Supporting Information for:

Design of a Small Molecule Against an Oncogenic Non-coding RNA

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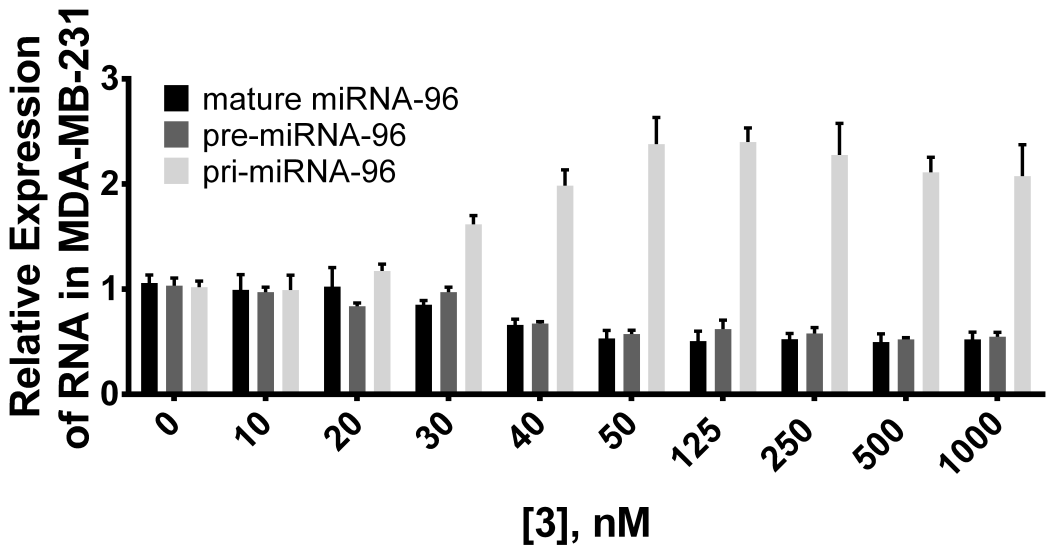


Figure S1: Dose response for **Targaprimir-96** (**3**) on pri-, pre-, and mature miR-96 levels in MDA-MB-231 triple negative breast cancer cells.

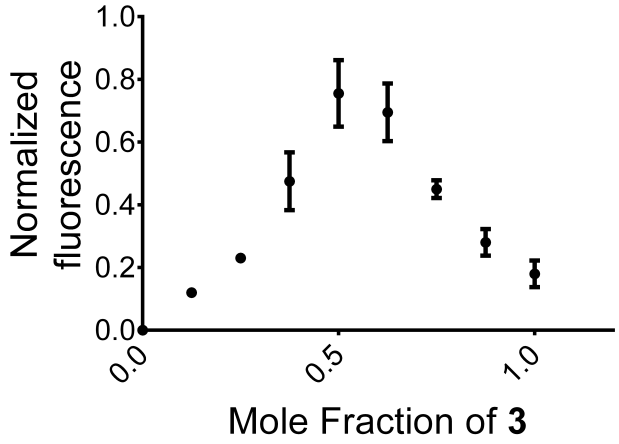


Figure S2: Job's plot to determine stoichiometry of **Targaprimir-96** (**3**) to **RNA3** (Figure 3).

Table S1: Sequences of primers used for qRT-PCR.	
hsa-miR-27a	TTCACAGTGGCTAAGTTCCGC
hsa-miR-9	TCTTTGGTTATCTAGCTGTATGA
hsa-miR-194	TGTAACAGCAACTCCATGTGGA
hsa-miR-15a	TAGCAGCACATAATGGTTTGTG
hsa-miR-16	TAGCAGCACGTAAATATTGGCG
hsa-miR-139-5p	TCTACAGTGCACGTGTCTCCAGT
hsa-miR-182	TTTGGCAATGGTAGAACTCACACT
hsa-miR-196a	TAGGTAGTTTCATGTTGTTGGG
hsa-miR-128a	TCACAGTGAACCGGTCTCTTT
hsa-miR-142-3p	TGTAGTGTTTCCTACTTTATGGA
hsa-miR-223	TGTCAGTTTGTCAAATACCCC
hsa-miR-101	TACAGTACTGTGATAACTGAA
hsa-miR-132	TAACAGTCTACAGCCATGGTCG
hsa-miR-212	TAACAGTCTCCAGTCACGGCC
hsa-miR-135a	TATGGCTTTTTATTCCTATGTGA
hsa-miR-1271	CTTGGCACCTAGCAAGCACTCA
hsa-miR-96	TTTGGCACTAGCACATTTTTGCT
RNU6	ACACGCAAATTCGTGAAGCGTTC
Universal Reverse	GAATCGAGCACCAGTTACGC
Pre-miRNA-96-F	ATTTTGGCACTAGCACATTTTTGCT
Pre-miRNA-96-R	CCATATTGGCACTGCACATGATT
Pri-miRNA-96-F	AGAGAGCCCGCACCAGT
Pri-miRNA-96-R	CTTGAGGAGGAGCAGGCT
18S-F	GTAACCCGTTGAACCCCATT
18S-R	CCATCCAATCGGTAGTAGCG

General Methods

DNA templates and PCR amplification: The RNA motifs (internal loops) used in these studies were embedded into a hairpin cassette, **RNAC** (Figure 3). The corresponding DNA templates (purchased from Integrated DNA Technologies, Inc. (IDT) and used without further purification) were PCR amplified in 1× PCR Buffer (10 mM Tris, pH 9.0, 50 mM KCl, and 0.1% (v/v) Triton X-100), 2 μM forward primer (5'-GGCCGGATCCTAATACGACTCACTATAGGGAGAG- GGTTTAAT), 2 μM reverse primer (5'-CCTTGCGGATCCAAT), 4.25 mM $MgCl_2$, 330 μM dNTPs, and 1 μL of Taq DNA polymerase in a 50 μL reaction. The cycling conditions used for PCR were 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min.

The miR-96 precursor used in nuclease protection assays was modified to contain a 5'-GGG overhang to facilitate transcription using T7 RNA polymerase, or GGG-pre-miR-96. It was shown previously that the additional nucleotides do not affect the RNA's secondary structure (10).

RNA transcription: RNA oligonucleotides were *in vitro* transcribed by T7 RNA polymerase in 1× Transcription Buffer (40 mM Tris HCl, pH 8.1, 1 mM spermidine, 0.001% (v/v) Triton X-100 and 10 mM DTT) containing 2.25 mM of each rNTP and 5 mM $MgCl_2$ at 37 °C overnight. The transcribed RNAs were purified on a denaturing 15% polyacrylamide gel and isolated as previously described (10). Concentrations were determined by absorbance at 260 nm and the corresponding extinction coefficient. Extinction coefficients were calculated using the HyTher server, which uses parameters based on the extinction coefficients of RNA nearest neighbors (38-40).

Binding affinity measurements: Dissociation constants were determined using an in solution fluorescence-based assay. The RNA of interest was folded in 1× Assay Buffer (8 mM Na_2HPO_4, pH 7.0, 190 mM NaCl, 1 mM EDTA and 40 μg/mL BSA) by heating at 60 °C for 5 min and slowly cooling to room temperature. Small molecule was added to a final concentration of 100 nM for **1-FI** (10), or 500 nM for **2** and **3**. Serial dilutions (1:2) were then completed in 1× Assay Buffer supplemented with 100 nM of **1-FI**, or 500 nM of **2** or **3**. The solutions were incubated for 30 min at room temperature and then transferred to a 96-well plate and fluorescence intensity measured. The change in fluorescence intensity as a function of RNA concentration was fit to the following equation:

$$I = I_0 + 0.5\Delta\varepsilon(([FL]_0 + [RNA]_0 + K_t) - (([FL]_0 + [RNA]_0 + K_t)^2 - 4[FL]_0[RNA]_0)^{0.5})$$

Where I and I_0 are the observed fluorescence intensity in the presence and absence of RNA respectively, Δε is the difference between the fluorescence intensity in the absence of RNA and in the presence of infinite RNA concentration, $[FL]_0$ and $[RNA]_0$ are the concentrations of small molecule and RNA, respectively, and K_t is the dissociation constant. Note: assays completed under molecular crowding conditions were supplemented with 20% (w/v) PEG-8000.

Cell culture: All cells were grown at 37 ^oC and 5% CO_2. MDA-MB-231-luc cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 1% penicillin/streptomycin and 10% FBS (complete growth medium). MDA-MB-231 cells

were cultured in Roswell Park Memorial Institute medium (RPMI) supplemented with 1% penicillin/streptomycin and 10% FBS (complete growth medium). MCF 10A cells were cultured in Dulbecco's modified eagle medium/F12 (DMEM/F12) supplemented with 20 ng/μL epidermal growth factor (EGF), 0.5 μg/mL hydrocortisone, cholera toxin 0.1 μg/mL, bovine insulin 10 μg/mL, 1% penicillin/streptomycin and 10% FBS (complete growth medium).

Preparation of cell extracts containing Drosha: HEK 293T cells were maintained in DMEM supplemented with 10% FBS in a 100 mm dish. After cells reached ~70% confluency, they were transfected with 10 μg of Drosha-cmyc, obtained from Addgene (plasmid #10828) using jetPRIME (Polyplus Transfection) per the manufacturer's protocol. Approximately 48 h post-transfection, the cells were collected by scraping them into 1 mL ice-cold 1× DPBS followed by centrifugation at 6000 rpm for 5 min at 4 ^{o}C. The cells were suspended in 500 μL of 1× Lysis Buffer (20 mM Tris HCl, pH 8.0, 100 mM KCl and 0.2 mM EDTA) and sonicated for 30 s. Cellular debris was pelleted by centrifugation (12000 rpm for 15 min at 4 ^{o}C), and the supernatant containing Drosha was transferred to a new tube.

