Synthetic Elaboration of Native DNA by RASS (SENDK)

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General Experimental
Tetrahydrofuran (THF), N,N-dimethylformamide (DMF), and dichloromethane (DCM) and acetonitrile (MeCN) were obtained by passing the previously degassed solvents through an activated alumina column. DMA was purchased from Sigma-Aldrich and used without further drying. (+)-Ψ and (−)-Ψ were obtained from Sigma-Aldrich. DIC (N,N'-disopropylcarbodiimide) was purchased from Oakwood. Deionized water was used in all the reactions, unless otherwise stated. All the other reagents were purchased at the highest commercial quality and used without further purification, unless otherwise stated. NaClO₄ was purchased at the highest commercial grade from Acros Organics. DNA tags was obtained from IDT, Inc., Coralville, IA. Recombinant All enzymes (Shrimp Alkaline Phosphatase (rSAP), ExoIII, T4PNK, and Lambda Exo) was obtained from New England Biolabs, Ipswich, MA. The Cut Smart buffer stock used enzymatic reactions was obtained from New England Biolabs, Ipswich, MA. UltraPure™ Agarose was obtained from Invitrogen, Carlsbad, CA. 50X TAE Buffer (Tris-acetate-EDTA) was obtained from Thermo Fisher Scientific, Waltham, MA. SYBR™ Safe DNA Gel Stain (10,000X) was obtained from Invitrogen, Carlsbad, CA. Gel Loading Dye, Purple (6X), no SDS was obtained from New England Biolabs, Ipswich, MA. Yields under normal organic conditions refer to chromatographically and spectroscopically (1H, 31P NMR) homogeneous material, unless otherwise stated. TLC was performed using 0.25 mm E. Merck silica plates (60F-254), using short-wave UV light as the visualizing agent, and phosphomolybdic acid or KMnO₄ and heat as developing agents. NMR spectra were recorded on Bruker DRX-600, DRX-500, and AMX-400 instruments and are calibrated using residual undeuterated solvent (CHCl₃ at 7.26 ppm 1H NMR, 77.16 ppm 13C NMR). The following abbreviations were used to explain multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. Column chromatography was performed using E. Merck silica gel (60, particle size 0.043–0.063 mm), and preparative TLC was performed on Merck silica plates (60F-254). High-resolution mass spectra (HRMS) were recorded on Agilent LC/MSD TOF mass spectrometer by electrospray ionization time of flight reflectron experiments. Melting points were recorded on a Fisher-Johns 12-144 melting point apparatus and are uncorrected.

No unexpected or unusually high safety hazards were encountered.

PBS: Phosphate buffered saline—was purchased from commercial and filtered before use. PBS is comprised of NaCl (137 mM), KCl (2.7 mM), Na₂HPO₄ (10 mM), KH₂PO₄ (1.8 mM), at pH 7.4.

Elute Buffer: DNA was eluted from resin using a sodium perchlorate buffer (1 M NaClO₄, 40 mM Tris, 20% MeOH, at pH 8.5). This was prepared from NaClO₄ (Acros Organics), and filtered after preparation. This buffer could be stored on the bench indefinitely.

HPLC-MS Analysis: Six microliter of the DNA solution was analyzed on a Waters H-Class LC with a Waters BEH C18 column (2.1x55 mm, 1.7 µm, 130 Å) using a gradient of 114 mM HFIP and 14 mM Et₃N in water (A) and methanol (B) (0.3 mL/min, 10-26 %B over 5 minutes) at 60º C. Peak identities were determined by ESI- using the 6- ion.

HPLC-TOF High Resolution Analysis: One microliter of the DNA solution was analyzed on a Waters I-Class LC with a Waters BEH C18 column (2.1x55 mm, 1.7 µm, 130 Å) using a gradient
of 114 mM HFIP and 14 mM Et₃N in water (A) and methanol (B) (0.3 mL/min, 10-26 %B over 10 minutes) at 60º C. Peak identities were determined by ESI- using the 3- ion.

**Deconvolution:** data visualization and integration were performed with Mass Lynx V4.1 software.

**Conversion determination:** the yields of on-DNA products were determined from UV absorbance trace (260 nm) peak area using the equation: Yield% = UV(prod)/UV(total DNA recovered), while ignoring UV coefficient difference for all DNA products and assuming 100% of DNA total recovery. While determining yields, any non-oligo material (as determined by close examination of mass spectra) that absorbed UV 260 nm was subtracted out of the final yield calculation. These peaks were determined to not contain oligo material when a DNA indicative mass spectra was not observed, and are usually attributed to small molecules not removed during the ethanol precipitation. These peaks were generally found at or before 1 minute retention time during analysis.

**Preparative HPLC:** Was performed on the Waters H Class instrument described above using customized gradients for each compound of interest and an automatic divert valve.
**DNA starting materials.** All DNA starting materials used in this paper and their structures are shown below. DNA was purchased from IDT.

Model DNA (1): 5’-TTCCGAGTCAAAAATGACTCGG/3Phos/-3’

Model DNA (2): /5Phos/CCGAGTCAAAAATGACTCGGTT

Model DNA (SI-1): CCGAGTCAAAAATGACTCGG/3Phos/

Model DNA (SI-2): TTTCCGAGTCAAAAATGACTCGG/3Phos/
Model DNA (SI-3): TCCGAGTCAAAAATGACTCGG/3Phos/

Model DNA (SI-4): ATCCGAGTCAAAAATGACTCGG/3Phos/

Model DNA (SI-5): CTCCGAGTCAAAAATGACTCGG/3Phos/

Model DNA (SI-6): GTCCGAGTCAAAAATGACTCGG/3Phos/

Model DNA (SI-7): /5Phos/CCGAGTCAAAAATGACTCGG
Model DNA (SI-8): 5Phos/CCGAGTCAAAAATGACTCGGT

Model DNA (SI-9): 5Phos/CCGAGTCAAAAATGACTCGGTTT

Model DNA (SI-10): 5Phos/CCGAGTCAAAAATGACTCGGTA

Model DNA (SI-11): 5Phos/CCGAGTCAAAAATGACTCGGTC

Model DNA (SI-12): 5Phos/CCGAGTCAAAAATGACTCGGTG
Vitravene Analog DNA (46): G*C*G*T*T*G*C*T*C*T*C*T*C*T*C*T*C*T*T*G*C*G/3phos/
* indicates phosphorothioate (PS) linkage

ATACCAGCTTATTCAATTAGCAACATGAGGGGGGATAGAGGGGGTGGGTCTCTCGG
CT

Chemical Formula: C_{236}H_{289}N_{35}O_{25}P_{11}
Molecular Weight: 6767.65
Exact Mass: 6707.55
Model DNA (60): /5Phos/GAATTCCAAAAATGGAATTCGG

cTegsedi (63): GGGATTTCATGTAACCAAGA/3Phos/

TaqMan Probe Starting Material (66): /5Phos/CCCCAGTCTCTGTCAGCACTCCCTTC

2019-nCov-N1-P Starting Material (SI-13): /5Phos/ACCCCGATTACGGTTGGTGGACC

2019-nCov-N2-P Starting Material (SI-14): /5Phos/ ACAATTTGCCCCCAGCGCTTCAG
2019-nCov-N3-P Starting Material (SI-15): /5Phos/CCCCAGTCTCTGTCAGCACTCCCTTC

Nonphosphorylated DNA (SI-16): CCGAGTAAAAATGACTCGGTT
High Tm DNA Hairpin (SI-17):
CACTAGGGATGCATCGTCATCTTTTTGATGACGATGCATCCCTAGTGTCCG/3Phos/

Chemical Formula: $C_{491}H_{630}N_{184}O_{313}P_{51}$
Exact Mass: 15719.56
Molecular Weight: 15727.12
hNE Aptamer (56): /5Phos/ aggacgatgcggCAGGCTAGTGCCTACGAGTTATTTGTCAC

Chemical Formula: C_{412}H_{516}N_{164}O_{253}P_{42}

Exact Mass: 13108.15

Molecular Weight: 13114.45
Template Strand (SI-18):
/5Phos/atagtgtcagatgacaaatggctactacgaagagctaccgacgagctgtagtggtgagtgggcaaaatgaagaagctca
gccgagatgtcactttctattacctaggaactgccagaagctactctctagcaacttggcatcacttgaactctgtagtggtgcaact
agggagccttgaatacacccaaa

Primer 1 (SI-19): /5Phos/aacccatacgatgccttttgt

Primer 2 (SI-20): ctactacggaagagctaccgac
Synthesis and Characterization of Ψ-modules

General Procedure 1 - Synthesis of Ψ-modules

The Ψ-modules were prepared as follow unless otherwise stated. Alcohol (1.0 equiv.) and (−)-Ψ (1.3 equiv.) were dissolved in anhydrous MeCN or DCM (0.1 M) in a flame-dried round-bottom flask. DBU (1.2 equiv.) was added to the reaction mixture while stirring. After 5-10 minutes, the crude reaction mixture was diluted with EtOAc or Et₂O and transferred to a separatory funnel. The organic layer was washed with H₂O, saturated aqueous KH₂PO₄ and brine. After drying over MgSO₄ and filtration, the solvent was removed in vacuo. The crude product was purified by silica gel column chromatography unless otherwise stated. Protocol adapted from.¹

No unexpected or unusually high safety hazards were encountered.
Source of Alcohols used in Synthesis of Ψ-modules

| Commercially Availible | Synthesized |
|------------------------|-------------|
| ![Image](image1.png)   | ![Image](image2.png) |

SI-49  SI-50  SI-51
SI-54  SI-57  SI-55
SI-56  SI-58  SI-59
SI-60  SI-62
Synthesis of Ψ-1 through Ψ-20

Ψ-1

Prepared according to **General Procedure 1** using 2-phenylethan-1-ol (2.44 g, 20.0 mmol). Purification using 50% DCM in hexanes afforded Ψ-1 (6.84 g, 18.6 mmol, 93% yield).

**Physical State:** Crystaline Solid;

**R_f = 0.50** (50% DCM/hexanes);

**1H NMR (600 MHz, Chloroform-d):** δ 7.32 (tt, J = 7.8, 1.4 Hz, 2H), 7.29 – 7.22 (m, 3H), 5.01 (q, J = 1.4 Hz, 1H), 4.89 – 4.84 (m, 1H), 4.47 – 4.38 (m, 2H), 4.36 (ddd, J = 14.2, 10.3, 7.1 Hz, 1H), 3.04 (t, J = 7.1 Hz, 2H), 2.60 (d, J = 6.2 Hz, 1H), 2.30 – 2.23 (m, 1H), 2.10 (td, J = 13.5, 4.3 Hz, 1H), 1.97 (ddt, J = 15.0, 4.3, 2.1 Hz, 1H), 1.93 – 1.84 (m, 2H), 1.83 – 1.74 (m, 4H), 1.72 (s, 3H);

**13C NMR (151 MHz, Chloroform-d):** δ 144.61, 136.67, 128.55, 128.05, 126.23, 111.33, 85.20, 68.65, 68.60, 64.76, 38.42, 36.27, 36.22, 33.31, 33.26, 27.34, 27.24, 22.95, 22.27, 21.25;

**31P NMR (162 MHz, Chloroform-d):** δ 101.30;

**HRMS (ESI-TOF, m/z):** Calcd for [C_{18}H_{25}O_{2}PS_{2}+H]^+ 369.1112; Found 369.1114.
Prepared according to General Procedure 1 using \textit{N-Boc-L-prolinol} (0.40 g, 2.0 mmol). Purification using a gradient of 50% DCM in hexanes afforded Ψ-2 (0.431 g, 0.96 mmol, 48% yield).

\textbf{Physical State}: Colorless Oil. 

$R_f = 0.40 \, (100\% \text{ DCM})$;  

$^{1}$H NMR (600 MHz, Chloroform-$d$): $\delta$ 4.99 (q, $J = 1.5$ Hz, 1H), 4.87 (s, 1H), 4.40 (d, $J = 12.3$ Hz, 1H), 4.16 (td, $J = 9.6, 3.4$ Hz, 2H), 3.98 (d, $J = 63.5$ Hz, 1H), 3.46 – 3.23 (m, 2H), 2.62 – 2.51 (m, 1H), 2.28 (dd, $J = 13.1, 3.7$ Hz, 1H), 2.10 (td, $J = 13.5, 4.2$ Hz, 1H), 2.02 – 1.83 (m, 6H), 1.83 – 1.66 (m, 8H), 1.47 (d, $J = 8.5$ Hz, 9H);  

$^{13}$C NMR (151 MHz, Chloroform-$d$): $\delta$ 154.66, 154.40, 145.39, 145.20, 111.83, 85.85, 80.03, 79.58, 68.70, 68.65, 68.23, 65.56, 65.37, 56.49, 46.98, 46.82, 39.04, 34.02, 33.96, 28.86, 28.66, 27.82, 27.74, 23.94, 23.57, 23.19, 22.82, 21.82;  

$^{31}$P NMR (162 MHz, Chloroform-$d$): $\delta$ 101.67, 101.19;  

HRMS (ESI-TOF, m/z): Calcd for [C$_{20}$H$_{34}$NO$_4$PS$_2$+H]$^+$ 464.1694; Found 464.1689.
\[ \Psi-3 \]

Prepared according to **General Procedure 1** using \textit{2-(2-(2-azidoethoxy)ethoxy)ethan-1-ol} (0.70 g, 4.0 mmol). Purification using a gradient of 50-100\% DCM in hexanes followed by 0-10\% EtOAc in DCM afforded \( \Psi-3 \) (0.809 g, 1.92 mmol, 48\% yield).

**Physical State:** Colorless oil;

\( R_f = 0.70 \) (100\% DCM);

**\( ^1H \) NMR (400 MHz, Chloroform-\textit{d}):** \( \delta \) 4.98 (s, 1H), 4.87 (s, 1H), 4.44 (dd, \( J = 12.8, 3.3 \) Hz, 1H), 4.41 – 4.32 (m, 1H), 4.32 – 4.20 (m, 1H), 3.80 – 3.60 (m, 9H), 3.39 (t, \( J = 4.1 \) Hz, 2H), 2.57 (s, 1H), 2.27 (d, \( J = 13.6 \) Hz, 1H), 2.21 – 2.02 (m, 1H), 2.00 – 1.83 (m, 3H), 1.78 (s, 4H), 1.69 (d, \( J = 2.7 \) Hz, 3H);

**\( ^{13}C \) NMR (151 MHz, Chloroform-\textit{d}):** \( \delta \) 145.25, 111.86, 85.83, 70.83, 70.79, 70.26, 70.21, 70.17, 67.67, 67.62, 65.20, 50.83, 39.00, 33.86, 33.80, 27.90, 27.79, 23.50, 22.81, 21.80;

**\( ^{31}P \) NMR (162 MHz, Chloroform-\textit{d}):** \( \delta \) 102.15;

**HRMS (ESI-TOF, \textit{m/z}):** Calcd for \([C_{16}H_{28}N_{3}O_{4}PS_2]+H\] 422.1337; Found 422.1327.
Ψ-4

Prepared according to General Procedure 1 using SI-49 (0.326 g, 2.0 mmol). Purification using 15% EtOAc in hexane afforded Ψ-4 (0.60 g, 1.5 mmol, 75% yield). Due to the instability of this compound, it was used directly after a crude purification. Crude spectroscopic data could be obtained but not in its pure form.

**Physical State:** Amourphous Crystalline Solid;

\[ R_f = 0.56 \text{ (15\% EtOAc/Hexane);} \]

\(^1\)H NMR (400 MHz, Chloroform-d): \( \delta \ 7.25 - 7.12 \text{ (m, 2H)}, \ 7.02 - 6.85 \text{ (m, 2H)}, \ 4.98 \text{ (s, 1H)}, \ 4.82 \text{ (s, 1H)}, \ 4.32 \text{ (ddt, } J = 17.8, 10.7, 6.6 \text{ Hz, 3H)}, \ 2.97 \text{ (q, } J = 5.1, 3.4 \text{ Hz, 2H)}, \ 2.56 \text{ (d, } J = 6.4 \text{ Hz, 1H)}, \ 2.22 \text{ (d, } J = 13.1 \text{ Hz, 1H)}, \ 2.05 \text{ (ddd, } J = 14.2, 10.9, 4.4 \text{ Hz, 1H)}, \ 1.88 \text{ (dtt, } J = 18.6, 13.3, 5.3 \text{ Hz, 3H)}, \ 1.80 - 1.58 \text{ (m, 7H)};\]

\(^3\)P NMR (162 MHz, Chloroform-d): \( \delta \ 101.32; \)

HRMS (ESI-TOF, m/z): Calcd for \([\text{C}_{18}\text{H}_{24}\text{N}_3\text{O}_2\text{PS}_2]^+\) 410.1126; Found 410.1135.
Prepared according to General Procedure 1 using 4-pentynol (1.26 g, 15.0 mmol). Purification using a gradient of 15-20% DCM in hexanes afforded Ψ-5 (4.56 g, 13.8 mmol, 92%).

Physical State: Colorless Oil;
Rf = 0.46 (50 % DCM in Hexanes);

^1H NMR (600 MHz, Chloroform-d): δ 5.01 (q, J = 1.5 Hz, 1H), 4.90 – 4.86 (m, 1H), 4.44 (dt, J = 12.7, 3.3 Hz, 1H), 4.32 – 4.25 (m, 1H), 4.27 – 4.20 (m, 1H), 2.58 (t, J = 6.4 Hz, 1H), 2.30 (qd, J = 8.4, 7.7, 2.3 Hz, 3H), 2.11 (td, J = 13.5, 4.3 Hz, 1H), 1.99 – 1.84 (m, 6H), 1.80 – 1.72 (m, 4H), 1.70 (s, 3H);

^13C NMR (151 MHz, Chloroform-d): δ 144.63, 111.32, 85.22, 82.34, 68.73, 66.76 (d, J = 7.9 Hz), 64.87, 38.44, 33.34 (d, J = 9.0 Hz), 28.54 (d, J = 7.5 Hz), 27.27 (d, J = 15.5 Hz), 22.97, 22.20, 21.24, 14.49;

^31P NMR (162 MHz, Chloroform-d): δ 101.32;

[a]_D^20 = -20 (c 2.0, CHCl3);

HRMS (ESI-TOF, m/z): Calcd for [C_{15}H_{23}O_{2}PS_{2}+H]^+ 331.0955; Found 331.0963.
Prepared according to General Procedure 1 using 2,2,3,3,4,4,4-heptafluorobutan-1-ol (0.40 g, 2.0 mmol). Purification using a gradient of 10-20% DCM in hexanes afforded Ψ-6 (0.249 g, 0.56 mmol, 28% yield).

