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5-Dihydroxyboryluridine Enhances Cytosolic Penetration of Antisense Oligonucleotides

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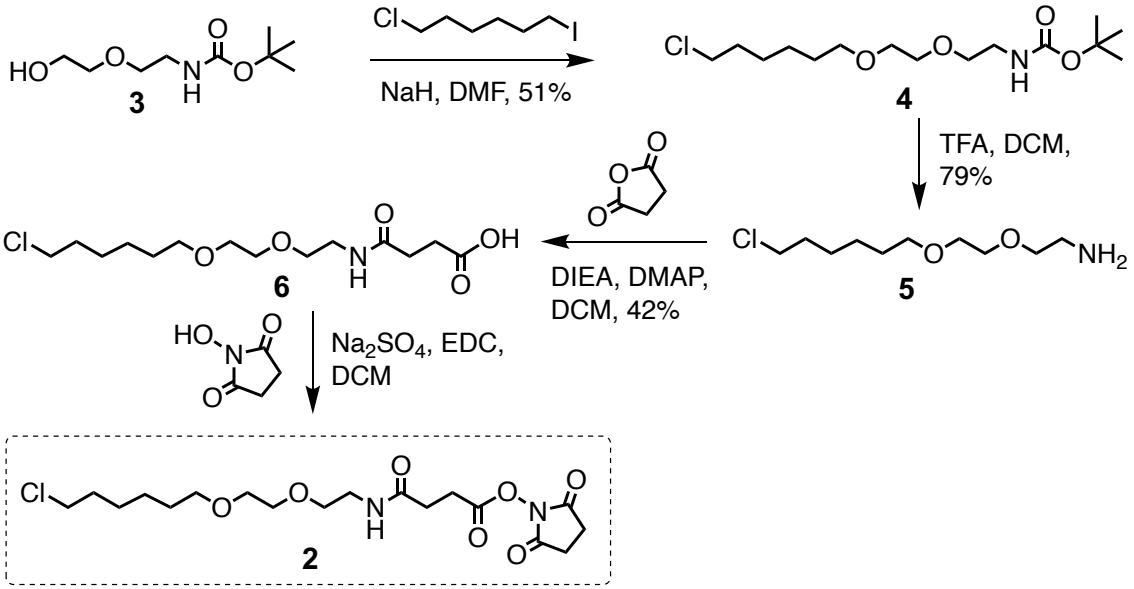
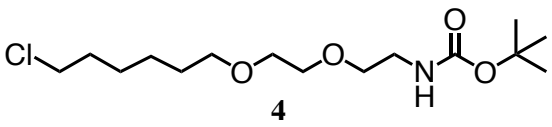
1. General materials, methods and equipment

Chemicals: All chemicals were purchased from established vendors (e.g., Sigma-Aldrich, Acros Organics, Glen Research) and used without purification and drying unless otherwise noted. Hexylamine phosphoramidite **7** was purchased form Glen Research (cat #133975-85-6). Optima grade acetonitrile was obtained from Fisher Scientific and degassed under vacuum prior to use during HPLC purification. All reactions to prepare uridine analogues were carried out in round bottom flasks and stirred with Teflon®-coated magnetic stir bars under Argon atmosphere when needed. Analytical thin layer chromatography (TLC) was performed using EMD 250 micron flexible aluminum backed, UV F_{254} pre-coated silica gel plates and visualized under UV light (254 nm) or by staining with $KMnO_4$ solution, phosphomolybdic acid, ninhydrin or anisaldehyde. Reaction solvents were removed by a Büchi rotary evaporator equipped with a dry ice-acetone condenser. Analytical and preparative HPLC was carried out on an Agilent 1220 Infinity HPLC with diode array detector. Concentration and lyophilization of aqueous samples were performed using Savant Sc210A SpeedVac Concentrator (Thermo), followed by Labconco Freeze-Dryer system.

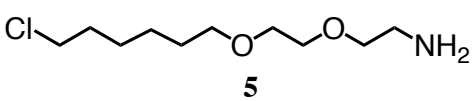
Proton nuclear magnetic resonance spectra (^{1}H NMR) were recorded on Bruker Ultrashield^{TM} Plus 600/500/400/300 MHz instruments at 24^{o}C. Chemical shifts of ^{1}H, ^{13}C NMR, ^{11}B and ^{31}P spectra are reported as δ in units of parts per million (ppm) relative to tetramethylsilane (δ 0.0) or residual solvent signals: chloroform-d (δ 7.26, singlet), methanol-d_4 (δ 3.30, quintet), and deuterium oxide-d_2 (δ 4.80, singlet). Coupling constants are expressed in Hz. MALDI mass spectra were collected at ultraFlextreme (Bruker) and the data was analyzed using flexAnalysis software. The ESI-MS were recorded on a Q-Exactive^{TM} Thermo Scientific LC-MS with electron spray ionization (ESI) probe.

2. Synthesis and characterization of activated N-hydroxysuccinimide (NHS) ester 2[1, 2]



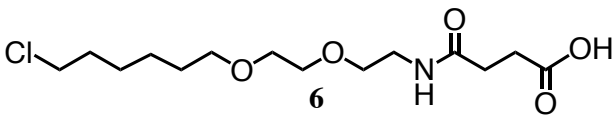


The starting 2-[2-(boc-amino)ethoxy]ethanol **3** (1.41 mL, 7.30 mmol) was dissolved in DMF (7.5 mL) under inert and dry conditions. Then sodium hydride (0.4 grams, 60% dispersion in mineral oil) was added to the reaction mixture on ice. After 30 minutes of stirring, the, 1-chloro-6-iodohexane (1.66 mL, 10.96 mmol) was added and the reaction was left stirring overnight at room temperature. The reaction was quenched with 1 M HCl and extracted with ethyl acetate, the organic portion was washed with water and brine and dried over sodium sulfate. The organic fraction was concentrated, and column chromatographed from 20 to 50% EtOAc in hexanes to give the chloro product **4** (1.20 grams, 50.8%). ^{1}H NMR (400 MHz, CHLOROFORM-d) δ = 3.48-3.62 (m, 8 H), 3,44 (t, *J*=6.65 Hz, 2 H), 3.29 (t, *J*=5.08 Hz, 2 H), 1.76 (quint, *J*=7.03 Hz, 2 H), 1.59 (quint, *J*=7.04 Hz, 2 H), 1.30-1.51 (m, 13 H) ppm; ^{13}C NMR (100 MHz, CHLOROFORM-d) δ = 156.09, 79.30, 71.37, 70.36, 70.30, 70.12, 45.13, 32.62, 29.52, 28.51, 26.78, 25.51 ppm; HRMS (ESI) calcd. for $C_{15}H_{31}O_4NCl$ $[M+H]^+$: 324.19417; found: 324.19651.



