DMF: MeCN to 0.12M. CDI was dissolved in anhydrous DMF until the solution was saturated. Solutions were stored in the dry environment of the robot.

ii. **Monomer coupling**. Using the single tip lookahead feature of the Freeslate CM3, 12 umol of starting (fluorous CbZ-protected) monomer was added to each well (100 µL of stock solution for monomers 3 and 5, 50 µL for other monomers). Wells were stirred at room temperature while 125 µL of saturated CDI solution was added to each well using the 6-channel tip or single tip lookahead feature. After addition, the wells were stirred for 1 h. Plates were

then cooled to 0°C and 5 μL of a 1:1 mixture of acetonitrile: water was added to each well. Alternatively, quenching of CDI could be done outside the robot by placing the plates on ice and adding MeCN: water using a multichannel pipette. After quenching, plates were removed from the robot and dried on a Genevac EZ-2 for ~1h. Benzene (50-100 μL) was added to each well using a multichannel pipette and the plates were stirred briefly before being dried again for ~45 min. Benzene was added one more time and the plates were dried thoroughly before being replaced in the robot's glove box. Deprotected monomer stock solution (125 μL) was added to each well using the robot's 6-tip pipette or single-tip lookahead feature. The wells were stirred overnight at 22°C.

iii. **Oligomer purification.** Oligomers were purified by solid phase extraction through fluorous silica in 96-well plate format. Before use, fluorous silica plates were washed with 1 mL THF per well, followed by 3 mL DMF, using a multichannel pipette to transfer solvents and vacuum to pull solvents through the silica. Each well was then conditioned with 3 mL of 85:15 DMF:water before sample loading. The reaction mixture was then loaded onto the silica in 125 μL DMF. The silica washed with 3 mL 85:15 DMF:water to remove excess reagents, and then the product was eluted with 4 mL of THF. Wells were then washed with 5 mL of 1:1:0.01 THF: MeOH:TFA. This fraction was collected and a selection of wells was analyzed by LCMS as it often contained additional product that could be combined with the previous fraction. Wells were then washed with an additional 2 mL of DMF before their next use. Product fractions were concentrated and transferred to glass 1-mL vials in 96-well plates for the next synthesis step. Yields ranged from 70 – 99%.

iv. **Coupling to Azido-PEG-amine.** CDI coupling was carried out in the same manner as for oligomer synthesis, with the exception that only 2 eq of amine (11-Azido-3,6,9-trioxaundecan-1-amine) were added to the mixed imidazolide intermediate.

v. **Purification of Oligomer-PEG-azides.** The same purification method was used as for oligomer purification, but with additional washing: after loading the crude

vi. **Removal of fluorous purification tag.** Protected oligomers were dissolved in 80 μL of 1:1 acetonitrile:benzene using anhydrous solvents. In a dry nitrogen environment, the reaction vessels were cooled to 0°C before the addition of 25 μL of a 1M solution of iodotrimethylsilane (TMSI) in dichloromethane. After stirring for 45 min, the reactions were quenched by the addition of 50 μL of methanol. Thiosulfate bound to polystyrene beads (Sigma) was added to each well and the solutions were incubated overnight to

remove residual iodine. The solution of deprotected product was then removed from the well with a pipette, concentrated, and purified by FSPE. Deprotected product was collected in the aqueous fraction. Deprotection was also achieved by heating to 50°C in trifluoroacetic acid for 3h.

III. **siRNA conjugation, purification, and characterization**

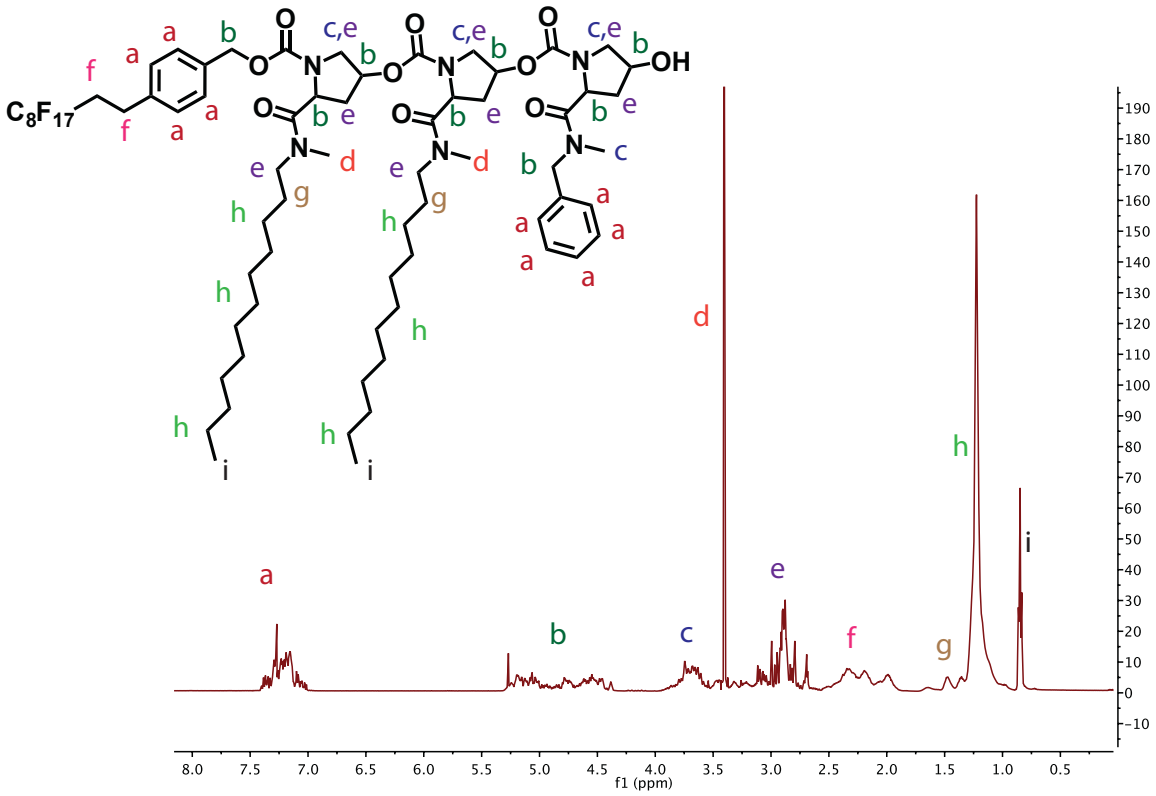
a. **siRNA conjugation conditions.** Trimer-PEG-azides were dissolved in 500 µL MeCN. The DBCO-modified siRNA sequence was dissolved in PBS and stored at a concentration of 1 mg/mL. Oligomer-PEG-azide stock solution was added to siRNA stock solution, PBS, and acetonitrile such that the final concentration of siRNA was 0.25 mg/mL in 30% MeCN. Oligomer-azide solution was added to a final concentration of 0.5 - 1 mg/mL. For high-throughput synthesis, reactions were prepared in PCR plates and allowed to stir on an orbital shaker overnight. Plates were then frozen at -20°C for 1-3h and allowed to thaw in a refrigerator.

b. **Purification of siRNA conjugates.** The reaction mixture was dried on a Genevac EZ-2 and then re-dissolved in 30% MeCN/PBS to a concentration of 1 mg/mL. One tenth volume of 3M sodium acetate buffer solution and then 5 volumes of 200 proof ethanol were added to this. The plates were covered and frozen at -20°C overnight, then moved to a -80°C freezer for 1-3 h. For high-throughput synthesis, the plates were then centrifuged at 3,200 rcf for 90 minutes, carefully decanted, and dried. For single-batch synthesis in microcentrifuge tubes, tubes were centrifuged at 15,000 rcf for 20 min before decanting.

c. **HPLC characterization of conjugates.** Samples were diluted in 0.1M triethylamino acetate (TEAA) buffer to a concentration of 1 µg/50 µL. Five hundred ng – 1 µg of siRNA was injected for each run on a Waters XBridge OST C18 (2.5 µm, 2.1 x 50 mm) column and run on a gradient of 10-95% B (A = 0.1M TEAA in water, B = 0.1M TEAA in MeCN) in 60 minutes at a flow rate of 0.2 mL/min.

3. Supplementary Data

3.1 ^{1}H NMR and ^{13}C NMR shifts

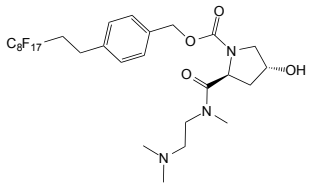


^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.40 – 7.01 (m, 9H), 5.21 – 4.45 (m, 10H), 3.84 – 3.57 (m, 6H), 3.40 (s, 6H), 3.11 – 2.68 (m, 13H), 2.39 – 2.30 (m, 2H), 2.23 – 2.13 (m, 2H), 1.75 – 1.34 (m, 3H), 1.34 – 0.89 (m, 36H), 0.85 (td, *J* = 6.9, 2.0 Hz, 6H).

Figure 3.1.1. Sample ^{1}HNMR analysis. Structure and ^{1}HNMR spectrum of trimer F443 is shown with interpretation of ^{1}HNMR shifts.

Monomer 1

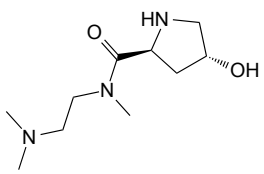
Fluorous protected:



^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.37 – 7.27 (m, 2H), 7.19 (m, 2H), 5.18 – 5.01 (m, 2H), 4.88 – 4.75 (m, 1H), 4.56 (d, *J* = 17.5 Hz, 1H), 3.80 – 3.71 (m, 1H), 3.70 – 3.52 (m, 2H), 3.47 – 3.23 (m, 1H), 3.15 (d, *J* = 0.9 Hz, 1H), 2.99 (d, *J* = 0.9 Hz, 1H), 2.90 (m, 3H), 2.50 – 2.30 (m, 2H), 2.29 – 2.16 (m, 7H), 2.16 – 2.03 (m, 3H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 173.09, 155.47, 139.58, 135.64, 129.70, 128.97, 120.66, 118.77, 116.76, 113.84, 111.52, 109.11, 70.20, 67.49, 57.79, 56.15, 50.85, 48.66, 46.94, 46.04, 39.38, 35.82, 33.51, 26.75.

Deprotected:

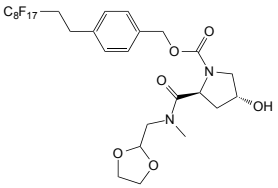


^{1}H NMR (500 MHz, Methanol-d_4) δ 4.41 (m, 1H), 4.19 (m, 1H), 3.60 – 3.51 (m, 1H), 3.55 – 3.41 (m, 1H), 3.32 – 3.21 (m, 1H), 3.09 (s, 2H), 2.97 (s, 1H), 2.78 (m, 1H), 2.64 – 2.41 (m, 2H), 2.30 (d, J = 10.6 Hz, 6H), 2.20 – 2.06 (m, 1H), 1.82 (m, 1H).

^{13}C NMR (126 MHz, Methanol-d_4) δ 173.89, 72.65, 57.22, 56.15, 55.08, 46.13, 45.17, 44.96, 40.01, 34.40.

Monomer 2

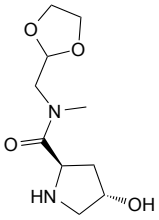
Fluorous protected:



^{1}H NMR (500 MHz, Chloroform-d) δ 7.35 – 7.24 (m, 2H), 7.24 – 7.17 (m, 2H), 5.19 – 4.76 (m, 4H), 4.67 – 4.55 (m, 1H), 4.03 – 3.72 (m, 4H), 3.71 – 3.53 (m, 2H), 3.51 – 3.26 (m, 1H), 3.24 (s, 1H), 3.07 (s, 1H), 3.02 (s, 1H), 2.96 – 2.86 (m, 3H), 2.44 – 2.34 (m, 2H), 2.34 – 2.00 (m, 2H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 173.41, 154.87, 139.00, 135.05, 128.97, 128.36, 120.22, 118.10, 116.01, 113.19, 110.77, 108.57, 102.26, 70.18, 69.41, 66.89, 65.03, 55.36, 51.89, 50.79, 39.01, 36.58, 32.88, 26.16.

Deprotected:

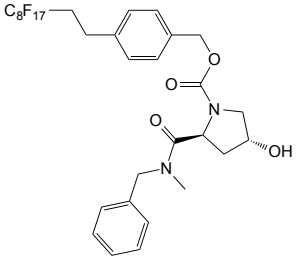


^{1}H NMR (500 MHz, Methanol-d_4) δ 5.08 – 4.93 (m, 1H), 4.49 – 4.40 (m, 1H), 4.31 (m, 1H), 4.03 – 3.91 (m, 2H), 3.91 – 3.80 (m, 2H), 3.71 – 3.46 (m, 2H), 3.33 – 3.23 (m, 1H), 3.16 (s, 1H), 3.03 (s, 1H), 2.80 (m, 1H), 2.22 – 2.06 (m, 1H), 1.80 (m, 1H).

^{13}C NMR (126 MHz, cd_3od) δ 175.27, 103.14, 73.25, 66.23, 65.89, 57.61, 55.76, 51.90, 40.61, 36.70.

Monomer 3

Fluorous protected:



^{1}H NMR (500 MHz, Chloroform-d) δ 7.54 – 6.91 (m, 9H), 5.24 – 4.99 (m, 2H), 4.99 – 4.70 (m, 2H), 4.68 – 4.38 (m, 2H), 3.86 – 3.53 (m, 2H), 3.04 (m, 2H), 2.91 (m, 2H), 2.85 (d, J = 19.5 Hz, 1H), 2.45 – 2.21 (m, 3H), 2.16 (m, 1H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 172.71, 155.04, 139.05, 136.71, 135.18, 129.07, 128.49, 128.18, 127.91, 126.54, 119.86, 118.35, 116.38, 113.19,

110.86, 108.71, 70.19, 66.91, 55.48, 53.21, 51.55, 38.93, 34.67, 31.87, 26.06.

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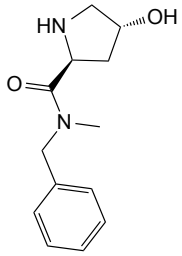
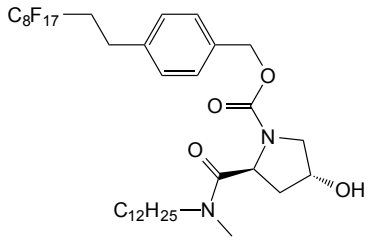


^{1}H NMR (500 MHz, Methanol-d_4) δ 7.35 – 7.13 (m, 5H), 4.77 (m, 1H), 4.66 – 4.41 (m, 3H), 3.39 (m, 1H), 3.35 – 3.22 (m, 1H), 2.88 (d, J = 4.1 Hz, 3H), 2.49 (m, 1H), 1.99 (m, 1H).