**Physical State:** Colorless Oil;

$R_f = 0.55$ (50% DCM/Hexane);

$^1$H NMR (400 MHz, Chloroform-$d$): $\delta$ 5.01 (d, $J = 2.3$ Hz, 1H), 4.86 (s, 1H), 4.64 (dd, $J = 25.1, 12.6$ Hz, 1H), 4.57 – 4.44 (m, 2H), 2.60 (s, 1H), 2.31 (d, $J = 13.6$ Hz, 1H), 2.14 (td, $J = 13.3, 4.0$ Hz, 1H), 2.04 – 1.65 (m, 10H);

$^{13}$C NMR (151 MHz, Chloroform-$d$): 144.78, 120.53, 118.85, 118.62, 118.40, 116.94, 116.72, 116.50, 115.79, 115.58, 115.53, 115.38, 115.32, 115.04, 114.82, 114.59, 114.08, 114.02, 113.88, 113.82, 113.67, 113.62, 112.37, 112.17, 112.11, 111.96, 110.89, 110.67, 110.41, 109.13, 108.91, 108.68, 108.65, 108.43, 107.37, 107.15, 106.89, 106.67, 86.06, 66.40, 63.13, 63.09, 62.94, 62.90, 62.76, 62.71, 39.01, 33.83, 33.77, 27.75, 27.65, 23.56, 22.54, 21.88;

$^{19}$F NMR (376 MHz, Chloroform-$d$): $\delta$-81.90 (t, $J = 9.2$ Hz), -121.62 (dq, $J = 11.3, 7.5, 5.7$ Hz), -128.39 (d, $J = 3.6$ Hz);

$^{31}$P NMR (162 MHz, Chloroform-$d$): $\delta$ 103.51;

HRMS (ESI-TOF, m/z): Calcd for $[C_{14}H_{18}F_7O_2PS_2+H]^+$ 447.0452; Found 447.0458.
**Ψ-7**

Prepared according to **General Procedure 1** using **SI-51** (0.90 g, 3.0 mmol). Purification using 25% EtOAc in hexanes afforded **Ψ-7** (1.0 g, 1.82 mmol, 61% yield).

**Physical State:** colorless solid; 
**Rf** = 0.28 (30% EtOAc/Hexane);

**1H NMR (400 MHz, Chloroform-d):** 8.17 – 8.11 (m, 1H), 7.90 – 7.84 (m, 1H), 7.80 – 7.72 (m, 2H), 5.25 (s, 1H), 4.99 (s, 1H), 4.86 (s, 1H), 4.45 – 4.37 (m, 1H), 4.13 – 4.10 (m, 2H), 3.10 (q, J = 6.8 Hz, 2H), 2.58 (s, 1H), 2.27 (d, J = 13.5 Hz, 1H), 2.15 – 2.05 (m, 1H), 1.90 (td, J = 21.4, 18.7, 14.3 Hz, 4H), 1.78 (s, 3H), 1.69 (d, J = 2.0 Hz, 3H), 1.62 (d, J = 6.5 Hz, 2H), 1.53 (d, J = 7.0 Hz, 2H), 1.33 (s, 4H);

**13C NMR (151 MHz, Chloroform-d):** 148.22, 145.25, 133.84, 133.73, 132.95, 131.21, 125.54, 111.83, 85.79, 68.72, 68.67, 65.41, 43.85, 39.00, 33.92, 33.86, 29.99, 29.95, 29.58, 27.91, 27.80, 26.08, 25.13, 23.52, 22.79, 21.80;

**31P NMR (162 MHz, Chloroform-d):** δ 100.65;

**HRMS (ESI-TOF, m/z):** Calcd for [C_{22}H_{33}N_{2}O_{6}PS_{3}+H]^+ 549.1317; Found 549.1326.
Prepared according to General Procedure 1 using SI-50 (1.35 g, 4.67 mmol). Purification using 100% DCM afforded Ψ-8 (1.54 g, 3.15 mmol, 68% yield).

**Physical State:** Light Yellow Oil; 

**Rf** = 0.50 (100% DCM); 

**1H NMR (600 MHz, Chloroform-d):** δ 8.45 (ddd, J = 4.9, 1.9, 0.9 Hz, 1H), 7.71 (dt, J = 8.1, 1.1 Hz, 1H), 7.64 (td, J = 7.7, 1.8 Hz, 1H), 7.08 (ddd, J = 7.4, 4.8, 1.1 Hz, 1H), 4.98 (q, J = 1.4 Hz, 1H), 4.87 – 4.84 (m, 1H), 4.42 (dt, J = 12.8, 3.3 Hz, 1H), 4.19 – 4.07 (m, 2H), 2.79 (t, J = 7.3 Hz, 2H), 2.57 (s, 1H), 2.27 (ddt, J = 15.0, 3.2, 1.7 Hz, 1H), 2.09 (td, J = 13.5, 4.2 Hz, 1H), 1.98 – 1.91 (m, 1H), 1.91 – 1.82 (m, 2H), 1.79 – 1.73 (m, 4H), 1.73 – 1.63 (m, 7H), 1.42 (pd, J = 7.1, 3.0 Hz, 2H), 1.41 – 1.31 (m, 2H);

**13C NMR (151 MHz, Chloroform-d):** δ 160.67, 149.73, 145.22, 137.10, 120.67, 119.72, 111.86, 85.71, 68.86 (d, J = 8.2 Hz), 65.35, 38.96 (d, J = 14.8 Hz), 33.90 (d, J = 8.8 Hz), 30.06 (d, J = 6.8 Hz), 28.86, 28.07, 27.91, 27.81, 25.30, 23.54, 22.81, 21.80;

**31P NMR (162 MHz, Chloroform-d):** δ 101.01;

**HRMS (ESI-TOF, m/z):** Calcd for [C_{21}H_{32}NO_2PS_4+H]^+ 490.1132; Found 490.1140.
Prepared according to General Procedure 1 using SI-54 (1.0 g, 4.67 mmol). Purification using a gradient of 20-30% EtOAc in hexanes afforded Ψ-9 (0.80 g, 1.3 mmol, 28% yield).

**Physical State:** White foam;

$R_f = 0.52$ (50% EtOAc/hexanes)

$^1$H NMR (400 MHz, Chloroform-$d$): $\delta$ 8.41 (s, 1H), 7.54 (s, 1H), 6.39 (dd, $J = 8.9$, 5.5 Hz, 1H), 5.34 (dd, $J = 11.7$, 5.7 Hz, 1H), 5.07 (s, 1H), 4.92 (s, 1H), 4.47 (d, $J = 12.7$ Hz, 1H), 4.26 (s, 1H), 3.97 – 3.85 (m, 2H), 2.59 (s, 1H), 2.49 (dd, $J = 13.8$, 5.5 Hz, 1H), 2.33 (d, $J = 13.5$ Hz, 1H), 2.23 – 2.07 (m, 2H), 2.04 – 1.86 (m, 6H), 1.80 (s, 3H), 1.77 – 1.60 (m, 4H), 0.92 (t, $J = 2.7$ Hz, 9H), 0.13 (t, $J = 2.8$ Hz, 6H);

$^{13}$C NMR (151 MHz, Chloroform-$d$): $\delta$ 163.73, 150.33, 144.77, 135.24, 112.34, 111.34, 86.26, 86.22, 86.17, 86.04, 84.78, 79.88, 79.83, 66.05, 63.55, 39.72, 39.69, 38.96, 33.81, 33.74, 28.32, 27.90, 27.80, 26.09, 23.50, 22.77, 21.89, 18.48, 12.62, -5.23, -5.25, -5.42;

$^{31}$P NMR (162 MHz, Chloroform-$d$): $\delta$ 101.18.

HRMS (ESI-TOF, m/z): Calcd for $[C_{26}H_{43}N_2O_6PS_2Si+H]^+$ 603.2148; Found 603.2147.
Prepared according to **General Procedure 1** using SI-56 (0.368 g, 1.0 mmol). Purification using a gradient of 20-50% EtOAc in hexanes afforded **Ψ-10** (0.382 g, 0.62 mmol, 62% yield).

**Physical State:** dark orange foam;

$R_f = 0.58$ (50% EtOAc/Hexane);

$^1$H NMR (400 MHz, Chloroform-\textit{d}): $\delta$ 8.06 – 7.68 (m, 6H), 6.76 (d, $J = 8.6$ Hz, 2H), 6.19 (s, 1H), 5.00 (s, 1H), 4.87 (s, 1H), 4.44 (d, $J = 12.9$ Hz, 1H), 4.17 (t, $J = 7.9$ Hz, 2H), 3.48 (d, $J = 6.9$ Hz, 3H), 3.11 (p, $J = 1.9$ Hz, 6H), 2.57 (s, 1H), 2.29 (s, 1H), 2.16 – 2.03 (m, 1H), 1.92 (dd, $J = 25.4$, 14.3 Hz, 2H), 1.71 (dd, $J = 32.2$, 18.3 Hz, 10H), 1.45 (s, 5H);

$^{13}$C NMR (151 MHz, Chloroform-\textit{d}): $\delta$ 167.17, 154.94, 152.98, 145.23, 143.73, 134.92, 127.90, 125.67, 122.34, 111.89, 111.73, 85.78, 68.90, 68.85, 65.38, 40.50, 40.13, 39.02, 33.93, 33.88, 30.11, 30.07, 29.73, 27.93, 27.82, 26.58, 25.39, 23.54, 22.81, 21.81;

$^{31}$P NMR (162 MHz, Chloroform-\textit{d}): $\delta$ 101.78;

HRMS (ESI-TOF, m/z): Calcd for $[\text{C}_{31}\text{H}_{43}\text{N}_{4}\text{O}_{3}\text{PS}_{2}+\text{H}]^+$ 615.2592; Found 615.2593.
Prepared according to General Procedure 1 using SI-57 (0.292 g, 1.0 mmol). Purification using a gradient of 75-100% EtOAc in hexanes afforded Ψ-11 (0.35 g, 0.65 mmol, 65% yield).

**Physical State:** Amorphous solid;

$R_f = 0.32$ (100% EtOAc);

$^1$H NMR (400 MHz, Chloroform-$d$): δ 8.52 (s, 1H), 7.94 (d, $J = 9.0$ Hz, 1H), 7.17 (d, $J = 8.9$ Hz, 1H), 6.24 (s, 1H), 4.97 (s, 1H), 4.84 (s, 1H), 4.40 (d, $J = 12.8$ Hz, 1H), 4.13 – 4.09 (m, 2H), 3.40 (q, $J = 7.0$ Hz, 2H), 2.55 (s, 1H), 2.25 (d, $J = 13.6$ Hz, 1H), 2.04 (s, 3H), 1.87 (d, $J = 15.8$ Hz, 6H), 1.71 (d, $J = 34.1$ Hz, 8H), 1.59 (s, 2H), 1.39 (d, $J = 6.5$ Hz, 4H);

$^{13}$C NMR (151 MHz, Chloroform-$d$): δ 165.64, 158.20, 152.28, 148.76, 147.96, 146.17, 145.22, 137.73, 135.23, 123.65, 121.76, 111.88, 107.05, 85.78, 68.90, 68.84, 65.38, 39.97, 39.02, 33.93, 33.87, 30.09, 30.05, 29.73, 27.92, 27.82, 26.54, 25.48, 25.35, 23.54, 22.81, 21.81, 16.36;

$^{31}$P NMR (162 MHz, Chloroform-$d$): δ 101.18;

HRMS (ESI-TOF, m/z): Calcd for [C$_{25}$H$_{39}$N$_4$O$_3$PS$_2$+H]$^+$ 539.2279; Found 539.2280.
Prepared according to General Procedure 1 using 5-Norbornene-2-methanol (1.24 g, 10 mmol) with the following modifications: The crude reaction was diluted with hexanes (50 mL) then passed through a plug of SiO$_2$ and concentrated to dryness, the solvent was swapped to methanol and upon standing overnight the product crystallized. Isolation by filtration to afford $\Psi$-12 (1.56 g, 4.2 mmol, 42% yield) as a mixture of endo and exo isomers.

Physical State: Crystalline solid;
$^1$H NMR (400 MHz, Chloroform-d): $\delta$ 6.16 (dt, $J = 6.2, 3.2$ Hz, 2H), 6.09 (s, 2H), 6.02 – 5.96 (m, 2H), 5.01 (s, 3H), 4.89 (s, 3H), 4.52 – 4.42 (m, 3H), 4.05 – 3.91 (m, 1H), 3.88 (d, $J = 7.4$ Hz, 1H), 3.72 (dt, $J = 35.2, 11.4$ Hz, 2H), 2.90 (d, $J = 8.7$ Hz, 2H), 2.83 (s, 2H), 2.74 (s, 1H), 2.59 (s, 3H), 2.47 (s, 2H), 2.29 (d, $J = 13.4$ Hz, 3H), 2.15 (s, 1H), 2.11 (d, $J = 13.5$ Hz, 2H), 1.98 (s, 1H), 1.96 – 1.83 (m, 7H), 1.54 (s, 3H), 1.46 (d, $J = 8.5$ Hz, 2H), 1.33 (d, $J = 9.2$ Hz, 2H), 1.26 (d, $J = 8.3$ Hz, 3H), 1.18 (d, $J = 15.4$ Hz, 1H), 0.54 (t, $J = 12.2$ Hz, 2H).

$^{13}$C NMR (151 MHz, CDCl$_3$): $\delta$ 144.67, 144.65, 144.64, 144.61, 137.32, 137.16, 136.64, 136.59, 135.73, 135.68, 131.75, 131.63, 111.28, 85.02, 84.99, 84.96, 84.92, 72.36, 72.30, 72.25, 72.19, 71.65, 71.60, 71.54, 64.83, 64.81, 64.77, 64.74, 48.85, 44.54, 44.50, 43.36, 43.31, 43.09, 42.97, 41.85, 41.81, 41.19, 41.15, 38.99, 38.95, 38.88, 38.84, 38.82, 38.77, 38.72, 38.48, 38.46, 33.40, 33.39, 33.36, 33.34, 33.33, 33.31, 28.90, 28.26, 28.16, 27.34, 27.32, 27.31, 27.24, 27.22, 27.20, 22.99, 22.19, 22.17, 21.26, 21.24.

$^{31}$P NMR (162 MHz, Acetone): $\delta$ 101.15, 100.93, 100.64, 100.55;
HRMS (ESI-TOF, m/z): Calcd for [C$_{18}$H$_{27}$O$_2$PS$_2$+H]$^+$ 371.1268; Found 371.1273.
Psi-13

Prepared according to General Procedure 1 using SI-55 (0.377 g, 1.0 mmol). Purification using a gradient of 70-100% EtOAc in hexanes afforded Psi-13 (0.35 g, 0.56 mmol, 56% yield).

Physical State: White foam;
Rf = 0.54 (100% EtOAc);

1H NMR (600 MHz, Chloroform-d): δ 7.71 (d, J = 7.6 Hz, 1H), 6.40 (t, J = 7.8 Hz, 1H), 5.81 (d, J = 7.6 Hz, 1H), 5.32 (dt, J = 13.9, 10.2, 7.4 Hz, 1H), 5.02 (s, 1H), 4.86 (d, J = 1.9 Hz, 1H), 4.53 (dt, J = 13.9, 7.4 Hz, 1H), 4.11 (dd, J = 7.3, 2.3 Hz, 1H), 3.98 (dd, J = 11.9, 2.2 Hz, 1H), 3.86 (dd, J = 11.9, 2.2 Hz, 1H), 2.57 (d, J = 6.0 Hz, 1H), 2.30 (dd, J = 13.6, 3.0 Hz, 1H), 2.14 (td, J = 13.5, 4.2 Hz, 1H), 1.97 (ddd, J = 17.1, 4.4, 2.0 Hz, 1H), 1.90 (dt, J = 13.2, 3.4 Hz, 1H), 1.84 (td, J = 13.0, 5.6 Hz, 1H), 1.71 (d, J = 47.0 Hz, 7H), 0.92 (s, 9H), 0.12 (d, J = 11.8 Hz, 6H);

13C NMR (151 MHz, Chloroform-d): δ 166.38, 164.46, 154.10, 143.92, 140.83, 122.42, 120.69, 118.95, 111.60, 95.04, 85.28, 83.80, 83.59, 83.38, 79.55, 79.52, 79.49, 71.97, 71.92, 71.81, 71.76, 71.65, 71.60, 66.44, 59.60, 38.35, 33.16, 33.10, 27.12, 27.02, 25.44, 22.95, 21.97, 21.44, 17.87, -5.76, -5.97;

19F NMR (376 MHz, Chloroform-d): δ -115.77;

31P NMR (162 MHz, Chloroform-d): δ 103.96;

HRMS (ESI-TOF, m/z): Calcd for [C25H40F2N3O5PS2Si]+ 624.1963; Found 624.1965.
Ψ-(Rp)-14

Prepared according to General Procedure 1 using phenol (0.94 g, 10 mmol) with the following modifications: After the standard workup, the crude reaction was solvent swapped to methanol (50 mL) and upon standing for several hours the product crystalized. Isolation by filtration afforded Ψ-(Rp)-14 (2.21 g, 6.5 mmol, 65% yield).

**Physical State:** crystalline colorless solid;

**$^1$H NMR (600 MHz, Chloroform-$d$):** $\delta$ 7.34 (t, $J = 7.9$ Hz, 2H), 7.20 (td, $J = 7.4$, 1.4 Hz, 1H), 7.20 – 7.13 (m, 2H), 4.90 (q, $J = 1.5$ Hz, 1H), 4.76 (d, $J = 1.9$ Hz, 1H), 4.37 (dt, $J = 12.8$, 3.3 Hz, 1H), 2.56 (t, $J = 6.2$ Hz, 1H), 2.32 – 2.25 (m, 1H), 2.05 (td, $J = 13.7$, 13.0, 4.7 Hz, 1H), 1.96 – 1.84 (m, 3H), 1.78 – 1.69 (m, 7H);

**$^{13}$C NMR (151 MHz, Chloroform-$d$):** $\delta$ 150.82, 150.74, 144.75, 129.64, 129.63, 125.67, 125.66, 121.71, 121.68, 111.86, 86.57, 86.55, 85.80, 38.85, 33.83, 33.78, 27.74, 27.64, 23.41, 22.68, 21.87;

**$^{31}$P NMR (162 MHz, Chloroform-$d$):** $\delta$ 96.40;

**HRMS (ESI-TOF, m/z):** Calcd for $[C_{16}H_{21}O_2P_2S_2+H]^+$ 341.0799; Found 341.0800.
$\Psi'$-(Sp)-14

Prepared according to General Procedure 1 using phenol (0.94 g, 10 mmol) with the following modifications: Using (+)-$\Psi$ (CAS Number : 2245335-71-9), after the standard workup, the crude reaction was solvent swapped to methanol (50 mL) and upon standing for several hours the product crystalized. Isolation by filtration afforded $\Psi'$-(Sp)-14 (2.38 g, 7.0 mmol, 70% yield).

The product had identical characterization data to $\Psi'$-(Rp)-14.
To a solution of (–)-Ψ (0.447 g, 1.0 mmol, 1 equiv.) in THF (10 mL) was added phenylethynylmagnesium bromide (2.0 mL, 2.0 mmol, 2.0 equiv., 1.0 m solution, THF). After stirring for 2 hours the reaction was diluted with 1 : 1 Et₂O/hexanes (50 mL) and washed consecutively with water, KH₂PO₄ (saturated aqueous) and brine. The organic layer was dried over MgSO₄, filtered and concentrated to a yellow oil. Purification in 10% Et₂O in hexanes afforded Ψ-15 (0.25 g, 0.72 mmol, 72% yield).