Boc protected chloro compound **4** (500 mg, 1.54 mmol) was dissolved in dichloromethane (5 mL) and TFA (1 mL) on ice. The reaction was stirred at room temperature for 2

hours. The reaction was concentrated, and the residue was dissolved in methanol and potassium carbonate was added until the solution was neutralized. The solution was filtered and concentrated, the remaining residue dissolved in EtOAc, washed with water and brine and concentrated to give the free amine **5** (272 mg, 78.9%). The next step was carried out without further purification.



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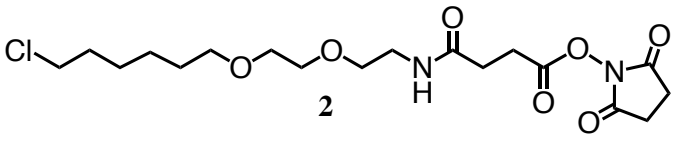
The starting free amine **5** (271 mg, 1.21 mmol) was dissolved in DCM (10 mL) and diisopropylethylamine (359.59 µL, 2.06 mmol) under inherit and dry conditions. Then 4-dimethylaminopyridine (148.35 mgs, 1.21 mmol) was added to the reaction and stirred vigorously Succinic anhydride (206.59 mg, 2.06 mmol) was added and the reaction was stirred for 2 hrs. at room temperature. The reaction was quenched with 1 M HCl and extracted with DCM, the organic portion was washed with water and brine and dried over sodium sulfate. The organic fraction was concentrated, and column chromatographed from 1 to 5% Methanol in DCM to give the free acid **6** (162 mg, 41.2%). ^{1}H NMR (400 MHz, CHLOROFORM-d) δ = 3.23-3.75 (m, 12 H), 2.44-2.74 (m, 4 H) 1.76 (quint, *J*=7.03 Hz, 2 H), 1.60 (quint, *J*=7.11 Hz, 2 H), 1.31-1.50 (m, 4 H) ppm; ^{13}C NMR (100 MHz, CHLOROFORM-d) δ = 175.65, 172.45, 71.46, 70.23, 70.16, 69.68, 45.15, 39.54, 32.58, 31.13, 30.18, 29.36, 26.74, 25.43 ppm; HRMS (ESI) calcd. for $C_{14}H_{25}O_5NCl$ [M-H]$^-$: 322.14158; found: 322.14408.



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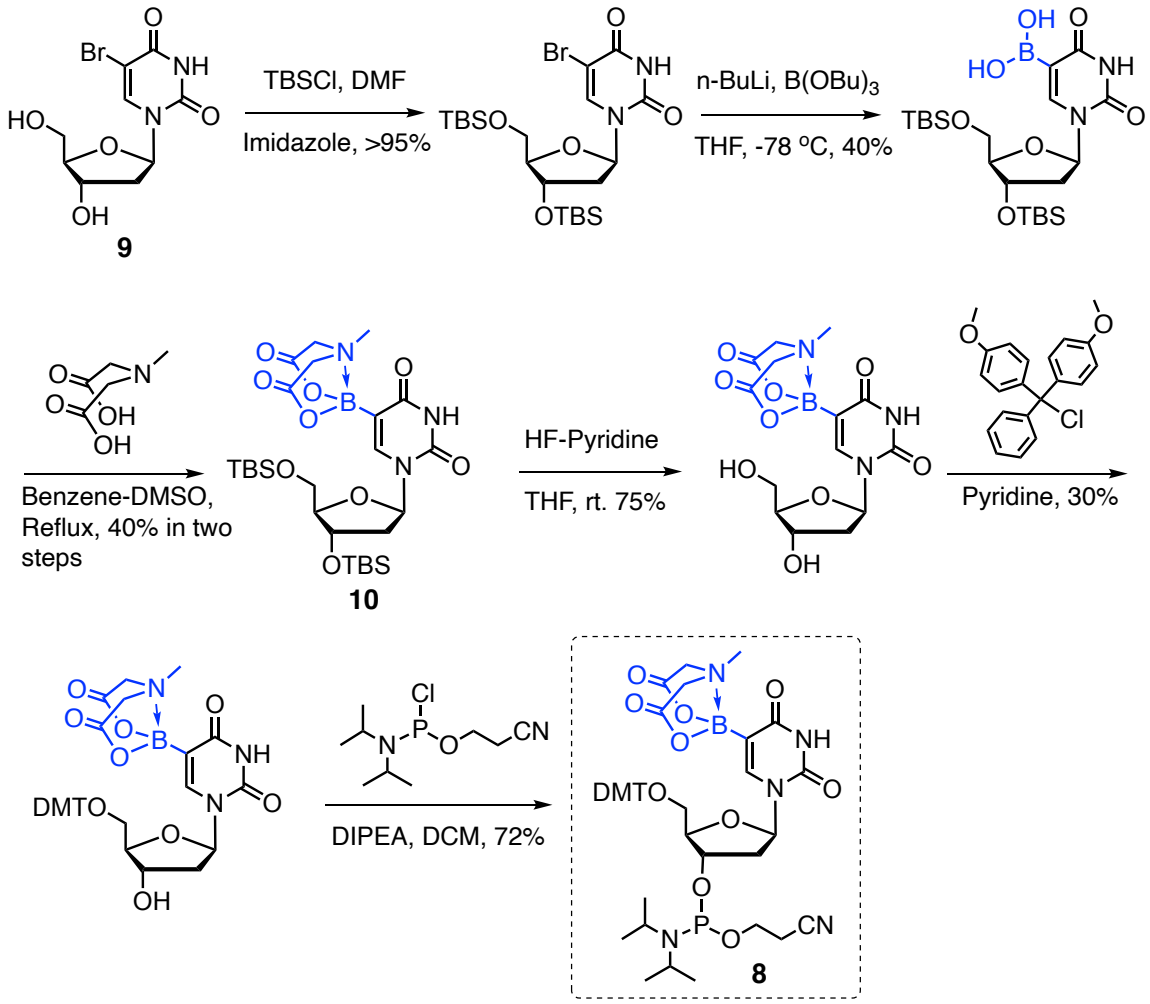
Under the dry conditions the free acid **6** (20 mg, 0.123 mmol) was dissolved in DCM (4 mL). N-hydroxysuccinimide (NHS) (23.94 mg, 0.185 mmol) and sodium sulfate (60 mg) was added to the solution and stirred for 1 hr at room temperature. EDC (14.38 mg, 0.092 mmol) was then added and stirred at r.t. overnight. The solution was concentrated and the residue was dissolved in 1:1 EtOAc:H2O, and extracted with EtOAc. The organic layer was concentrated and used the activated NHS ester **2** immediately to couple with purified oligonucleotides as described in section 5.