^{13}C NMR (126 MHz, cd$_3$od) δ 174.95, 137.99, 129.95, 129.68, 128.75, 128.49, 127.67, 73.41, 57.80, 55.82, 52.78, 40.90, 34.65.

Monomer 4

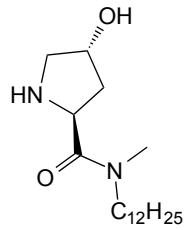
Fluorous protected:



^{1}H NMR (500 MHz, Chloroform-d) δ 7.35 – 7.28 (m, 1H), 7.27 – 7.23 (m, 1H), 7.23 – 7.14 (m, 2H), 5.28 – 4.93 (m, 2H), 4.91 – 4.67 (m, 1H), 4.59 (d, J = 18.2 Hz, 1H), 3.92 – 3.74 (m, 1H), 3.74 – 3.57 (m, 1H), 3.57 – 3.42 (m, 1H), 3.36 – 3.19 (m, 1H), 3.12 (s, 1H), 3.04 – 2.79 (m, 4H), 2.47 – 2.30 (m, 2H), 2.28 – 2.06 (m, 2H), 1.63 – 1.49 (m, 2H), 1.44 – 1.03 (m, 18H), 0.89 (t, J = 6.9, 2.6 Hz, 3H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 172.32, 154.89, 138.91, 135.05, 128.91, 128.31, 120.08, 118.12, 116.01, 113.17, 110.80, 108.51, 69.68, 66.90, 55.46, 50.03, 48.55, 38.79, 35.03, 34.15, 32.87, 31.94, 29.66, 29.37, 28.79, 28.45, 26.92, 26.17, 22.68, 13.97.

Deprotected:

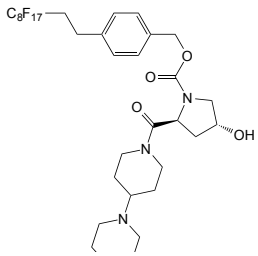


^{1}H NMR (500 MHz, Methanol-d_4) δ 4.81 – 4.70 (m, 1H), 4.63 – 4.57 (m, 1H), 3.52 – 3.25 (m, 4H), 3.06 (s, 2H), 2.98 (s, 1H), 2.59 – 2.43 (m, 1H), 2.05 – 1.90 (m, 1H), 1.72 – 1.52 (m, 2H), 1.36 – 1.27 (m, 18H), 0.94 – 0.86 (m, 3H).

^{13}C NMR (126 MHz, cd$_3$od) δ 168.97, 71.30, 58.65, 54.95, 50.24, 38.99, 34.47, 32.89, 30.63, 30.60, 30.55, 30.53, 30.50, 30.31, 28.89, 27.57, 23.56, 14.40.

Monomer 5

Fluorous protected:

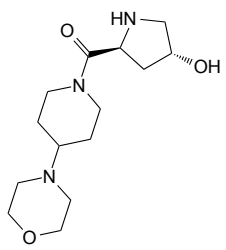


^{1}H NMR (500 MHz, Chloroform-d) δ 7.37 – 7.25 (m, 2H), 7.25 – 7.15 (m, 2H), 5.30 – 4.96 (m, 2H), 4.95 – 4.78 (m, 1H), 4.70 – 4.42 (m, 2H), 4.18 – 3.83 (m, 1H), 3.82 – 3.64 (m, 5H), 3.61 – 3.45 (m, 1H), 3.31 – 2.96 (m, 1H), 2.95 – 2.76

(m, 2H), 2.75 – 2.48 (m, 4H), 2.47 – 2.30 (m, 3H), 2.29 – 1.99 (m, 3H), 1.97 – 1.69 (m, 2H), 1.62 – 1.05 (m, 2H).

^{13}C NMR (126 MHz, cd_3od) δ 171.29, 155.67, 139.55, 135.66, 128.96, 128.51, 121.17, 118.75, 116.61, 113.33, 111.55, 108.84, 69.86, 67.59, 62.33, 55.78, 50.11, 45.20, 42.29, 39.17, 33.51, 29.68, 28.88, 26.77.

Deprotected:

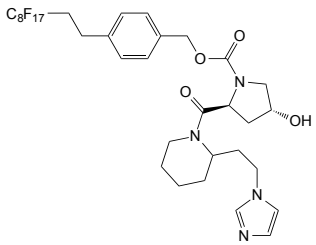


^{1}H NMR (500 MHz, Methanol-d_4) δ 4.57 – 4.48 (m, 1H), 4.46 – 4.36 (m, 1H), 4.29 – 4.20 (m, 1H), 4.06 – 3.96 (m, 1H), 3.72 – 3.66 (m, 4H), 3.33 – 3.24 (m, 2H), 3.18 – 3.05 (m, 1H), 2.88 – 2.78 (m, 1H), 2.77 – 2.64 (m, 1H), 2.65 – 2.55 (m, 4H), 2.54 – 2.42 (m, 1H), 2.18 – 2.07 (m, 1H), 2.08 – 1.92 (m, 1H), 1.92 – 1.72 (m, 1H), 1.53 – 1.25 (m, 2H).

^{13}C NMR (126 MHz, cd_3od) δ 172.51, 73.23, 67.88, 62.95, 62.88, 57.56, 55.66, 50.97, 50.85, 45.24, 42.55, 40.80, 29.75, 28.97.

Monomer 6

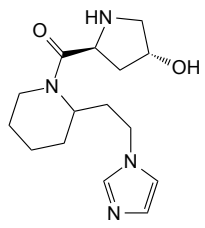
Fluorous protected:



^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.98 (d, *J* = 33.5 Hz, 1H), 7.39 – 7.27 (m, 2H), 7.20 – 7.13 (m, 2H), 7.10 (s, 1H), 7.05 (d, *J* = 1.7 Hz, 1H), 5.11 – 5.02 (m, 2H), 4.92 – 4.79 (m, 2H), 4.58 (dq, *J* = 4.9, 2.3 Hz, 1H), 4.11 – 3.91 (m, 2H), 3.84 (d, *J* = 14.0 Hz, 1H), 3.81 – 3.73 (m, 1H), 3.63 (dt, *J* = 11.5, 1.8 Hz, 1H), 3.21 – 3.10 (m, 1H), 2.95 – 2.80 (m, 2H), 2.42 – 2.25 (m, 4H), 2.25 – 2.18 (m, 1H), 2.17 – 2.03 (m, 1H), 1.81 – 1.69 (m, 2H), 1.69 – 1.50 (m, 3H), 1.49 – 1.27 (m, 1H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 171.50, 155.07, 139.04, 136.89, 135.14, 128.95, 128.49, 128.25, 125.55, 119.62, 118.23, 116.07, 113.26, 110.85, 108.63, 69.91, 66.92, 60.53, 55.63, 50.12, 45.48, 40.96, 37.86, 32.89, 31.47, 29.33, 26.07, 21.16, 19.18.

Deprotected:



^{1}H NMR (500 MHz, Methanol-d_4) δ 7.79 – 7.65 (m, 1H), 7.27 – 7.11 (m, 1H), 7.01 – 6.89 (m, 1H), 4.41 (ddq, *J* = 11.0, 5.5, 2.7 Hz, 2H), 4.25 – 4.01 (m, 2H), 3.95 (dt, *J* = 12.6, 7.3 Hz, 2H), 3.80 – 3.73 (m, 1H), 3.33 – 3.17 (m, 2H), 2.85 – 2.72 (m, 1H), 2.43 – 2.27 (m, 1H), 2.25 – 2.08 (m, 1H), 2.07 – 1.87 (m, 1H), 1.87 – 1.68 (m, 2H), 1.67 – 1.37 (m, 4H).

^{13}C NMR (126 MHz, CD_3OD) δ 172.80, 138.56, 128.96, 120.66, 72.97, 58.02, 55.58, 49.85, 48.08, 45.16, 41.15, 32.15, 29.81, 27.12, 19.96.

Trimers

F122

^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.37 – 7.27 (m, 2H), 7.23 – 7.13 (m, 2H), 5.41 – 4.94 (m, 5H), 4.94 – 4.55 (m, 4H), 4.56 – 4.37 (m, 1H), 4.20 – 4.00 (m, 1H), 4.00 – 3.82 (m, 7H), 3.82 – 3.57 (m, 7H), 3.57 – 3.36 (m, 3H), 3.36 – 3.19 (m, 2H), 3.19 – 3.09 (m, 3H), 3.09 – 2.77 (m, 10H), 2.57 – 2.42 (m, 2H), 2.42 – 2.22 (m, 8H), 2.22 – 1.99 (m, 4H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 174.02, 172.20, 155.21, 154.22, 139.57, 135.44, 128.90, 118.41, 116.90, 113.64, 111.34, 109.07, 103.12, 102.64, 75.24, 73.44, 70.60, 69.97, 67.58, 65.40, 58.24, 56.79, 55.95, 53.41, 51.05, 49.05, 47.25, 46.20, 40.37, 36.39, 33.47, 29.94, 26.85.

F144

^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.35 – 7.24 (m, 2H), 7.22 – 7.15 (m, 2H), 5.25 – 4.93 (m, 4H), 4.92 – 4.42 (m, 6H), 3.88 – 3.57 (m, 8H), 3.54 – 3.35 (m, 2H), 3.30 – 3.04 (m, 5H), 3.00 – 2.95 (m, 2H), 2.94 – 2.86 (m, 4H), 2.85 – 2.68 (m, 2H), 2.55 – 2.42 (m, 2H), 2.40 – 2.19 (m, 9H), 2.14 – 1.94 (m, 3H), 1.75 – 1.44 (m, 4H), 1.44 – 1.00 (m, 36H), 0.86 (td, *J* = 7.0, 2.3 Hz, 6H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 172.35, 154.24, 139.69, 135.50, 129.09, 120.88, 118.79, 116.51, 113.65, 111.45, 108.94, 78.00, 77.94, 77.74, 77.49, 74.32, 73.03, 70.17, 67.59, 57.60, 56.62, 55.68, 53.28, 51.33, 50.50, 48.90, 46.38, 40.79, 40.15, 39.61, 37.79, 36.31, 35.24, 34.49, 33.54, 32.60, 30.32, 30.04, 29.46, 29.12, 27.75, 27.47, 26.86, 23.36, 14.78.

F146

^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.50 (d, *J* = 16.9 Hz, 1H), 7.37 – 7.28 (m, 2H), 7.19 (d, *J* = 7.6 Hz, 2H), 7.07 – 7.01 (m, 1H), 6.95 (d, *J* = 11.4 Hz, 1H), 5.26 – 4.94 (m, 4H), 4.87 – 4.35 (m, 5H), 4.17 – 4.00 (m, 2H), 3.94 – 3.44 (m, 10H), 3.22 – 3.10 (m, 3H), 3.02 – 2.82 (m, 6H), 2.76 – 2.45 (m, 5H), 2.38 – 2.20 (m, 10H), 2.15 – 1.83 (m, 6H), 1.75 – 1.61 (m, 4H), 1.46 – 1.37 (m, 2H), 1.25 (s, 18H), 0.91 – 0.83 (m, 3H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 172.32, 154.92, 139.80, 137.99, 135.14, 129.10, 129.05, 119.51, 116.45, 113.23, 110.97, 108.87, 106.88, 103.84, 70.94, 69.75, 67.56, 64.46, 56.77, 55.80, 54.71, 53.26, 46.35, 46.21, 43.82, 41.56, 36.96, 32.61, 31.09, 30.35, 30.06, 26.88, 24.55, 23.89, 23.39, 23.37, 19.49, 14.81.

F214

^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.31 – 7.22 (m, 2H), 7.19 – 7.14 (m, 2H), 5.26 – 5.00 (m, 4H), 4.98 – 4.60 (m, 4H), 4.42 (s, 1H), 3.94 – 3.55 (m, 11H), 3.39 (s, 4H), 3.25 – 3.15 (m, 2H), 3.11 – 3.00 (m, 3H), 2.98 – 2.78 (m, 9H), 2.48 – 2.39 (m, 1H), 2.38 – 2.07 (m, 12H), 2.08 – 1.85 (m, 1H), 1.47 (s, 2H), 1.24 (d, *J* = 31.8 Hz, 18H), 0.90 – 0.78 (m, 3H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 173.04, 154.70, 139.67, 135.41, 129.13, 120.59, 118.78, 116.63, 113.69, 111.38, 109.18, 102.86, 74.48, 70.59, 69.81, 67.65, 65.68, 57.82, 56.93, 55.83, 53.06, 51.02, 49.34, 46.31, 39.84, 38.42, 37.08, 35.66, 34.67, 33.51, 32.56, 30.16, 27.34, 26.83, 23.33, 14.74.

F215

^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.35 – 7.26 (m, 2H), 7.22 – 7.18 (m, 2H), 5.41 – 5.17 (m, 2H), 5.16 – 5.02 (m, 2H), 5.01 – 4.61 (m, 4H), 4.60 – 4.37 (m, 2H), 4.28 – 4.02 (m, 1H), 4.01 – 3.55 (m, 15H), 3.55 (s, 3H), 3.32 – 3.11 (m, 3H), 3.11 – 2.77 (m, 10H), 2.76 – 2.62 (m, 1H), 2.54 (s, 4H), 2.48 – 2.14 (m, 11H), 2.13 – 1.78 (m, 5H), 1.77 – 1.58 (m, 1H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 173.01, 155.41, 139.63, 135.90, 129.10, 120.98, 118.80, 116.66, 114.02, 111.45, 109.27, 102.88, 74.91, 74.12, 70.36, 67.77, 65.70, 62.36, 57.62, 55.79, 53.46, 52.51, 51.44, 50.51, 45.91, 42.37, 38.45, 37.16, 33.52, 30.39, 29.99, 29.39, 28.84, 28.52, 26.87, 23.39, 22.15.

F341

^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.38 – 7.28 (m, 3H), 7.26 – 6.95 (m, 6H), 5.32 – 4.96 (m, 4H), 4.92 – 4.60 (m, 3H), 4.58 – 4.39 (m, 3H), 3.83 – 3.51 (m, 6H), 3.41 (s, 4H), 3.28 – 3.17 (m, 1H), 3.10 – 2.58 (m, 12H), 2.49 – 2.20 (m, 11H), 2.16 – 2.03 (m, 2H), 1.99 – 1.87 (m, 1H), 1.70 – 1.38 (m, 2H), 1.38 – 0.94 (m, 18H), 0.86 (t, J = 8.3, 4.7, 1.4 Hz, 3H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 172.47, 154.63, 139.65, 137.35, 135.49, 129.65, 129.31, 129.07, 128.47, 127.42, 120.81, 118.71, 116.50, 113.91, 111.55, 109.10, 74.40, 70.81, 69.77, 67.52, 57.94, 56.69, 55.84, 53.36, 52.00, 51.19, 50.23, 48.59, 46.29, 40.19, 39.53, 38.99, 37.67, 36.61, 34.74, 33.59, 32.58, 30.31, 30.03, 29.47, 27.51, 26.95, 23.34, 14.75.