Inhibition of Drosha cleavage *in vitro*: The cDNA template for pri-miRNA-96 was PCR amplified from MCF7 genomic DNA using the following primers: forward primer, 5'-GGCCGAATTCTAATACGACTCACTATAGGCACCA- GTGCCATCTGCTT; and reverse primer, 5'-CGCAGCTGCGGGTCCT. The forward primer contains a T7 promoter (underlined) that was employed to produce pri-miR-96 via run-off transcription, as

described above. The RNA was 5'-end labeled with [g-^{32}P]ATP as previously described (10) and purified using a denaturing 10% polyacrylamide gel. In order to determine if **3** inhibits Drosha cleavage *in vitro*, 1 µL of ^{32}P-labeled pri-miR-96 (~10,000 counts) and varying concentrations of **3** (500, 50, 5 nM) were incubated in 6.4 mM $MgCl_2$ (30 µL total volume) at room temperature for 10 min. Then, 1 µL of the Drosha-cmyc lysate was added, and the samples were incubated at 37 ˚C for 3 h. The reactions were quenched by phenol-chloroform extraction followed by ethanol precipitation. The resulting pellet was dissolved in 10 µL of 2× Gel Loading Buffer (8 M urea, 50 mM EDTA, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol), and the reaction products were separated on a denaturing 10% acrylamide gel.

S1 nuclease protection assay: RNA (5'-end labeled) was folded in 20 mM sodium acetate, pH 4.5 and 150 mM NaCl by heating at 65 ˚C for 5 min and slowly cooling to room temperature. Zinc sulfate was added to a final concentration of 1 mM followed by addition of serially diluted concentrations of **3.** After incubating the samples at room temperature for 15 min, 0.05 units/µL of S1 nuclease was added and the samples were incubated at 37 ˚C for 30 min. A T1 ladder was generated by folding the RNA in 1× RNA Sequencing Buffer (Ambion; denaturing conditions) and incubating the sample at 55 °C for 10 min followed by slowly cooling to room temperature. RNase T1 was added to a final concentration of 0.3 units/µL and the solution was incubated at room temperature for 15 min. A hydrolysis ladder was generated by incubating RNA in 1× Hydrolysis Buffer (Ambion) for 10 min at 95 ˚C. Cleavage products were separated on a denaturing 15% polyacrylamide gel.

Western blotting: Cells were grown in 6-well plates to ~80% confluency in complete growth medium and then incubated with 50 nM of **3** for 48 h. Total protein was extracted using M-PER Mammalian Protein Extraction Reagent (Pierce Biotechnology) using the manufacturer's protocol. Extracted total protein was quantified using a Micro BCA Protein Assay Kit (Pierce Biotechnology). Approximately 40 µg of total protein was resolved on a 4-12% (Genscript) SDS-polyacrylamide gel, and then transferred to a PVDF membrane.

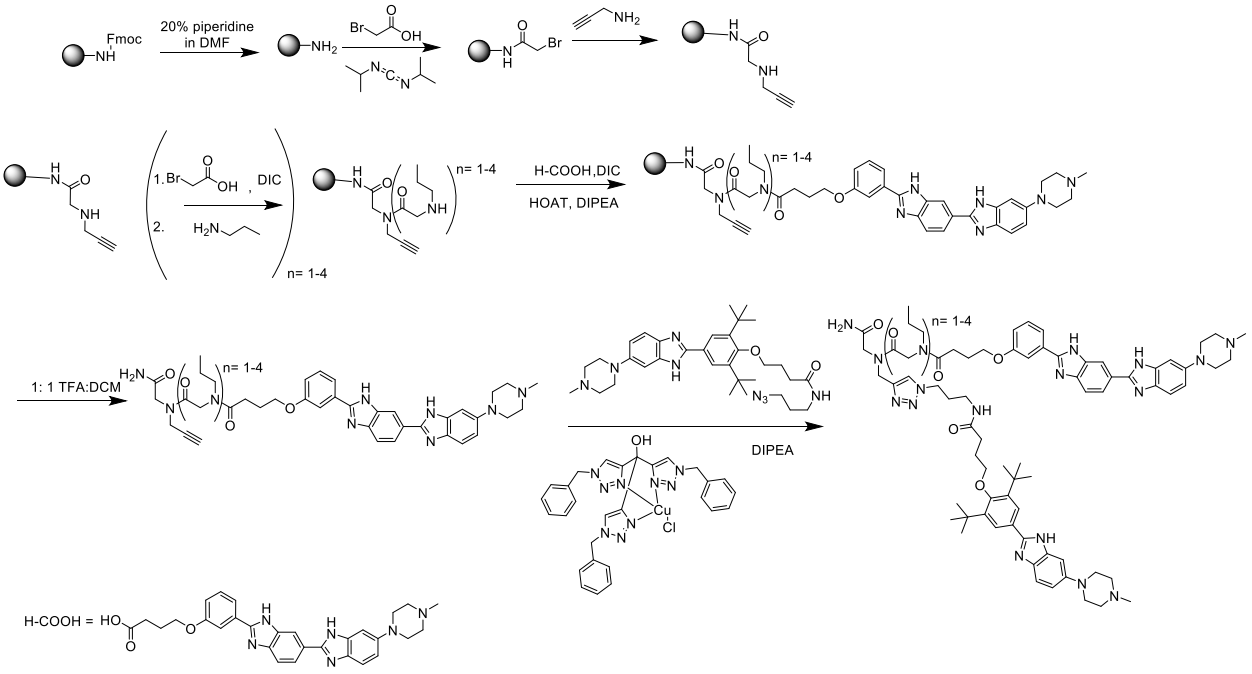
The membrane was briefly washed with 1× Tris-buffered saline (TBS) and blocked in 5% milk in 1× TBST (1× TBS containing 0.1% Tween-20) for 1 h at room temperature. The membrane was then incubated in 1:1000 FOXO1 primary antibody (Cell Signaling Technology) in 1× TBST containing 3% BSA overnight at 4 ^{o}C. The membrane was washed with 1× TBST and incubated with 1:2000 anti-rabbit IgG horseradish-peroxidase secondary antibody conjugate (Cell Signaling Technology) in 1× TBS for 1 h at room temperature. After washing with 1× TBST, protein expression was quantified using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology) per the manufacturer's protocol.

The membrane was then stripped using 1× Stripping Buffer (200 mM glycine, pH 2.2 and 0.1% SDS) followed by washing in 1× TBS. The membrane was blocked and probed for β-actin following the same procedure described above using 1:5000 β-actin primary antibody (Cell Signaling Technology) in 1× TBST containing 3% BSA overnight at 4 ^{o}C. The membrane was washed with 1× TBST and incubated with 1:10,000 anti-rabbit IgG horseradish-peroxidase secondary antibody conjugate (Cell Signaling

Technology) in 1× TBS for 1 h at room temperature. ImageJ software from the National Institutes of Health was used to quantify band intensities.

Annexin V/PI Assays: Cells were grown in 6-well plates to ~60% confluency and then incubated with 50 nM of **3** for 48 h. The cells were then detached from the surface using accutase and washed twice each with ice-cold 1× DPBS and 1× Annexin Binding Buffer (50 mM Hepes (pH 7.4), 700 mM NaCl and 12.5 mM $CaCl_2$). The cells were suspended in 100 µL 1× Annexin Binding Buffer containing 5 µL Annexin V-APC (eBioscience). The solution was incubated for 10 min at room temperature followed by washing with 1× Annexin Binding Buffer. The cells were then stained with 1 µg/mL propidium iodide in 300 µL of 1× Annexin Binding Buffer for 15 min at room temperature. Flow cytometry was performed using a BD LSRII instrument (BD Biosciences). At least 10,000 events were used for analysis.

Abbreviations: Boc, tert-butyloxycarbonyl; DCM, dichloromethane; DIC, *N*,*N*′-Diisopropylcarbodiimide; DIEA, *N*,*N*-Diisopropylethylamine; DMF, dimethylformamide; dDMF, dry dimethylformamide; EDC, *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride; HOAT, 1-Hydroxy-7-azabenzotriazole; HPLC, high performance liquid chromatography; MALDI, matrix-assisted laser desorption/ionization; TFA, trifluoroacetic acid



Scheme S1: General protocol for dimer synthesis.

General protocol for peptoid synthesis: Peptoids were synthesized via standard resin-supported oligomerization protocol. Fmoc-protected rink amide resin (200 mg, 138 μmol) with a substitution level of 0.69 mmol/g was allowed to swell for 5 min each in DCM and DMF with shaking. The resin was deprotected with 20% piperidine in DMF (3 mL, 2x 20 min) at room temperature.

Coupling step: The resin was then washed with DMF (3x 5 min). Bromoacetic acid was coupled to the resin bound amine in the presence of 5 equivalents of bromoacetic acid and 5 equivalents of DIC in 3 mL dDMF. The reaction mixture was heated in a Panasonic microwave at 10% power (70 watts) (2x 30 s). The resin was then washed with dDMF (3x 5 min).

Displacement step: (a) Introduction of click counterpart: The resin was then treated with 10 equivalents of propargylamine (1.38 mmol, 88 µL) in 3 mL of DMF in a Panasonic microwave at 10% power (70 watts) (1x 30 s) and shaken at room temperature for 2 h. (b) Chain extension with spacer (propylamine): Coupling with bromoacetic acid was repeated after the introduction of propargylamine. The resin was then treated with 10 equivalents of propylamine (1.38 mmol, 113 µL) in 3 mL of DMF in a Panasonic microwave at 10% power (70 watts) (1x 30 s) and shaken at room temperature for 20 min. The resin was then washed with dDMF and reaction with propylamine was repeated one more time. Step (b) was repeated until the required length (n= 1 – 4) of the peptoid was obtained.