3. Synthesis and characterization of 5-dihydroxyboryldexoyuridine phosphoramidite 8

The synthesis of **8** as detailed in the scheme below was accomplished following the reported method.[3]



4. Synthesis and purification of oligonucleotides 11a-d. Antisense oligonucleotides were synthesized using standard RNA phosphoramidite monomers carrying 2'-OCH_3 (Glen Research) in an Expedite Nucleic Acid Synthesis System (PerSeptive Biosystems). Standard synthesis protocols were followed including standard coupling time (2 min) for the natural bases. To ensure good coupling with the modified phosphoramidites **7** and **8**, elongated coupling time (4 min 30 sec) was applied. Oxidation with 3-[(Dimethylaminomethylene)amino]-3H-1,2,4-dithiazole-5-thione (DDTT) in each cycle provided the thiophosphate linkages. Upon completion of the sequences, the protected oligonucleotides were treated with ammonium hydroxide (28% v/v) at room temperature for overnight for global deprotection and cleavage from the solid support. The final purification was carried out on HPLC on a C-18 column with the following gradient: Solvent A: 0.1 M TEAA pH 7, Solvent B: Acetonitrile; 0 min 5% B, 10 min 40% B, 15 min 100% B with a flow rate of 4 mL/min. All fractions were evaporated using a SpeedVac concentrator followed by freeze-drying with a lyophilizer. All ASOs were re-dissolved in nuclease free water and were confirmed by ESI LC-HRMS.

5. Synthesis and purification of fully assembled ASOs 1a-d. The oligonucleotides **11a-d** carrying a free amine at the 5'-end were coupled to the activated NHS ester **2** under following reaction conditions. *Coupling reaction of RNAs containing no 5boU unit:* 100 µL reaction scale with 100 µM RNA, 1 mM NHS-ester **2** dissolved in DMSO. Final volume reached by diluting with 100 mM sodium carbonate buffer pH 8.3. the reaction mixture was agitated at 700 rpm at room temperature overnight. *Coupling reaction of RNAs containing one 5boU:* The same procedure as no 5boU containing RNA was employed, except the reaction was performed at 37°C for 48 hrs. *Coupling reaction of RNAs containing two or more 5boU units:* 200 µL of 327 µM RNA was diluted with 700 µL of 100 mM sodium carbonate buffer pH 8.3 followed by addition of 100 µL of 600 mM NHS-ct linker **2**. The reaction mixture was agitated at 700 rpm at 37°C for 72 hrs. upon completion, the final products **1a-d** were purified by HPLC using the following method. Solvent A: 100 mM TEAA, Solvent B: ACN. 0 min 5% B, 10 min 40% B, 15 min 100% B with a flow rate of 4 mL/min, 100% B at 18 min, and returned to 5% B at 20 min. All the fully assembled ASOs were concentrated on a SpeedVac concentrator followed by freeze-drying with a lyophilizer and confirmed by ESI LC-HRMS.

6. Methods for CAPA Assay.[4]

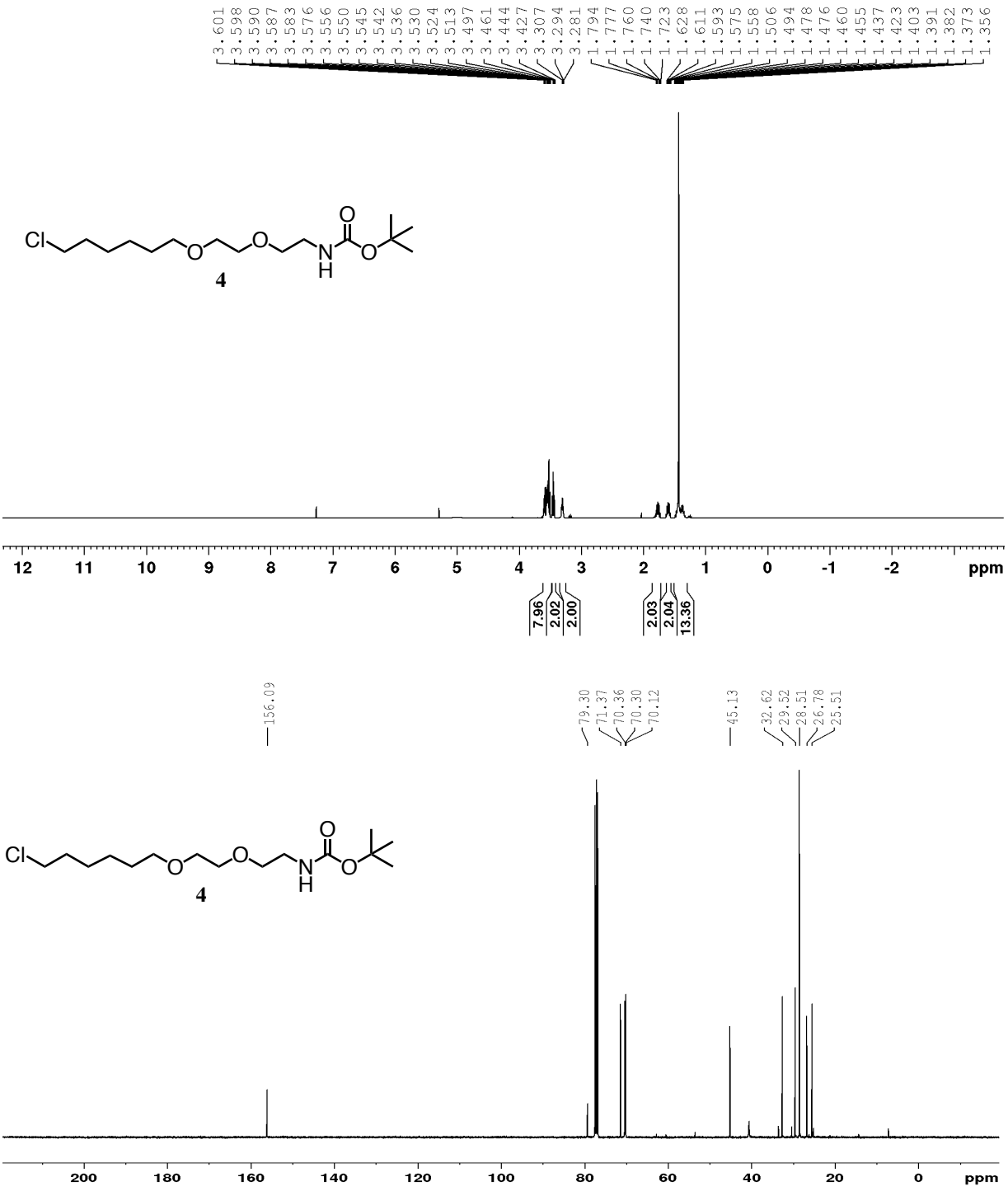
Chloroalkane Penetration Assay. CAPA was performed as described previously on adhered HaloTag-expressing cells (HGM) in a 96-well tissue culture treated plate.[5, 6] Briefly, serial dilutions of ct-oligonucleotides were prepared in nuclease-free sterile water. A sample of 25 μL of each concentration was added to the adhered cells with 100 μL of optiMEM. The molecules were incubated with cells at 37 ºC for the indicated incubation time to allow for internalization. Cells were washed with 50 μL fresh optiMEM for 15 min, chased with 50 μL of 5 mM ct-TMR for 15 min, and then washed with fresh optiMEM for 30 min. After the washes, cells were trypsinized with 40 μL of 0.05% clear trypsin, resuspended in 180 mL of PBS, and then analyzed by flow cytometry as described.[5, 6]