F365

^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.49 – 6.63 (m, 12H), 5.39 – 5.03 (m, 4H), 5.01 – 4.64 (m, 3H), 4.62 – 4.37 (m, 4H), 4.23 – 3.88 (m, 3H), 3.88 – 3.46 (m, 11H), 3.45 – 3.38 (m, 3H), 3.19 – 2.95 (m, 3H), 2.95 – 2.73 (m, 4H), 2.71 – 2.20 (m, 11H), 2.18 – 1.76 (m, 6H), 1.74 – 1.42 (m, 4H), 1.43 – 1.16 (m, 3H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 171.78, 154.63, 140.19, 137.44, 136.86, 135.46, 130.05, 129.59, 129.32, 129.08, 128.33, 127.22, 120.45, 120.04, 118.92, 116.63, 113.80, 111.43, 109.22, 74.35, 70.67, 67.81, 62.50, 55.99, 53.35, 52.11, 51.23, 50.42, 45.29, 44.30, 42.51, 42.14, 39.37, 37.87, 34.90, 33.40, 32.30, 30.09, 28.87, 26.96, 25.31, 19.26.

F425

^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.33 – 7.20 (m, 2H), 7.20 – 7.12 (m, 2H), 5.29 – 5.00 (m, 4H), 4.97 – 4.65 (m, 3H), 4.63 – 4.35 (m, 3H), 4.06 – 3.79 (m, 5H), 3.77 – 3.50 (m, 10H), 3.39 (d, J = 1.0 Hz, 5H), 3.17 – 2.94 (m, 4H), 2.93 – 2.85 (m, 4H), 2.84 – 2.73 (m, 2H), 2.61 – 2.12 (m, 11H), 2.11 – 1.71 (m, 5H), 1.67 – 1.38 (m, 2H), 1.36 – 1.03 (m, 18H), 0.83 (t, J = 6.3, 1.8 Hz, 3H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 171.92, 154.47, 139.80, 135.48, 129.68, 129.07, 120.87, 118.74, 116.60, 113.40, 111.29, 109.19, 102.50, 74.12, 70.66, 69.80, 67.65, 65.69, 62.48, 55.90, 53.03, 51.30, 50.45, 49.05, 44.52, 42.18, 39.92, 37.55, 36.21, 34.57, 33.47, 32.55, 30.25, 30.16, 30.00, 29.02, 27.48, 26.82, 23.31, 14.72.

F443

^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.40 – 7.01 (m, 9H), 5.21 – 4.45 (m, 10H), 3.84 – 3.57 (m, 6H), 3.40 (s, 6H), 3.11 – 2.68 (m, 13H), 2.39 – 2.30 (m, 2H), 2.23 – 2.13 (m, 2H), 1.75 – 1.34 (m, 3H), 1.34 – 0.89 (m, 36H), 0.85 (td, *J* = 6.9, 2.0 Hz, 6H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 172.61, 154.63, 139.61, 136.74, 135.41, 129.90, 129.47, 129.02, 128.28, 127.00, 120.44, 118.75, 116.64, 114.01, 111.56, 109.45, 74.51, 70.21, 67.52, 55.95, 53.39, 50.98, 48.83, 39.75, 37.98, 34.71, 33.51, 32.57, 30.30, 30.14, 30.11, 30.02, 29.45, 27.53, 26.83, 23.33, 14.73.

F465

^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.42 – 7.32 (m, 1H), 7.33 – 7.22 (m, 2H), 7.23 – 7.13 (m, 2H), 7.12 – 6.90 (m, 2H), 5.41 – 4.96 (m, 5H), 4.97 – 4.68 (m, 2H), 4.68 – 4.36 (m, 3H), 4.27 – 3.89 (m, 3H), 3.89 – 3.38 (m, 15H), 3.37 – 2.98 (m, 4H), 2.98 – 2.75 (m, 5H), 2.53 (d, *J* = 7.8 Hz, 6H), 2.43 – 2.15 (m, 6H), 2.14 – 1.78 (m, 5H), 1.78 – 1.44 (m, 5H), 1.46 – 1.26 (m, 2H), 1.27 – 1.07 (m, 18H), 0.89 – 0.76 (m, 3H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 172.11, 170.75, 154.66, 139.95, 136.94, 135.29, 129.97, 129.07, 120.46, 120.07, 118.92, 116.50, 113.55, 111.43, 109.20, 74.55, 70.43, 67.80, 62.54, 55.91, 53.20, 51.21, 50.45, 48.89, 45.28, 44.30, 42.32, 39.40, 37.77, 35.47, 33.55, 32.57, 30.21, 29.00, 27.55, 26.85, 25.72, 23.34, 19.39, 14.75.

F534

^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.55 – 6.78 (m, 9H), 5.41 – 4.93 (m, 4H), 4.93 – 4.30 (m, 6H), 4.24 – 3.91 (m, 1H), 3.91 – 3.51 (m, 9H), 3.42 (d, *J* = 0.6 Hz, 4H), 3.14 – 2.76 (m, 8H), 2.74 – 2.45 (m, 7H), 2.25 (s, 6H), 2.15 – 1.59 (m, 6H), 1.28 (s, 20H), 0.91 – 0.76 (m, 3H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 172.66, 170.53, 154.52, 139.77, 137.29, 135.67, 130.09, 129.39, 129.05, 128.24, 127.18, 120.63, 118.77, 116.63, 113.58, 111.27, 109.19, 74.71, 71.43, 69.96, 67.67, 62.28, 55.90, 53.28, 51.36, 50.33, 45.07, 42.30, 39.77, 37.24, 35.37, 34.37, 33.48, 32.59, 30.32, 30.03, 29.05, 27.17, 23.62, 14.78.

F542

^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.37 – 7.22 (m, 2H), 7.20 – 7.11 (m, 2H), 5.23 – 5.05 (m, 3H), 5.04 – 4.71 (m, 3H), 4.68 – 4.34 (m, 4H), 4.18 – 3.81 (m, 5H), 3.81 – 3.49 (m, 11H), 3.38 (s, 6H), 3.19 – 2.92 (m, 4H), 2.91 – 2.80 (m, 4H), 2.77 – 2.53 (m, 3H), 2.52 – 2.22 (m, 9H), 2.05 – 1.80 (m, 4H), 1.66 – 1.37 (m, 2H), 1.37 – 0.94 (m, 18H), 0.83 (t, *J* = 7.1, 3.6, 1.7 Hz, 3H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 172.93, 170.53, 154.93, 154.19, 139.57, 135.54, 128.98, 120.65, 118.70, 116.61, 113.66, 111.32, 109.17, 102.81, 74.34, 70.17, 67.68, 65.72, 62.35, 55.86, 52.88, 51.17, 50.42, 48.45, 45.12, 42.24, 39.77, 37.37, 34.84, 33.59, 32.55, 30.05, 28.92, 27.19, 23.31, 14.71.

F632

^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.60 – 7.39 (m, 1H), 7.38 – 6.67 (m, 11H), 5.37 – 4.88 (m, 5H), 4.86 – 4.59 (m, 4H), 4.58 – 4.48 (m, 1H), 4.47 – 4.31 (m, 1H), 3.98 – 3.65 (m, 9H), 3.65 – 3.52 (m, 3H), 3.41 (d, *J* = 0.5 Hz, 8H), 3.23 – 3.12 (m, 1H), 3.10 – 2.73 (m, 8H), 2.73 – 2.47 (m, 3H), 2.42 – 2.17 (m, 5H), 2.15 – 2.10 (m, 1H), 2.06 – 1.89 (m, 2H), 1.77 (s, 1H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 171.54, 154.31, 139.73, 137.67, 137.23, 135.53, 129.63, 129.33, 128.95, 128.34, 127.09, 119.70, 118.90, 116.63, 114.00, 111.38, 109.47, 107.03, 102.71, 74.55, 69.60, 67.55, 65.71, 56.04, 52.76, 51.31, 47.16, 44.83, 41.40, 40.17, 37.79, 35.16, 33.05, 30.14, 27.02, 19.84.

3.2 LCMS analysis of oligomer synthesis

Trimers synthesized in Chapter 2: LC traces @ 214 nm. Mass spectra show ions contributing to peak in MS trace of trimer.

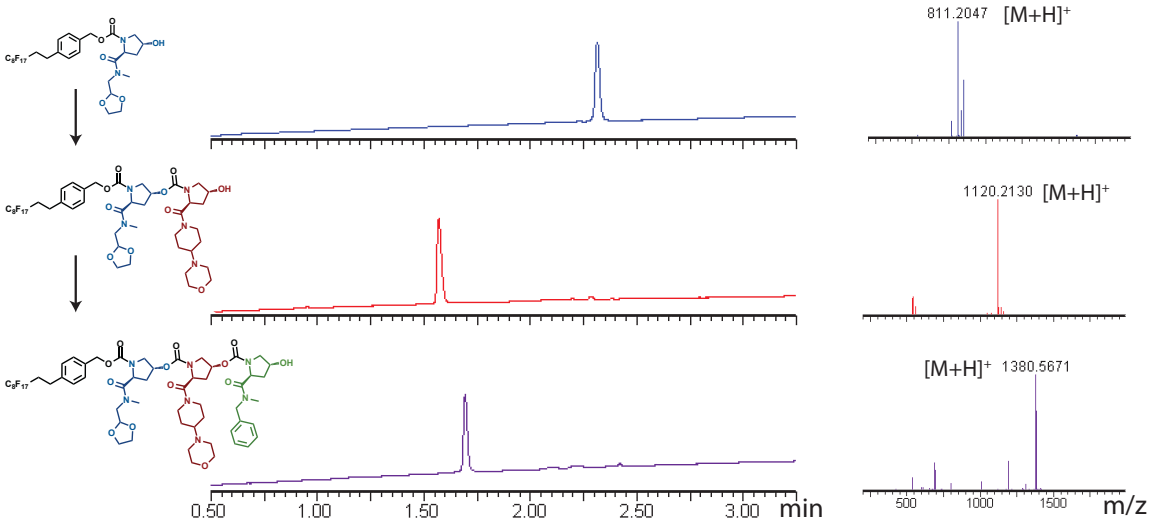


Figure 3.2.1. Representative LCMS analysis of trimer synthesis. LC traces of monomer, dimer, and trimer show UV absorbance at 214 nm. Mass spectra show ions contributing to the corresponding peak in the MS trace. Calculated masses: 810.16 (monomer), 1119.33 (dimer), 1379.44 (trimer). Mass spec zooms and detailed analysis can be found in Figure 3.2.2.

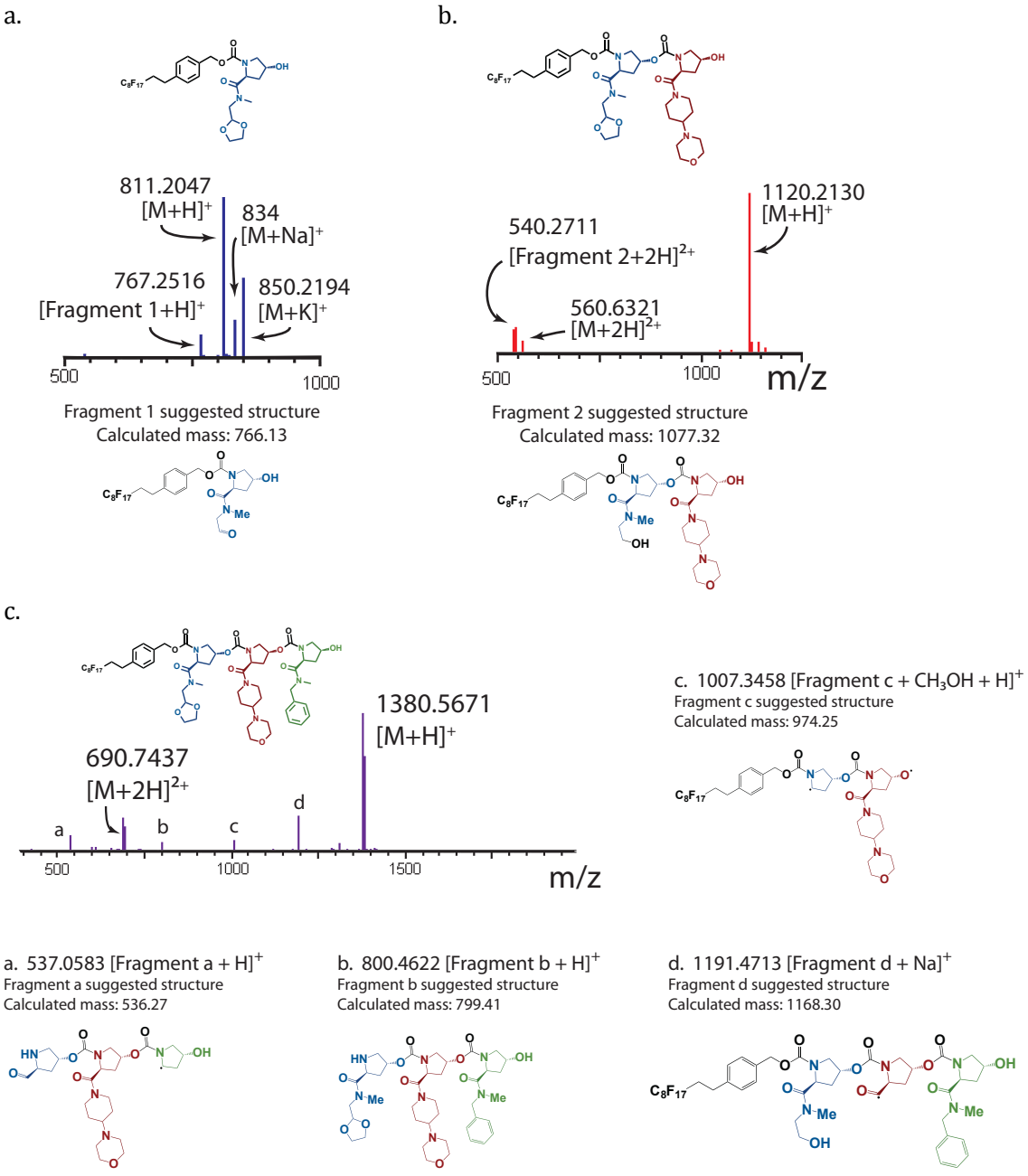
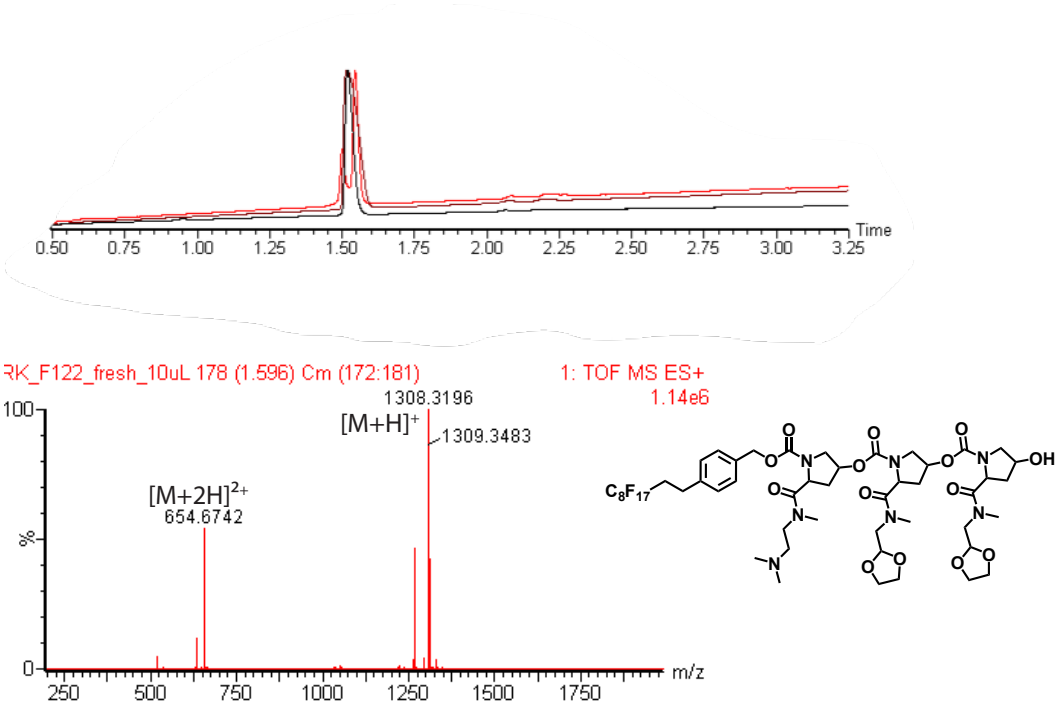