Conjugation of ligand modules to the peptoid scaffold: The following process was employed: (a) Hoechst carboxylate (28) was coupled to the end of the peptoid backbone. The peptoid (50 mg, 34.5 µmol) from the previous step was directly treated with a solution of Hoechst carboxylate (27 mg, 52 µmol), EDC (8 mg, 52 µmol), HOAT (7 mg, 52 µmol) and DIEA (66 µL, 345 µmol) in 2 mL dDMF in a microwave vial in a Biotage Initiator+ at 75 ˚C for 2 h. The resin was then washed with DMF (3 x 5 min). (b) **1** (Figure 1B) was added onto the peptoid from the previous step via Huisgen

dipolar cycloaddition reaction (HDCR). The peptoid (50 mg, 34.5 μmol) from previous step was directly treated with a solution of **1** (7 mg, 8.6 μmol), Cu(I) catalyst (1.5 mg; 2.6 μmol) and DIEA (66 μL, 345 μmol) in 2 mL dDMF in a microwave vial at 120 ˚C for 2 h in a Biotage Initiator+. The resin was then washed with DMF (3 x 5 min) followed by DCM (3 x 5 min) before cleaving the peptoid off the resin in (1:1) TFA:DCM for 15 min at room temperature. The solvent was removed under vacuum and the crude product was purified via HPLC. The product was purified by preparative HPLC using either a reverse phase Atlantis®Prep T3 C18 5 μM column or a SunfirePrep C18 5 μM 19 × 150 mm column. HPLC separations were completed using a Waters 1525 Binary HPLC Pump equipped with a Waters 2487 Dual Absorbance Detector system. A linear gradient from 0% to 100% B in A over 60 min and a flow rate of 5 mL/min were employed. (A: water + 0.1% (v/v) trifluoroacetic acid (TFA); B: methanol or acetonitrile + 0.1% (v/v) TFA.). The purity was evaluated on a reverse phase Waters Symmetry C18 5 μm 4.6 × 150 mm column at room temperature. A flow rate of 1 mL/min and a linear gradient of 0–100% B in A. Absorbance was monitored at 254 nm. All dimers were ≥95% pure. Mass spectra were recorded on a 4800 plus MALDI TOF/TOF analyzer. NMR spectra were collected on either a 400 MHz or a 700 MHz Bruker.

Characterization of designer dimers:

Designer dimer, n =1: HRMS MALDI: $M+H^+$ calculated: 1292.7568; $M+H^+$ observed: 1292.7554. ^1H NMR (400 MHz, CD_3OD) δ 0.80 - 1.03 (3H, m), 1.05 - 1.40 (2H, m), 1.41 - 1.56 (18H, m), 1.57 - 1.82 (2H, m), 1.90 - 2.28 (7H, m), 2.28 - 2.40 (2H, m), 2.40 - 2.56 (1H, m), 2.58 - 2.89 (3H, m), 2.95 - 3.06 (5H, m), 3.08 - 3.27 (7H, m), 3.33 - 3.54 (5H,

m), 3.55 - 3.71 (4H, m), 3.71 - 3.85 (3H, m), 3.86 - 3.99 (3H, m), 3.99 - 4.27 (6H, m), 4.29 - 4.55 (4H, m), 4.57 - 4.69 (1H, m), 4.69 - 4.85 (2H, m), 7.15 (2H, br d, *J*=10.36), 7.29 (2H, br d, *J*=6.82), 7.32 - 7.43 (2H, m), 7.44 - 7.59 (2H, m), 7.59 - 7.78 (4H, m), 7.78 - 7.97 (2H, m), 7.99 - 8.08 (3H, m), 8.08 - 8.23 (1H, m), 8.40 (1H, s). ^{13}C NMR (175 MHz, CD_3OD) δ 11.5, 21.77, 22.83, 26.21, 26.68, 29.94, 30.39, 31.17, 31.12, 32.38, 33.01, 37.99, 37.52, 40.48, 43.60, 43.63, 44.25, 44.65, 48.56, 50.18, 52.20, 52.30, 54.68, 54.70, 61.06, 68.43, 77.56, 101.08, 101.11, 114.02, 115.29, 115.42, 115.58, 116.91, 116.99, 118.61, 119.48, 120.74, 123.87, 125.07, 127.77, 127.83, 128.18, 131.74, 134.35, 134.63, 140.11, 141.14, 141.86, 144.34, 147.57, 150.72, 150.77, 150.85, 156.20, 158.88, 159.12, 161.07, 161.14, 171.32, 171.46, 173.61, 175.16, 175.49, 176.22.

Designer dimer, n =2: HRMS MALDI: $M+H^+$ calculated: 1391.8252; $M+H^+$ observed: 1391.8220. ^{1}H NMR (400 MHz CD_3OD) δ ppm 0.79 - 0.99(3H, m), 1.00 - 1.26 (1H, m), 1.27 - 1.41 (18H, m), 1.41 - 1.64 (9H, m), 2.32 (1H, br d, *J*=4.04), 2.46 (1H, br d, *J*=7.07), 2.66 (2H, s), 2.90 - 3.12 (2H, m), 3.12 - 3.32 (6H, m), 3.34 - 3.58 (6H, m) 3.72 (4H, d, *J*=6.61), 4.00 - 4.27 (2H, m), 4.28 - 4.50 (2H, m), 4.52 - 4.78 (1H, m), 4.90 (3H, s), 7.09 - 7.25 (1H, m), 7.49 - 7.61 (1H, m), 7.61 - 7.74 (1H, m), 7.90 - 8.08 (1H, m), 8.24 (1H, br s). ^{13}C (175 MHz, CD_3OD) δ 11.48, 11.53, 11.60, 13.16, 17.31, 18.74, 19.32, 21.65, 21.74, 22.48, 22.63, 22.75, 26.22, 26.75, 26.66, 31.06, 32.54, 37.16, 43.67, 43.70, 43.81, 54.95, 54.97, 55.85, 68.30, 77.17, 102.80, 113.65, 117.45, 122.93, 123.46, 123.84, 124.99, 125.14, 126.91, 131.43, 131.88, 133.68, 134.13, 138.62,

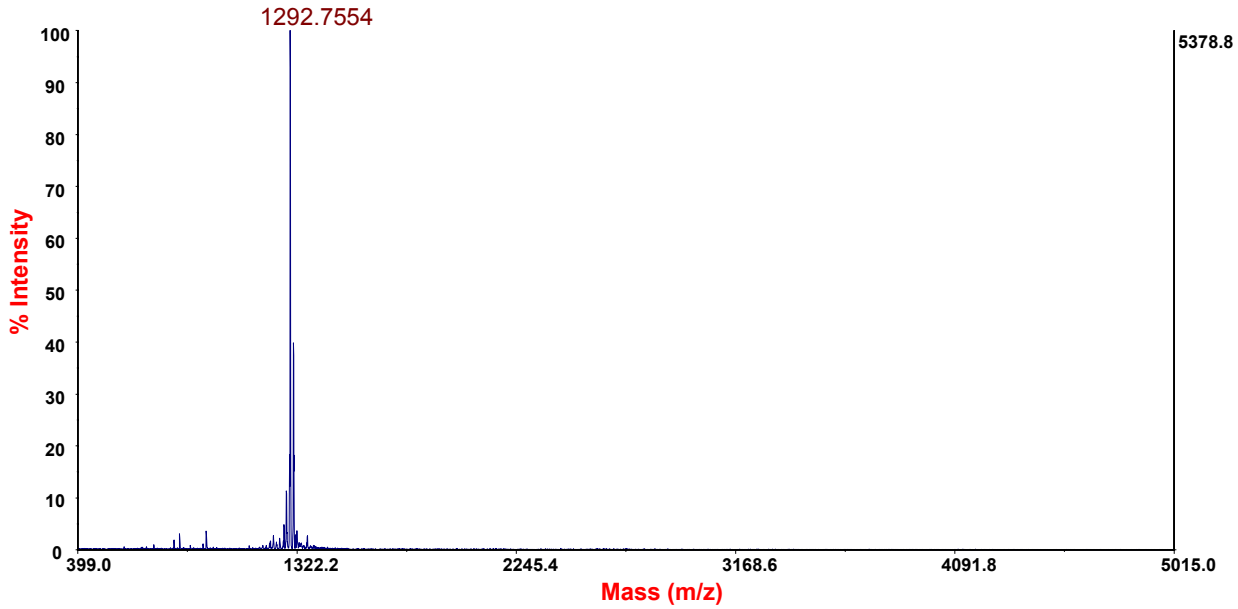
139.02, 143.92, 144.13, 146.31, 148.80, 153.01, 155.42, 160.89, 161.96, 170.82, 171.74, 172.44, 172.65, 173.49, 175.23, 176.04, 176.21.

Designer dimer, n =3: HRMS MALDI: $M+H^+$ calculated: 1490.8936; $M+H^+$ observed: 1490.8934. ^1H NMR (400 MHz, CD_3OD) δ 0.78 - 1.00 (9H, m), 1.26 - 1.44 (1H, m), 1.49 (18H, m), 2.01 - 2.16 (4H, m) 2.22 (2H, br d, *J*=6.82) 2.31 - 2.42 (2H, m), 2.48 (1H, br d, *J*=7.07), 2.67 (1H, br d, *J*=6.32), 3.00 (6H, d, *J*=8.59), 3.12 - 3.26 (7H, m), 3.60 - 3.75 (4H, m), 3.75 - 3.85 (2H, m), 3.86 - 3.95 (3H, m), 3.96 - 4.11 (4H, m), 4.12 - 4.50 (9H, m), 4.53 - 4.74 (3H, m), 5.02 (4H, br s), 5.51 -5.57 (2H, m), 7.04 - 7.24 (1H, m), 7.24-7.40 (8H, m), 7.46 (1H, br dd, *J*=7.45, 3.66), 7.56 - 7.64 (1H, m), 7.64 - 7.71 (3H, m), 7.84 - 8.00 (2H, m), 8.00 - 8.11 (4H, m), 8.37 (1H, br d, *J*=11.87). ^{13}C (100 MHz, CD_3OD) δ 11.38, 11.55, 11.74, 20.28, 21.77, 22.63, 26.05, 26.7, 29.87, 30.38, 31.13, 32.39, 33.01, 37.38, 43.61, 43.66, 54.64, 55.03, 68.54, 68.82, 77.53, 101.07, 113.14, 113.84, 114.67, 115.41, 115.66, 116.98, 117.5, 118.57, 118.89, 119.42, 119.63, 121.02, 121.76, 125.06, 126.06, 127.78, 129.24, 129.64, 130.06, 131.98, 134.29, 134.63, 143.93, 147.54, 150.62, 150.85, 158.79, 159.21, 164.10, 171.01, 171.43, 171.73, 175.19,176.16