7. Methods for Splice-correcting Assay.[5, 7]

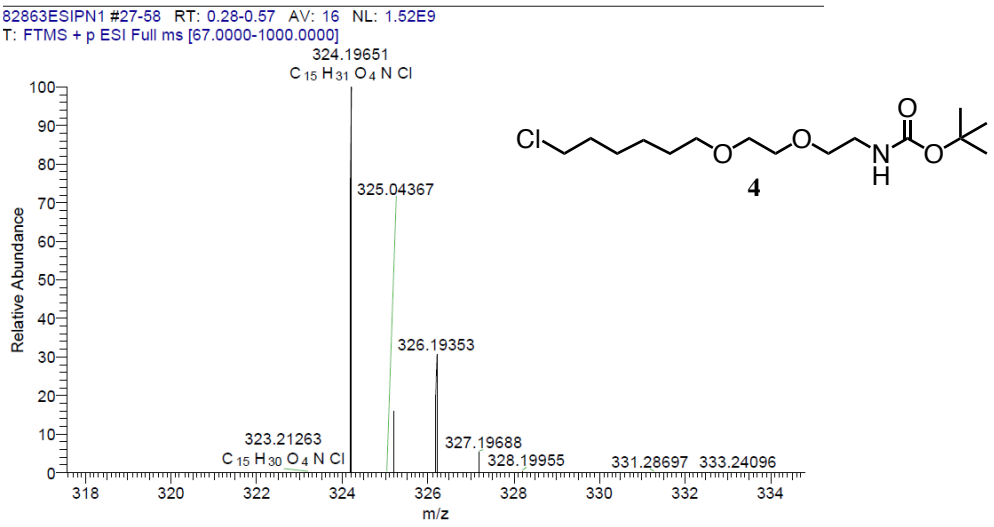
The splice-switching reporter cell line was a HeLa cell line that stably expresses a pre-mRNA transcript of the luciferase gene interrupted by a β-globin mRNA sequence, as described previously.[7, 8] his cell line is referred to as HeLa-Luc705, and was obtained from the UNC Tissue Culture Facility. The oligonucleotides (Sequence C: CCUCUUACCUCAGUUACA) were designed to splice out this transcript.[7, 8] HeLa-Luc705 cells were seeded in 96-well tissue culture treated plates at a density of 1.2 x 10^4 cells per well. At the start of the experiment, the cells were treated with serial dilutions of oligonucleotides in 100 μL optiMEM, and then incubated at 37 ºC for either 24 or 48 h. After the indicated incubation period, cells were washed with fresh optiMEM for 15 min, then washed with PBS for 15 min. Cells were lysed directly in the 96-well plate with 50 mL of 1x cell culture lysis reagent (Promega). 55 μL of luciferase assay reagent was added (Promega), samples were mixed well by pipetting and then incubated for 2 minutes at room temperature. Luciferase activity was measured with a Tecan Spark plate reader. Relative

luminescence units (RLU) for each sample were measured for each sample. The value for RLU for each treated sample was used to calculate a fold change over that of the untreated sample.