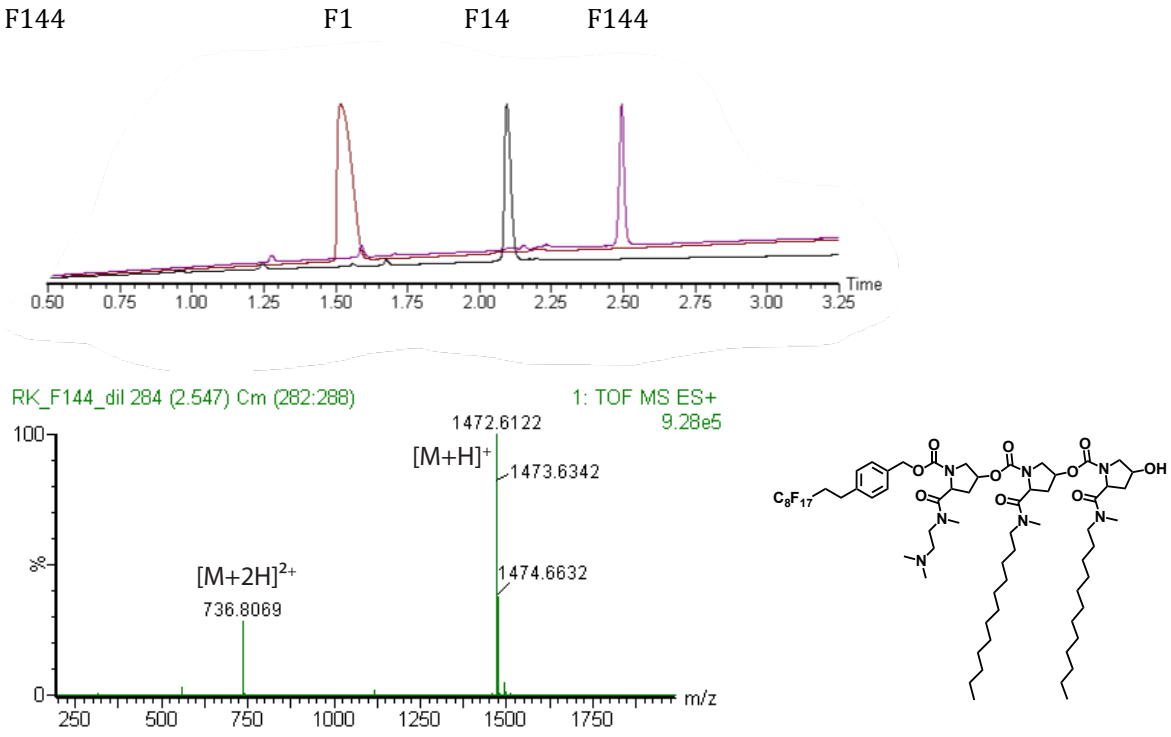
Figure 3.2.2. Mass spec zooms corresponding to trimer synthesis in Figure 3.2.1. a.) Monomer F2, structure shown above MS spectrum. b.) Dimer F25 c.) Trimer F23.

Below: **Figures 3.2.3.** LCMS analysis of all 14 trimers. UV traces (absorbance @ 214 nm) show monomer, dimer, and trimer traces overlaid. MS spectrum of the final trimer product is included below the UV trace. For naming abbreviations (F1, F122, etc.) see section 1 of this appendix.

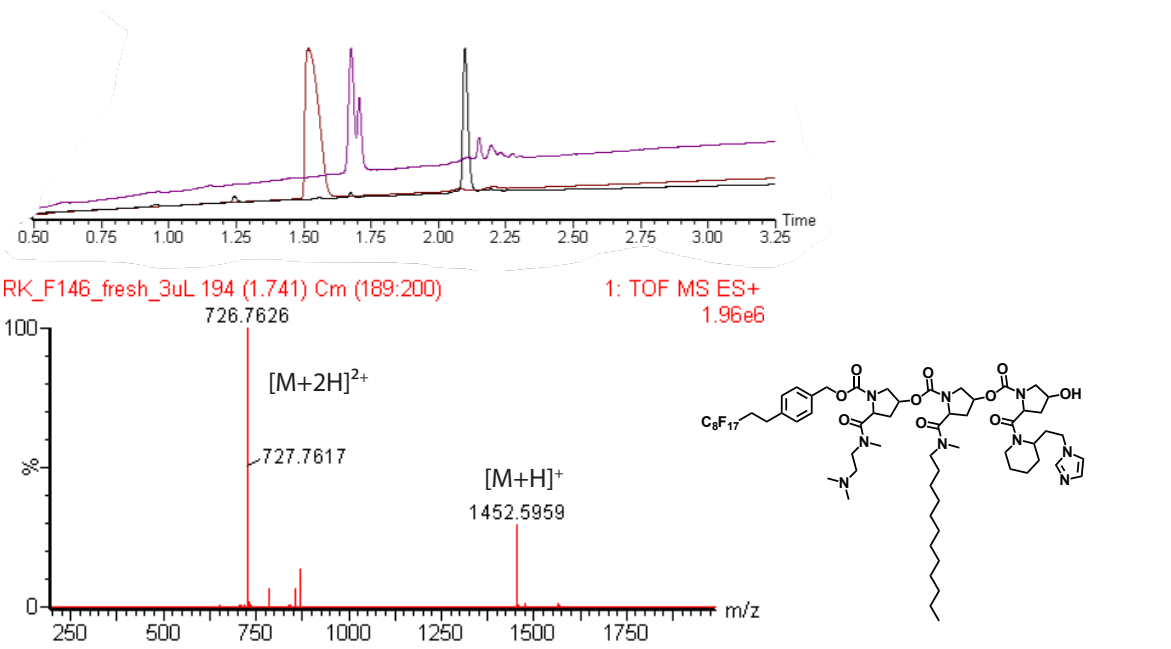
F122



Trimer F122. Top: UV traces of monomer F1, dimer F12, and trimer F122. Some degradation of the acetal in the dioxolane side chain is observed, resulting in a split peak for the trimer F122. This degradation may have resulted from purification conditions, in which product was eluted in a solvent doped with TFA and not immediately evaporated. Bottom: Mass spectrum and structure of trimer F122. Calculated trimer mass: 1307.41 Calculated mass of aldehyde degradant/fragment (1219.36) is also detected.

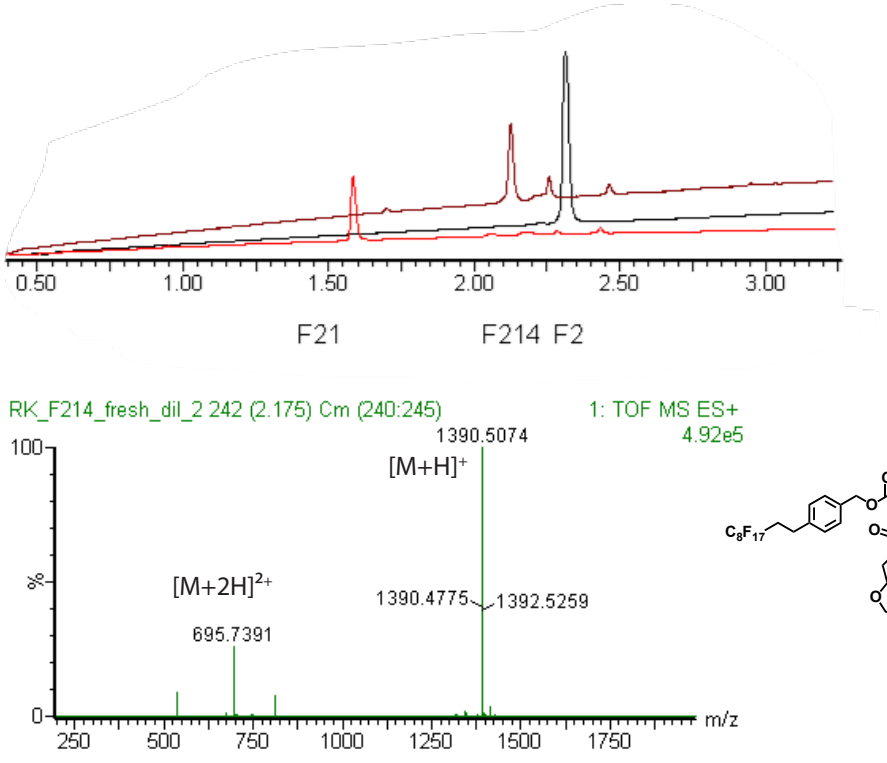


Trimer F144. Top: UV traces of monomer F1, dimer F14, and trimer F144. Bottom: Mass spectrum and structure of trimer F144. Calculated trimer mass: 1471.71



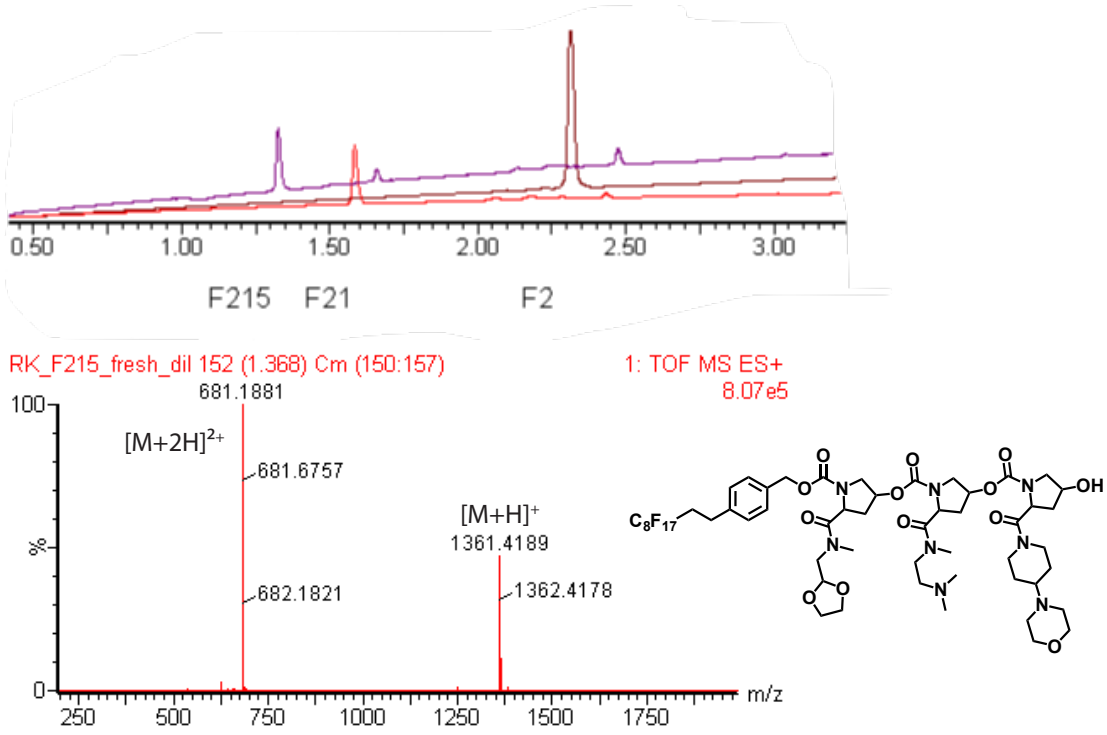
Trimer F146. Top: UV traces of monomer F1, dimer F14, and trimer F146. Peak splitting in F146 can be explained by rotational isomers of monomer 6. Residual dimer, dimer-CDI intermediate also detectable. Bottom: Mass spectrum and structure of trimer F146. Calculated trimer mass: 1451.62