Designer dimer, n =4: HRMS MALDI: $M+H^+$ calculated: 1589.9620; $M+H^+$ observed: 1589.9626. ^1H NMR (400 MHz, CD_3OD) δ 0.76 - 1.00 (12H, m), 1.44 - 1.59 (21H, m), 1.59 - 1.69 (3H, m), 2.01 - 2.27 (6H, m) 2.28 - 2.41 (2H, m), 2.41 - 2.61 (1H, m), 2.63 - 2.79 (1H, m), 3.01 (6H, d, *J*=8.08), 3.69 (4H, br s), 3.74 - 3.88 (3H, m), 3.88 - 3.96 (3H, m), 3.96 - 4.24 (9H, m), 4.25 - 4.47 (5H, m), 4.54 - 4.80 (3H, m), 5.56 (1H, d, *J*=10.86),

7.10 (1H, br s), 7.25 - 7.50 (6H, m), 7.57 - 7.75 (4H, m), 7.76 - 7.95 (2H, m), 7.95 - 8.14 (3H, m), 8.21 - 8.40 (1H, m). ^{13}C NMR (175 MHz, CD_3OD) δ 11.46, 11.53, 11.71, 21.74, 21.85, 22.40, 22.63, 22.70, 26.14, 26.70, 29.77, 29.91, 31.21, 32.39, 33.01, 37.39, 37.52, 37.86, 43.60, 43.65, 52.10, 54.66, 54.68, 55.03, 68.43, 68.73, 77.54, 101.02, 101.08, 113.80, 115.42, 118.61, 118.73, 119.44, 120.70, 123.87, 125.32, 127.77, 130.06, 131.69, 134.33, 134.51, 136.65, 139.66, 143.90, 144.17, 144.55, 147.55, 150.49, 150.67, 150.73, 155.91, 158.87, 159.11. 159.87, 160.97, 164.10, 170.40, 170.81, 171.45, 171.64, 173.19, 173.51, 175.17, 175.26, 176.19, 176.40.

A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70
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50
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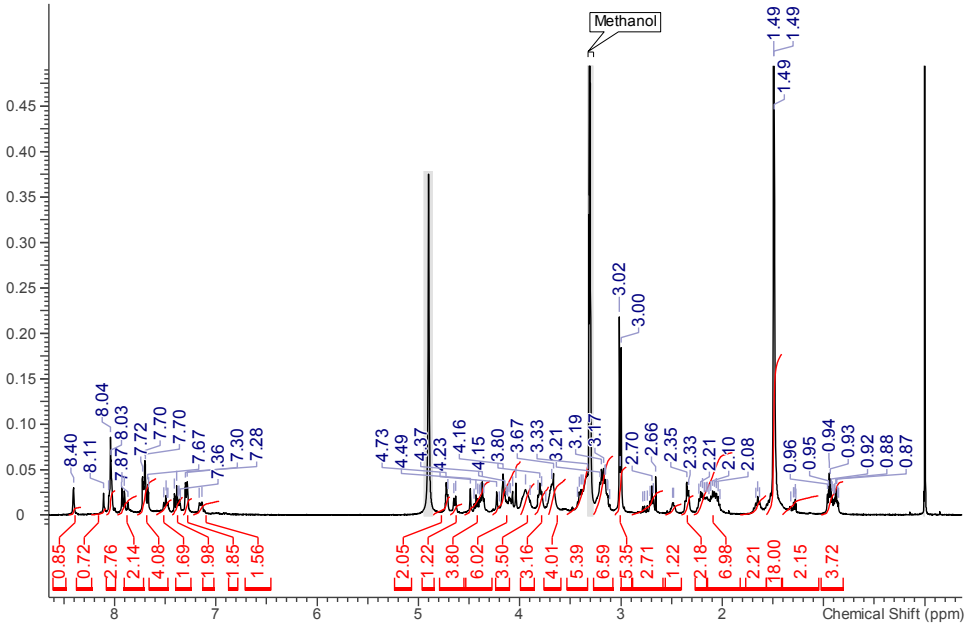
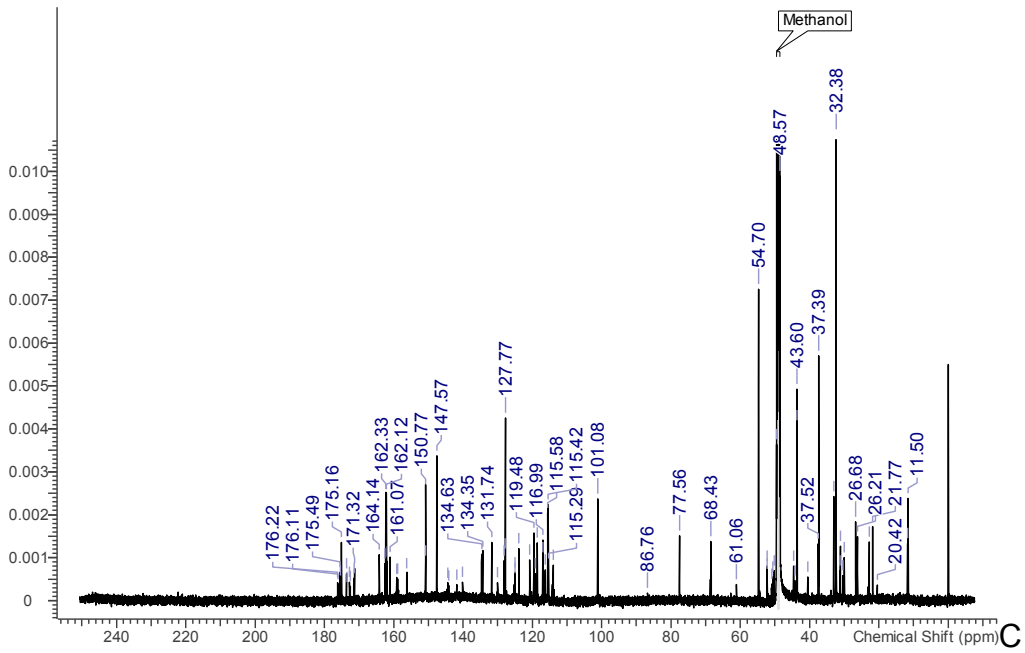
B


Methanol
Chemical Shift (ppm)

C



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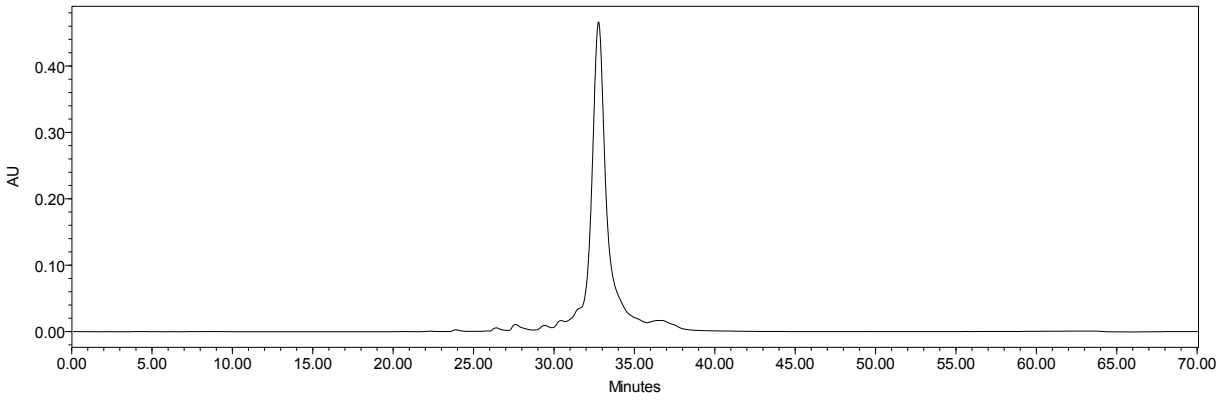
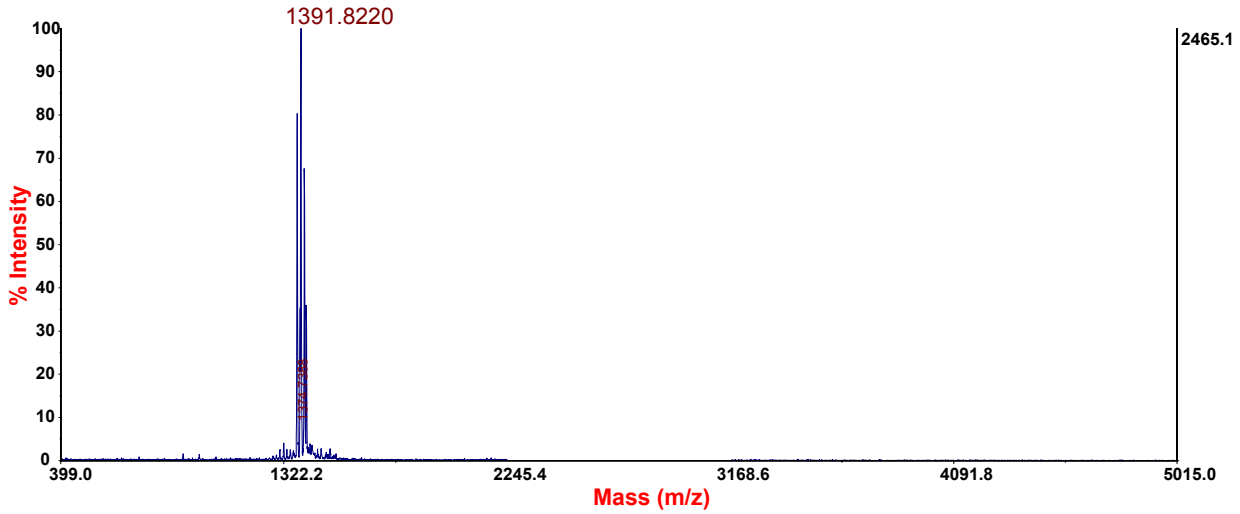


Figure S3: Characterization of designer dimer (n=1). **A**, HRMS MALDI-TOF. **B**, ^{1}H NMR in CD_3OD at 400 MHz. **C**, ^{13}C NMR in CD_3OD at 175 MHz **D**, Analytical HPLC trace. A linear gradient from 0% to 100% B in A over 60 min and a flow rate of 1 mL/min were employed. (A: water + 0.1% (v/v) trifluoroacetic acid (TFA); B: methanol + 0.1% (v/v) TFA).