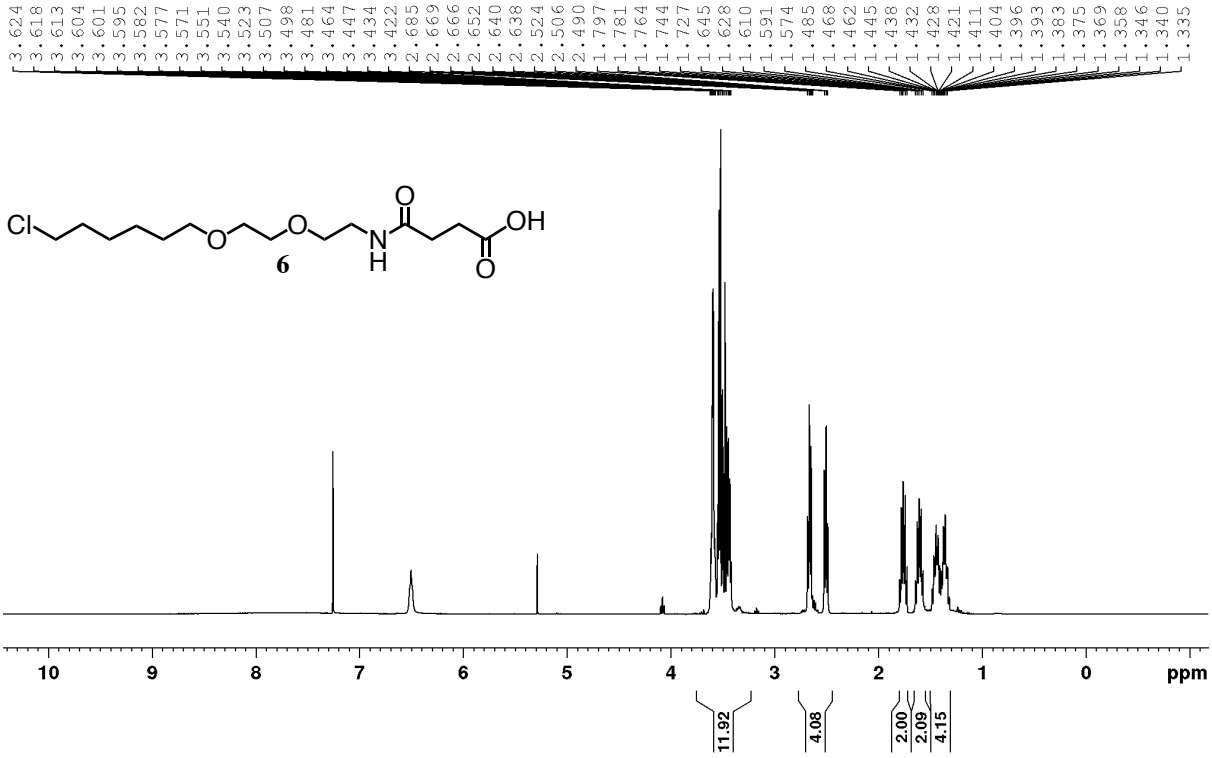
8. Supplementary Figures



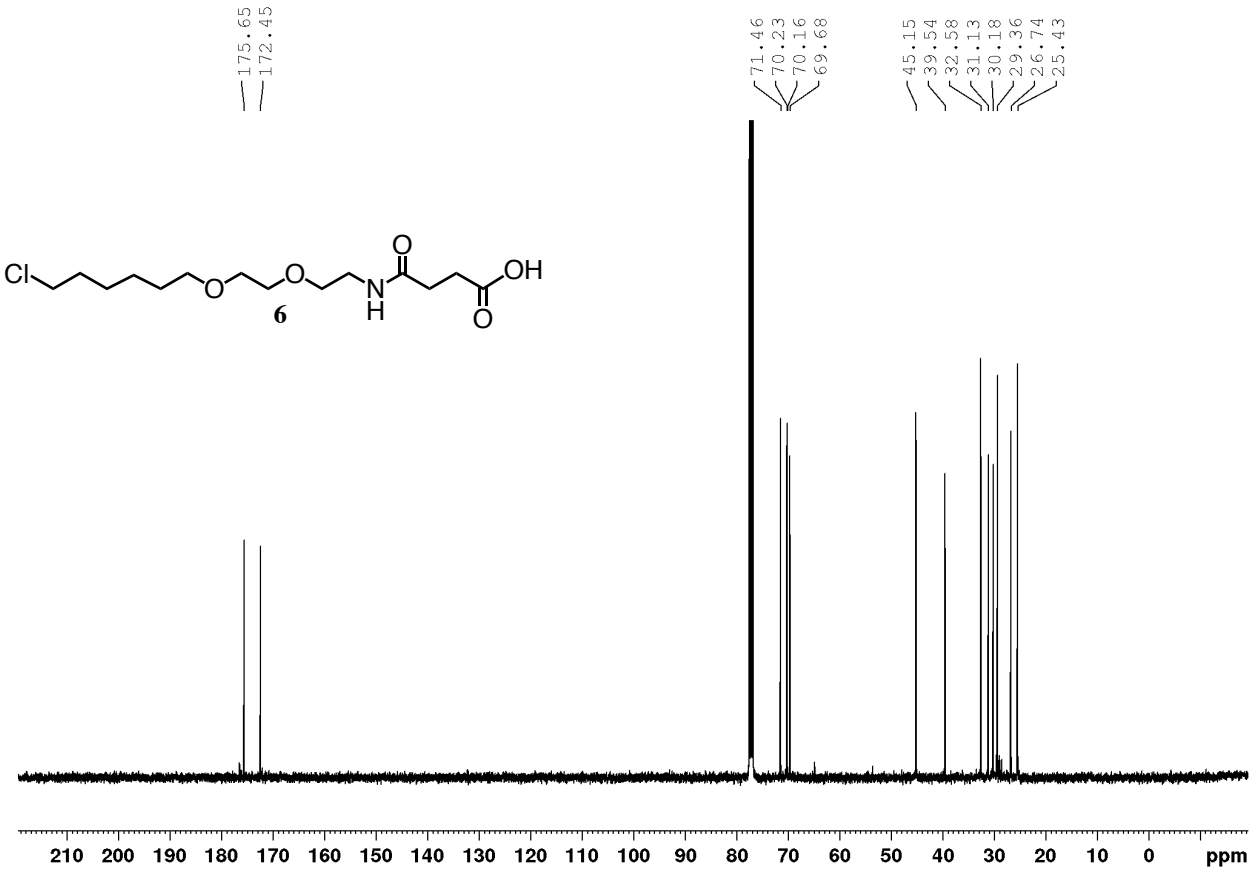
Supplementary Figure S1. ^{1}H and ^{13}C NMR spectra of compound **4** in $CDCl_3$.



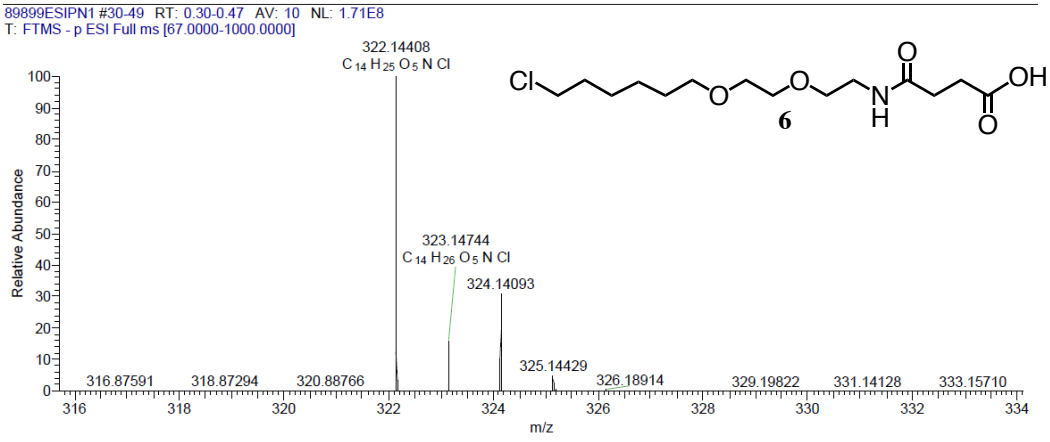
Supplementary Figure S2. ESI HRMS spectrum of compound **4**.



Supplementary Figure S3. ^{1}H NMR spectrum of compound **6** in $CDCl_3$.