F214

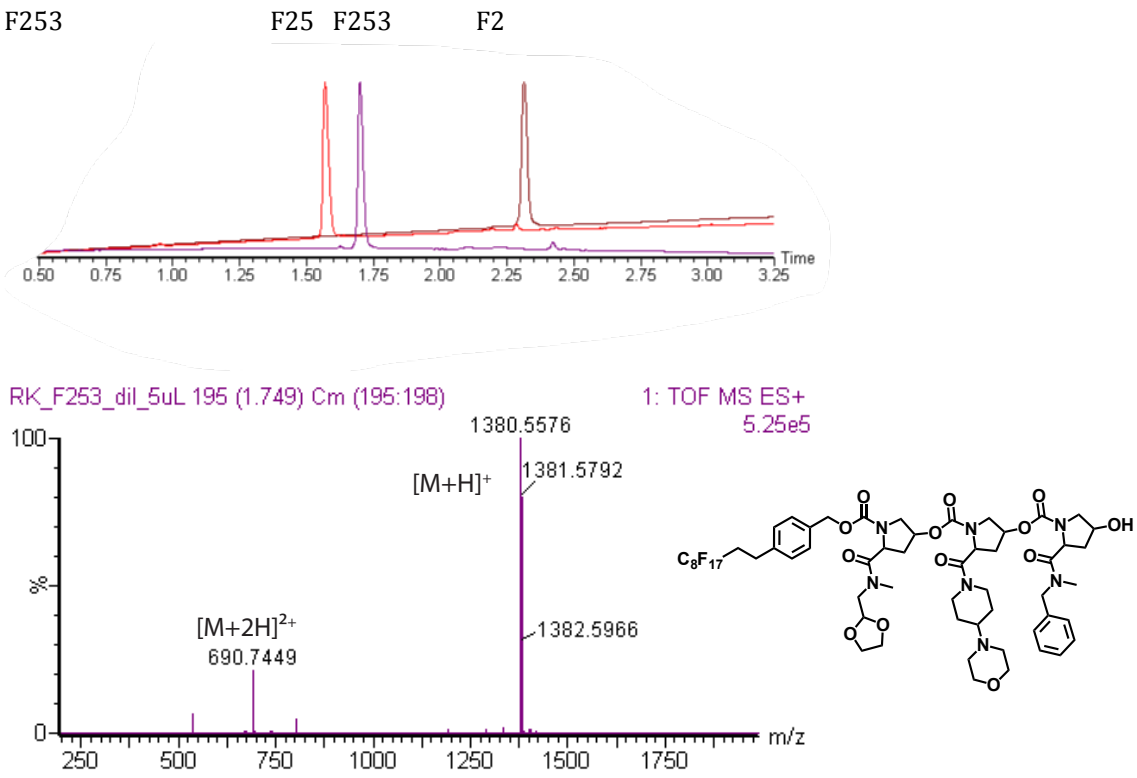


Trimer F214. Top: UV traces of monomer F2, dimer F21, and trimer F214. Degradation of the acetal side chain during purification may contribute to impurities in F21 and F214. Acetal degradation may have resulted from purification conditions in which product was eluted in solvent doped with TFA and not immediately evaporated. Bottom: Mass spectrum and structure of trimer F214. Calculated trimer mass: 1389.56

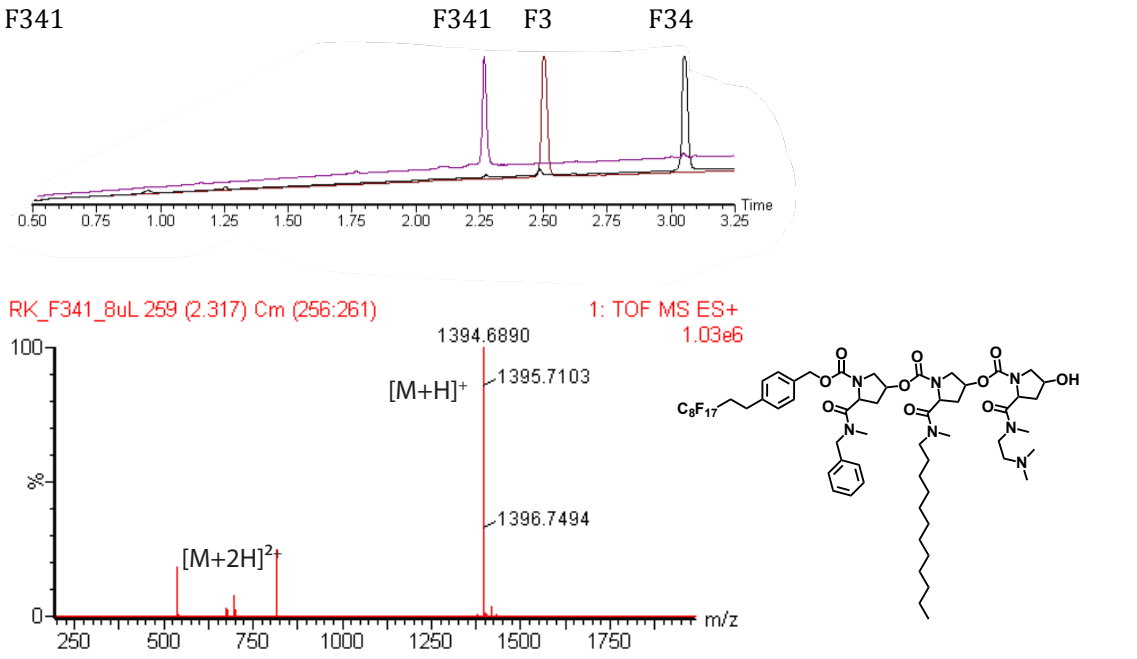
F215



Trimer F215. Top: UV traces of monomer F2, dimer F21, and trimer F215. Degradation of the acetal side chain during purification may contribute to impurities in F21 and F215. Acetal degradation may have resulted from purification conditions in which product was eluted in solvent doped with TFA and not immediately evaporated. Bottom: Mass spectrum and structure of trimer F215. Calculated trimer mass: 1360.47

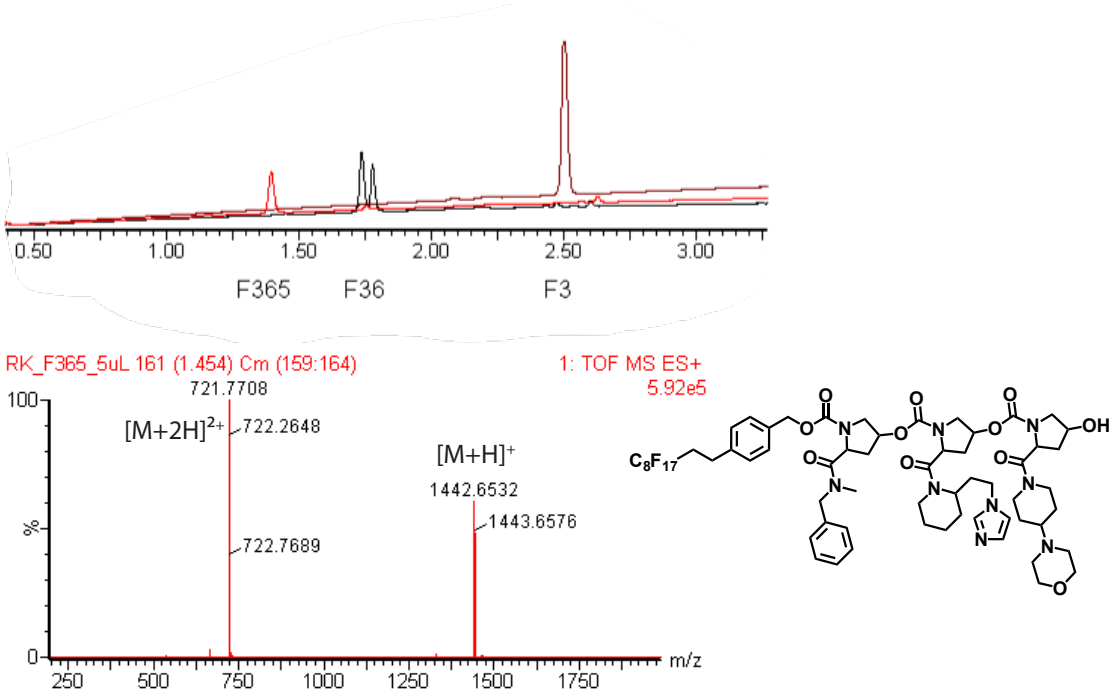


Trimer F253. Top: UV traces of monomer F2, dimer F25, and trimer F253. Bottom: Mass spectrum and structure of trimer F253. Calculated trimer mass: 1379.44



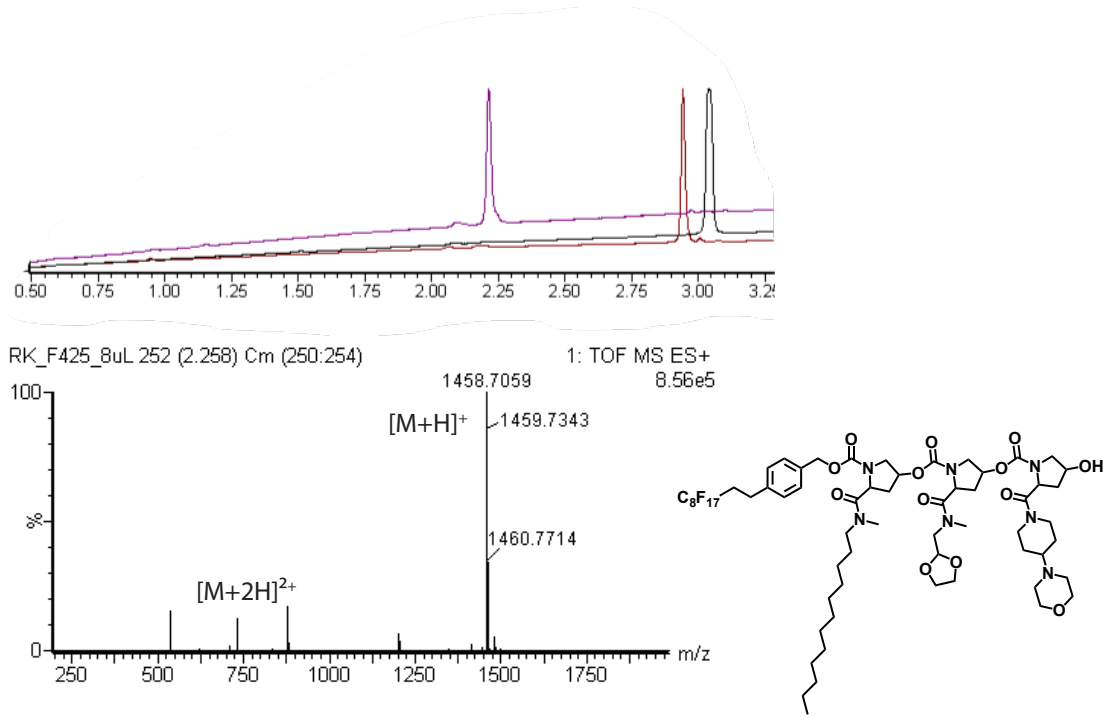
Trimer F341. Top: UV traces of monomer F3, dimer F34, and trimer F341. Bottom: Mass spectrum and structure of trimer F341. Calculated trimer mass: 1393.5

F365

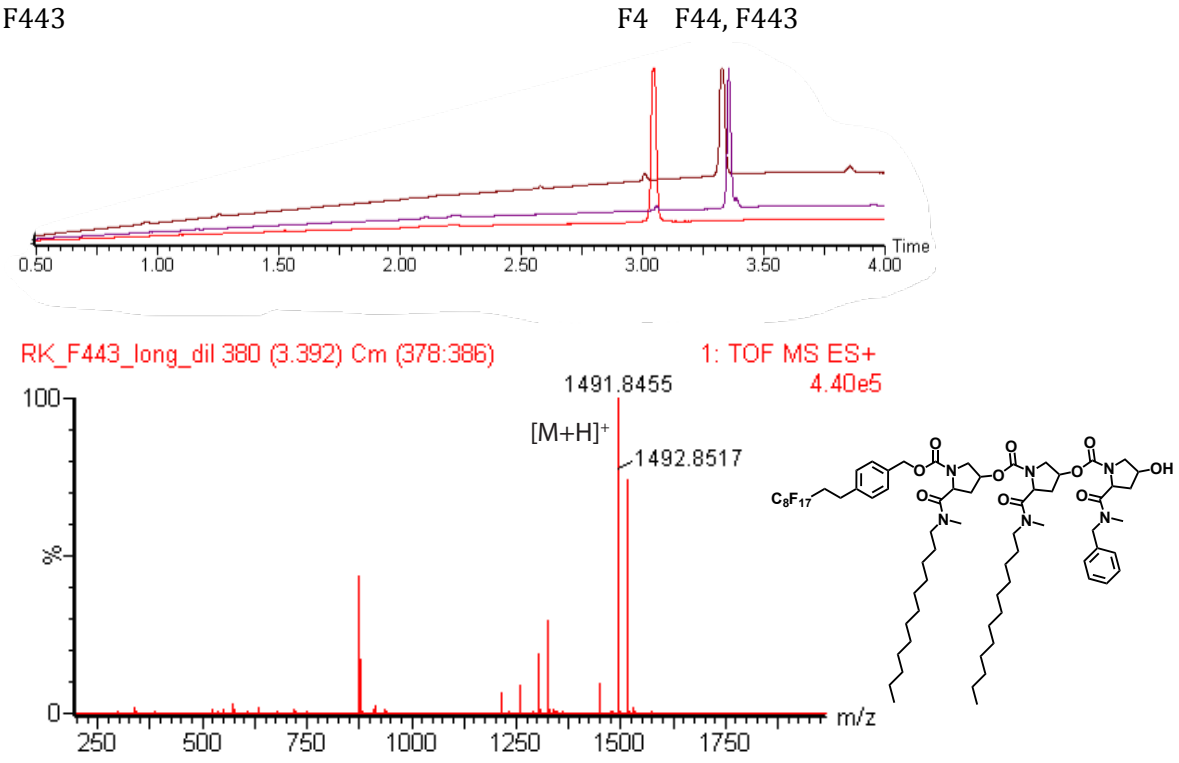


Trimer F365. Top: UV traces of monomer F3, dimer F36, and trimer F365. Peak splitting in F36 due to rotational isomers of monomer 6. Bottom: Mass spectrum and structure of trimer F365. Calculated trimer mass: 1441.51

F425 F425 F42 F4

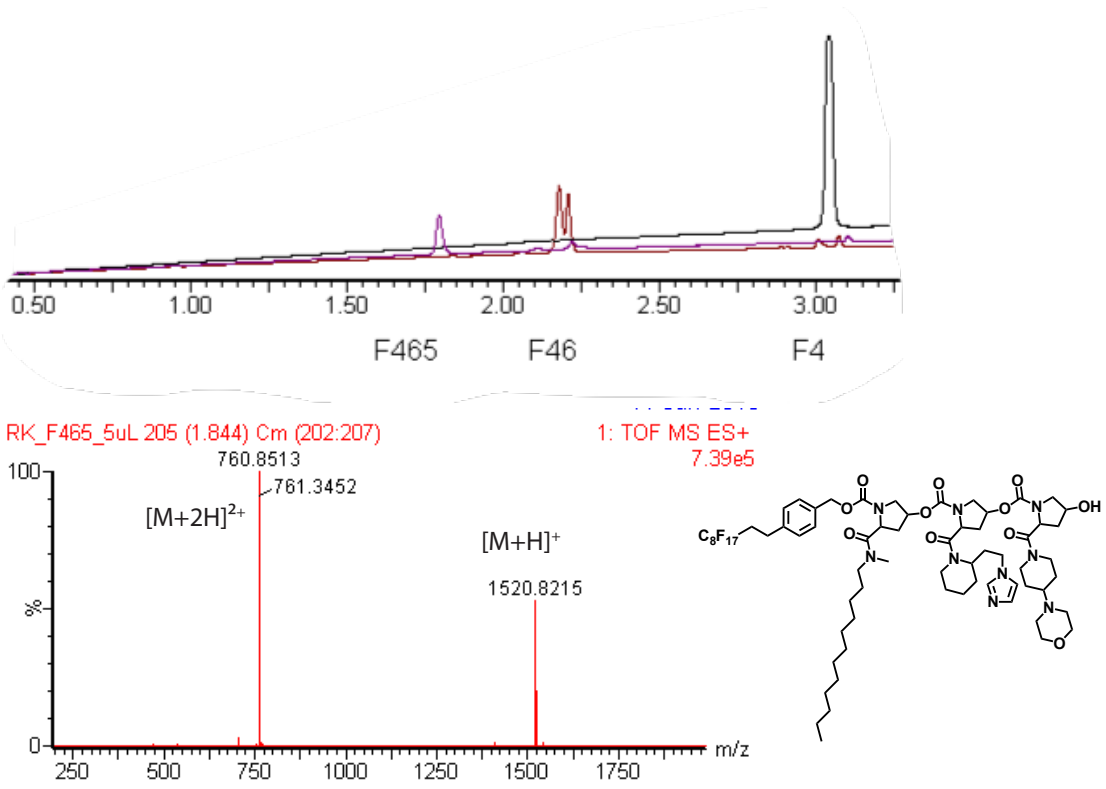


Trimer F425. Top: UV traces of monomer F4, dimer F42, and trimer F425. Bottom: Mass spectrum and structure of trimer F425. Calculated trimer mass: 1457.59