A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% Intensity
Mass (m/z)
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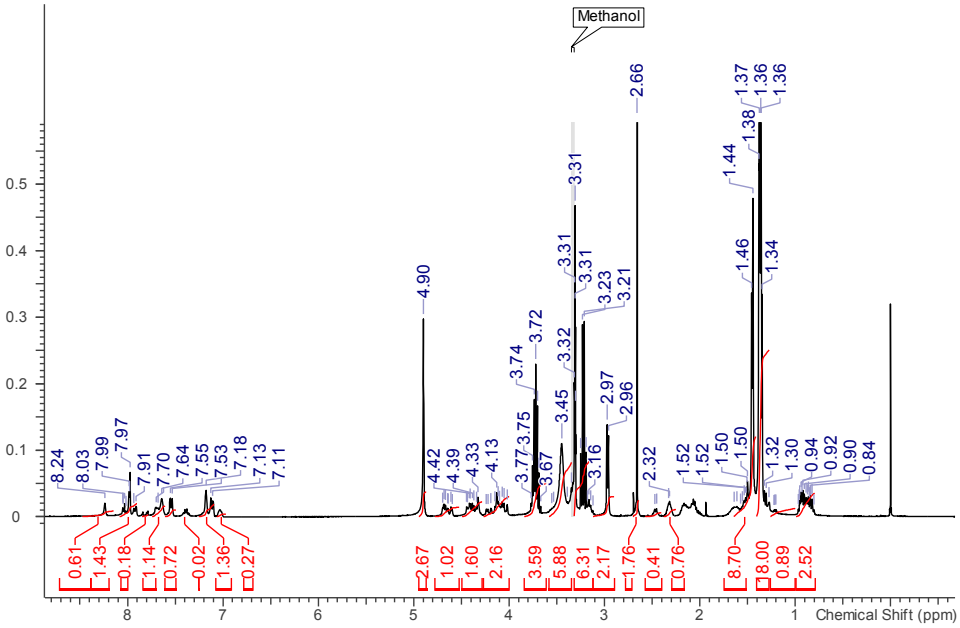
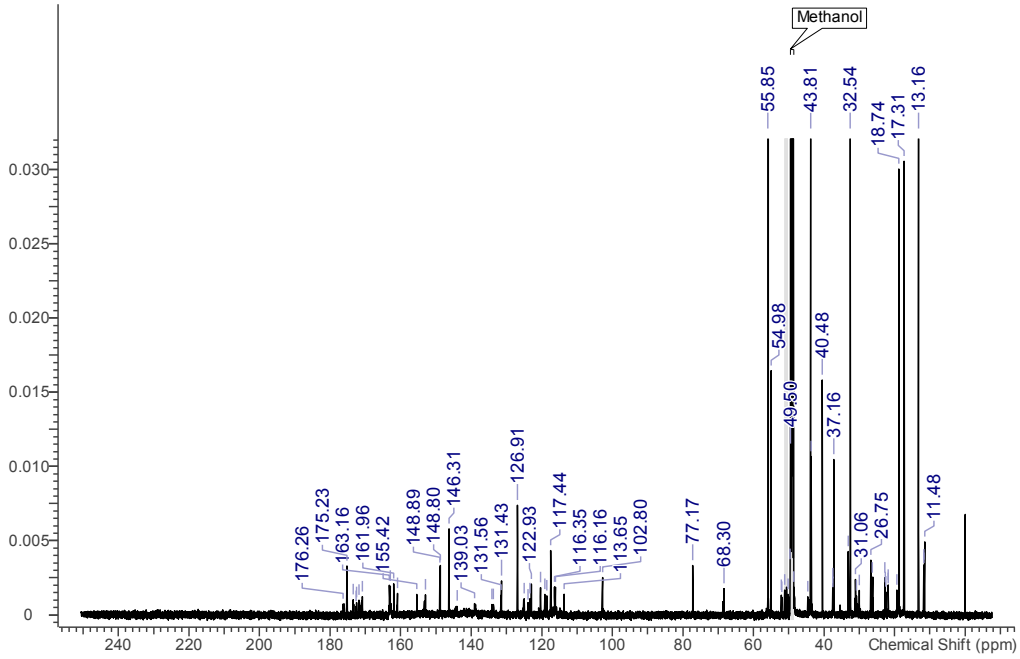
1322.2
2245.4
3168.6
4091.8
5015.0

B


Methanol
Chemical Shift (ppm)

C



D

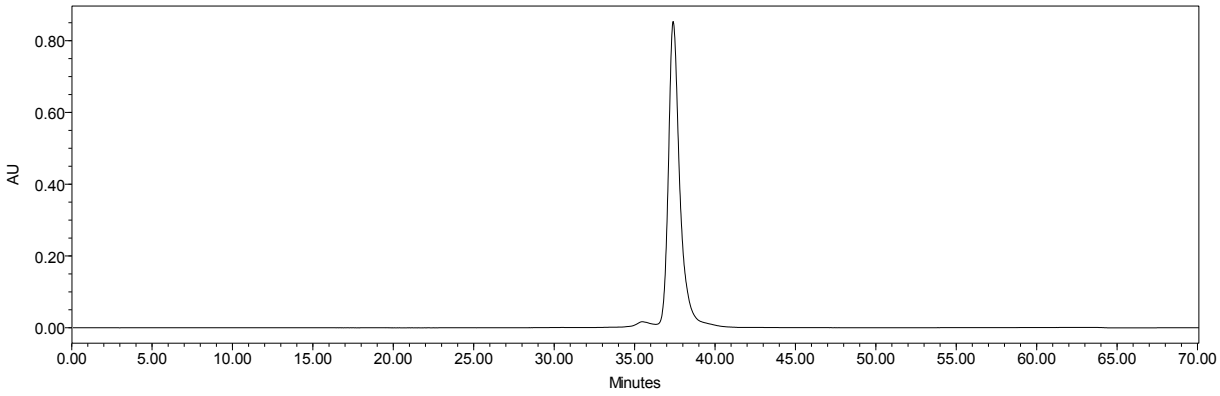
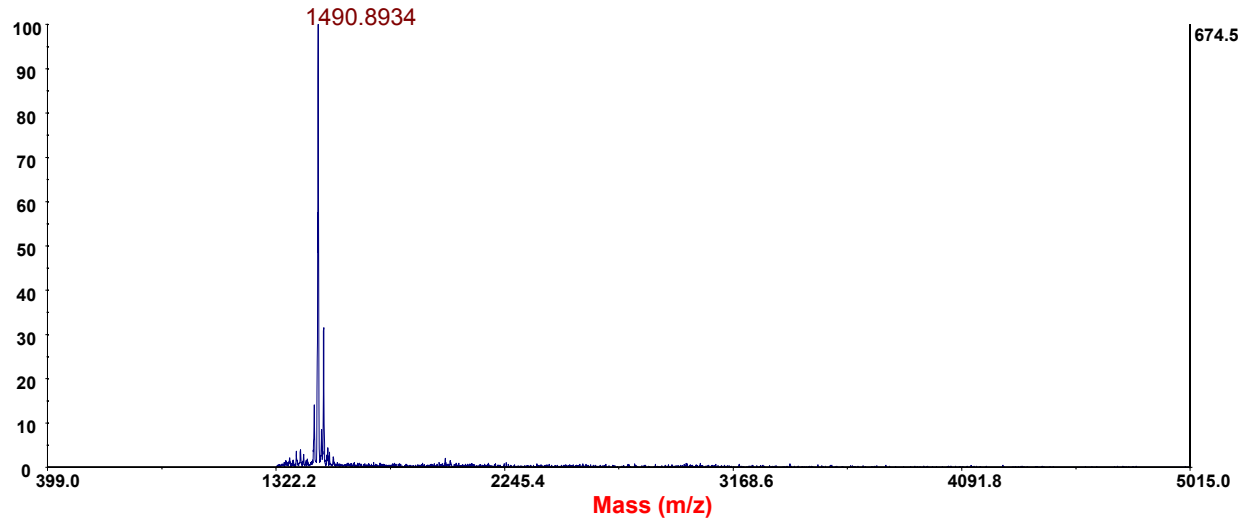
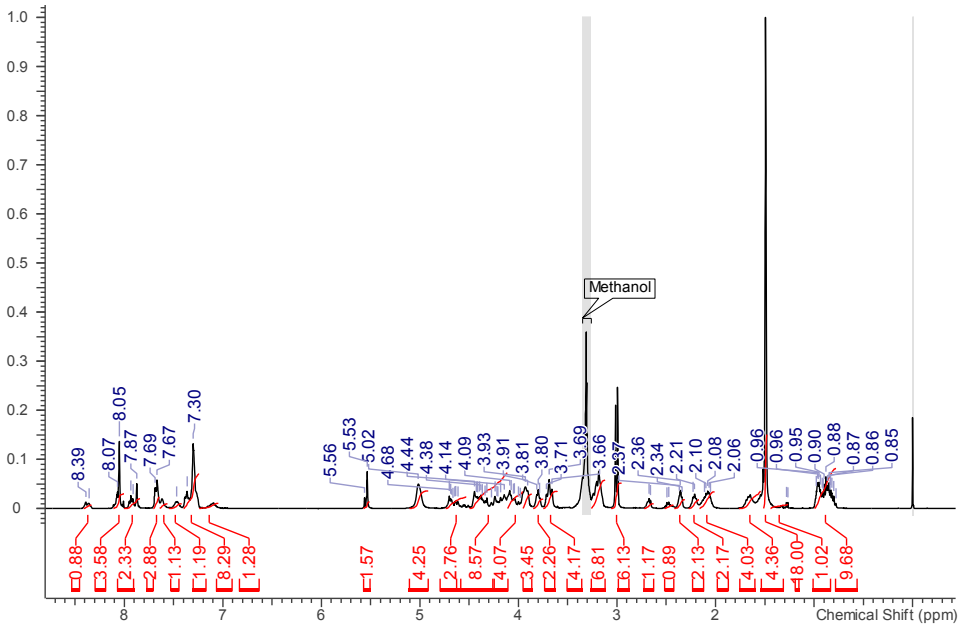