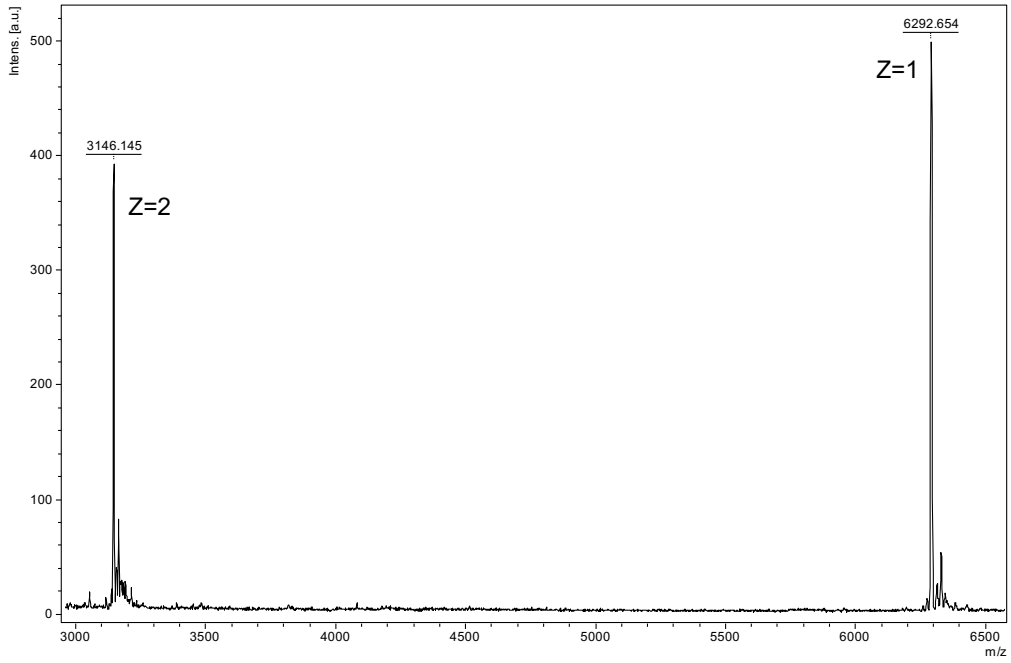


Supplementary Figure S4. ^{13}C spectrum of compound **6** in $CDCl_3$.



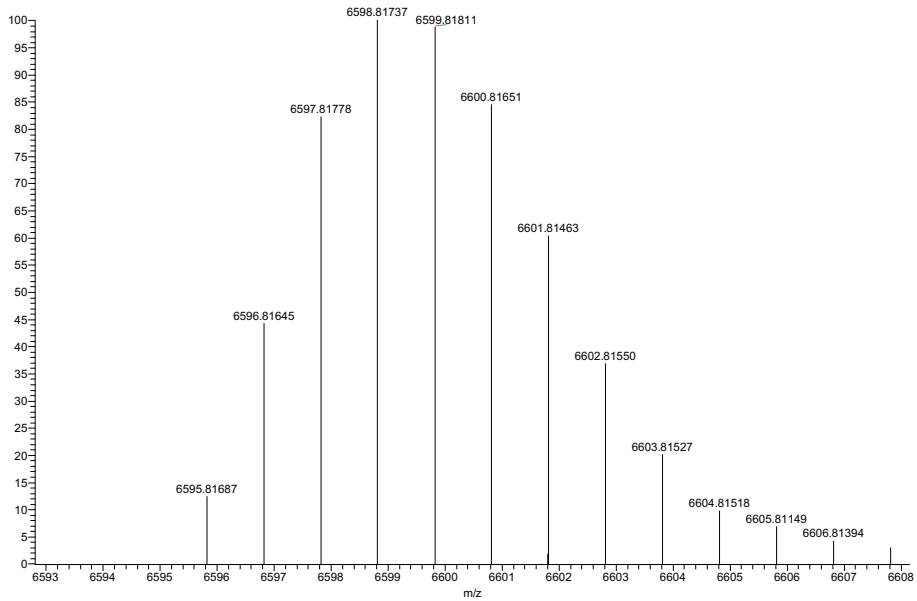
Supplementary Figure S5. ESI HRMS spectrum of compound **6**.

11a

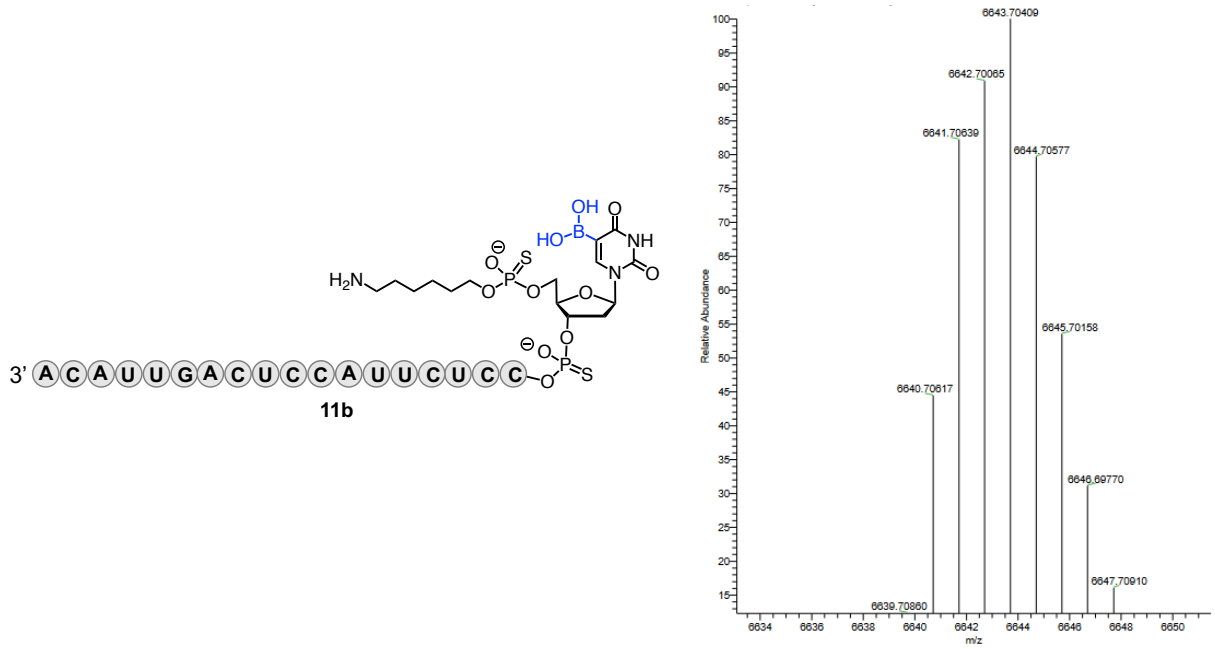


Supplementary Figure S6. ESI HRMS spectrum of compound **11a**. Chemical formula = $C_{191}H_{262}N_{59}O_{111}P_{18}S_{18}$; Expected Mol. Weight = 6294.098