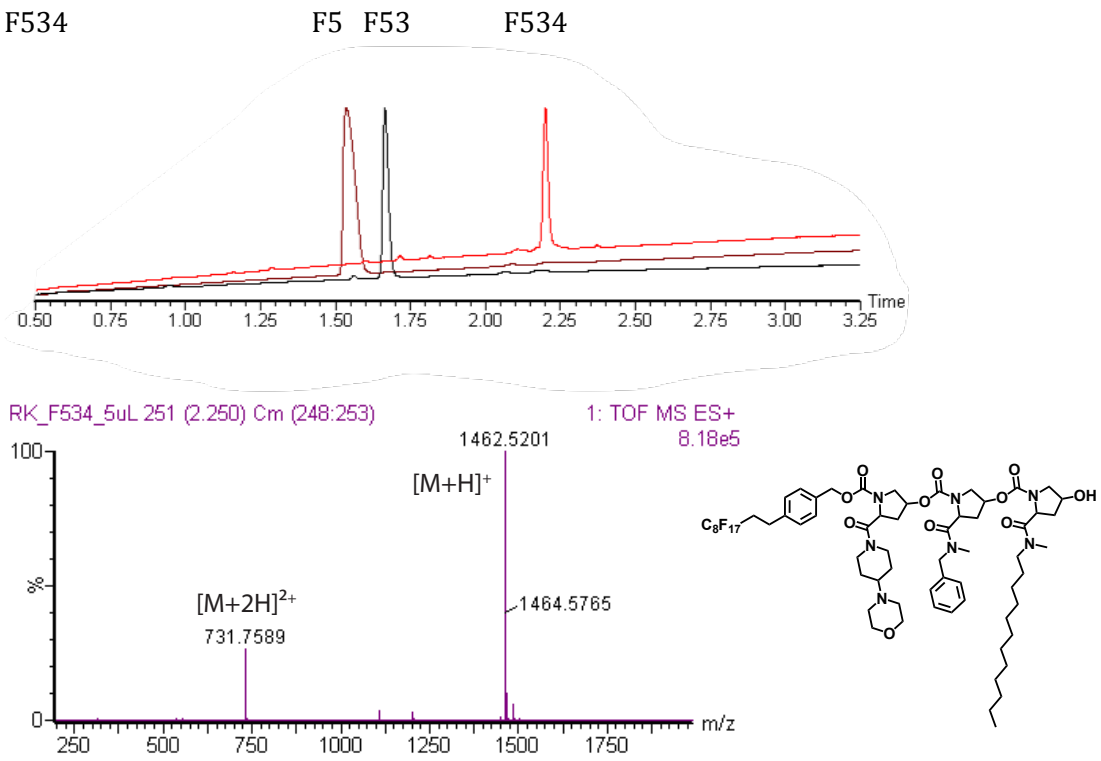


Trimer F443. Top: UV traces of monomer F4, dimer F44, and trimer F443. Trace unreacted monomer (F4) are detected in dimer and trimer. Unreaced monomer may be a result of poor monomer solubility. Bottom: Mass spectrum and structure of trimer F443. Calculated trimer mass: 1490.68

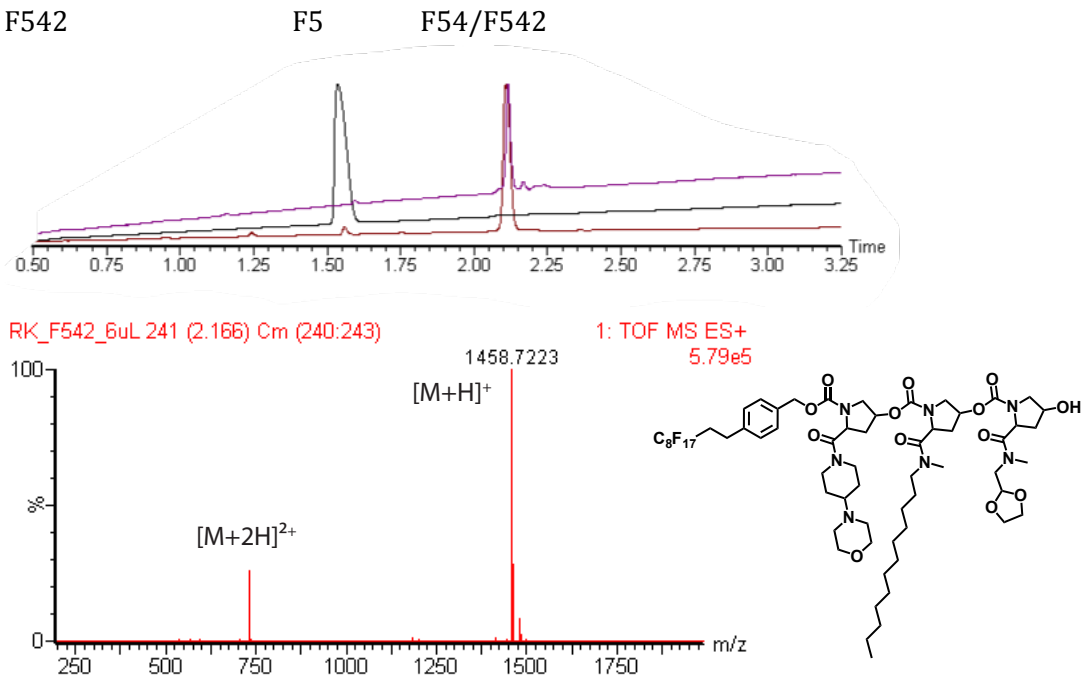
F465



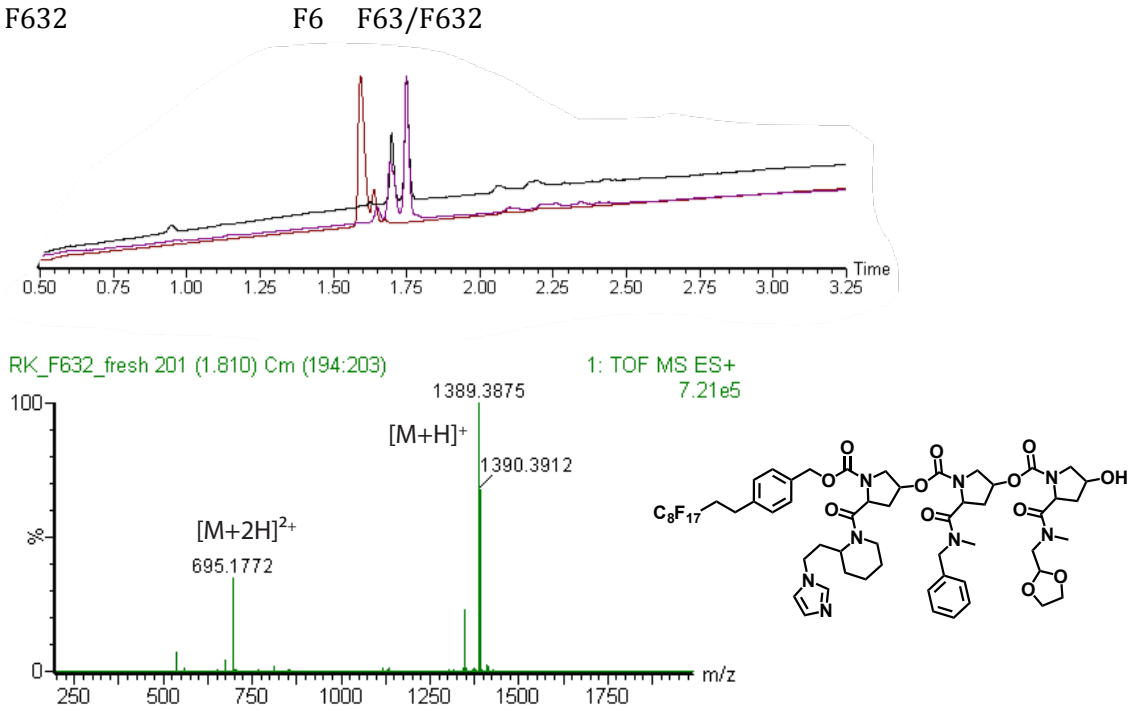
Trimer F465. Top: UV traces of monomer F4, dimer F46, and trimer F465. Trace unreacted monomer (F4) are detected in dimer and trimer. Unreaced monomer may be a result of poor monomer solubility. Peak splitting in F46 due to rotational isomers of monomer 6 Bottom: Mass spectrum and structure of trimer F465. Calculated trimer mass: 1519.65



Trimer F534. Top: UV traces of monomer F5, dimer F53, and trimer F534. Bottom: Mass spectrum and structure of trimer F534. Calculated trimer mass: 1461.60



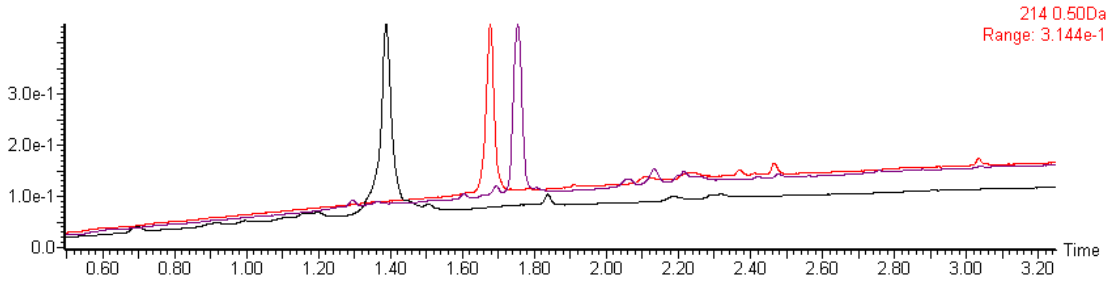
Trimer F542. Top: UV traces of monomer F5, dimer F54, and trimer F542. Trace unreacted monomer 5 due to poor solubility of this monomer. Bottom: Mass spectrum and structure of trimer F542. Calculated trimer mass: 1457.59

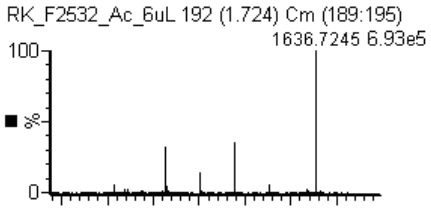


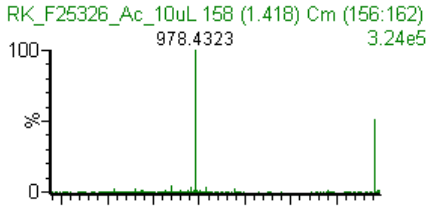
Trimer F632. Top: UV traces of monomer F6, dimer F63, and trimer F632. Peak splitting due to rotational isomers of monomer 6. Unreacted monomer 6 also detected in dimer due to hydrolysis of CDI intermediate. Bottom: Mass spectrum and structure of trimer F632. Calculated trimer mass: 1388.45

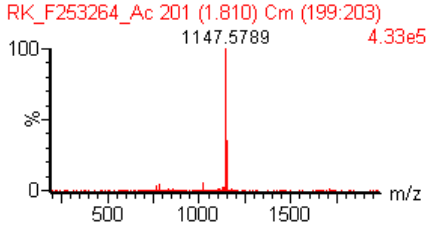
Below: **Figures 3.2.4.** LCMS analysis of 3 hexamers not shown in main text. UV traces (absorbance @ 214 nm) show tetramer, pentamer, and hexamer traces overlaid. MS spectra for each step of synthesis is included below the UV trace. For naming abbreviations (F1, F122, etc.) see section 1 of this appendix.