**Physical State:** yellow amorphous solid;

Rᵣ = 0.4 (10% Et₂O/hexanes);

**¹H NMR (600 MHz, Chloroform-d):** δ 7.54 (dd, J = 7.1, 2.0 Hz, 2H), 7.44 (td, J = 7.4, 1.9 Hz, 1H), 7.37 (td, J = 7.8, 2.0 Hz, 2H), 5.02 – 4.97 (m, 1H), 4.91 (s, 1H), 4.56 (ddt, J = 12.8, 6.1, 2.8 Hz, 1H), 2.61 (d, J = 6.4 Hz, 1H), 2.41 – 2.35 (m, 1H), 2.19 – 2.08 (m, 1H), 1.97 (td, J = 11.0, 9.3, 3.4 Hz, 3H), 1.83 – 1.75 (m, 1H), 1.80 (s, 3H), 1.71 (s, 1H);

**¹³C NMR (151 MHz, Chloroform-d):** δ 145.29, 132.59, 132.57, 130.84, 128.64, 119.88, 119.85, 111.85, 101.85, 101.60, 86.73, 86.62, 85.33, 64.71, 38.98, 33.99, 33.93, 28.36, 28.26, 23.63, 22.84, 22.54;

**³¹P NMR (162 MHz, Chloroform-d):** δ 61.01;

**HRMS (ESI-TOF, m/z):** Calcd for [C₁₈H₂₁OPS₂+H]⁺ 349.0850; Found 349.0844.
Prepared according to **General Procedure 1** using 2-(2-((6-chlorohexyl)oxy)ethoxy)ethan-1-ol (0.224 g, 1.00 mmol). Purified in 10-20% EtOAc in hexanes (0.146 g, 0.31 mmol, 31% yield).

**Physical State:** Yellow Oil

$R_f = 0.33$ (15% EtOAc/Hexanes)

$^1$H NMR (600 MHz, Chloroform-($d_2$)): $\delta$ 4.97 (q, $J = 1.5$ Hz, 1H), 4.85 (d, $J = 2.0$ Hz, 1H), 4.43 (dt, $J = 12.7$, 3.2 Hz, 1H), 4.33 (dddd, $J = 13.3$, 11.4, 6.2, 4.0 Hz, 1H), 4.25 (dddd, $J = 12.6$, 11.4, 5.5, 3.9 Hz, 1H), 3.76 – 3.66 (m, 2H), 3.67 – 3.58 (m, 2H), 3.56 (dd, $J = 5.5$, 4.2 Hz, 2H), 3.52 (t, $J = 6.7$ Hz, 2H), 3.45 (t, $J = 6.7$ Hz, 2H), 2.59 – 2.54 (m, 1H), 2.26 (ddq, $J = 13.5$, 3.4, 1.7 Hz, 1H), 2.08 (td, $J = 13.5$, 4.3 Hz, 1H), 1.93 (ddt, $J = 14.9$, 4.4, 2.2 Hz, 1H), 1.92 – 1.82 (m, 2H), 1.80 – 1.71 (m, 6H), 1.68 (s, 3H), 1.62 – 1.52 (m, 2H), 1.48 – 1.40 (m, 2H), 1.42 – 1.32 (m, 2H);

$^{13}$C NMR (151 MHz, Chloroform-($d_2$)): $\delta$ 145.18, 111.85, 85.76, 71.38, 70.72, 70.19, 70.09, 70.05, 67.63, 67.58, 65.18, 45.17, 38.97, 33.83, 33.77, 32.63, 29.56, 27.86, 27.76, 26.79, 25.52, 23.47, 22.78, 21.78;

$^{31}$P NMR (162 MHz, Chloroform-($d_2$)): $\delta$ 102.07;

HRMS (ESI-TOF, $m/z$): Calcd for [C$_{20}$H$_{36}$ClO$_4$PS$_2$ +H]$^+$ 471.1559; Found 471.1553
Prepared according to **General Procedure 1** using **SI-58** (1.4 g, 5.24 mmol). Purified in 30-50% EtOAc in hexanes (2.3 g, 4.48 mmol, 85% yield).

**Physical State:** white foam;

$R_f = 0.66$ (60% EtOAc/Hexanes);

$^1$H NMR (600 MHz, Chloroform-$d$): $\delta$ 8.34 (s, 1H), 7.36 (q, $J = 1.3$ Hz, 1H), 6.35 (dd, $J = 8.6$, 5.7 Hz, 1H), 5.26 (ddt, $J = 11.5$, 6.7, 2.4 Hz, 1H), 5.06 (q, $J = 1.4$ Hz, 1H), 4.92 – 4.88 (m, 1H), 4.45 (dt, $J = 12.7$, 3.2 Hz, 1H), 4.26 (q, $J = 3.0$ Hz, 1H), 3.80 – 3.70 (m, 2H), 2.60 (t, $J = 6.1$ Hz, 1H), 2.49 (ddd, $J = 14.2$, 5.7, 2.1 Hz, 1H), 2.35 – 2.23 (m, 2H), 2.13 (td, $J = 13.5$, 4.2 Hz, 1H), 1.98 – 1.85 (m, 6H), 1.80 (s, 3H), 1.80 – 1.72 (m, 1H), 1.70 (s, 3H);

$^{13}$C NMR (151 MHz, Chloroform-$d$): $\delta$ 162.71, 149.64, 144.28, 134.36, 111.67, 111.36, 85.74, 83.88, 82.59, 82.55, 77.86, 77.81, 65.68, 51.75, 38.36, 38.16, 38.13, 33.27, 33.21, 27.31, 27.21, 22.93, 22.20, 21.32, 12.26;

$^{31}$P NMR (162 MHz, Chloroform-$d$): $\delta$ 102.22;

**HRMS (ESI-TOF, m/z):** Calcd for $[C_{20}H_{28}N_5O_5PS_2+H]^+$ 514.1348; Found 514.1350
Prepared according to **General Procedure 1** using **SI-59** (1.4 g, 5.00 mmol). Purified in 50% EtOAc in hexanes (2.13 g, 4.05 mmol, 83% yield).

**Physical State:** white foam; 
**R**$_f$ = 0.27 (50% EtOAc/Hexanes);  
**$^1$H NMR (600 MHz, Chloroform-$d$):** δ 8.30 (s, 1H), 7.44 (q, J = 1.2 Hz, 1H), 6.30 (dd, J = 8.4, 5.7 Hz, 1H), 4.97 (q, J = 1.5 Hz, 1H), 4.83 (dd, J = 2.1, 1.1 Hz, 1H), 4.51 (ddd, J = 12.5, 11.5, 3.4 Hz, 1H), 4.43 (ddd, J = 12.8, 3.6, 2.5 Hz, 1H), 4.40 – 4.30 (m, 2H), 4.28 – 4.23 (m, 1H), 4.23 – 4.18 (m, 1H), 4.21 – 4.09 (m, 1H), 2.60 (s, 1H), 2.51 – 2.44 (m, 2H), 2.28 (ddt, J = 13.4, 3.2, 1.7 Hz, 1H), 2.08 (td, J = 13.4, 4.2 Hz, 1H), 2.04 (s, 1H), 2.01 – 1.93 (m, 5H), 1.95 – 1.90 (m, 1H), 1.92 – 1.85 (m, 1H), 1.82 – 1.72 (m, 7H);  
**$^{13}$C NMR (151 MHz, Chloroform-$d$):** δ 163.60, 150.04, 144.52, 134.86, 111.34, 110.95, 85.99, 84.42, 82.32, 82.27, 78.52, 78.01, 75.10, 67.08, 67.02, 65.50, 56.49, 38.34, 36.93, 33.31, 33.26, 27.27, 27.17, 22.85, 22.14, 21.23, 12.31;  
**$^{31}$P NMR (162 MHz, Chloroform-$d$):** δ 102.69;  
**HRMS (ESI-TOF, m/z):** Calcd for [C$_{23}$H$_{31}$N$_2$O$_6$PS$_2$+H]$^+$ 527.1439; Found 527.1431
Prepared according to **General Procedure 1** using **SI-60** (0.60 g, 2.26 mmol). Purified in 50% EtOAc in hexanes (0.769 g, 1.50 mmol, 66% yield).

**Physical State:** yellow oil;  
\( R_f = 0.42 \) (50% EtOAc/hexanes);

\[ ^1H \text{ NMR (600 MHz, Chloroform-}d\text{):} \delta \]
- 6.50 (s, 2H), 5.25 (s, 2H), 4.99 (s, 1H), 4.90 – 4.82 (m, 1H), 4.41 (dt, \( J = 12.8, 3.3 \) Hz, 1H), 4.12 (m, 2H), 3.46 (t, \( J = 7.3 \) Hz, 2H), 2.83 (s, 2H), 2.60 – 2.53 (m, 1H), 2.27 (m, 1H), 2.09 (td, \( J = 13.5, 4.3 \) Hz, 1H), 1.94 (m, 1H), 1.90 – 1.81 (m, 2H), 1.80 – 1.70 (m, 4H), 1.70 – 1.61 (m, 5H), 1.55 (m, 2H), 1.40 – 1.33 (m, 2H), 1.29 (m, 2H);

\[ ^{13}C \text{ NMR (151 MHz, Chloroform-}d\text{):} \delta \]
- 176.4, 145.2, 136.7, 111.9, 85.7, 81.0, 68.9, 68.8, 65.3, 47.5, 39.0, 38.9, 33.9, 33.9, 30.0, 30.0, 27.9, 27.8, 27.6, 26.3, 25.2, 23.5, 22.8, 21.8;

\[ ^{31}P \text{ NMR (162 MHz, Chloroform-}d\text{):} \delta \]
- 101.03;

**HRMS (ESI-TOF, m/z):** Calcd for \([\text{C}_{24}\text{H}_{34}\text{NO}_{5}\text{PS}_2\text{H}]^+\) 512.1694; Found 512.1694
Prepared according to **General Procedure 1** using **SI-62** (0.60 g, 2.26 mmol). The crude solution was used directly for the SENDR reaction without workup or isolation.
NMR Spectra

Compound Ψ-1 $^1$H NMR

Compound Ψ-1 $^{13}$C NMR
Compound Ψ-1 $^{31}$P NMR

Compound Ψ-2 $^1$H NMR
Compound Ψ-2 $^{13}$C NMR

Compound Ψ-2 $^{31}$P NMR
Compound Ψ-3 $^{1}H$ NMR

Compound Ψ-3 $^{13}C$ NMR
Compound Ψ-3 $^{31}$P NMR

Compound Ψ-5 $^1$H NMR
Compound Ψ-5 $^{13}$C NMR

[Graph of $^{13}$C NMR spectrum]

Compound Ψ-5 $^{31}$P NMR

[Graph of $^{31}$P NMR spectrum]
Compound Ψ-6 $^1$H NMR

Compound Ψ-6 $^{13}$C NMR
Compound Ψ-6 $^{19}$F NMR

Compound Ψ-6 $^{31}$P NMR
Compound Ψ-7 $^1$H NMR

Compound Ψ-7 $^{13}$C NMR
Compound Ψ-7 $^{31}$P NMR

Compound Ψ-8 $^1$H NMR
Compound Ψ-8 $^{13}$C NMR

Compound Ψ-8 $^{31}$P NMR
Compound Ψ-9 ³¹P NMR

Compound Ψ-9 ¹H NMR
Compound Ψ-10 $^{13}$C NMR

Compound Ψ-10 $^{31}$P NMR
Compound Ψ-11 $^1$H NMR

Compound Ψ-11 $^{13}$C NMR
Compound Ψ-11 $^{31}$P NMR

Compound Ψ-12 $^1$H NMR
Compound Ψ-12 $^{13}$C NMR

Compound Ψ-12 $^{31}$P NMR
Compound Ψ-13 $^1$H NMR

Compound Ψ-13 $^{13}$C NMR
Compound $Ψ$-13 $^{19}$F NMR

Compound $Ψ$-13 $^{31}$P NMR
Compound $\Psi-(Rp)-14\ H$ NMR

Compound $\Psi-(Rp)-14\ C$ NMR
Compound Ψ-(R)p-14 $^{31}$P NMR

Compound Ψ-15 $^1$H NMR
Compound \( \Psi-15 \)\(^{13}\text{C} \) NMR

\[
\begin{array}{c}
\text{Compound } \Psi-15 \text{\(^{31}\text{P} \) NMR}
\end{array}
\]
Compound Ψ-16 $^1$H NMR

Compound Ψ-16 $^{13}$C NMR
Compound Ψ-16 $^{31}$P NMR

Compound Ψ-17 $^1$H NMR
Compound ψ-18 ¹H NMR

Compound ψ-18 ¹³C NMR
Compound Ψ-18 $^{31}$P NMR

Compound Ψ-19 $^1$H NMR
Compound Ψ-19 $^{13}$C NMR

Compound Ψ-19 $^{31}$P NMR
SENDRI Protocol

General Procedure 2: SENDR in Microcentrifuge Tubes

Resin Preparation and DNA Loading
1. Cut the tip off of a 1 mL pipette tip and transfer resin (100 µL resin equilibrate in 1:1 PBS:MeCN, ~25 mg dry resin) with an adjustable volume pipette—Phenomenex Strata-XAL—into a 2 mL microcentrifuge tube.
2. Wash Resin with 500-1000 µL PBS.
   a. Spin down the resin slurry and aspirate the resin bed (by sucking off the supernatant with a glass pipet).
3. Add DNA (up to 50 nmol) in 100-500 µL PBS, vortex and incubate at room temp for 5-15 min.

Resin Washing and Drying
4. Wash loaded resin twice with DMA (500 µL)
5. Wash loaded resin three times with dry THF (500 µL)
6. Dry the resin for >2h in a speed vac or stuff a ball of paper in the top (so no resin can escape) and dry under vacuum for >2 h.
7. Resin Washing and Drying

Running the Reaction
8. Dissolve Ψ-Module in dry MeCN (150 mM, 250 µM), and add it to the dry resin tube.
9. Add DBU (450 mM total, 18 µL) to the reaction tube.
10. Vortex for 30 seconds and incubate for 60 minutes at room temperature or 37 ºC.

Working up Reaction
11. Aspirate reaction mixture and discard.
12. Wash reaction with MeCN or DMA (500 µL)
13. Wash with PBS or 1:1 PBS:MeCN (500 µL)

Elute DNA
14. Add 300 uL Elute Buffer (1 M NaClO₄, 20% MeOH, 40 mM tris, pH 8.5) to resin bed.
15. Vortex for 30 seconds and agitate for 5-10 minutes.
16. Carefully collect the elution butter with micropipette without sucking up any resin.
17. Desalt with your preferred method.
Note: Strata-XAL if designed for large analytes. Strata-XAL is a 100 µm particle with 300 Å pore size.

Note: “Washing the resin” refers to adding 500 to 1000 µL of solvent to the resin, vortexing (30 sec), spinning the resin down, and removing the supernatant with a pipette.

Note: Dry acetonitrile should be used for best reaction conversion.

Note: The elute buffer which contains 1 M NaClO₄, 20% MeOH, 40 mM tris, pH 8.5 should be accessed with high quality NaClO, Acros Organics is our preferred supplier (part number AC197122500)
Troubleshooting and Frequently Asked Questions

1. When I try to pellet my resin, it is too flocculant and is still in solution after taking out of the centrifuge.
   Allow the resin tube to sit for 5-10 minutes undisturbed to allow the resin to settle to the bottom of the tube.

2. How do I remove the supernatant with a pipette?
   Gently suck off the supernatant while trying to leave the resin bed undisturbed. Keeping the pipette tip a few millimeters off the top of the resin bed usually helps with this, also allowing a small amount of liquid to remain on the top of the resin bed is alright.

3. My Ψ-Module is not dissolving, what do I do?
   To get the Ψ-Module dissolved you may need to sonicate the solution for up to 10 minutes. Usually this is sufficient to dissolve all of the PSI-module. If this does not work, transfer heterogeneous PSI-module solution to the reaction solution and add the DBU. Usually upon addition of DBU the rest of the PSI-module will dissolve.

4. My reaction resulting in low conversions, what should I do.
   Try to heat the reaction to 37 °C.
General Procedure 3: SENDR in Fritted Cartridges (Kit Format)

Resin Preparation
1. Break off bottom of preloaded (with 100 µL Strata-XAL resin in 1:1 PBS:MeCN) Biorad column. Or transfer equilibrated resin (100 µL) to the column as above.
2. Allow packing solution to flow through into waste.
3. Add PBS (500 µL) and allow to flow through into waste.

DNA Loading:
4. Cap the bottom spout and add DNA (up to 50 nmol in 100-500 µL PBS) to column.
5. Cap the top, vortex and agitate for 5-15 min.

Resin Washing and Drying
6. Wash loaded resin twice with DMA (500 µL) and allow to flow to waste.
7. Wash loaded resin three times with dry THF (500 µL) and allow to flow to waste.
8. Replace the top cap but not the bottom and dry the resin for >2h in under vacuum.
**Running the Reaction**

9. Add a NEW, CLEAN, and DRY cap to the bottom of the column.

10. Dissolve Ψ-Module in dry MeCN (300 mM, 125 µL) or add the supplied PSI-Module solution to dry resin tube.

11. Add DBU in dry MeCN (300 mM, 125 µL) or add the supplied DBU solution to resin tube.

12. Replace both caps, vortex for 30 seconds, and incubate for 60 minutes at 37 °C.

**Working up Reaction**

13. Remove both caps and allow the reaction mixture to drain to waste.

14. Wash reaction with MeCN or DMA (500 µL) allow to drain to waste.

15. Wash with PBS or 1:1 PBS:MeCN (500 µL) allow to drain to waste.

**Elute DNA**

16. Replace bottom cap. Add 300 uL Elute Buffer (1 M NaClO₄, 20% MeOH, 40 mM tris, pH 8.5) to resin bed and then replace top cap.

17. Vortex for 30 seconds and agitate for 5-10 minutes.

18. Remove both caps and allow elute buffer to drain into a collection tube.

19. Desalt with your preferred method.
Note: Allow flow throughs to occur under gravity not under vacuum.

Note: Agitating the resin can be done with an end over end tumbler or an orbital shaker.

Note: Drying the resin under vacuum can be performed using a lyophilizer, high vac, or speed vac (if the columns fit).
Troubleshooting and Frequently Asked Questions

1. The storage solution in the column takes a long time to drain, what do I do?
Sometimes, an air bubble forms at the bottom of the column and surface tension prevents the solution from flowing through. Put gentle pressure on top of the column using a gloved finger. Once you see a single drop drip out of the bottom of the column, allow the rest of the solution flow through by gravity.

2. When I add the PBS to wash the resin, it takes a long time to drip out?
See above.

3. Why do I need a dry bottom cap while running the reaction?
Any water remaining on the cap might quench the Ψ-Module. Using a new, clean, dry cap ensures that no water enters the reaction mixture.