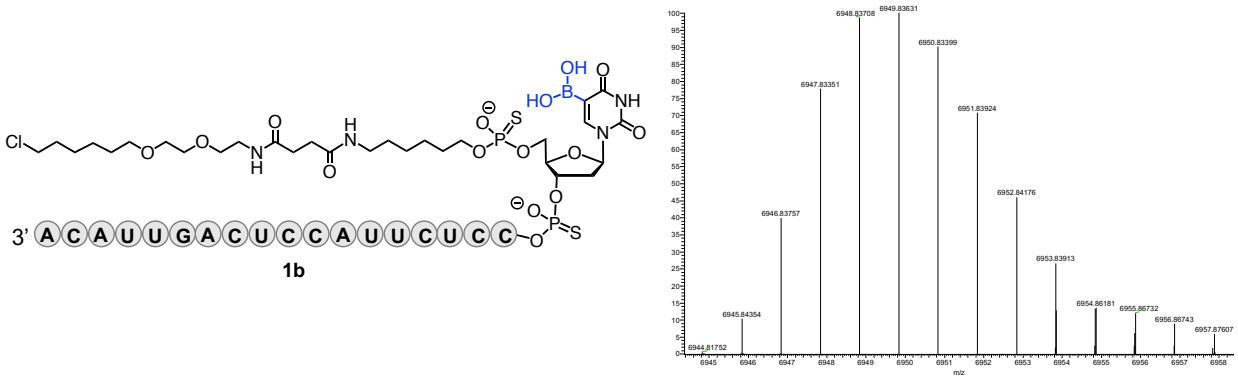
1a



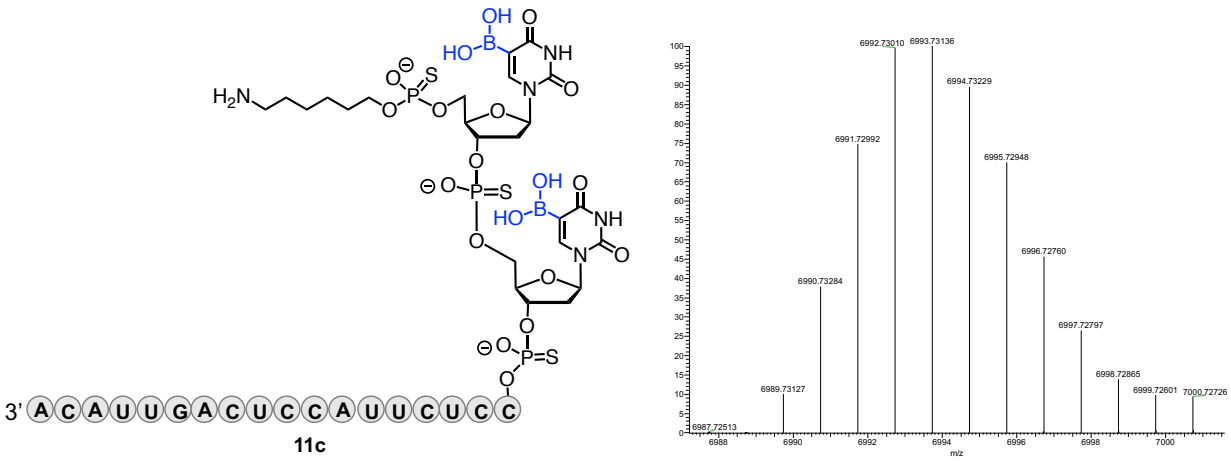
Supplementary Figure S7. ESI HRMS spectrum of compound **1a**. Chemical formula = $C_{205}H_{285}ClN_{60}O_{115}P_{18}S_{18}$; Expected Mol. Weight = 6599.897



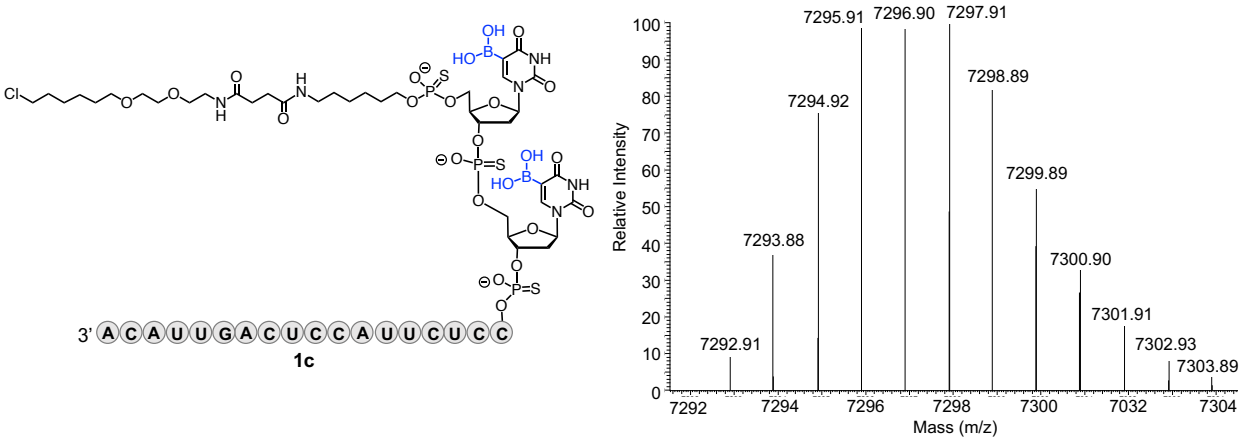
Supplementary Figure S8. ESI HRMS spectrum of compound **11b**. Chemical formula = $C_{200}H_{273}BN_{61}O_{119}P_{19}S_{19}$; Expected Mol. Weight = 6644.143