Figure S4: Characterization of designer dimer (n=2). **A**, HRMS MALDI-TOF. **B**, ^{1}H NMR in CD_3OD at 400 MHz. **C**, ^{13}C NMR in CD_3OD at 175 MHz **D**, Analytical HPLC trace. A linear gradient from 0% to 100% B in A over 60 min and a flow rate of 1 mL/min were employed. (A: water + 0.1% (v/v) trifluoroacetic acid (TFA); B: methanol + 0.1% (v/v) TFA).

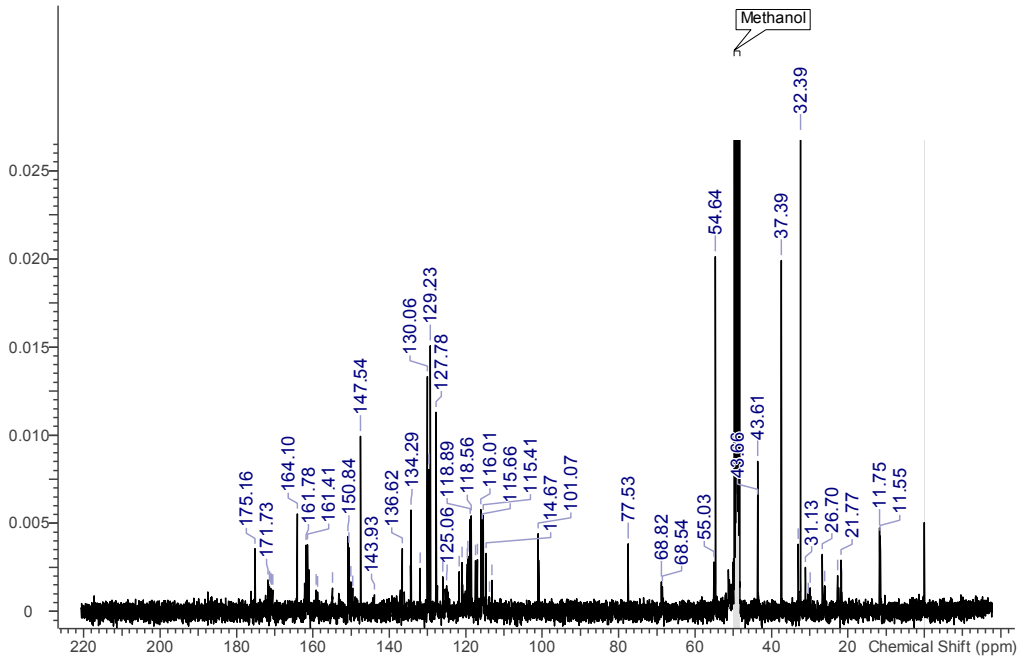
A


1490.8934
674.5
100
90
80
70
60
50
40
30
20
10
0
399.0
1322.2
2245.4
3168.6
4091.8
5015.0
Mass (m/z)

B


Methanol
Chemical Shift (ppm)

C



D

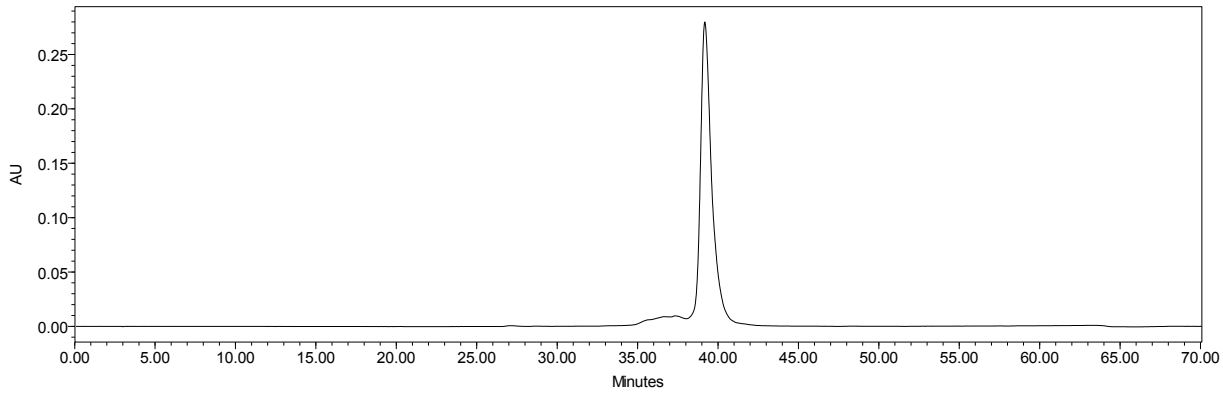
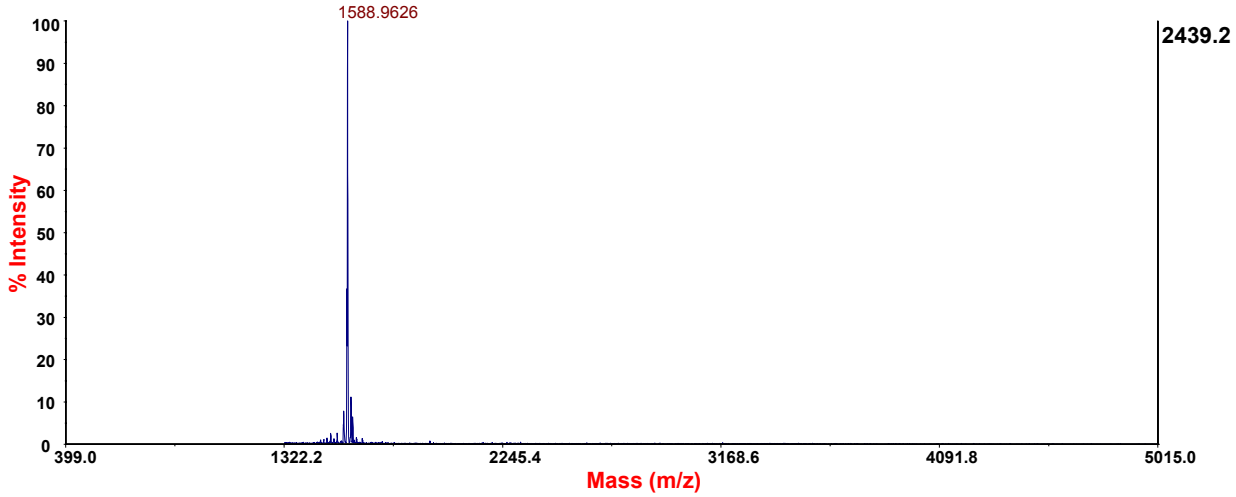
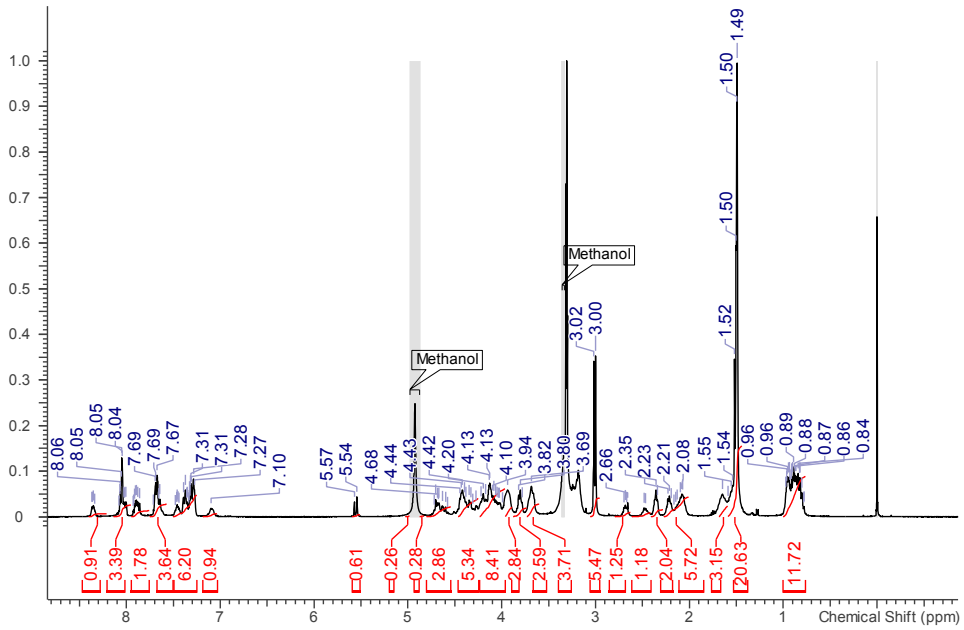


Figure S5: Characterization of designer dimer (n=3). **A**, HRMS MALDI-TOF. **B**, ^{1}H NMR in CD_3OD at 400 MHz. **C**, ^{13}C NMR in CD_3OD at 175 MHz **D**, Analytical HPLC trace. A linear gradient from 0% to 100% B in A over 60 min and a flow rate of 1 mL/min were employed. (A: water + 0.1% (v/v) trifluoroacetic acid (TFA); B: methanol + 0.1% (v/v) TFA).