4. How do I get my DNA out of the elute buffer?
Almost any standard DNA desalting method will work. We tend to use ethanol precipitations. Some prefer other methods that are faster but require different DNA consumable kits. In our hands both silica-based desalting columns and gel filtration worked for desalting of modified DNA.
General Procedure 4: In Situ Module Formation

*In some instances, it may be advantageous to directly prepare a module in solution and immediately use it in the SENDR process without isolation.*

To a solution of alcohol (1.0 equiv.) and (–)-Ψ (1.3 equiv.) in anhydrous MeCN in a flame-dried round-bottom flask was added DBU (1.3 equiv.). After mixing for 5 minutes, the reaction was directly used in the SENDR protocol via General Procedure 2 or 3.
Optimization of SENDR protocol and conditions

Optimization Procedure

SENDR was optimized by performing reactions in a microcentrifuge tube as described in General Procedure 2 with various reaction conditions (as described in the main text and Figure 2B). Briefly, 5 nmol of DNA 1 or 2 was loaded onto 100 µL of equilibrated resin. This loaded resin was washed with DMA (500 µL twice) and dry THF (500 µL three times) and dried under vacuum. In the case in which stringent drying was not performed (Figure 2B, Entry 8) the loaded resin was simply washed with acetonitrile (500 µL three times). Ψ-1 in MeCN (varying concentration, 250 µL) was added to the loaded and dried resin. Then DBU (various concentration) was added to the reaction mixture. The reaction tube was vortexed for 30 seconds and incubated at room temperature for various amounts of time. The reactions were worked up by aspirating and discarding the reaction solution, and washing the resin bed with MeCN (500 µL) and 1:1 MeCN:PBS (500 µL). Elute buffer (300 µL) was added to the resin bed and the tube was agitated by orbital shaker for 5-10 minutes. The DNA containing elution buffer was collected by carefully pipetting the supernatant. DNA was isolate via ethanol precipitation. Ethanol precipitation was performed by adding 10% v/v of a NaCl solution (30 µL, 5M) to the elute buffer and three volumes of cold ethanol (-20 ºC) were added to the tube (~1000µL) and incubated for 18 hours at -20 ºC. The tube was then centrifuged at 13,000 rpm for 15 minutes to pellet the DNA, and the supernatant ethanol was decanted off. The tubes were dried via speed vacuum, the DNA was dissolved and HPLC-MS analysis was performed.
Characterization of SENDR Optimization

LCMS Characterization of 3

Produced from 1 by General Procedure 2:
Conversion (A260): 91%
Expected Mass: 7023.61
Expected (M-6)/6 Ion: 1169.60
Found Deconvoluted Mass: 7024.00
Found (M-6)/6 Ion: 1169.61

A260 Chromatogram
Mass Spectrum (RT= 1.68 min)
Deconvoluted Mass Spectrum
LCMS Characterization of 4
Produced from 2 by General Procedure 2:
Conversion (A260): 72%
Expected Mass: 7023.61
Expected (M-6)/6 Ion: 1169.60
Found Deconvoluted Mass: 7024.00
Found (M-6)/6 Ion: 1169.01

5’-C-C-G-A-G-T-C-A-A-A-A-A-A-T-G-A-C-T-C-G-G-T-3’

Exact Mass: 7020.14
Molecular Weight: 7023.61

A260 Chromatogram
Mass Spectrum (RT= 2.76 min)
Deconvoluted Mass Spectrum
Process for Mass Fragmentation of 3

A crude solution of 3 in water (100 µM) was injected on the Waters I-Class ToF for peak identification. The MS cone voltage was then increased from 5 mV to 30 mV and the same sample was reinjected to fragment the modified oligonucleotide. Diagnostic mass fragments were then extracted, and their presence confirmed in the total ion current. They are plotted below along with total ion current and UV absorbance. UV absorbance is slightly out of alignment because there is a non-zero-time difference between when the compound passes through the UV detector and when it enters the mass spec. This is because of the plumbing between the two modules.
Analysis of Optimization Reactions

Entry 1: 5’

LCMS Characterization of 3
Produced from 1 by General Procedure 3:
Conversion (A260): 56%
Expected Mass: 7023.61
Expected (M-6)/6 Ion: 1169.60
Found Deconvoluted Mass: 7024.00
Found (M-6)/6 Ion: 1169.54

A260 Chromatogram
Mass Spectrum (RT = 3.26 min)
Deconvoluted Mass Spectrum
Entry 1: 3’

LCMS Characterization of 4
Produced from 2 by General Procedure 2:
Conversion (A260): 17%
Expected Mass: 7023.61
Expected (M-6)/6 Ion: 1169.60
Found Deconvoluted Mass: 7023.00
Found (M-6)/6 Ion: 1169.21

\[
\text{Exact Mass: 7020.14} \\
\text{Molecular Weight: 7023.61}
\]

A260 Chromatogram
Mass Spectrum (RT= 2.19 min)
Deconvoluted Mass Spectrum
Entry 2: 5’

LCMS Characterization of 3
Produced from 1 by General Procedure 2:
Conversion (A260): 72%
Expected Mass: 7023.61
Expected (M-6)/6 Ion: 1169.60
Found Deconvoluted Mass: 7023.00
Found (M-6)/6 Ion: 1169.54

Exact Mass: 7020.14
Molecular Weight: 7023.61
Mass Spectrum (RT = 2.21 min)
Entry 2: 3’

LCMS Characterization of 4
Produced from 2 by General Procedure 2:
Conversion (A260): 51%
Expected Mass: 7023.61
Expected (M-6)/6 Ion: 1169.60
Found Deconvoluted Mass: 7024.00
Found (M-6)/6 Ion: 1169.41

5’-C-G-A-G-T-C-A-A-A-A-T-G-A-C-T-C-G-G-3’

Exact Mass: 7020.14
Molecular Weight: 7023.61

A260 Chromatogram
Mass Spectrum (RT= 2.19 min)
Deconvoluted Mass Spectrum
Entry 3: 5’

LCMS Characterization of 3
Produced from 1 by General Procedure 2:
Conversion (A260): 91%
Expected Mass: 7023.61
Expected (M-6)/6 Ion: 1169.60
Found Deconvoluted Mass: 7024.00
Found (M-6)/6 Ion: 1169.61

A260 Chromatogram
Entry 3: 3’

LCMS Characterization of 4
Produced from 2 by General Procedure 2:
Conversion (A260): 72%
Expected Mass: 7023.61
Expected (M-6)/6 Ion: 1169.60
Found Deconvoluted Mass: 7024.00
Found (M-6)/6 Ion: 1169.01

\[
\begin{align*}
5’ &- \text{C-G-A-G-T-C-A-A-A} - \text{A-A-T-G-A-C-T-C-G} - \text{T} \\
\end{align*}
\]

Exact Mass: 7020.14
Molecular Weight: 7023.61

A260 Chromatogram
Mass Spectrum (RT = 2.76 min)
Deconvoluted Mass Spectrum
Entry 4: 5’

LCMS Characterization of 3
Produced from 1 by General Procedure 2:
Conversion (A260): 84%
Expected Mass: 7023.61
Expected (M-6)/6 Ion: 1169.60
Found Deconvoluted Mass: 7023.00
Found (M-6)/6 Ion: 1169.61

A260 Chromatogram
Deconvoluted Mass Spectrum
Entry 4: 3’

LCMS Characterization of 4
Produced from 2 by General Procedure 2:
Conversion (A260): 62%
Expected Mass: 7023.61
Expected (M-6)/6 Ion: 1169.60
Found Deconvoluted Mass: 7023.00
Found (M-6)/6 Ion: 1169.54

\[ \text{Exact Mass: 7020.14} \]
\[ \text{Molecular Weight: 7023.61} \]

A260 Chromatogram
Mass Spectrum (RT= 2.69 min)
Entry 5: 5’
LCMS Characterization of 3
Produced from 1 by General Procedure 2:
Conversion (A260): 0%
Expected Mass: 7023.61
Expected (M-6)/6 Ion: 1169.60
Found Deconvoluted Mass: 6824.00 (Starting material 1)
Found (M-6)/6 Ion: 1135.99 (Starting material 1)

A260 Chromatogram
Mass Spectrum (RT= 1.69 min)
Deconvoluted Mass Spectrum

Mass: 6824.00
Intensity: 1.3567
Entry 5: 3’

LCMS Characterization of 4
Produced from 2 by General Procedure 2:
Conversion (A260): 0%
Expected Mass: 7023.61
Expected (M-6)/6 Ion: 1169.60
Found Deconvoluted Mass: 6824.00 (Starting material 2)
Found (M-6)/6 Ion: 1136.12(Starting material 2)

A260 Chromatogram
Mass Spectrum (RT= 1.64 min)
Deconvoluted Mass Spectrum
Entry 6: 5’

LCMS Characterization of 3
Produced from 1 by General Procedure 2:
Conversion (A260): 0%
Expected Mass: 7023.61
Expected (M-6)/6 Ion: 1169.60
Found Deconvoluted Mass: 6823.00 (Starting material 1)
Found (M-6)/6 Ion: 1135.92 (Starting material 1)

A260 Chromatogram
Mass Spectrum (RT = 1.69 min)
Deconvoluted Mass Spectrum

![Deconvoluted Mass Spectrum Image]
Entry 6: 3’
LCMS Characterization of 4
Produced from 2 by General Procedure 2:
Conversion (A260): 0%
Expected Mass: 7023.61
Expected (M-6)/6 Ion: 1169.60
Found Deconvoluted Mass: 6823 (Starting material 2)
Found (M-6)/6 Ion: 1135.85 (Starting material 2)

5’-C-G-A-G-T-C-A-A-A-A-A-T-G-A-C-T-C-G-G-

Exact Mass: 7020.14
Molecular Weight: 7023.61

A260 Chromatogram
Mass Spectrum (RT= 1.63 min)
Deconvoluted Mass Spectrum
Entry 7: 5’

LCMS Characterization of 3
Produced from 1 by General Procedure 2:
Conversion (A260): 0%
Expected Mass: 7023.61
Expected (M-6)/6 Ion: 1169.60
Found Deconvoluted Mass: 6823.00 (Starting material 1)
Found (M-6)/6 Ion: 1136.12

A260 Chromatogram
Mass Spectrum (RT = 1.69 min)
Deconvoluted Mass Spectrum
Entry 7: 3’

LCMS Characterization of 4
Produced from 2 by General Procedure 2:
Conversion (A260): 56%
Expected Mass: 7023.61
Expected (M-6)/6 Ion: 1169.60
Found Deconvoluted Mass: 6823.00 (Starting material 2)
Found (M-6)/6 Ion: 1136.26 (Starting material 2)

5’-C-C-G-A-G-T-C-A-A-A-A-A-T-G-A-C-T-C-G-G-3’

A260 Chromatogram
Mass Spectrum (RT= 1.64 min)
Deconvoluted Mass Spectrum
Entry 8: 5’

LCMS Characterization of 3
Produced from 1 by General Procedure 2:
Conversion (A260): 0%
Expected Mass: 7023.61
Expected (M-6)/6 Ion: 1169.60
Found Deconvoluted Mass: 6824.00 (Starting material 1)
Found (M-6)/6 Ion: 1135.79 (Starting material 1)

A260 Chromatogram
Mass Spectrum (RT = 1.62 min)
Deconvoluted Mass Spectrum
Entry 8: 3’

LCMS Characterization of 4
Produced from 2 by General Procedure 2:
Conversion (A260): 0%
Expected Mass: 7023.61
Expected (M-6)/6 Ion: 1169.60
Found Deconvoluted Mass: 6824.00 (Starting material 2)
Found (M-6)/6 Ion: 1136.32 (Starting material 2)

5’-C-C-G-A-G-T-C-A-A-A-A-A-T-G-A-C-T-C-G-G-T-HO-O-P-O-S-HO

Exact Mass: 7020.14
Molecular Weight: 7023.61

A260 Chromatogram
Mass Spectrum (RT = 1.62 min)
Deconvoluted Mass Spectrum
Entry 9: 5’

LCMS Characterization of 3
Produced from 1 by General Procedure 2:
Conversion (A260): <10%
Expected Mass: 7023.61
Expected (M-6)/6 Ion: 1169.60
Found Deconvoluted Mass: 7024.00
Found (M-6)/6 Ion: 1169.54

A260 Chromatogram
Mass Spectrum (RT= 2.21 min)
Deconvoluted Mass Spectrum
Entry 9: 3’
LCMS Characterization of 4
Produced from 2 by General Procedure 2:
Conversion (A260): <10%
Expected Mass: 7023.61
Expected (M-6)/6 Ion: 1169.60
Found Deconvoluted Mass: 7024.00
Found (M-6)/6 Ion: 1169.54

5’—C—G—A—G—T—C—A—A—A—A—T—G—A—C—T—C—G—G—

Exact Mass: 7020.14
Molecular Weight: 7023.61

A260 Chromatogram
Mass Spectrum (RT= 2.19 min)
Deconvoluted Mass Spectrum
Entry 10: 5’
LCMS Characterization of 3
Produced from 1 by General Procedure 2:
Conversion (A260): trace
Expected Mass: 7023.61
Expected (M-6)/6 Ion: 1169.60
Found Deconvoluted Mass: 7023.00
Found (M-6)/6 Ion: 1169.34

Exact Mass: 7020.14
Molecular Weight: 7023.61

A260 Chromatogram

[Image of chromatogram]
Mass Spectrum (RT = 1.70 min)
Deconvoluted Mass Spectrum
Entry 10: 3’

LCMS Characterization of 4
Produced from 2 by General Procedure 2:
Conversion (A260): trace
Expected Mass: 7023.61
Expected (M-6)/6 Ion: 1169.60
Found Deconvoluted Mass: 70234.00
Found (M-6)/6 Ion: 1169.27

5’—C–C–G–A–G–T–C–A–A–A–A–A–T–G–A–C–T–C–G—G—T

Exact Mass: 7020.14
Molecular Weight: 7023.61

A260 Chromatogram

Range: 1.000
Deconvoluted Mass Spectrum
SENDR on High Tm Hairpin

Hairpin oligo SI-17 was incubated for 10 minutes at either RT or 66°C in 100 µL PBS. This solution was pipetted directly onto washed support (50 µL) and quickly vortexed. The reactions were allowed to cool to room temperature before washing and drying was performed according to general procedure 2. SENDR derivatization was performed under standard conditions.

### Lableing of High Tm Oligo

**Parent Sequence**

```
[Nonoverhanging Terminus] [Tm = 74.3°C]
```

**Optimization**

| Entry | Conditions         | Conversion (%) |
|-------|--------------------|----------------|
| 1     | Adsorb at RT       | 63             |
| 2     | Adsorb at 66°C     | 70             |

**SENDR Derivatization**

- Adsorb To Strata XL-A: RT or 66°C
- DBU (450 mM)
- MeCN, 60 min, RT
Entry 1:
LCMS Characterization of **SI-21**
Produced from **SI-17**:
Conversion (A260): 63%
Retention Time: 2.29 min
Expected Mass: 15927.31
Found Deconvoluted Mass: 15930

5’-C-A-C-T-A-G-G-A-T-G-C-A-T-C-G-T-C-A-T-C-T-T-T-T-G-A-T-G-A-C-G-A-T-G-C-A-T-C-

![Molecular structure](image)

Molecular Weight: 15927.31
Raw Mass Spectrum Retention Time 2.57 minutes
Deconvoluted Mass Spectrum Retention Time 2.57 Minutes
Entry 2:
LCMS Characterization of SI-21
Produced from SI-17:
Conversion (A260): 70%
Retention Time: 2.29 min
Expected Mass: 15927.31
Found Deconvoluted Mass: 15928

5’-C-A-C-T-A-G-G-A-T-G-C-A-T-C-G-T-C-A-T-C-T-T-G-A-T-G-A-C-G-A-T-G-C-A-T-C-

Molecular Weight: 15927.31

A260

TIC
Raw Mass Spectrum Retention Time 2.57 minutes
Deconvoluted Mass Spectrum Retention Time 2.57 Minutes
Alcohol Selective Reactions: An Investigation

Phosphoramidate Formation

DNA 2 was dissolved (90 µM) in MES buffer (50 mM) at pH 6.2 containing phenethylamine (5 mM) and imidazole (20 mM). to this solution was added EDC (10 µL) from a stock solution in DMA (50 mM). This reaction was quickly vortexed and allowed to incubate at room temperature for 60 minutes. After 60 minutes the DNA was isolated by ethanol precipitation (previously described) and analyzed by LCMS.
DNA 1 was loaded onto the support according to General Procedure 2 and dried accordingly. Phosphoramidite (150 mM) and tetrazole (450 mM) were added in dry MeCN and the reaction was allowed to incubate at room temperature for 60 minutes. After 60 minutes the resi was washed once with dry MeCN (500 µL) and oxidation solution was added (iodine, pyridine, water in THF). This was reaction incubated for 15 minutes and then the resin bed washed with MeCN and PBS. The DNA was eluted and precipitated as previously described and finally analyzed by LCMS.
Tosylation reaction

DNA 1 was loaded onto the support according to **General Procedure 2** and dried accordingly. Tosyl Chloride (150 mM) and Collidine (450 mM) were added in dry MeCN and the reaction was allowed to incubate at room temperature for 60 minutes. After 60 minutes the resin was washed once with dry MeCN (500 µL). The DNA was eluted and precipitated as previously described and finally analyzed by LCMS.

**A260**

**TIC**
Raw Mass Spectrum RT = 1.73 (Starting material 1)
Deconvoluted Mass Spectrum RT = 1.73 (Starting material 1)
DNA 1 was loaded onto the support according to General Procedure 2 and dried accordingly. Nirtopheol (150 mM) and Triphenylphosphine (150 mM) were added in dry MeCN. DIAD (150 mM) was then spiked in and the reaction was allowed to incubate at room temperature for 60 minutes. After 60 minutes the resin was washed once with dry MeCN (500 µL). The DNA was eluted and precipitated as previously described and finally analyzed by LCMS. Low DNA recovery was observed by UV Vis absorbance (nanodrop).
Raw Mass Spectrum RT = 1.93 (SI-22)
Deconvoluted Mass Spectrum RT = 1.93 (SI-22)
Williamson Ether Synthesis

DNA 1 was loaded onto the support according to **General Procedure 2** and dried accordingly. DBU (450 mM) was added in dry MeCN and this was added to the resin from 5 minutes. After 5 minutes benzyl bromide (150 mM) was then spiked in and the reaction was allowed to incubate at room temperature for 60 minutes. After 60 minutes the resin was washed once with dry MeCN (500 µL). The DNA was eluted and precipitated as previously described and finally analyzed by LCMS.

A260

![Graph of A260](image)

TIC

![Graph of TIC](image)
Labeling of DNA Without Phosphate Blocking Groups

General Protocol and Optimization

SENDR on non-phosphate DNA (SI-16) was optimized by performing reactions in a microcentrifuge tube as described in General Procedure 2 with various reaction conditions (as described in the main text and Figure 2B). Briefly, 5 nmol of DNA (SI-16) was loaded onto 100 µL of equilibrated resin. This loaded resin was washed with DMA (500 µL twice) and dry THF (500 µL three times) and dried under vacuum. In the case in which stringent drying was not performed (Figure 2B, Entry 8) the loaded resin was simply washed with acetonitrile (500 µL three times).