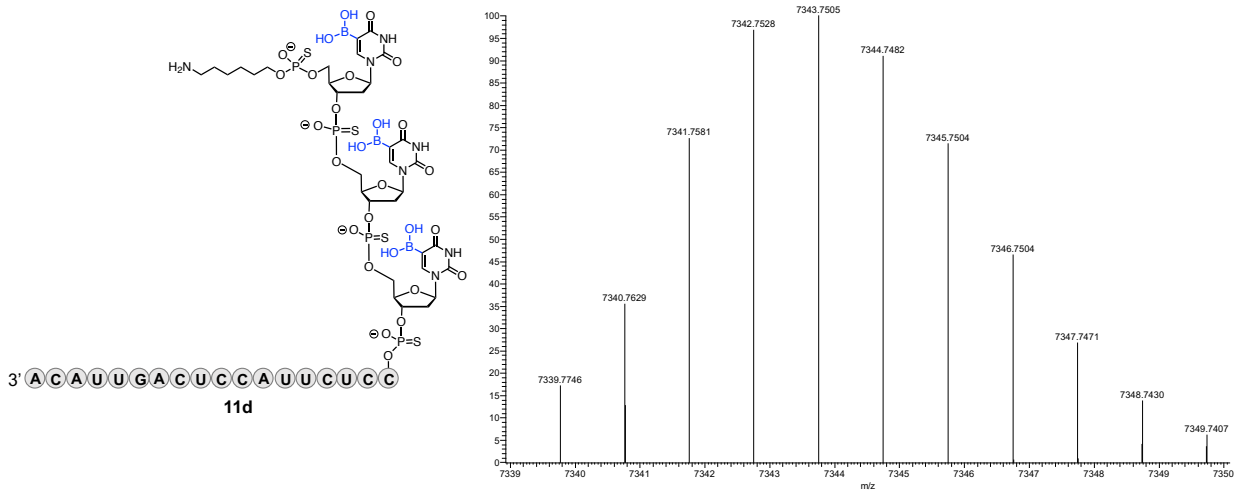
Supplementary Figure S9. ESI HRMS spectrum of compound **1b**. Chemical formula = $C_{214}H_{297}BClN_{62}O_{123}P_{19}S_{19}$; Expected Mol. Weight = 6949.942



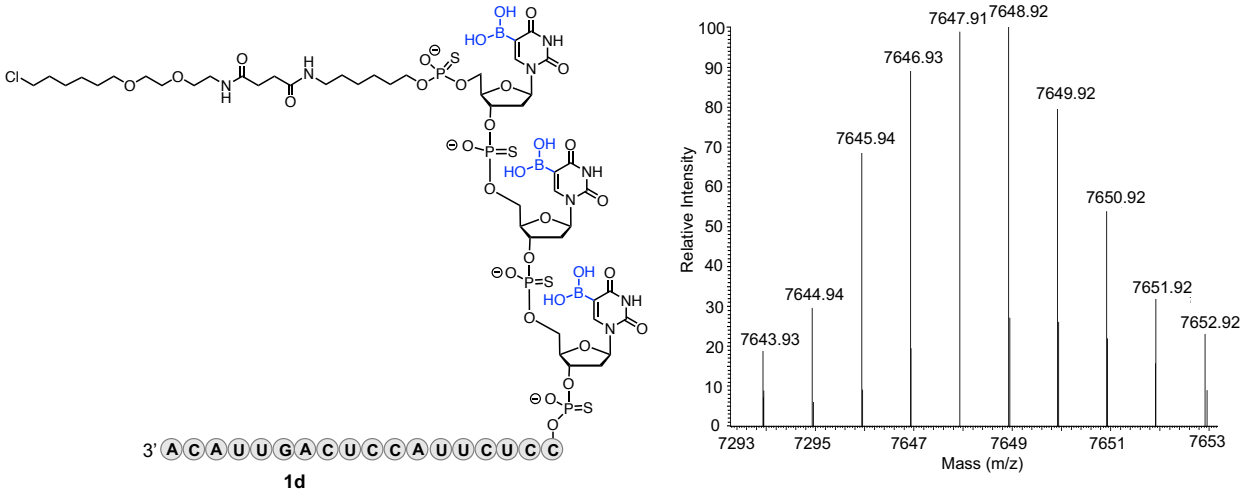
Supplementary Figure S10. ESI HRMS spectrum of compound **11c**. Chemical formula = $C_{209}H_{285}B_2N_{63}O_{127}P_{20}S_{20}$; Expected Mol. Weight = 6994.188



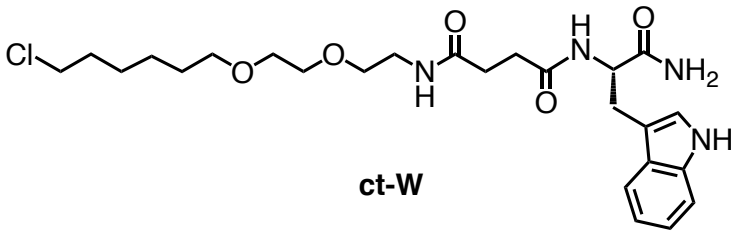
Supplementary Figure S11. ESI HRMS spectrum of compound **1c**. Chemical formula = $C_{223}H_{309}B_2ClN_{64}O_{131}P_{20}S_{20}$; Expected Mol. Weight = 7299.987



Supplementary Figure S12. ESI HRMS spectrum of compound **11d**. Chemical formula = $C_{218}H_{297}B_3N_{65}O_{135}P_{21}S_{21}$; Expected Mol. Weight = 7344.233



Supplementary Figure S13. ESI HRMS spectrum of compound **1d**. Chemical formula = $C_{232}H_{321}B_3ClN_{66}O_{139}P_{21}S_{21}$; Expected Mol. Weight = 7650.032



Supplementary Figure S14. Chemical structure of **ct-W**

9. References

1. G. V. Los, L. P. Encell, M. G. McDougall, D. D. Hartzell, N. Karassina, C. Zimprich, M. G. Wood, R. Learish, R. F. Ohana, M. Urh, D. Simpson, J. Mendez, K. Zimmerman, P. Otto, G. Vidugiris, J. Zhu, A. Darzins, D. H. Klaubert, R. F. Bulleit and K. V. Wood, *ACS chemical biology*, 2008, **3**, 373-382.
2. Y. Zhang, M. K. So, A. M. Loening, H. Yao, S. S. Gambhir and J. Rao, *Angewandte Chemie (International ed. in English)*, 2006, **45**, 4936-4940.
3. S. Kavoosi, D. Dey and K. Islam, *Organic letters*, 2019, **21**, 6614-6618.
4. L. Peraro, K. L. Deprey, M. K. Moser, Z. Zou, H. L. Ball, B. Levine and J. A. Kritzer, *Journal of the American Chemical Society*, 2018, **140**, 11360-11369.
5. K. Deprey, N. Batistatou, M. F. Debets, J. Godfrey, K. B. VanderWall, R. R. Miles, L. Shehaj, J. Guo, A. Andreucci, P. Kandasamy, G. Lu, M. Shimizu, C. Vargeese and J. A. Kritzer, *ACS chemical biology*, 2022, **17**, 348-360.
6. K. Deprey and J. A. Kritzer, *Methods in enzymology*, 2020, **641**, 277-309.
7. S. H. Kang, M. J. Cho and R. Kole, *Biochemistry*, 1998, **37**, 6235-6239.
8. Z. Dominski and R. Kole, *Proceedings of the National Academy of Sciences of the United States of America*, 1993, **90**, 8673-8677.