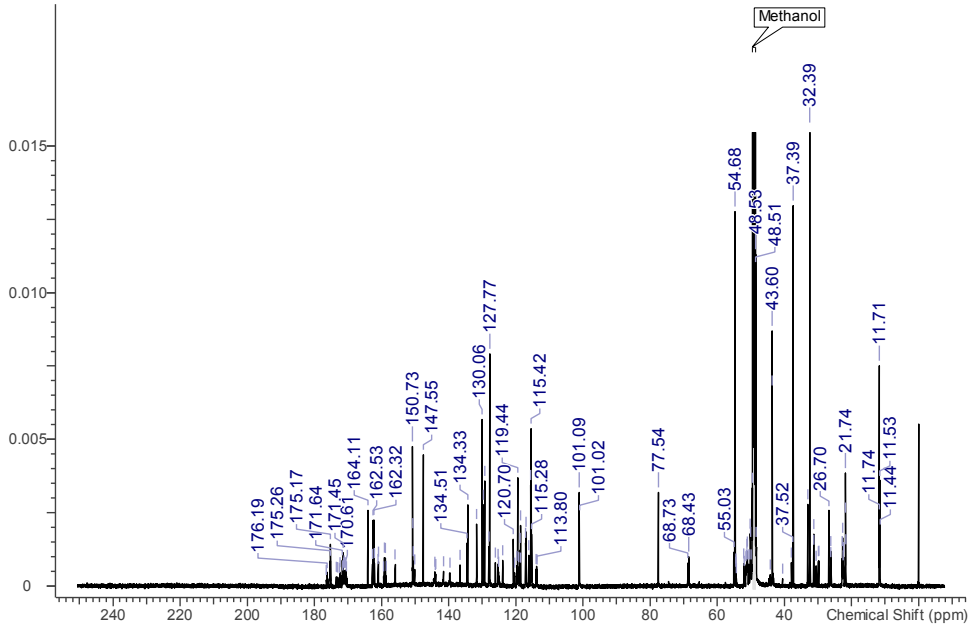
A


1588.9626
2439.2
% Intensity
Mass (m/z)
399.0
1322.2
2245.4
3168.6
4091.8
5015.0

B


Methanol
Methanol
Chemical Shift (ppm)

C



D

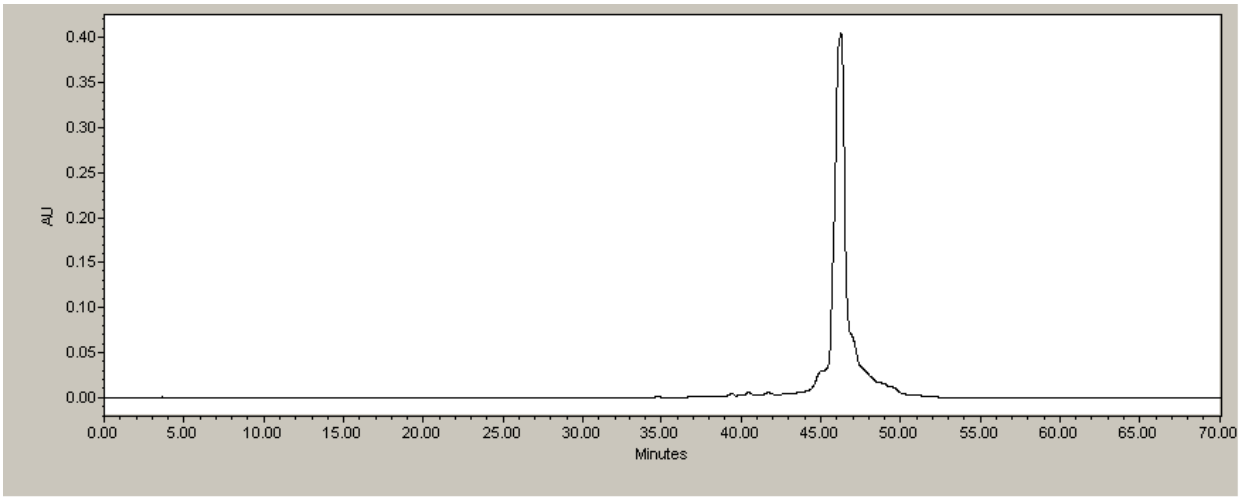
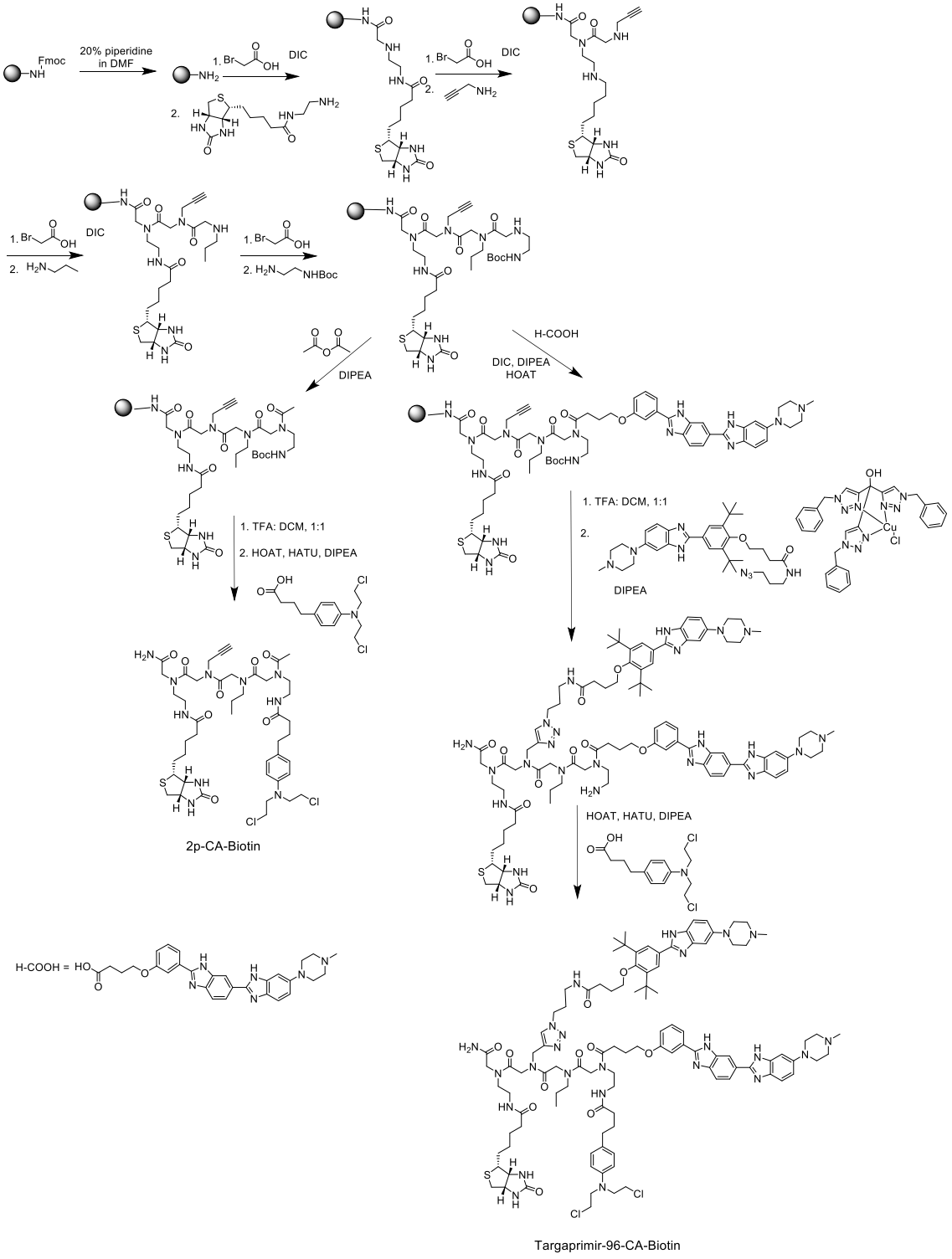


Figure S6: Characterization of designer dimer (n=4). **A**, HRMS MALDI-TOF. **B**, ^{1}H NMR in CD_3OD at 400 MHz. **C**, ^{13}C NMR in CD_3OD at 175 MHz **D**, Analytical HPLC trace. A linear gradient from 0% to 100% B in A over 60 min and a flow rate of 1 mL/min were employed. (A: water + 0.1% (v/v) trifluoroacetic acid (TFA); B: methanol + 0.1% (v/v) TFA).

Synthesis of Targaprimir-96-CA-Biotin and 2p-CA-Biotin:



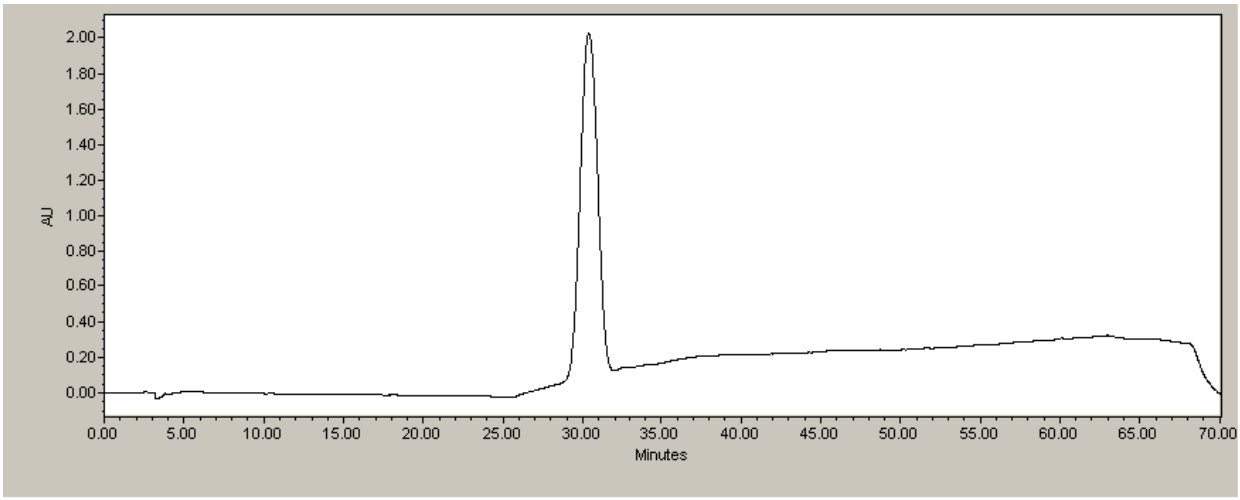
Scheme S2: Synthesis of **Targaprimir-96-CA-Biotin** (**4**) and **2p-CA-Biotin** (**5**).