F253262 **F25326** **F2532, F253262**

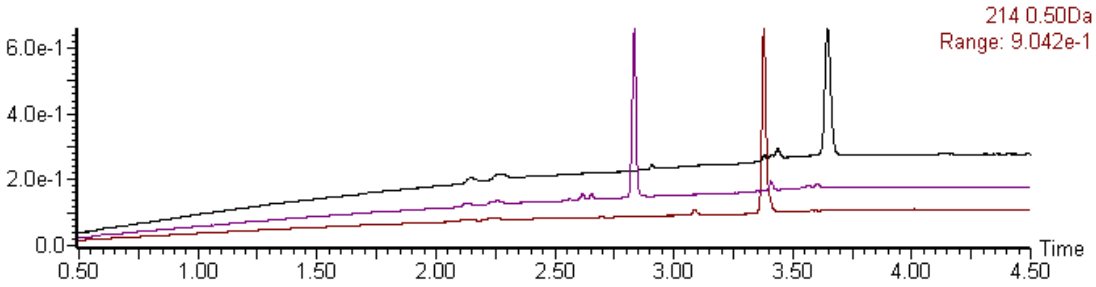


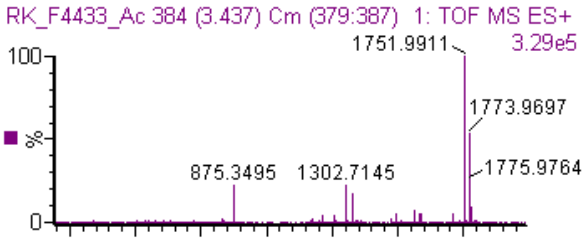


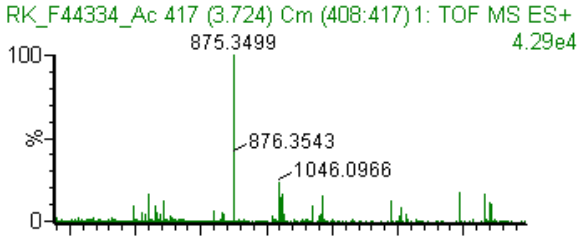


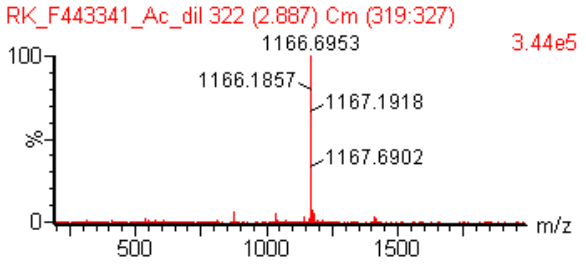


F443341 **F4433 F443341, F44334**

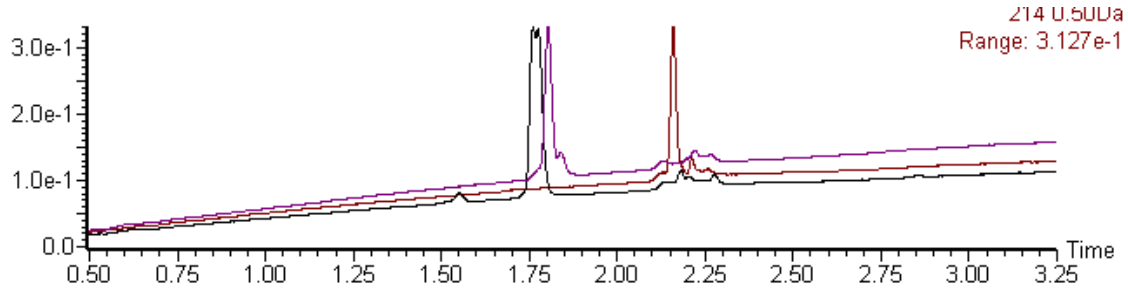


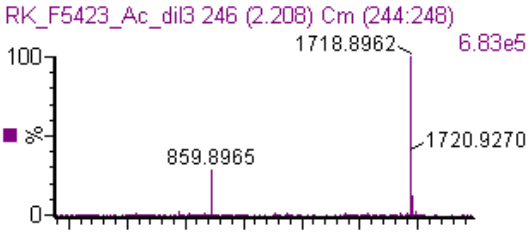


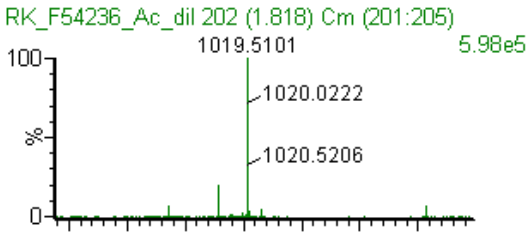


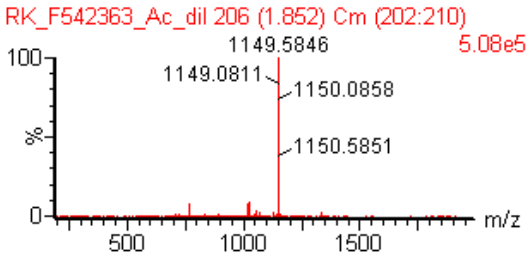


F542363 **F54236, F542363 F5423**

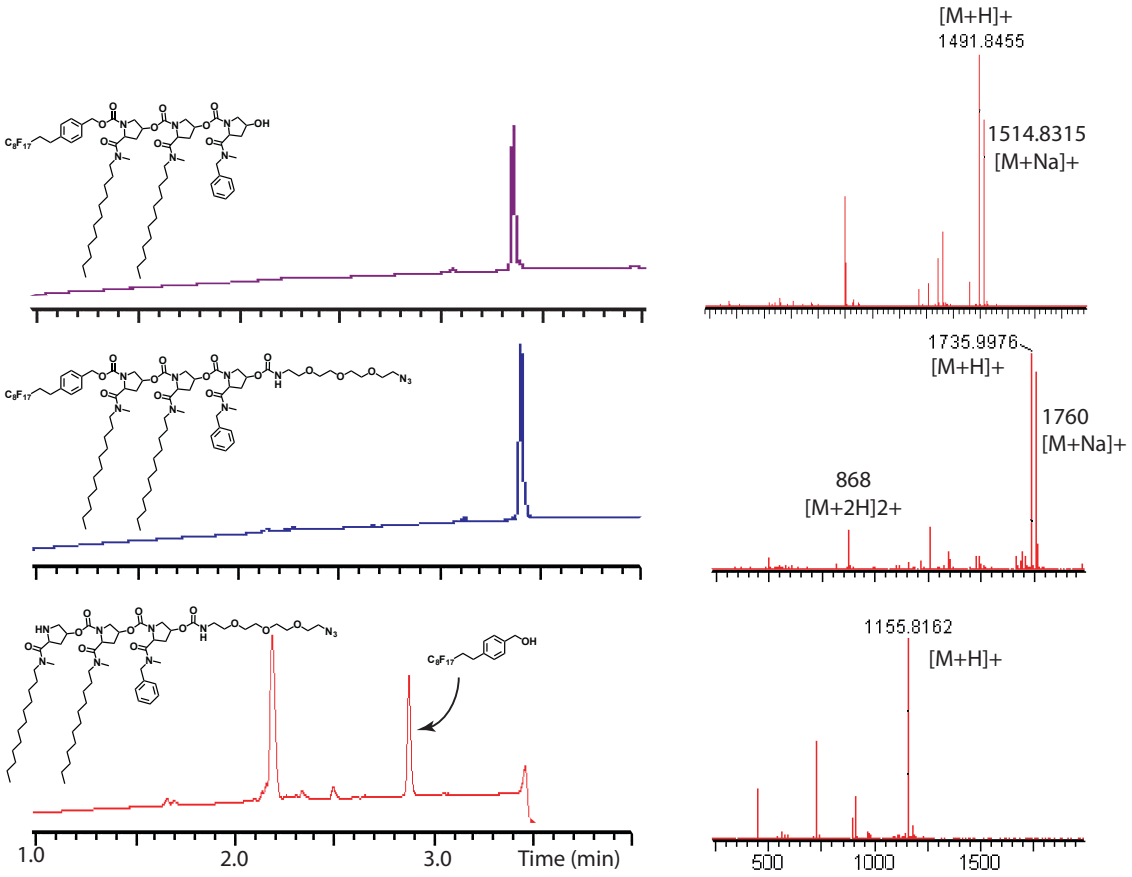




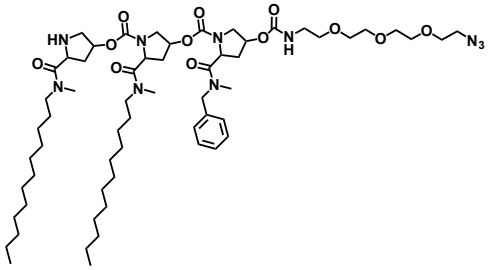




a.



b.



^{1}H NMR (500 MHz, Methanol-d_4) δ 7.52 – 7.12 (m, 5H), 5.45 – 4.94 (m, 3H), 3.79 – 3.55 (m, 20H), 3.55 – 3.46 (m, 5H), 3.26 – 3.17 (m, 3H), 3.17 – 3.10 (m, 3H), 3.10 – 2.91 (m, 10H), 2.70 (s, 4H), 2.58 – 2.51 (m, 1H), 1.96 – 1.86 (m, 2H), 1.73 – 1.35 (m, 4H), 1.35 – 1.22 (m, 36H), 0.96 – 0.80 (m, 6H).

Figure 3.2.5. Trimer coupling to azido PEG and removal of purification tag. a. LC traces show UV absorbance at 214 nm. Mass spectra show ions contributing to the product peak in the MS trace. Deprotected trimer-azides were coupled to siRNA without purification. **b.** ^{1}HNMR shifts of trimer 443-azide after removal of purification tag.

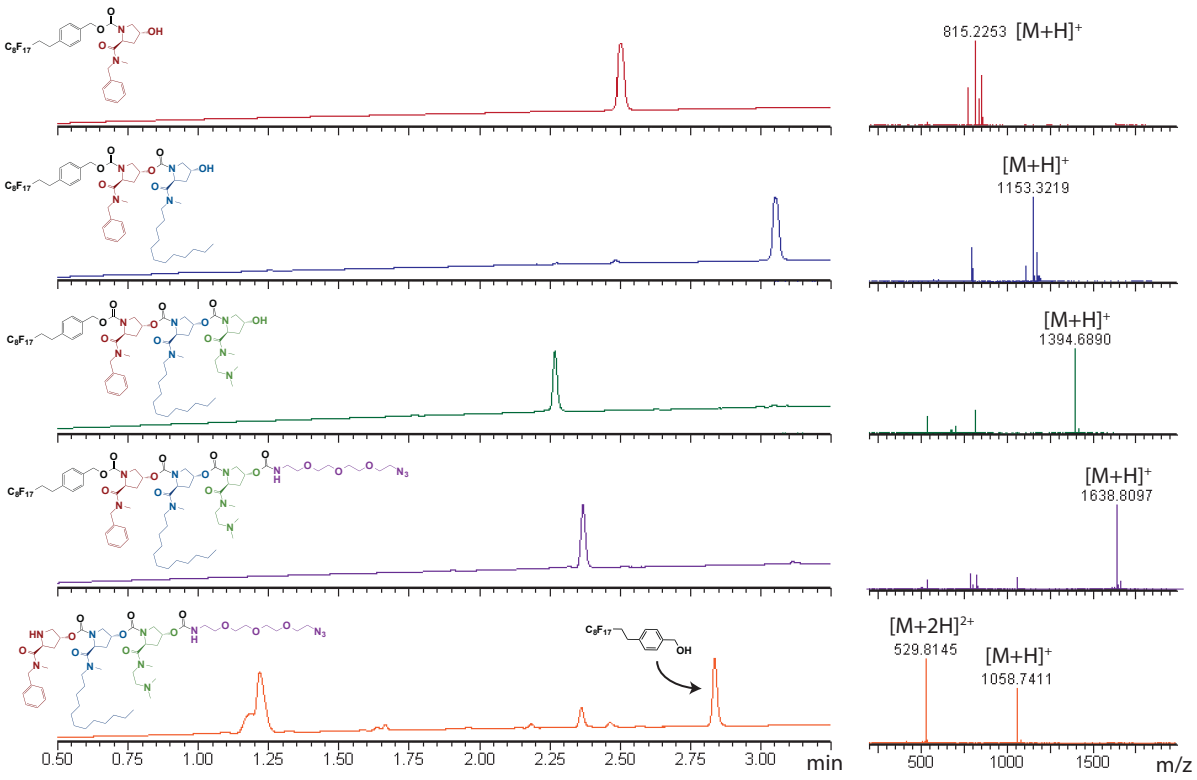
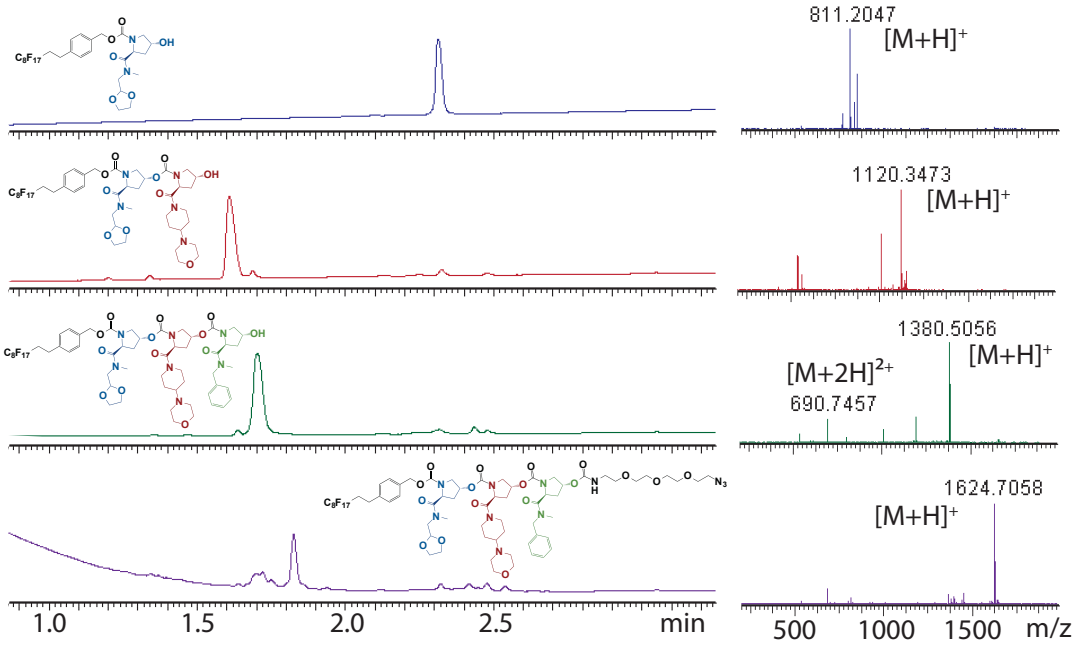


Figure 3.2.6. LCMS analysis of trimer synthesis, coupling to azido PEG, and removal of purification tag. LC traces show UV absorbance at 214 nm. Mass spectra show ions contributing to the product peak in the MS trace. The final LC trace shows peaks representing deprotected product (1.2 min), fluorous-tagged starting material (2.35 min), and free fluorous tag (2.8 min).

3.3 LCMS analysis of oligomers made by high throughput synthesis



3.3.1 LCMS analysis of high-throughput trimer synthesis and attachement of azido-PEG. LC traces show UV absorbance at 214 nm. Mass spectra show ions contributing to the corresponding peak in the MS trace. Some residual monomer (F2) is present in the dimer and is carried over into subsequent steps, forming impurity F23 in the trimer (elution time = 2.4 min). Similarly, trace unreacted dimer (F25) is present in the trimer trace and goes on to form other products in the subsequent steps. Incomplete reaction is due to hydrolysis of the imidazole-N-carboxylic ester intermediate, resulting from longer handling times during high throughput synthesis. See main text and supplemental figure 3.3.2 for discussion.

Table 3.3.1. Purity of trimers synthesized in high-throughput format. Purities are based on integration of the LC trace at 214 nm.