Ψ-1 in MeCN (varying concentration, 250 µL) was added to the loaded and dried resin. Then DBU (various concentration) was added to the reaction mixture. The reaction tube was vortexed for 30 seconds and incubated at room temperature for various amounts of time. The reactions were worked up by aspirating and discarding the reaction solution, and washing the resin bed with MeCN (500 µL) and 1:1 MeCN:PBS (500 µL). Elute buffer (300 µL) was added to the resin bed and the tube was agitated by orbital shaker for 5-10 minutes. The DNA containing elution buffer was collected by carefully pipetting the supernatant. DNA was isolate via ethanol precipitation. Ethanol precipitation was performed by adding 10% v/v of a NaCl solution (30 µL, 5M) to the elute buffer and three volumes of cold ethanol (-20 ºC) were added to the tube (~1000µL) and incubated for 18 hours at -20 ºC. The tube was then centrifuged at 13,000 rpm for 15 minutes to pellet the DNA, and the supernatant ethanol was decanted off. The tubes were dried via speed vacuum, the DNA was dissolved and HPLC-MS analysis was performed.

| Entry | Conditions | (SI-23) Single Label (%) | (SI-24) Dual Label (%) |
|-------|------------|--------------------------|------------------------|
| 1     | 150 mM Ψ-1, 450 mM DBU, 60 min | 30 | 61 |
| 2     | 150 mM Ψ-1, 450 mM DBU, 30 min | 33 | 51 |
| 3     | 150 mM Ψ-1, 450 mM DBU, 60 min, 37ºC | 24 | 76 |
| 4     | 75 mM Ψ-1, 225 mM DBU, 60 min | 53 | <5 |
| 5     | 75 mM Ψ-1, 225 mM DBU, 30 min x 2 | 53 | 21 |
| 6     | 75 mM Ψ-1, 225 mM DBU, 60 min x 2 | 50 | 25 |
| 7     | 75 mM Ψ-1, 225 mM DBU, 120 min | 48 | 17 |
Entry 1
LCMS Characterization of **Single Label SI-23**
Produced from **SI-16** by General Procedure **3:**
Conversion (A260): 30%
Retention Time: 2.29 min
Expected Mass: 7552.02
Expected (M-8)/8 Ion: 943.0
Found Deconvoluted Mass: 7552.00
Found (M-6)/6 Ion: 942.78

![Molecular structure](image)

**A260**

![Graph showing A260 with peaks at 1.81, 2.29, 2.72](image)

**TIC**

![Graph showing TIC with peaks at 0.76, 1.15, 1.44, 1.82, 2.28, 2.37, 2.81, 3.16, 3.51, 3.78, 4.18, 4.67, 5.035, 5.275, 5.32](image)
Raw Mass Spectrum Retention Time (2.3 minutes)
Deconvoluted Mass Spectrum Retention Time (2.3 minutes)
LCMS Characterization of **Double Label SI-24**
Produced from **SI-16** by General Procedure **3**:  
Conversion (A260): 61%  
Retention Time: 2.72 min  
Expected Mass: 7752.2  
Expected (M-8)/8 Ion: 968.02  
Found Deconvoluted Mass: 7552.00  
Found (M-6)/6 Ion: 968.06

![Molecular structure image](image)

Molecular Weight: 7752.21
Raw Mass Spectrum (Retention Time 2.7 minutes)
Deconvoluted Mass Spectrum (Retention Time 2.7 minutes)
Entry 2
LCMS Characterization of Single Label SI-23
Produced from SI-16 by General Procedure 3:
Conversion (A260): 33%
Retention Time: 2.29 min
Expected Mass: 7552.02
Expected (M-8)/8 Ion: 943.0
Found Deconvoluted Mass: 7551.00
Found (M-6)/6 Ion: 942.92

Molecular Weight: 7552.02
Raw Mass Spectrum (Retention Time 2.3 minutes)
Deconvoluted Mass Spectrum (Retention Time 2.3 minutes)
LCMS Characterization of **Double Lable SI-24**
Produced from **SI-16** by General Procedure 3:
Conversion (A260): 51%
Retention Time: 2.72 min
Expected Mass: 7752.2
Expected (M-8)/8 Ion: 968.02
Found Deconvoluted Mass: 7553.00
Found (M-6)/6 Ion: 967.72

Molecular Weight: 7752.21
Raw Mass Spectrum (Retention Time 2.7 Minutes)
Deconvoluted Mass Spectrum (Retention Time 2.7 Minutes)
LCMS Characterization of **Double Label SI-24**
Produced from **SI-16** by General Procedure 3:
Conversion (A260): 76%
Retention Time: 2.71 min
Expected Mass: 7752.2
Expected (M-8)/8 Ion: 968.02
Found Deconvoluted Mass: 7552.00
Found (M-6)/6 Ion: 967.9

A260

Molecular Weight: 7752.21

TIC

SI176
Raw Mass Spectrum (Retention Time 2.7 Minutes)
Deconvoluted Mass Spectrum (Retention Time 2.7 Minutes)
Entry 4
LCMS Characterization of Single Label SI-23
Produced from SI-16 by General Procedure 3:
Conversion (A260): 53%
Retention Time: 2.29 min
Expected Mass: 7552.02
Expected (M-8)/8 Ion: 943.0
Found Deconvoluted Mass: 7552.00
Found (M-6)/6 Ion: 942.92

Molecular Weight: 7552.02

A260

TIC
Raw Mass Spectrum (Retention Time 2.3 minutes)
Deconvoluted Mass Spectrum (Retention Time 2.3 minutes)
Entry 5
LCMS Characterization of Single Label SI-23
Produced from SI-16 by General Procedure 3:
Conversion (A260): 53%
Retention Time: 2.29 min
Expected Mass: 7552.02
Expected (M-8)/8 Ion: 943.0
Found Deconvoluted Mass: 7552.00
Found (M-6)/6 Ion: 942.78

Molecular Weight: 7552.02

A260

TIC
Raw Mass Spectrum (Retention Time 2.3 minutes)
Deconvoluted Mass Spectrum (Retention Time 2.3 minutes)
LCMS Characterization of **Double Label SI-24**

Produced from **SI-16** by General Procedure 3:

Conversion (A260): 21%
Retention Time: 2.72 min
Expected Mass: 7752.2
Expected (M-8)/8 Ion: 968.02
Found Deconvoluted Mass: 7552.00
Found (M-6)/6 Ion: 968.19

Molecular Weight: 7752.21
Raw Mass Spectrum (Retention Time 2.7 Minutes)
Deconvoluted Mass Spectrum (Retention Time 2.7 Minutes)
Entry 6
LCMS Characterization of Single Label SI-23
Produced from SI-16 by General Procedure 3:
Conversion (A260): 50%
Retention Time: 2.29 min
Expected Mass: 7552.02
Expected (M-8)/8 Ion: 943.0
Found Deconvoluted Mass: 7551.00
Found (M-6)/6 Ion: 942.92

Molecular Weight: 7552.02
Raw Mass Spectrum (Retention Time 2.3 minutes)
Deconvoluted Mass Spectrum (Retention Time 2.3 minutes)
LCMS Characterization of **Double Label SI-24**

Produced from **SI-16** by General Procedure 3:

- Conversion (A260): 25%
- Retention Time: 2.72 min
- Expected Mass: 7752.2
- Expected (M-8)/8 Ion: 968.02
- Found Deconvoluted Mass: 7552.00
- Found (M-6)/6 Ion: 967.86

![Molecular Structure](image)

Molecular Weight: 7752.21
Raw Mass Spectrum (Retention Time 2.7 Minutes)
Deconvoluted Mass Spectrum (Retention Time 2.7 Minutes)
Entry 7
LCMS Characterization of Single Label SI-23
Produced from SI-16 by General Procedure 3:
Conversion (A260): 33%
Retention Time: 2.29 min
Expected Mass: 7552.02
Expected (M-8)/8 Ion: 943.0
Found Deconvoluted Mass: 7552.00
Found (M-6)/6 Ion: 943.18

A260

DTF-6.197-H1
Oligo03528

TIC
Oligo03528

1. Scan ESI-TIC
1.15e7

2: Diode Array
260 4.0000Da
Range: 1.394

Molecular Weight: 7552.02
Deconvoluted Mass Spectrum (Retention Time 2.3 minutes)
Structure Confirmation of SI-23

A crude solution of **SI-23** in water (100 µM) was injected on the Waters I-Class ToF for peak identification. The MS cone voltage was then increased from 5 mV to 30 mV and the same sample was reinjected to fragment the modified oligonucleotide. Diagnostic mass fragments were then extracted, and their presence confirmed in the total ion current. They are plotted below.
Mass Fragmentation of SI-23

Exact Mass: 7548.27
Molecular Weight: 7552.02

Fragment 1
Chemical Formula: C_{18}H_{22}N_{2}O_{7}PS⁻
Exact Mass: 441.09
Molecular Weight: 441.42

Fragment 2
Chemical Formula: C_{28}H_{35}N_{4}O_{14}P_{2}S⁻
Exact Mass: 745.14
Molecular Weight: 745.61

Fragment 3
Chemical Formula: C_{37}H_{47}N_{7}O_{20}P_{3}S⁻
Exact Mass: 1034.18
Molecular Weight: 1034.79

Time (min)
Intensity

Extracted Mass: 1043.181
Extracted Mass: 745.135
Extracted Mass: 441.089
**T4PNK Phosphorylation then SENDR**

**Protocol**

Nonphosphorylated DNA **SI-16** was phosphorylated at the 5’ end by T4PNK. Briefly, 5 nMol DNA was added to a PCR tube containing CutSmart® (1X), 1 mM ATP, and 5 mM DTT (from 5x stocks). T4PNK was added (2 uL) and the reaction volume was brought to 50 µL total. The reaction tube was allowed to incubate for 30 minutes at 37°C. After 30 minutes the reaction mixture was added directly to the Strata XL-A support in PBS (500 uL) and vortexed. The support was dried according to General Procedure 2, and standard SENDR conditions were applied.
LCMS Characterization of **SI-25**
Produced from **SI-16**:  
Conversion (A260): 75%  
Retention Time: 2.28 min  
Expected Mass: 7632.00  
Expected (M-8)/8 Ion: 953.0  
Found Deconvoluted Mass: 7632.00  
Found (M-6)/6 Ion: 952.78

Molecular Weight: 7632.00

**A260**

**TIC**

*Raw Mass Spectrum (Retention Time 2.30)*
Deconvoluted Mass Spectrum (Retention Time 2.30)

![Graph showing a deconvoluted mass spectrum with retention time 2.30.]
Sequence Independence

Procedure
SENDR was optimized by performing reactions in microcentrifuge tube as described in General Procedure 2. Briefly, 5 nmol of DNA 1 or 2 was loaded onto 100 µL of equilibrated resin. This loaded resin was washed with DMA (500 µL twice) and dry THF (500 µL three times) and dried under vacuum. Ψ-I in MeCN (150 mM, 250 µL) was added to the loaded and dried resin. Then DBU (450 mM, 18 µL) was added to the reaction mixture. The reaction tube was vortexed for 30 seconds and incubated at room temperature for various amounts of time. The reactions were worked up by aspirating and discarding the reaction solution, and washing the resin bed with MeCN (500 µL) and 1:1 MeCN:PBS (500 µL). Elute buffer (300 µL) was added to the resin bed and the tube was agitated by orbital shaker for 5-10 minutes. The DNA containing elution buffer was collected by carefully pipetting the supernatant. DNA was isolated via ethanol precipitation. Ethanol precipitation was performed by adding 10% v/v of a NaCl solution (30 µL, 5M) to the elute buffer and three volumes of cold ethanol (-20 ºC) were added to the tube (~1000µL) and incubated for 18 hours at -20 ºC. The tube was then centrifuged at 13,000 rpm for 15 minutes to pellet the DNA, and the extra ethanol was decanted off. The tubes were dried via speed vacuum and the DNA was dissolved and HPLC-MS analysis was performed.
Characterization of Sequence Independence

LCMS Characterization of SI-26

Produced from SI-1 by General Procedure 2:
Yield (A260): 52%
Expected Mass: 6415.22
Expected (M-6)/6 Ion: 1068.2
Found Deconvoluted Mass: 6415.00
Found (M-6)/6 Ion: 1068.16

![Molecular Structure]

Exact Mass: 6412.05
Molecular Weight: 6415.22

A260 Chromatogram
Mass Spectrum (RT= 2.58 min)
Deconvoluted Mass Spectrum
LCMS Characterization of SI-27

Produced from SI-2 by General Procedure 2:
Yield (A260): 76%
Expected Mass: 7327.81
Expected (M-6)/6 Ion: 1220.2
Found Deconvoluted Mass: 7328.00
Found (M-6)/6 Ion: 1220.11

![A260 Chromatogram]

Exact Mass: 7324.19
Molecular Weight: 7327.81
Mass Spectrum (RT=2.70 min)
Deconvoluted Mass Spectrum

100

7328.00

4.01e6

7385.00

7193.00

7404.00

mass
LCMS Characterization of 3

Produced from 1 by General Procedure 2:
Yield (A260): 91%
Expected Mass: 7023.61
Expected (M-6)/6 Ion: 1169.60
Found Deconvoluted Mass: 7024.00
Found (M-6)/6 Ion: 1169.61

A260 Chromatogram
Mass Spectrum (RT= 1.68 min)
Deconvoluted Mass Spectrum
LCMS Characterization of SI-28

Produced from **SI-3** by General Procedure 2:
Yield (A260): 73%
Expected Mass: 6719.42
Expected (M-8)/8 Ion: 838.93
Found Deconvoluted Mass: 6720.00
Found (M-8)/8 Ion: 838.95

![Chemical Structure](image)

**Exact Mass**: 6716.10
**Molecular Weight**: 6719.42

A260 Chromatogram

![Chromatogram](image)
Mass Spectrum (RT = 2.66 min)
Deconvoluted Mass Spectrum

- Mass: 6720.00
- Mass: 6777.00
- Intensity: 1.82e7
LCMS Characterization of SI-29

Produced from SI-4 by General Procedure 2:
Yield (A260): 76%
Expected Mass: 6732.63
Expected (M-6)/6 Ion: 1171.105
Found Deconvoluted Mass: 6733.00
Found (M-6)/6 Ion: 1171.07

A260 Chromatogram
Deconvoluted Mass Spectrum
LCMS Characterization of SI-30

Produced from SI-5 by General Procedure 2:
Yield (A260): 66%
Expected Mass: 7008.6
Expected (M-6)/6 Ion: 1167.1
Found Deconvoluted Mass: 7008.00
Found (M-6)/6 Ion: 1166.7

A260 Chromatogram
Mass Spectrum (RT = 2.64 min)
Deconvoluted Mass Spectrum
**LCMS Characterization of SI-31**

Produced from **SI-6** by General Procedure 2:
Yield (A260): 76%
Expected Mass: 7048.63
Expected (M-6)/6 Ion: 1173.77
Found Deconvoluted Mass: 7049.00
Found (M-6)/6 Ion: 1173.61

![Exact Mass: 7045.15 Molecular Weight: 7048.63](image)

**A260 Chromatogram**

![A260 Chromatogram Image]
Mass Spectrum (RT = 2.57 min)
Deconvoluted Mass Spectrum
LCMS Characterization of SI-32

Produced from SI-7 by General Procedure 2:
Yield (A260): 61%
Expected Mass: 6415.22
Expected (M-6)/6 Ion: 1068.20
Found Deconvoluted Mass: 6145.00
Found (M-6)/6 Ion: 1067.82

5′—C-C-G-A-G-T-C-A-A—A-A-T-G-A-C-T-C-G

Exact Mass: 6412.05
Molecular Weight: 6415.22

A260 Chromatogram
Mass Spectrum (RT = 2.18 min)
LCMS Characterization of SI-33

Produced from SI-8 by General Procedure 2:
Yield (A260): 73%
Expected Mass: 6719.42
Expected (M-6)/6 Ion: 1118.9
Found Deconvoluted Mass: 6719.00
Found (M-6)/6 Ion: 1118.45

A260 Chromatogram
Deconvoluted Mass Spectrum
LCMS Characterization of 4

Produced from 2 by General Procedure 1:
Yield (A260): 72%
Expected Mass: 7023.61
Expected (M-6)/6 Ion: 1169.6
Found Deconvoluted Mass: 7023.00
Found (M-6)/6 Ion: 1169.54

\[
5'\text{-}C\text{-}C\text{-}G\text{-}A\text{-}G\text{-}T\text{-}C\text{-}A\text{-}A\text{-}A\text{-}A\text{-}A\text{-}T\text{-}G\text{-}A\text{-}C\text{-}T\text{-}C\text{-}G\text{-}G\text{-}T\text{-}O\text{-}R\text{-}OH
\]

Exact Mass: 7020.14
Molecular Weight: 7023.61

A260 Chromatogram
Mass Spectrum (RT = 2.76 min)
Deconvoluted Mass Spectrum
LCMS Characterization of SI-34

Produced from SI-8 by General Procedure 2:
Yield (A260): 74%
Expected Mass: 7327.81
Expected (M-6)/6 Ion: 1220.3
Found Deconvoluted Mass: 7328.00
Found (M-6)/6 Ion: 1219.84

5’-C-C-G-A-G-T-C-A-A-A-A-A-T-G-A-C-T-C-G-G-T-T-3’

Exact Mass: 7324.19
Molecular Weight: 7327.81

A260 Chromatogram
Deconvoluted Mass Spectrum
LCMS Characterization of SI-35

Produced from SI-10 by General Procedure 2:
Yield (A260): 69%
Expected Mass: 7032.63
Expected (M-6)/6 Ion: 1171.1
Found Deconvoluted Mass: 7032.00
Found (M-6)/6 Ion: 1170.94

A260 Chromatogram

Exact Mass: 7029.16
Molecular Weight: 7032.63
Mass Spectrum (RT = 2.39 min)
Deconvoluted Mass Spectrum
LCMS Characterization of SI-36

Produced from SI-11 by General Procedure 2:
Yield (A260): 72%
Expected Mass: 7008.6
Expected \((M-6)/6\) Ion: 1167.1
Found Deconvoluted Mass: 7008.00
Found \((M-6)/6\) Ion: 1166.94

A260 Chromatogram

Exact Mass: 7005.14
Molecular Weight: 7008.60
Mass Spectrum (RT = 2.29 min)
Deconvoluted Mass Spectrum
LCMS Characterization of SI-37

Produced from SI-12 by General Procedure 2:
Yield (A260): 62%
Expected Mass: 7048.63
Expected (M-6)/6 Ion: 1173.8
Found Deconvoluted Mass: 7049.00
Found (M-6)/6 Ion: 1173.74

A260 Chromatogram
Substrate scope:

Procedure
SENDR substrate scope was analyzed by performing reactions in microcentrifuge tube as described in General Procedure 2. Briefly, 5 nmol of DNA 1 or 2 was loaded onto 100 µL of equilibrated resin. This loaded resin was washed with DMA (500 µL twice) and dry THF (500 µL three times) and dried under vacuum. Ψ-module in MeCN (150 mM, 250 µL) was added to the loaded and dried resin. Then DBU (450 mM, 18 µL) was added to the reaction mixture. The reaction tube was vortexed for 30 seconds and incubated at room temperature or 37°C for 60 minutes. The reactions were worked up by aspirating and discarding the reaction solution, and washing the resin bed with MeCN (500 µL) and 1:1 MeCN:PBS (500 µL). Elute buffer (300 µL) was added to the resin bed and the tube was agitated by orbital shaker for 5-10 minutes. The DNA containing elution buffer was collected by carefully pipetting the supernatant. DNA was isolate via ethanol precipitation. Ethanol precipitation was performed by adding 10% v/v of a NaCl solution (30 µL, 5M) to the elute buffer and three volumes of cold ethanol (-20 °C) were added to the tube (~1000µL) and incubated for 18 hours at -20 °C. The tube was then centrifuged at 13,000 rpm for 15 minutes to pellet the DNA, and the extra ethanol was decanted off. The tubes were dried via speed vacuum, the DNA was dissolved, and HPLC-MS analysis was performed.
Characterization of Substrate Scope

LCMS Characterization of 5

Produced from 1 by General Procedure 2:

Using Ψ-2

Yield (A260): 94%

Expected Mass: 7102.72

Expected (M-6)/6 Ion: 1182.20

Found Deconvoluted Mass: 7102.0

Found (M-6)/6 Ion: 1182.15

![Chemical structure](image)