Fmoc-protected rink amide resin (200 mg, 138 μmol) with a substitution level of 0.69 mmol/g was allowed to swell for 5 min each in DCM and DMF with shaking. The resin was deprotected with 20% piperidine in DMF (3 mL, 2 x 20 min) at room temperature. The resin was then treated with 10 equivalents of Biotin ethylenediamine (1.38 mmol) in 3 mL of dDMF in a Panasonic microwave at 10% power (70 watts) (1 x 30 s) and shaken at room temperature for 2 h. Coupling with bromoacetic acid was repeated after the introduction of Biotin ethylene diamine. The resin was then treated with 10 equivalents of propargylamine (1.38 mmol, 88 μL) in 3 mL of dDMF in a Panasonic microwave at 10% power (70 watts) (1 x 30 s) and shaken at room temperature for 2 h. Coupling with bromoacetic acid was repeated after the introduction of propargylamine. The resin was then treated with 10 equivalents of propylamine (1.38 mmol; 113 μL) in 3 mL of dDMF in a Panasonic microwave at 10% power (70 watts) (1 x 30 s) and shaken at room temperature for 20 min. The resin was washed with dDMF and reaction with propylamine was repeated one more time. Coupling with bromoacetic acid was repeated after the introduction of propylamine. The resin was then treated with 10 equivalents of N-boc-ethylenediamine (1.38 mmol) in 3 mL of dDMF in a Panasonic microwave at 10% power (70 watts) (1 x 30 s) and shaken at room temperature for 2 h. The resin was next either treated with 100 μL 1:1 mixture of acetic anhydride and DIEA in 3 mL dDMF for 5 min and then washed with DMF or the resin was subjected to conjugation with ligand modules as described above and purified via preparative HPLC. Chlorambucil (CA) was then coupled using HOAT (3 mg; 15 μmol), HATU (5.5 mg; 15 μmol) and DIEA (10 μL; 50 μmol) in 200 μL dDMF at RT for overnight to obtain either

2p-CA-Biotin (**5**) or **Targaprimir-96-CA-Biotin** (**4**). The purity of both compounds was evaluated on a reverse phase Waters Sunfire C18 3.5 μm 4.6 × 150 mm column at room temperature. A flow rate of 1 mL/min and a linear gradient of 0–100% B in A. Absorbance was monitored at 254 nm. **Targaprimir-96-CA-Biotin** (**4**) and **2p-CA-Biotin** (**5**) were ≥95% pure.

2p-CA-Biotin (**5**): HRMS MALDI: $M+H^+$ calculated: 965.4263; $M+H^+$ observed: 965.4203, 10 % yield. **Targaprimir-96-CA-Biotin** (**4**): HRMS MALDI: $M+H^+$ calculated: 2004.0304; $M+H^+$ observed: 2004.0396, 5 % yield.

A



B

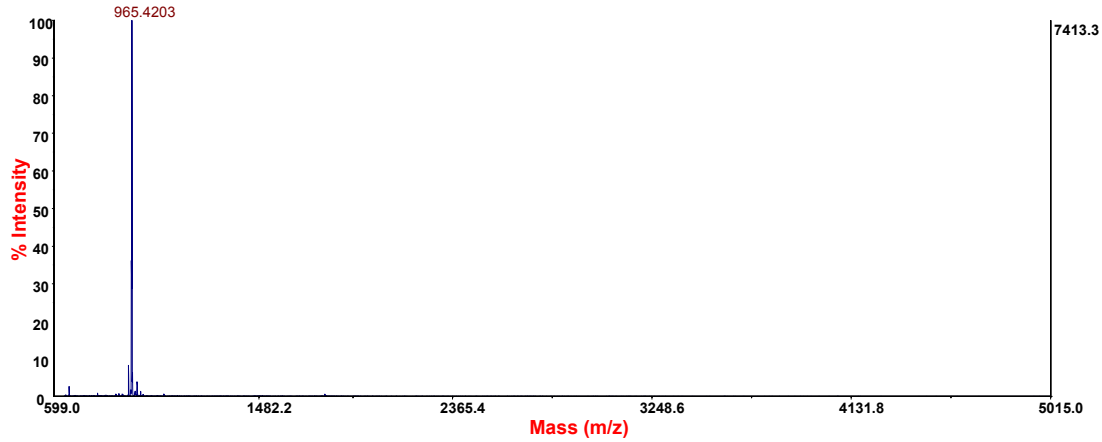
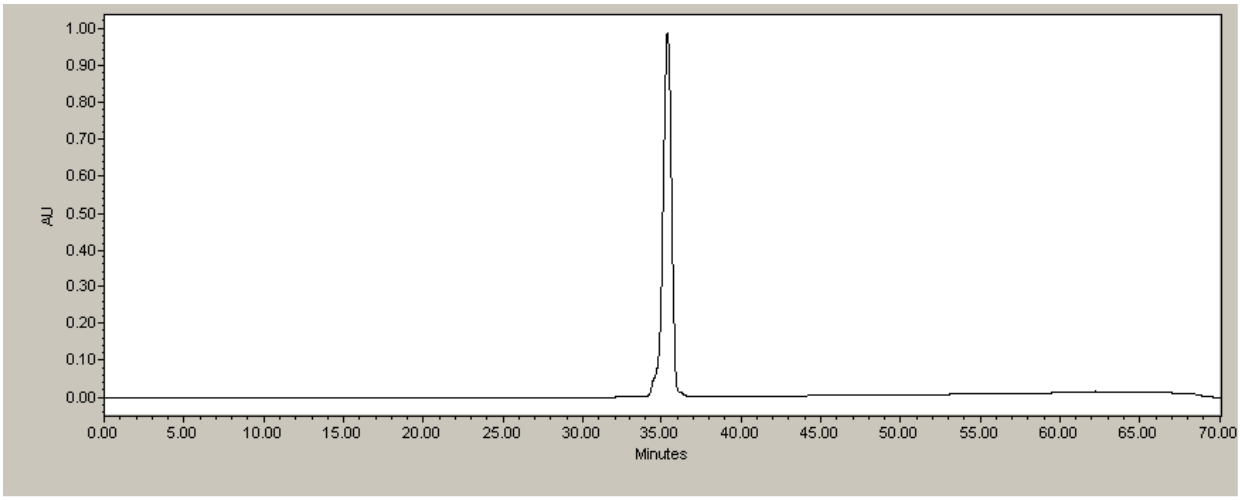


Figure S7: Analytical HPLC trace for **2p-CA-Biotin** (**5**). A linear gradient from 0% to 100% B in A over 60 min and a flow rate of 1 mL/min were employed. (A: water + 0.1% (v/v) trifluoroacetic acid (TFA); B: acetonitrile + 0.1% (v/v) TFA). **B**, MALDI-TOF of **2p-96-CA-Biotin** (**5**).

A



B

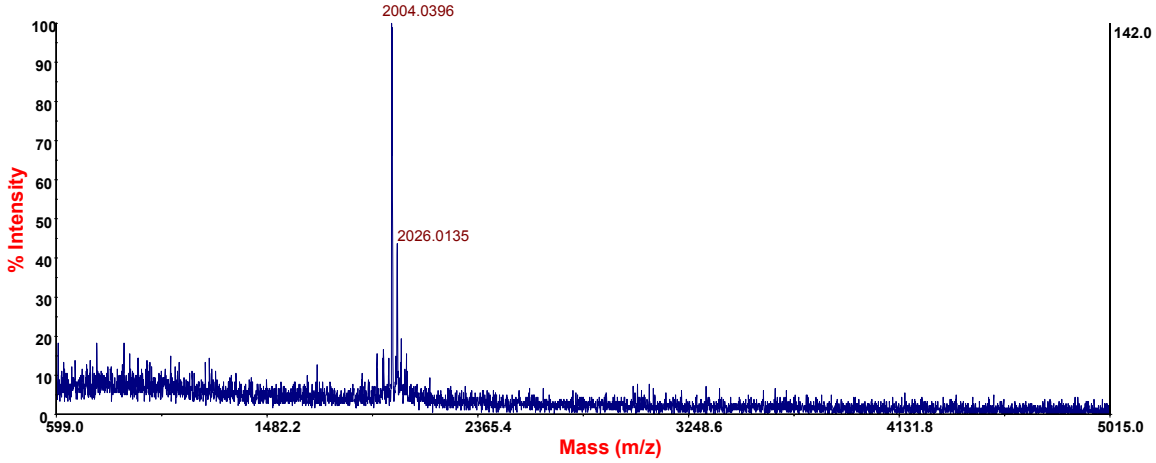


Figure S8: **A**, Analytical HPLC trace for **Targaprimir-96-CA-Biotin** (**4**). A linear gradient from 0% to 100% B in A over 60 min and a flow rate of 1 mL/min were employed. (A: water + 0.1% (v/v) trifluoroacetic acid (TFA); B: acetonitrile + 0.1% (v/v) TFA). **B**, MALDI-TOF of **Targaprimir-96-CA-Biotin** (**4**).

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