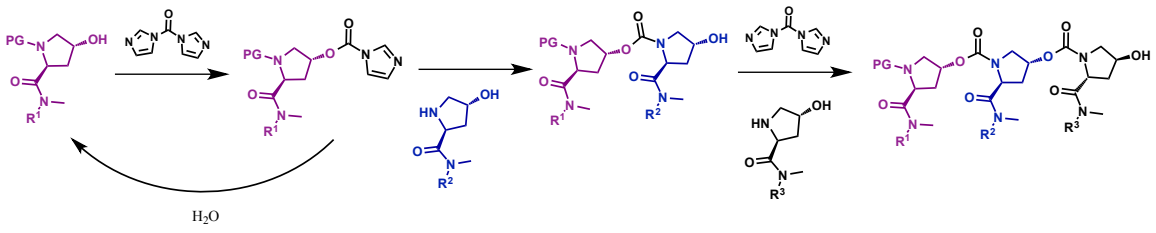
Monomer sequence	% purity
F122	92
F136	65
F253	90
F262	64
F325	91
F415	69
F431	73
F521	60
F534	43
F623	67
Average	71

3.3.2 Impurities detected in high-throughput synthesis.

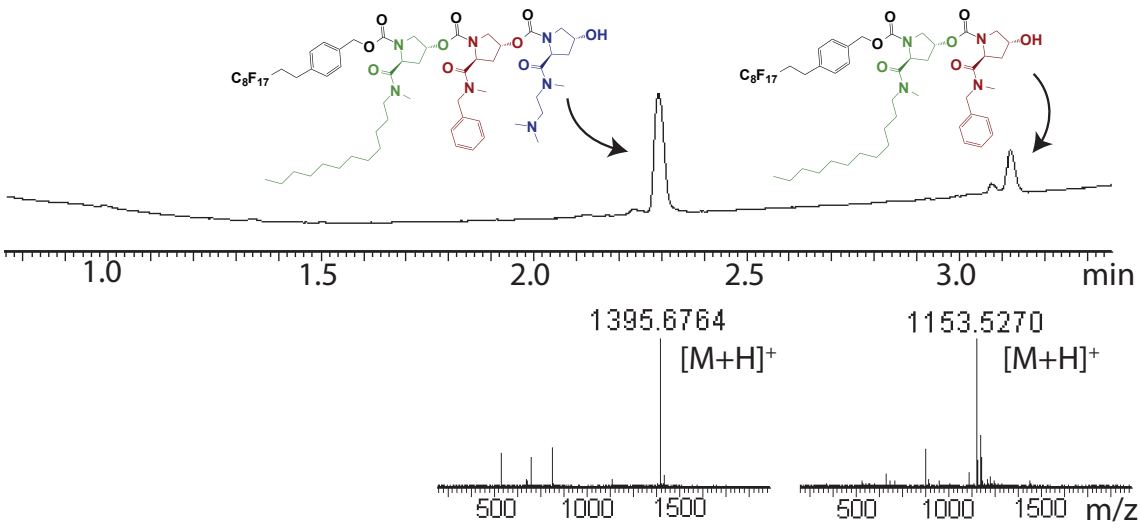
a. The activated imidazole-N-carboxylic ester is susceptible to hydrolysis upon exposure to moisture, preventing the addition of the subsequent monomer. This resulted in the presence of unreacted starting material in purified oligomers, the most common impurity observed during oligomer synthesis.

b. LCMS analysis of impure trimer F431 synthesized by high throughput methods. The most common impurity detected in the library was unreacted starting material. In this case, LCMS analysis shows the presence of unreacted dimer. Hydrolysis happened more frequently during high-throughput synthesis than during conventional synthesis, due to longer handling times and increased exposure to moisture during workup in multiwell plates. A simple solution to this issue may be to use a more volatile solvent during the CDI activation step. Dimethylformamide was used as the solvent here, requiring long times for evaporation steps. Tetrahydrofuran may be a more suitable solvent.

a.)

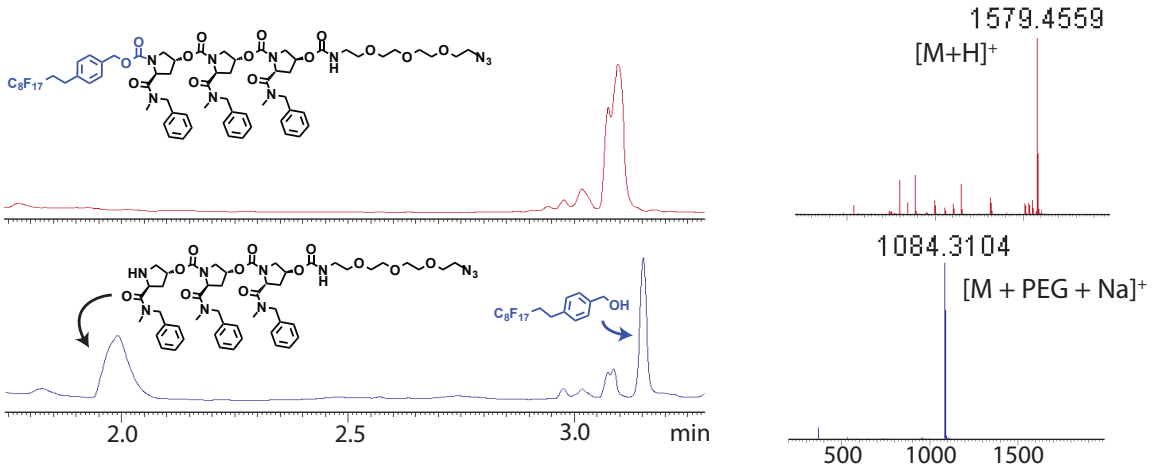


b.)



3.3.3 Removal of fluorous purification handle in high-throughput synthesis.

An example of LCMS analysis of a trimer-azide material before and after deprotection of the fluorous-CbZ group. For each of the 216 trimer-azides in the library, the material in each well was divided in half and one half of the material was deprotected. With both fluorous-protected and deprotected trimers, the total number of unique materials in the library was doubled to 432.



3.3.4 Recovered yield of trimer-azides synthesized in high-throughput format

After high-throughput synthesis of trimer-azides was complete, product was recovered, dried, and weighed from a selection of wells to determine overall yield after the three-step synthesis. Yield was calculated in molar percent of the starting (fluorous-protected) monomer in each well. Results are shown below.

The causes of well-to-well variation in recovered yield are unclear, but may result from variations in a number of handling steps involved in 96-well plate manipulations. One example of such procedures is the transfer of the reaction mixture to the purification plate. As the reaction mixture must be relatively concentrated during loading on to the silica matrix, small variations in pipetting volumes across the plate may translate to significant changes in material recovery after purification.

Structure	% yield based on starting monomer
F122N3	55
F136N3	42
F166N3	33
F231N3	45
F323N3	63
F364N3	52
F423N3	74
F446N3	53
F511N3	40
F564N3	55
F633N3	60
Average recovered yield	**52%**
s.d.	**11.5%**

3.4. High-throughput purification of siRNA conjugates: Yield

After conjugation of trimer-azides to siRNA, excess azide material was separated from siRNA conjugates by ethanol precipitation (see Materials and Methods). Average recovery of nucleic acid was then characterized by UV absorbance of the purified samples.

UV absorbance was measured using a NanoDrop spectrophotometer. Nucleic acid concentration was calculated based on sample absorbance at 260 and 280 nm using the built in RNA method. Yield was determined by comparing RNA concentration in purified samples to control samples at a concentration representing 100% yield. To determine whether the addition of trimer-azide material to the siRNA may interfere with this measurement, an additional control of the trimer-azide material alone at a high concentration (~10 mg/mL) was run to observe its absorbance spectrum (sample labeled "trimer-azide F333"). Trimer-azides showed maximum absorbance at below 230 nm and had little absorbance at 260 nm (Figure 3.4a), indicating that addition of trimer-azide to the siRNA should have minimal effect on the measurement of siRNA conjugate concentration.

Yield was studied using two different siRNA sequences: siLuc-DBCO, which bears a DBCO group on the 3' end of the sense strand, and an additional sequence targeting the transthyretin gene (TTR). The siTTR sequence included a single DBCO group for conjugation and as well as a covalently attached targeting ligand containing a trivalent N-acetylgalactosamine (GalNAc) structure. An example reaction is shown below (Figure 3.4a).

Average yield of all conjugates was 95.5%. Recovery was slightly higher for the siLuc-DBCO sequence (97.5%) than for the GalNAc-siTTR sequence (93.5%).

Figure 3.4a

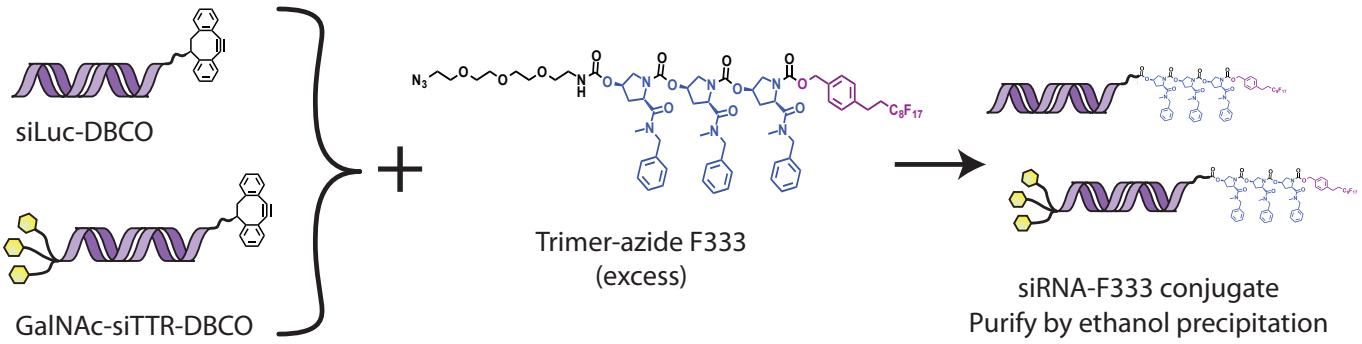
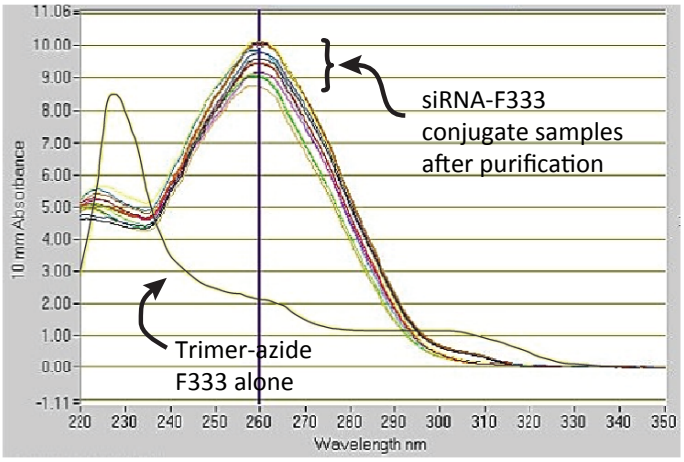


Figure 3.4b

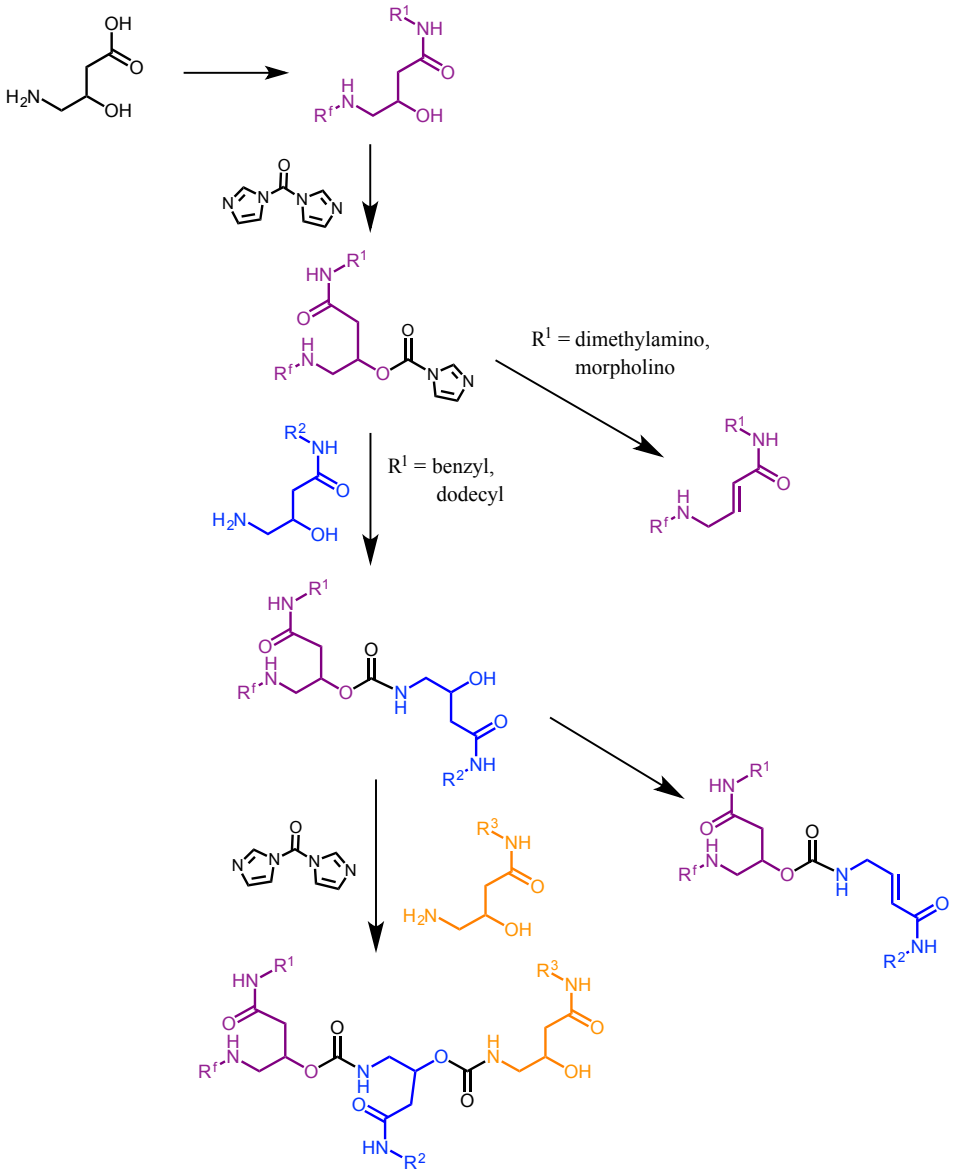


Average recovery of siRNA conjugates after purification: **95.5%**

3.5. Alternate strategies for CDI-linked oligomer synthesis

Oligomer synthesis using a linear monomer backbone was explored. In each case, purity was limited by side reactions, including uncontrolled cyclization of the oligomer backbone. The work summarized below motivated the choice of cyclic hydroxyproline as the monomer backbone structure in the rest of this work. A) Using 1-amino-2-hydroxybutyric acid as a monomer backbone, elimination of the CDI-activated hydroxyl group limited the efficiency of monomer coupling. Elimination was observed most frequently when monomer side chains included basic groups. B) Using 1-amino-3-hydroxybutyric acid as a monomer backbone, various cyclization products limited the efficiency of monomer coupling and/or the stability of monomers themselves.

a.)



b.)