A260 Chromatogram

```
  2.62
  1.78

Range: 2.307e-1
Area: 7069
```
Mass Spectrum (RT= 2.62 min)

Deconvoluted Mass Spectrum
LCMS Characterization of 6

Produced from 2 by General Procedure 2:
Using Ψ-2
Yield (A260): 74%
Expected Mass: 7102.71
Expected (M-6)/6 Ion: 1182.8
Found Deconvoluted Mass: 7102.0
Found (M-6)/6 Ion: 1182.69

A260 Chromatogram
Mass Spectrum (RT = 2.56 min)

Deconvoluted Mass Spectrum
LCMS Characterization of 7

Produced from 1 by General Procedure 2:
Using Ψ-3
Yield (A260): 88%
Expected Mass: 7076.63
Expected (M-6)/6 Ion: 1177.86
Found Deconvoluted Mass: 7075.00
Found (M-6)/6 Ion: 1178.01

Exact Mass: 7073.17
Molecular Weight: 7076.63
Mass Spectrum (RT= 2.05 min)

Deconvoluted Mass Spectrum
LCMS Characterization of 8

Produced from 2 by General Procedure 2:
Using Ψ-3
Yield (A260): 74%
Expected Mass: 7076.63
Expected (M-6)/6 Ion: 1177.86
Found Deconvoluted Mass: 7076.0
Found (M-6)/6 Ion: 1177.55
Mass Spectrum (RT = 2.02 min)

Deconvoluted Mass Spectrum
LCMS Characterization of 9

Produced from 1 by General Procedure 2:
Using Ψ-4
Yield (A260): 78%
Expected Mass: 7064.62
Expected (M-6)/6 Ion: 1175.85
Found Deconvoluted Mass: 7064.00
Found (M-6)/6 Ion: 1176.14

A260 Chromatogram

[Image of A260 Chromatogram]
Mass Spectrum (RT = 1.61 min)
Deconvoluted Mass Spectrum
LCMS Characterization of 10
Produced from 2 by General Procedure 2:
Using Ψ-4
Yield (A260): 78%
Expected Mass: 7064.62
Expected (M-6)/6 Ion: 1175.85
Found Deconvoluted Mass: 7065.00
Found (M-6)/6 Ion: 1175.75

A260 Chromatogram (Small Molecule at RT=1 min)

Exact Mass: 7061.15
Molecular Weight: 7064.62
Mass Spectrum (RT = 2.33 min)

Deconvoluted Mass Spectrum
LCMS Characterization of 11

Produced from 1 by General Procedure 2 (Reaction run at 45°C):
Using Ψ-8
Yield (A260): 76%
Expected Mass: 7144.83
Expected (M-6)/6 Ion: 1189.8
Found Deconvoluted Mass: 7144.00
Found (M-6)/6 Ion: 1189.42

A260 Chromatogram
Mass Spectrum (RT = 2.81 min)
LCMS Characterization of 12

Produced from 2 by General Procedure 2 (reaction run at 45°C):
Using \( \Psi - 8 \)
Yield (A260): 62%
Expected Mass: 7144.83
Expected (M-6)/6 Ion: 1189.8
Found Deconvoluted Mass: 7144.0
Found (M-6)/6 Ion: 1189.4

Exact Mass: 7141.15
Molecular Weight: 7144.83

A260 Chromatogram
Mass Spectrum (RT = 2.77 min)
LCMS Characterization of 13
Using Ψ-19
Produced from 1 by General Procedure 2 (Reaction Run at 37ºC):
Yield (A260): 84%
Expected Mass: 7166.76
Expected (M-6)/6 Ion: 1193.5
Found Deconvoluted Mass: 7166.00
Found (M-6)/6 Ion: 1193.42

A260 Chromatogram

TIC

1: Scan ESI-
TIC
tic 2.54e7
Mass Spectrum (RT= 2.23 min)
LCMS Characterization of 14

Using Ψ-19
Produced from 2 by General Procedure 2 (Reaction Run at 37°C):
Yield (A260): 62%
Expected Mass: 7166.76
Expected (M-6)/6 Ion: 1193.5
Found Deconvoluted Mass: 7166.0
Found (M-6)/6 Ion: 1193.82

Molecular Weight: 7166.75
Mass Spectrum (RT= 2.22 min)
Deconvoluted Mass Spectrum
LCMS Characterization of 15

Produced from 1 by General Procedure 2:
Using Ψ-12
Yield (A260): 81%
Expected Mass: 7025.6
Expected (M-6)/6 Ion: 1169.36
Found Deconvoluted Mass: 7026.0
Found (M-6)/6 Ion: 1169.41

A260 Chromatogram

Exact Mass: 7022.16
Molecular Weight: 7025.63
Mass Spectrum (RT = 2.36 min)

Deconvoluted Mass Spectrum
LCMS Characterization of 16

Produced from 2 by General Procedure 2:
Using \( \Psi - 12 \)
Yield (A260): 68%
Expected Mass: 7025.63
Expected (M-6)/6 Ion: 1169.94
Found Deconvoluted Mass: 7025.0
Found (M-6)/6 Ion: 1169.74

A260 Chromatogram
Mass Spectrum (RT= 2.32 min)

Deconvoluted Mass Spectrum
LCMS Characterization of 17

Produced from 1 by General Procedure 2:
Using Ψ-13
Yield (A260): 51%
Expected Mass: 7278.91
Expected (M-6)/6 Ion: 1211.5
Found Deconvoluted Mass: 7279.0
Found (M-6)/6 Ion: 1211.7

A260 Chromatogram
Mass Spectrum (RT = 3.06 min)
Deconvoluted Mass Spectrum
**LCMS Characterization of 18**

Produced from 2 by General Procedure 2:

Using Ψ-13

Yield (A260): 45%

Expected Mass: 7278.23

Expected (M-6)/6 Ion: 1211.5

Found Deconvoluted Mass: 7279.00

Found (M-6)/6 Ion: 1211.77

![Chemical Structure of 18](image)

**A260 Chromatogram**

![A260 Chromatogram Graph](image)

Exact Mass: 7275.23

Molecular Weight: 7278.91
Mass Spectrum (RT = 2.93 min)
Deconvoluted Mass Spectrum
LCMS Characterization of 19

Using $\Psi$-14
Produced from 1 by General Procedure 2 (Reaction run with 75 mM $\Psi$-14 and 225 mM DBU):
Yield (A260): 68%
Expected Mass: 6995.11
Expected (M-6)/6 Ion: 1164.9
Found Deconvoluted Mass: 6995.0
Found (M-6)/6 Ion: 1165.0

Exact Mass: 6992.11
Molecular Weight: 6995.56

A260 Chromatogram
Mass Spectrum (RT = 2.29 min)
Deconvoluted Mass Spectrum
LCMS Characterization of 20

Using Ψ-14
Produced from 2 by General Procedure 2 (Reaction run with 75 mM Ψ-14 and 225 mM DBU):
Yield (A260): 76%
Expected Mass: 6995.11
Expected (M-6)/6 Ion: 1164.9
Found Deconvoluted Mass: 6995.0
Found (M-6)/6 Ion: 1164.54

A260 Chromatogram
Mass Spectrum (RT= 2.04 min)
Deconvoluted Mass Spectrum
LCMS Characterization of 21

Using \( \Psi-15 \)
Produced from 1 by General Procedure 2 (Reaction run with 75 mM \( \Psi-15 \) and 225 mM DBU):
Yield (A260): 77%
Expected Mass: 7003.6
Expected (M-6)/6 Ion: 1166.3
Found Deconvoluted Mass: 7004.0
Found (M-6)/6 Ion: 1166.2

A260 Chromatogram
Mass Spectrum (RT= 2.27 min)
Deconvoluted Mass Spectrum
LCMS Characterization of 22

Using Ψ-15
Produced from 1 by General Procedure 2 (Reaction run with 75 mM Ψ-15 and 225 mM DBU):
Yield (A260): 56%
Expected Mass: 7003.6
Expected (M-6)/6 Ion: 1166.3
Found Deconvoluted Mass: 7003.0
Found (M-6)/6 Ion: 1166.2

5′—C-C-G-A-G-T-C-A-A-A—A-A-T-G-A-C-T-C-G-G—T-T—

Molecular Weight: 7003.58

A260 Chromatogram

TIC

1: Scan ES-TIC
1.4167
LCMS Characterization of 23

Using Ψ-17
Produced from 1 by General Procedure 2 (Reaction run at 37ºC):
Yield (A260): 91%
Expected Mass: 7168.69
Expected (M-6)/6 Ion: 1193.8
Found Deconvoluted Mass: 7169.00
Found (M-6)/6 Ion: 1193.69

A260 Chromatogram

![A260 Chromatogram](image)

TIC

![TIC](image)
Mass Spectrum (RT = 2.1 min)
LCMS Characterization of 24

Using Ψ-17
Produced from 2 by General Procedure 2 (reaction run with 200 mM Ψ-17 at 50°C)
Yield (A260): 89%
Expected Mass: 7168.69
Expected (M-6)/6 Ion: 1193.8
Found Deconvoluted Mass: 7169.00
Found (M-6)/6 Ion: 1193.56

Exact Mass: 7165.17
Molecular Weight: 7168.69

A260 Chromatogram

TIC

2: Diode Array
260 4.80000Da
Range: 2.383

1: Scan ESI-TIC
3.92e8
Mass Spectrum (RT = 2.04 min)

1: Scan ES-

3.41e5
Deconvoluted Mass Spectrum
LCMS Characterization of 23
Using Ψ-18
Produced from 1 by General Procedure 2 (Reaction run at 37°C):
Yield (A260): 85%
Expected Mass: 7181.72
Expected (M-6)/6 Ion: 1195.6
Found Deconvoluted Mass: 7182.0
Found (M-6)/6 Ion: 1195.69

A260 Chromatogram

TIC
Mass Spectrum (RT= 2.06 min)
Deconvoluted Mass Spectrum
LCMS Characterization of 26

Using **Ψ-18**
Produced from 1 by General Procedure 2 (Reaction run with **Ψ-18** (200 mM) at 50ºC):
Yield (A260): 69%
Expected Mass: 7181.72
Expected (M-6)/6 Ion: 1195.6
Found Deconvoluted Mass: 7182.0
Found (M-6)/6 Ion: 1195.69

Exact Mass: 7178.18
Molecular Weight: 7181.72

A260 Chromatogram

TIC

SI303
Mass Spectrum (RT= 1.98 min)
Deconvoluted Mass Spectrum
LCMS Characterization of 27

Using Ψ-5
Produced from 1 by General Procedure 2:
Yield (A260): 79%
Expected Mass: 6985.563
Expected (M-6)/6 Ion: 1163.26
Found Deconvoluted Mass: 6986.00
Found (M-6)/6 Ion: 1163.01

Exact Mass: 6982.129
Molecular Weight: 6985.563

A260 Chromatogram
Mass Spectrum (RT= 3.38 min)

Deconvoluted Mass Spectrum
LCMS Characterization of 28

Produced from 2 by General Procedure 2:
Using Ψ-5
Yield (A260): 84%
Expected Mass: 6985.53
Expected (M-6)/6 Ion: 1163.255
Found Deconvoluted Mass: 6986.00
Found (M-6)/6 Ion: 1163.07

A260 Chromatogram
Mass Spectrum (RT= 1.92 min)

Deconvoluted Mass Spectrum
LCMS Characterization of 29
Produced from 1 by General Procedure 2:
Using Ψ-9
Yield (A260): 85%
Expected Mass: 7257.939
Expected (M-6)/6 Ion: 1208.65
Found Deconvoluted Mass: 7258.00
Found (M-6)/6 Ion: 1208.23

A260 Chromatogram
Mass Spectrum (RT = 3.38 min)

Deconvoluted Mass Spectrum
LCMS Characterization of 30

Produced from 2 by General Procedure 2:

Using Ψ-9

Yield (A260): 78%
Expected Mass: 7257.939
Expected (M-6)/6 Ion: 1208.662
Found Deconvoluted Mass: 7258.00
Found (M-6)/6 Ion: 1208.57

A260 Chromatogram

Not DNA
Mass Spectrum (RT = 3.26 min)

Deconvoluted Mass Spectrum
LCMS Characterization of 31

Produced from 1 by General Procedure 2:
Using Ψ-6
Yield (A260): 75%
Expected Mass: 7101.50
Expected (M-6)/6 Ion: 1182.6
Found Deconvoluted Mass: 7102.00
Found (M-6)/6 Ion: 1182.21

A260 Chromatogram

Exact Mass: 7098.08
Molecular Weight: 7101.50
Mass Spectrum (RT = 2.43 min)

Deconvoluted Mass Spectrum
LCMS Characterization of 32

Produced from 2 by General Procedure 2:
Using Ψ-6
Yield (A260): 74%
Expected Mass: 7101.5
Expected (M-6)/6 Ion: 1182.01
Found Deconvoluted Mass: 7102.00
Found (M-6)/6 Ion: 1182.2

A260 Chromatogram
Mass Spectrum (RT = 2.39 min)

Deconvoluted Mass Spectrum
LCMS Characterization of 33

Produced from 1 by General Procedure 2:

Using Ψ-7

Yield (A260): 90%
Expected Mass: 7203.790
Expected (M-6)/6 Ion: 1199.63
Found Deconvoluted Mass: 7204.00
Found (M-6)/6 Ion: 1199.23

A260 Chromatogram

Not DNA
Mass Spectrum (RT= 2.37 min)

Deconvoluted Mass Spectrum
LCMS Characterization of 34

Produced from 2 by General Procedure 1:
Using Ψ-7
Yield (A260): 84%
Expected Mass: 7203.79
Expected (M-6)/6 Ion: 1199.63
Found Deconvoluted Mass: 7204.00
Found (M-6)/6 Ion: 199.29

Exact Mass: 7200.165
Molecular Weight: 7203.790

A260 Chromatogram
Mass Spectrum (RT=2.43 min)

Deconvoluted Mass Spectrum
LCMS Characterization of 35

Using Ψ-16
Produced from 1 by General Procedure 2:
Yield (A260): 88%
Expected Mass: 7126.17
Expected (M-6)/6 Ion: 1186.03
Found Deconvoluted Mass: 7126.00
Found (M-6)/6 Ion: 1186.28

A260 Chromatogram

TIC
Mass Spectrum (RT= 2.70 min)
Deconvoluted Mass Spectrum
LCMS Characterization of 35

Using Ψ-16
Produced from 2 by General Procedure 2:
Yield (A260): 64%
Expected Mass: 7126.17
Expected (M-6)/6 Ion: 1186.03
Found Deconvoluted Mass: 7126.0
Found (M-6)/6 Ion: 1186.42

A260 Chromatogram

TIC

2: Diode Array
250 4.8000Da
Range: 9.992e-1
Mass Spectrum (RT = 2.27 min)
LCMS Characterization of 37

Produced from 1 by General Procedure 2:

Using Ψ-10

Yield (A260): 76%

Expected Mass: 7269.9

Expected (M-6)/6 Ion: 1210.65

Found Deconvoluted Mass: 7270.0

Found (M-6)/6 Ion: 1210.5

A260 Chromatogram
Deconvoluted Mass Spectrum
LCMS Characterization of 38

Produced from 2 by General Procedure 2:
Using Ψ-10
Yield (A260): 68%
Expected Mass: 7269.9
Expected (M-6)/6 Ion: 1210.65
Found Deconvoluted Mass: 7270.0
Found (M-6)/6 Ion: 1210.5

Molecular Weight: 7269.93

A260 Chromatogram
Mass Spectrum (RT = 3.30 min)

Deconvoluted Mass Spectrum
LCMS Characterization of 39

Produced from 1 by General Procedure 2:

Using Ψ-11

Yield (A260): 55%

Expected Mass: 7193.83

Expected (M-6)/6 Ion: 1197.97

Found Deconvoluted Mass: 1197.69

Found (M-6)/6 Ion: 7194 and 7232.00 (potassium adduct)

A260 Chromatogram

Mass Spectrum (RT= 2.45 min)
Deconvoluted Mass Spectrum
LCMS Characterization of 40

Produced from 1 by General Procedure 2:
Using Ψ-20 produced in situ
Yield (A260): 72%
Expected Mass: 7285.92
Expected (M-6)/6 Ion: 1213.32
Found Deconvoluted Mass: 7287.0
Found (M-6)/6 Ion: 1213.23

Exact Mass: 7282.28
Molecular Weight: 7285.92
Mass Spectrum (RT= 3.58 min)
Deconvoluted Mass Spectrum

DTF-6-5-INSITU

Oligo: 3943 854 (3.506) M1 [Ev0.1#10] (G3:1, 1500, 600: 1249, 1.00: L33, R33). Cm (844.877)

1: Scan ES- 1.67e6
LCMS Characterization of 41

Produced from 2 by General Procedure 2:
Using Ψ-20 produced in situ
Yield (A260): 65%
Expected Mass: 7285.92
Expected (M-6)/6 Ion: 1213.32
Found Deconvoluted Mass: 7287.0
Found (M-6)/6 Ion: 1213.41

Molecular Weight: 7269.93

A260 Chromatogram and TIC
Mass Spectrum (RT = 3.39 min)
Deconvoluted Mass Spectrum

DTF-6-3'-INSITU

Oligo03644 796 (3.400) M1 [Ev00,110] (G61,1.500,600,1250,1.00,1.33,1.33); Cm (776.825)

1: Scan ES- 1.45e6
Secondary Manipulations:

Azide Reduction (42):

Compound 21 (5 nmol) was synthesized by General Procedure 2 and isolated crude after ethanol precipitation. Solid 21 was dissolved (100 µL) in tris buffer (50 mM) containing TCEP (5 mM) at pH 8.5. This solution was vortexed and transferred to an HPLC vial. This reaction was injected onto the H-Class HPLC at different timepoints. The reaction was found to be complete after 35 minutes at room temperature. Reduction product 42 was characterized below.
LCMS Characterization of 42

Produced from 21
Yield (A260): 78% (total yield including 1)
Expected Mass: 7038.63
Expected (M-6)/6 Ion: 1171.5
Found Deconvoluted Mass: 7039.0
Found (M-6)/6 Ion: 1171.7

A260 Chromatogram

Exact Mass: 7035.16
Molecular Weight: 7038.63
Mass Spectrum (RT = 1.38 min)

Deconvoluted Mass Spectrum
43 SPAAC Reaction:

Compound 19 (5 nmol) was synthesized by General Procedure 2 and isolated crude after ethanol precipitation. Solid 19 was dissolved (98 µL) in tris buffer (50 mM) at pH 8.5. To this solution BDP-FL (2 µL) was added from stock solution (5 mM) in DMA, to a final concentration of 100 µM. This solution was vortexed and transferred to an HPLC vial. This reaction was injected onto the H-Class HPLC after 60 minutes. SPAAC product 43 was characterized below.
LCMS Characterization of 43

Produced from 19
Yield (A260): 86%
Expected Mass: 7627.05
Expected (M-8)/8 Ion: 951.925
Found Deconvoluted Mass: 7627.0
Found (M-6)/6 Ion: 952.05

*The two peaks at 3.91 and 4.14 minutes correspond to the two regioisomers of 43.

![A260 Chromatogram](image)

**Exact Mass: 7623.40**
**Molecular Weight: 7627.05**
Mass Spectrum (RT = 3.91 min)
Deconvoluted Mass Spectrum (RT = 3.91)

Deconvoluted Mass Spectrum (RT = 4.14)
44 CuAAC Reaction:

Compound 24 (5 nmol) was synthesized by General Procedure 2 and isolated crude after ethanol precipitation. Solid 24 was dissolved (92.5 µL) in tris buffer (50 mM) at pH 8.5. To this solution TAMRA Azide (2 µL) was added from stock solution (5 mM) in DMA, (final concentration of 100 µM). Next, BTTP (2 µL) was added from a stock solution (40 mM in water). Next, CuSO₄ (1 µL) was added from a stock solution (40 µM in water) (a final concentration of 400 µM). The solution was capped and vortexed. Finally, sodium ascorbate (2.5 uL) was added from a stock solution (100 mM in water). This reaction solution was vortexed and incubated for 1 hour at 37 ºC. This reaction was injected onto the H-Class HPLC after 60 minutes. CuAAC product 44 was characterized below.
LCMS Characterization of 44

Produced from 24
Yield (A260): >90%
Expected Mass: 7630.3
Expected (M-8)/8 Ion: 952.75
Found Deconvoluted Mass: 7631.00
Found (M-8)/8 Ion: 952.92

A260 Chromatogram
Mass Spectrum (RT = 3.38 min)
Deconvoluted Mass Spectrum
**CuAAC Reaction:**

Compound 24 (5 nmol) was synthesized by *General Procedure 2* and isolated crude after ethanol precipitation. Solid 24 was dissolved (92.5 µL) in tris buffer (50 mM) at pH 8.5. To this solution Biotin Azide (2 µL) was added from stock solution (5 mM) in DMA, (final concentration of 100 µM). Next, BTTP (2 µL) was added from a stock solution (40 mM in water). Next, CuSO$_4$ (1 µL) was added from a stock solution (40 µM in water) (a final concentration of 400 µM). The solution was capped and vortexed. Finally, sodium ascorbate (2.5 uL) was added from a stock solution (100 mM in water). This reaction solution was vortexed and incubated for 1 hour at 37 ºC. This reaction was injected onto the H-Class HPLC after 60 minutes. CuAAC product 43 was characterized below.
LCMS Characterization of 45

Produced from 24
Yield (A260): 90%
Expected Mass: 7387.04
Expected (M-8)/8 Ion: 921.9
Found Deconvoluted Mass: 7387
Found (M-6)/6 Ion: 922.4

A260 Chromatogram
Deconvoluted Mass Spectrum
Phosphorothioate (PS) compatibility:

Protocol for SENDR on PS DNA:
SENDR phosphorothioate (PS) compatibility was analyzed by performing reactions in microcentrifuge tube as described in General Procedure 2. Briefly, 5 nmol of PS DNA 46 was loaded onto 100 µL of equilibrated resin. This loaded resin was washed with DMA (500 µL twice) and dry THF (500 µL three times) and dried under vacuum. To the loaded and dried resin, PSI-module in MeCN (150 mM, 250 µL) was added. Then DBU (450 mM, 18 µL) was added to the reaction mixture. The reaction tube was vortexed for 30 seconds and incubated at 37ºC for 60 minutes. The reactions were worked up by aspirating and discarding the reaction solution, and washing the resin bed with MeCN (500 µL) and 1:1 MeCN:PBS (500 µL). Elute buffer (300 µL) was added to the resin bed and the tube was agitated by orbital shaker for 5-10 minutes. The DNA containing elution buffer was collected by carefully pipetting the supernatant. DNA was isolate via ethanol precipitation. Ethanol precipitation was performed by adding 10% v/v of a NaCl solution (30 µL, 5M) to the elute buffer and three volumes of cold ethanol (-20 ºC) were added to the tube (~1000µL) and incubated for 18 hours at -20 ºC. The tube was then centrifuged at 13,000 rpm for 15 minutes to pellet the DNA, and the extra ethanol was decanted off. The tubes were dried via speed vacuum, the DNA was dissolved, and HPLC-MS analysis was performed.
LCMS Characterization of 47

Produced from 46 by General Procedure 2:
Using Ψ-1
Yield (A260): 70%
Expected Mass: 6962.52
Expected (M-6)/6 Ion: 1159.6
Found Deconvoluted Mass: 6963.0
Found (M-6)/6 Ion: 1159.4

A260 Chromatogram
Mass Spectrum (RT = 2.61 min)

Deconvoluted Mass Spectrum
LCMS Characterization of 48

Produced from 46 by General Procedure 2:
Using \( \Psi -2 \)
Yield (A260): 71%
Expected Mass: 7041.62
Expected (M-6)/6 Ion: 1172.6
Found Deconvoluted Mass: 7040
Found (M-6)/6 Ion: 1172.7

A260 Chromatogram
LCMS Characterization of 49

Produced from 46 by General Procedure 2:

Using Ψ-12

Yield (A260): 74%

Expected Mass: 6964.54

Expected (M-6)/6 Ion: 1158.9

Found Deconvoluted Mass: 6964.0

Found (M-6)/6 Ion: 1159.2

A260 Chromatogram
Mass Spectrum (RT = 2.69 min)

Deconvoluted Mass Spectrum
LCMS Characterization of 50

Produced from 46 by General Procedure 2:

Using Ψ-3

Yield (A260): 81%

Expected Mass: 7015.54

Expected (M-6)/6 Ion: 1168.3

Found Deconvoluted Mass: 7015

Found (M-6)/6 Ion: 1168.07

A260 Chromatogram
Mass Spectrum (RT= 2.47 min)

Deconvoluted Mass Spectrum
LCMS Characterization of 52

Produced from 51 by General Procedure 3:
Using Ψ-16
Yield (A260): 66%
Expected Mass: 6604
Expected (M-6)/6 Ion: 1099.6
Found Deconvoluted Mass: 6601.0
Found (M-6)/6 Ion: 1099.7

MW: 6616
Raw Mass Spectrum Retention Time 3.08 min
Deconvoluted Mass Spectrum Retention Time 3.08 Minutes
LCMS Characterization of 53

Produced from 51 by General Procedure 3:
Using Ψ-8
Yield (A260): 68%
Expected Mass: 6632
Expected (M-8)/8 Ion: 828
Found Deconvoluted Mass: 6636.0
Found (M-8)/6 Ion: 828.42

MW: 6632

A260

| Time (min) | Absorbance (260 nm) |
|------------|---------------------|
| 2          |                    |
| 3          |                    |
| 4          |                    |
Raw Mass Spectrum Retention Time 3.32 Minutes
Deconvoluted Mass Spectrum Retention Time 3.32 Minutes
SENDR on Aptamers:

**Aptamer compatibility protocol:**
SENDR Aptamer compatibility was analyzed by performing reactions in microcentrifuge tube as described in *General Procedure 2*. Briefly, 5 nmol of aptamer 54 was loaded onto 100 µL of equilibrated resin. This loaded resin was washed with DMA (500 µL twice) and dry THF (500 µL three times) and dried under vacuum.

To the loaded and dried resin, Using Ψ-4 in MeCN (150 mM, 250 µL) was added. Then DBU (450 mM, 18 µL) was added to the reaction mixture. The reaction tube was vortexed for 30 seconds and incubated at 37°C for 60 minutes. The reactions were worked up by aspirating and discarding the reaction solution, and washing the resin bed with MeCN (500 µL) and 1:1 MeCN:PBS (500 µL). Elute buffer (300 µL) was added to the resin bed and the tube was agitated by orbital shaker for 5-10 minutes. The DNA containing elution buffer was collected. DNA was isolate *via* ethanol precipitation. Ethanol precipitation was performed by adding 10% v/v of a NaCl solution (30 µL, 5M) to the elute buffer and three volumes of cold ethanol (-20 °C) were added to the tube (~1000µL) and incubated for 18 hours at -20 °C. The tube was then centrifuged at 13,000 rpm for 15 minutes to pellet the DNA, and the extra ethanol was decanted off. The tubes were dried *via* speed vacuum and the DNA was dissolved and HPLC-MS analysis was performed.
LCMS Characterization of 55

Produced from 54 by General Procedure 2:

Using Ψ-4

Yield (A260): 70%

Expected Mass: 18353.88

Expected (M-15)/15 Ion: 1221.99

Found Deconvoluted Mass: 18350

Found (M-6)/6 Ion: 1221.84

\[ \text{Exact Mass: } 18344.98 \]

\[ \text{Molecular Weight: } 18353.88 \]

Starting material

Product

A260 Chromatogram

TIC Chromatogram
Mass Spectrum (RT = 3.05 min)
Deconvoluted Mass Spectrum
Synthesis of Electrophile Modified hNE Aptamers R/S-(57)

Aptamer 56 was used in two SENDR protocols according to General Procedure 3 with both enantiomers of Ψ-14. Reaction conditions were slightly altered to increase conversion, PSI was used at 150 mM and the reaction was run at 37°C for 60 minutes. Modified aptamers R-(57) and S-(57) were isolated by ethanol precipitation. After ethanol precipitation unlabeled aptamer 56 was selectively degraded by ExoIII. In short, 10 µg of S-(57) or R-(57) were loaded into a PCR tube in 40 µL water. To each tube CutSmart® (5 µL, 10x), water (3 µL), and ExoIII (2 µL) was added. The reactions were vortexed and incubated at 37°C for 60 minutes at which point the ExoIII was thermally inactivated at 75°C for 10 minutes. The resulting DNA constructs were isolated by ZymoSpin oligo clean and concentrator column.
Analysis of S-57

LCMS Characterization of S-57/ After SENDR

Produced from 56 by General Procedure 2:
Using (-)-Ψ-14
Yield (A260): 76%
Expected Mass: 13286.6
Found Deconvoluted Mass: 13288.0

\[
5'\text{---A-G-A-C-G-A-T-G-C---G-G-C-A-C-G-T-A-G---T-G-C-T-A-C-C-A-G-A---T-G-G-T-T-A-T-G-T-T---A-C---SH} \]

Exact Mass: 13280.13
Molecular Weight: 13286.59

A260

TIC
Raw Mass Spectrum RT = 2.24
Analysis of S-57
LCMS Characterization of S-57/ After ExoIII Digestion

Using (-)Ψ-14
Yield (A260): 91%
Expected Mass: 13286.6
Found Deconvoluted Mass: 13287.0

S'—A-G-G-A-C-G-A-T-G-C—G-G-C-A-C-G-T-A-G—T-G-C-T-A-C-C-A-G-A—T-G-G-T-A-T-G-T-T—A-C

Exact Mass: 13280.13
Molecular Weight: 13286.59

A260

TIC
Deconvoluted Mass Spectrum RT = 2.25
Analysis of R-57
LCMS Characterization of R-57/ SEDNR

Using (+)Ψ-14
Yield (A260): 63%
Expected Mass: 13286.6
Found Deconvoluted Mass: 13286.0

5′-A-G-G-A-T-G-C-G-G-G-A-C-G-T-A-G-T-G-C-T-A-C-C-A-G-A-T-G-G-T-A-T-G-T-T-A-C

Exact Mass: 13280.13
Molecular Weight: 13286.59

A260

TIC

Oligo003603

1: Scan ES-
TIC
4.40e7
Raw Mass Spectrum RT = 2.22 minutes
Deconvoluted Mass Spectrum RT = 2.22 Minutes
Analysis of **R-57**

**LCMS Characterization of R-57/ After EXOIII**

Using $(+)^\Psi-14$

Yield (A260): 83%
Expected Mass: 13286.6
Found Deconvoluted Mass: 13287.0

```
5'-A-G-G-A-C-G-A-T-G-C---G-G-C-A-C-G-T-A-G---T-G-C-T-A-C-C-A-G-A---T-G-G-T-A-T-G-T-T---A-C---G
```

Exact Mass: 13280.13
Molecular Weight: 13286.59

![A260 Graph](image)

![TIC Graph](image)
Deconvoluted Mass Spectrum RT = 2.21
**hNE Inhibition Assay**

**Protocol**

R/S-(57) were used directly in the assay after isolation, without further purification. Activity of human neutrophil elastase (hNE) was measured using the method described previously with slight modifications in a total volume of 10 μL in a reaction buffer of PBS (pH 7.4) and 0.05% (v/v) Nonidet™ P 40 Substitute (Sigma). Final composition of each reaction was 5 nM hNE (Elastin Products Corp.), 50 μM AAPV-aminomethylcoumarin (AMC) substrate (Millipore), and various concentrations of compounds. hNE was incubated with inhibitors for 10 min at room temperature before addition of AAPV-AMC. Residual proteolytic activity was measured kinetically at 25 °C using a Synergy H1 microplate reader (BioTek) for a total of 30 min at 30 sec intervals (Excitation: 380 nm, Emission: 460 nm). Only data points reflecting linear substrate conversion were used to determine relative protease activity. IC₅₀ values were obtained by fitting the data to a concentration-response inhibition, log (inhibitor) vs. response – variable slope (four parameters) using GraphPad Prism.

The same protocols were used to elicit the ability for small molecule electrophiles to inhibit hNe. All of the small molecules below proved inactive.
## Raw Data

### Raw data for R-(57)

| Log [M] | R-(55) | R-(55) |
|---------|--------|--------|
| -6      | 0.08443| 0.09821| 0.1046 |
| -6.30103| 0.08173| 0.09096| 0.09701|
| -6.60206| 0.1038 | 0.1246 | 0.1173 |
| -6.90309| 0.09301| 0.1166 | 0.1337 |
| -7.20412| 0.1193 | 0.144  | 0.1471 |
| -7.50515| 0.1201 | 0.1623 | 0.1527 |
| -7.80618| 0.1355 | 0.1876 | 0.1953 |
| -8.10721| 0.1741 | 0.2032 | 0.2049 |
| -8.40824| 0.2159 | 0.2269 | 0.2179 |
| -8.70927| 0.2347 | 0.2387 | 0.2242 |
| -9.0103 | 0.2237 | 0.248  | 0.2462 |

### Raw data for S-(57)

| Log [M] | S-(55) | S-(55) | S-(55) |
|---------|--------|--------|--------|
| -6      | 0.1285 | 0.1268 | 0.1318 |
| -6.301  | 0.1127 | 0.1152 | 0.1286 |
| -6.602  | 0.13   | 0.1229 | 0.1433 |
| -6.903  | 0.1256 | 0.1381 | 0.1447 |
| -7.204  | 0.1514 | 0.1531 | 0.1821 |
| -7.505  | 0.177  | 0.1934 | 0.2086 |
| -7.806  | 0.2347 | 0.2358 | 0.2644 |
| -8.107  | 0.253  | 0.2727 | 0.298  |
| -8.408  | 0.3032 | 0.3237 | 0.3083 |
| -8.709  | 0.3229 | 0.3286 | 0.3441 |
| -9.010  | 0.3415 | 0.3645 | 0.3692 |

### Raw data for 56

| Log [M] | 54 |
|---------|----|
| -6      | 0.1285 | 0.1268 | 0.1318 |
| -6.301  | 0.1127 | 0.1152 | 0.1286 |
| -6.602  | 0.13   | 0.1229 | 0.1433 |
| -6.903  | 0.1256 | 0.1381 | 0.1447 |
| -7.204  | 0.1514 | 0.1531 | 0.1821 |
| -7.505  | 0.177  | 0.1934 | 0.2086 |
| -7.806  | 0.2347 | 0.2358 | 0.2644 |
| -8.107  | 0.253  | 0.2727 | 0.298  |
| -8.408  | 0.3032 | 0.3237 | 0.3083 |
| -8.709  | 0.3229 | 0.3286 | 0.3441 |
| -9.010  | 0.3415 | 0.3645 | 0.3692 |
Plotted Data

Plotted Data for R-(57)

Non Linear Fit Parameters

| Best-fit values | Value       |
|-----------------|-------------|
| Bottom          | 0.08754     |
| Top             | 0.2613      |
| LogIC50         | -7.820      |
| HillSlope       | -0.7621     |
| IC50            | 1.513e-008  |
| Span            | 0.1737      |

95% CI (profile likelihood)

|                |            |
|----------------|------------|
| Bottom         | 0.002397 to 0.1062 |
| Top            | 0.2324 to 0.5491  |
| LogIC50        | -9.462 to -7.536  |
| HillSlope      | -1.303 to -0.2260 |
| IC50           | 3.451e-010 to 2.912e-008 |

Goodness of Fit

|                |            |
|----------------|------------|
| Degrees of Freedom | 29        |
| R squared        | 0.9290     |
| Sum of Squares  | 0.006909   |
| Sy.x             | 0.01543    |
**Plotted Data for S-(57)**

![Graph showing D(-) (S) vs Log [M]](image)

**Non-Linear Fit Parameters**

| Best-fit values |  |
|-----------------|--|
| Bottom          | 0.1175 |
| Top             | 0.3686 |
| LogIC50         | -7.858 |
| HillSlope       | -1.005 |
| IC50            | 1.386e-008 |
| Span            | 0.2510 |

| 95% CI (profile likelihood) |  |
|-----------------------------|--|
| Bottom                      | 0.1021 to 0.1292 |
| Top                         | 0.3464 to 0.4059 |
| LogIC50                     | -8.016 to -7.742 |
| HillSlope                   | -1.305 to -0.7440 |
| IC50                        | 9.635e-009 to 1.811e-008 |

| Goodness of Fit |  |
|-----------------|--|
| Degrees of Freedom | 29 |
| R squared        | 0.9793 |
| Sum of Squares  | 0.005105 |
| Sy.x             | 0.01327 |
Plotted Data for 56

Nonlinear Fit Parameters

|                           |       |
|---------------------------|-------|
| **Best-fit values**       |       |
| Bottom                    | 0.1175|
| Top                       | 0.3686|
| LogIC50                   | -7.858|
| HillSlope                 | -1.005|
| IC50                      | 1.386e-008|
| Span                      | 0.2510|
| **95% CI (profile likelihood)** |     |
| Bottom                    | 0.1021 to 0.1292|
| Top                       | 0.3464 to 0.4059|
| LogIC50                   | -8.016 to -7.742|
| HillSlope                 | -1.305 to -0.7440|
| IC50                      | 9.635e-009 to 1.811e-008|

**Goodness of Fit**

|                           |       |
|---------------------------|-------|
| Degrees of Freedom        | 29    |
| R squared                 | 0.9793|
| Sum of Squares            | 0.005105|
| Sy.x                      | 0.01327|
Small Molecule Electrophiles

The same protocols were used to elicit the ability for small molecule electrophiles to inhibit hNe. All of the small molecules below proved inactive.

![Chemical structures and graphs](image-url)
SENDRon Biosynthetically Derived DNA

Construction of 58

PCR Amplification:

To a PCR tube template strand (SI-18) (2 µL, 5 ng from 2 ng/µL stock), primer 1 (SI-19) (2.5 µL from a 10 µM stock), primer 2 (SI-20) (2.5 µL from a 10 µM stock), water (18 µL) were added on ice. To this mixture Q5® High Fidelity 2x Master Mix (NEB) was added (25 µL). Tubes were place into a preheated (95°C) thermocycler. An initial denaturing step of 95 °C for 30 was performed. This was followed by 30 rounds standard PCR protocol, with a denaturing step at 95 °C for 10 seconds, an annealing step at 59 °C for 15 seconds and an elongation step at 72 °C for 20 minute. A final extension step was performed at 752 °C for 2 minutes. The PCR products were purified by ZymoSpin Oligo Clean and Concentrator and eluted in 50 µL and carried forward.

Template Strand: SI-18
/5Phos/ata tgtgtgttcccatagacaaattgctactacggaagagctacccgagctacccgacgagttggttggtggtgacggcaaaatgaaagctcagccccagatggtacttctattacctaggaactggcccagaagcttcacttccctacgcgctaaacacaaagaagccatgatgaggtggtgaactagggaggctctaatgaggaggccctgaatcactctacccgac

Primer 1: SI-19
/5Phos/ aacccatactgatgccttcttttgt

Primer 2: SI-20
cctactacggaagagctacccgac

PCR Map
Analysis of Ampicons

5’ Phosphorylated Amplicon

5’-A-A-C-C-C-A-T-A-C-G-A-T-G-C-C-T-T-C-T-T-T-G-T-T-A-G-C-G-C-C-G-T-A-G-G-G-A-A-G-T-
G-A-A-G-C-T-T-C-T-G-G-G-C-C-A-G-T-T-C-C-T-A-G-G-T-A-G-T-A-G-A-A-G-T-A-C-C-A-T-C-
T-G-G-G-G-C-T-G-A-G-C-T-C-T-T-C-A-T-T-T-T-G-G-C-G-T-C-A-C-C-A-C-A-C-G-A-A-C-
T=C=G=T=C=G=G=T=G=C=T=C=T=C=G=T=A=G=T=A=G-3’

HPLC

5’ OH Amplicon
SI-38

5’ Phos Amplicon
SI-39
Deconvoluted Mass Spectrum RT=2.28 min

5’ OH Amplicon SI-38

![](image)

Molecular Weight: 44761.98
Deconvoluted Mass Spectrum RT=2.79 min

5’ Phosphorylated Amplicon **SI-39**

5’-A-A-C-C-C-A-T-A-C-G—A-T-G-C-C-T-T-C-T-T—T-G-T-T-A-G-C-G-C-C—G-T-A-G-G-G-A-A-G-T-
G-A-A-G-C-T-T-C-T-G—G-G-C-C-A-G-T-T-C-C—T-A-G-G-T-A-A-T-A-G—A-A-G-T-A-C-C-A-T-C-
T-G-G-G-G-C-T-G-A-G—C-T-C-T-T-C-A-T-T—T-T-G-C-G-T-C-A-C—C-A-C-C-A-C-G-A-A-C-
T-C-G-T-C-G-G-G-T—G—C-T-C-T-C-G-G-T—A-G-T-A-G—3’

Molecular Weight: 44791.83
Lambda Exonuclease Degradation:

To the DNA mixture from the previous step (40 µL), CutSmart® (5 µL, 10x), water (3 µL), and Lambda Exonuclease (2 µL) was added in a PCR tube. This mixture was vortexed and incubated at 37°C for 30 minutes at which point the lambda exonuclease was thermally denatured at 75°C for 10 minutes. The resulting ssDNA (not phosphorylated) was isolated using a ZymoSpin Oligo clean and concentrator column.

Analysis of Lambda Exonuclease Digestion:
Remaining ssDNA SI-38

Molecular Weight: 44761.98
T4PNK Phosphorylation:

To the DNA mixture from the previous step (30 µL), CutSmart® (5 µL, 10x), DTT (5 µL, 50 mM Stock), ATP (5 µL from 10 mM stock), water (3 uL) and T4PNK (2 µL) was added in a PCR tube. This mixture was vortexed and incubated at 37ºC for 60 minutes at which point the T4PNK was thermally denatured at 75ºC for 10 minutes. The resulting ssDNA 58 was isolated using a ZymoSpin Oligo clean and concentrator column.

Analysis of T4PNK Phosphorylation 58:
DNA 58

5'-CTACTACGACGAGCTACTACCTGACTG-3'

Molecular Weight: 44841.96
Analysis of PCR and Enzymatic Modifications by DNA Gel:

Without purification, 1 µL of each DNA sample was added to 4 µL water and 1 µL Gel Loading Dye, Purple (6X), no SDS. Ladder was Thermofisher O’RangeRuler 10 bp DNA Ladder. Then, 5 µL of each sample was loaded onto a 5% agarose gel, which had been freshly cast with 7.5 g UltraPure Agarose, 150 mL 1X TAE buffer and 15 µL 10,000X SYBR™ Safe DNA Gel Stain. The gel was run in 1X TAE buffer at 150 V for 30 minutes.
SENDRA: 58→59

DNA 58 was used in SENDR Protocol without variation.

Analysis of 59:
Produced from 58 by General Procedure 2:
Using Ψ-7
Yield (A260): 54%
Expected Mass: 45222.23
Found Deconvoluted Mass: 45224.00

Molecular Weight: 45222.33
Deconvoluted Mass Spectrum RT=2.48

MW: 45224.00
DNA BSA Conjugation

Procedure for the creation of 61

DNA 61 was synthesized using General Procedure 2. Briefly, 100 nmol of DNA 60 was loaded onto two tubes each containing 100 µL of equilibrated resin. This loaded resin was washed with DMA (500 µL twice) and dry THF (500 µL three times) and dried under vacuum. These tubes were manipulated in parallel. Ψ-8 in MeCN (150 mM, 250 µL) was added to the loaded and dried resin. Then DBU (450 mM, 18 µL) was added to the reaction mixture. The reaction tube was vortexed for 30 seconds incubated at 37 ºC for 60 minutes. The reactions were worked up by aspirating and discarding the reaction solution, and washing the resin bed with MeCN (500 µL) and 1:1 MeCN:PBS (500 µL). Elute buffer (300 µL) was added to the resin bed and the tube was agitated by orbital shaker for 5-10 minutes. The DNA containing elution buffer was collected. DNA was isolate via ethanol precipitation. Ethanol precipitation was performed by adding 10% v/v of a NaCl solution (30 µL, 5M) to the elute buffer and three volumes of cold ethanol (-20ºC) were added to the tube (~1000µL) and incubated for 18 hours at -20 ºC. The tube was then centrifuged at 13,000 rpm for 15 minutes to pellet the DNA, and the extra ethanol was decanted off. The tubes were dried via speed vacuum and the DNA was dissolved and HPLC-MS analysis was performed.
LCMS Characterization of 61

Produced from 60 by General Procedure 2:
Using Ψ-8
Conversion (A260): 48%
Expected Mass: 7192.88
Expected (M-6)/6 Ion: 1197.81
Found Deconvoluted Mass: 7193.0
Found (M-6)/6 Ion: 1197.74
Total DNA Recovery: 60 nmol (60%)

5'-G-A-A-T-T-C-C-A-A-A-A-T-G-A-A-T-T-C-3'
Mass Spectrum (RT = 2.76 min)
Deconvoluted Mass Spectrum
Procedure for the creation of 62

Compound 61 was synthesized by General Procedure 2 and isolated crude after ethanol precipitation. DNA 61 was dissolved to 300 µM in PBS. Solid BSA was dissolved in PBS (2.5 mg/mL, ~40 µM). These two solutions were combined in a PCR tube (25 µL of each for a reaction volume of 50 µL) and the resulting reaction mixture was incubated in a thermocycler for 4 hours. The crude ligation solution was diluted to 0.5 mg/mL with respect to BSA and injected for intact protein analysis. Deconvolution across the entire mass peak showed no detectible unmodified BSA remaining.
Analysis of 62

5' H₂O₃P-G-A-A-T-T-C-C-A-A-A-A

A-T-G-G-A-A-T-T-C-G-G

3'

Bovine Serum Albumin (20 µM)

DNA (150 µM)

PBS, 37ºC, 4 h

Deconvoluted M/Z

BSA 66,429 Da

Intesity

60000 65000 70000 75000 80000

Deconvoluted M/Z

DNA-BSA Conjugate

Expected Mass: 73,510.7 Da

Observed Mass: 73,511 Da

Intesity

60000 65000 70000 75000 80000

Deconvoluted M/Z
DNA DVD Conjugation

Procedure for the creation of SI-40

DNA SI-40 was synthesized using General Procedure 2. Briefly, 100 nmol of DNA 63 was loaded onto two tubes containing 100 µL of equilibrated resin. This loaded resin was washed with DNA (500 µL twice) and dry THF (500 µL three times) and dried under vacuum. These tubes were manipulated in parallel. Ψ-5 in MeCN (150 mM, 250 µL) was added to the loaded and dried resin. Then DBU (450 mM, 18 µL) was added to the reaction mixture. The reaction tube was vortexed for 30 seconds incubated at 37 ºC for 60 minutes. The reaction was worked up by aspirating and discarding the reaction solution, and washing the resin bed with MeCN (500 µL) and 1:1 MeCN:PBS (500 µL). Elute buffer (300 µL) was added to the resin bed and the tube was agitated by orbital shaker for 5-10 minutes. The DNA containing elution buffer was collected. DNA was isolate via ethanol precipitation. Ethanol precipitation was performed by adding 10% v/v of a NaCl solution (30 µL, 5M) to the elute buffer and three volumes of cold ethanol (-20 ºC) were added to the tube (~1000µL) and incubated for 18 hours at -20 ºC. The tube was then centrifuged at 13,000 rpm for 15 minutes to pellet the DNA, and the extra ethanol was decanted off. The tubes were dried via speed vacuum and the DNA was dissolved and HPLC-MS analysis was performed.
LCMS Characterization of SI-40

Produced from 63 by General Procedure 2:
Crude Conversion (A260): 73%
Expected Mass: 6407.2
Expected (M-6)/6 Ion: 1066.34
Found Deconvoluted Mass: 6407.0
Found (M-6)/6 Ion: 1066.5
Crude Isolated Recovery: 44%
Crude isolated DNA SI-40 was purified using RP HPLC. After purification 15 nMol (15% total starting material) of pure SI-40 was obtained. To SI-40 a CuAAC reaction was performed. Compound SI-40 (15 nmol) was dissolved (92.5 µL) in tris buffer (50 mM) at pH 8.5. To this solution the β-lactam azide SI-53 (2 µL) was added from stock solution (30 mM) in DMA, (final concentration of 300 µM). Next, BTTP (2 µL) was added from a stock solution (40 mM in water). Next, CuSO₄ (1 µL) was added from a stock solution (40 µM in water) (a final concentration of 400 µM). The solution was capped and vortexed. Finally, sodium ascorbate (2.5 uL) was added from a stock solution (100 mM in water). This reaction solution was vortexed and incubated for 1 hour at 37 ºC. DNA 64 was ethanol precipitated, to remove excess small molecules, as previously described. Mass recovery and conversion for this transformation was assumed to be quantitative for subsequent protein conjugation.
LCMS Characterization of 64

Produced from SI-40:
Crude Conversion (A260): >85%
Expected Mass: 6826.63
Expected (M-6)/6 Ion: 1136.20
Found Deconvoluted Mass: 6827 + Cu Adducts
Found (M-6)/6 Ion: 1136.4

A260 Chromatogram
Mass Spectrum (RT= 3.26 min)
Deconvoluted Mass Spectrum
DNA-DVD conjugation procedure

DVD in storage solution was buffer exchanged into PBS by amicon spin filter (30 kDA) and concentrated to 25 µM (~5 mg/mL). This solution was used to dissolve (50 µL) solid DNA 64 to a presumptive concentration of 300 µM. In addition, a second tube containing 50 µL of DVD was aliquoted and taken through an identical process to serve as a positive control. Dissolving 64 with DVD solution was performed by vortexing the solution. This reaction mixture was incubated at 37 ºC for 8 hours to produce DNA-DVD conjugate 65. The crude reactions mixture was analyzed for conjugation efficiency as previously described by Rader et. al.3
Methodol Assay

The methodol assay for conjugation confirmation was performed as described by Rader and co-workers. Briefly, aliquots (12.5 µL) of the reaction and control solutions were diluted (0.2 mg/mL relative to original antibody concentration) in PBS to a final volume of 310 µL. Each sample were dispensed (98 µL) in triplicate into a black 96-well plate. Three blank wells, containing PBS were also dispensed (98 µL) into the black plate. A plate reader was prepared, the wavelength of excitation (λ<sub>ext</sub>) was set to 330 nm and wavelength of emission (λ<sub>em</sub>) was set to 452 nm. The instrument was programed to record every minute for 60 minutes and shake the plate in between. Finally, methodol (10 mM in ethanol) was added (2µL) to each well using a multichannel pipette and the plate was immediately loaded into the plate reader and data collection initiated. Signal was determined by normalizing against the blank wells. Measurements in triplicate were averaged and plotted along with standard deviation. Standard deviation was usually smaller than the marker size. Protocol adapted from: A. R. Nanna and C. Rader, Methods Mol Biol, 2019, 2033, 39-52.
## Methodol Assay Raw Data:

| Time (min) | Control   | DVD       | DVD+DNA   |
|------------|-----------|-----------|-----------|
| 0          | 4.813     | 6.357     | 5.112     |
| 1          | 5.779     | 3.962     | 9.547     |
| 2          | 5.863     | 5.366     | 5.509     |
| 3          | 5.548     | 6.566     | 6.439     |
| 4          | 5.777     | 4.774     | 6.747     |
| 5          | 6.511     | 4.014     | 6.368     |
| 6          | 4.227     | 5.487     | 5.596     |
| 7          | 5.7       | 5.688     | 7.117     |
| 8          | 7.151     | 4.483     | 7.073     |
| 9          | 6.632     | 6.401     | 5.278     |
| 10         | 6.318     | 5.399     | 7.315     |
| 11         | 6.58      | 4.204     | 6.119     |
| 12         | 5.014     | 6.556     | 6.253     |
| 13         | 5.269     | 6.264     | 6.618     |
| 14         | 4.601     | 7.207     | 5.439     |
| 15         | 6.21      | 4.36      | 6.972     |
| 16         | 6.395     | 6.288     | 6.027     |
| 17         | 6.729     | 5.695     | 5.602     |
| 18         | 4.902     | 7.298     | 5.627     |
| 19         | 6.954     | 6.659     | 5.49      |
| 20         | 6.867     | 5.246     | 6.748     |
| 21         | 5.383     | 5.222     | 6.976     |
| 22         | 6.852     | 6.222     | 6.126     |
| 23         | 5.513     | 6.902     | 6.138     |
| 24         | 5.68      | 7.073     | 6.87      |
| 25         | 6.004     | 7.747     | 6.449     |
| 26         | 6.952     | 4.948     | 4.724     |
| 27         | 5.418     | 7.521     | 4.372     |
| 28         | 5.892     | 6.805     | 7.725     |
| 29         | 5.745     | 5.655     | 4.88      |
| 30         | 4.098     | 6.869     | 5.767     |
| 31         | 5.641     | 5.135     | 5.41      |
| 32         | 7.755     | 6.277     | 4.241     |
| 33         | 4.777     | 6.663     | 6.414     |
| 34         | 5.165     | 7.52      | 6.512     |
| 35         | 6.156     | 4.771     | 6.389     |
| 36         | 7.009     | 5.765     | 5.657     |
| 37         | 7.032     | 5.683     | 5.893     |
| 38         | 4.559     | 8.857     | 5.67      |
| 39         | 5.461     | 7.194     | 5.186     |
| 40         | 6.613     | 6.042     | 7.168     |
| 41         | 5.345     | 6.697     | 5.75      |
| 42         | 7.773     | 6.158     | 5.62      |
| 43         | 5.055     | 6.443     | 7.933     |
| 44         | 4.025     | 6.35      | 5.12      |
| 45         | 5.898     | 5.723     | 6.062     |
| 46         | 6.305     | 6.259     | 6.21      |
| 47         | 5.971     | 6.406     | 4.709     |
| 48         | 5.629     | 7.202     | 5.665     |
| 49         | 6.416     | 8.093     | 6.528     |
| 50         | 6.283     | 5.27      | 6.002     |
| 51         | 5.623     | 6.58      | 5.649     |
| 52         | 7.091     | 6.33      | 4.083     |
| 53         | 4.3       | 5.85      | 4.851     |
| 54         | 5.291     | 7.323     | 7.095     |
| 55         | 6.488     | 5.384     | 6.398     |
| 56         | 6.149     | 4.663     | 7.725     |
| 57         | 6.4       | 6.638     | 6.978     |
| 58         | 6.732     | 5.518     | 6.723     |
| 59         | 7.388     | 4.446     | 5.885     |
| 60         | 6.68      | 4.384     | 7.986     |

SI419
**SDS PAGE Analysis**

SDS Page was performed to confirm mass increase of the DVD-DNA construct. For this analysis 1 µg of the reaction and the control were aliquoted into PCR tubes. To these tubes 6X Lamelli buffer (6µL) was added and finally the tubes were diluted with water (to 24 µL). These reactions were heated at 95 °C for 10 minutes before being loaded into separate lanes on a precast Bio-Rad 4-20% SDS PAGE Gel. To another lane of Bio-Rad Precision Plus Protein standard was added (7 µL). The rest of the lanes were loaded with 6x Lamelli buffer (6µL). The gel was run at 200V for 30 minutes at which point it was stained with Coomassie protein stain and destained with water over night. Finally, the gel was imaged on a Bio-Rad gel imager. Protocol adapted from: A. R. Nanna and C. Rader, *Methods Mol Biol*, 2019, 2033, 39-52.
Creation of Dual Labeled Probes:

Procedure For the Creation of 67-68

66→SI-41
SENDR was used to access the dual label probes. The first tag was appended to the 3’ end of DNA 66 using General Procedure 2. Briefly, 25 nmol of precursor 66 was loaded onto 100 µL of equilibrated resin. This loaded resin was washed with DMA (500 µL twice) and dry THF (500 µL three times) and dried under vacuum. Ψ-5 in MeCN (150 mM, 250 µL) was added to the loaded and dried resin. Then DBU (450 mM, 18 µL) was added to the reaction mixture. The reaction tube was vortexed for 30 seconds and incubated at 37 °C for various amounts of time. The reactions were worked up by aspirating and discarding the reaction solution, and washing the resin bed with MeCN (500 µL) and 1:1 MeCN:PBS (500 µL). Elute buffer (300 µL) was added to the resin bed and the tube was agitated by orbital shaker for 5-10 minutes. The DNA containing elution buffer was collected by carefully pipetting the supernatant. DNA was isolate via ethanol precipitation. Ethanol precipitation was performed by adding 10% v/v of a NaCl solution (30 µL, 5M) to the elute buffer and three volumes of cold ethanol (-20°C) were added to the tube (~1000µL) and incubated for 18 hours at -20 °C. The tube was then centrifuged at 13,000 rpm for 15 minutes to pellet the DNA, and the extra ethanol was decanted off. The tubes were dried via speed vacuum and the DNA was dissolved and HPLC-MS analysis was performed.

SI-41→67
Dephosphorylation of the 5’ end of SI-41 to generate 67 along with degradation of unlabeled 3’ DNA was carried out as follows. The crude reaction mixture from the previous step was dissolved in water (120 µL). This solution was aliquoted (40µL) into three separate PCR tubes. To these tubes, Cut Smart 10X Buffer was added (5µL) and the tubes were vortexed. Finally, rSAP (2.5 µL) and ExoIII (2.5 uL) was added to each tube. These tubes were vortexed, spun down and placed in a thermocycler. They were incubated at 37 ºC for 60 min, then the enzymes were deactivated at 70 ºC for 10 minutes. The reaction mixtures were isolated by zymo spin column.

67→68
67 was loaded onto resin and modified with SENDR by General Procedure 2 using Ψ-17. After this reaction, the crude reaction mixture is ethanol precipitated, pelleted, dried, dissolved and analyzed by HPLC-MS.

Lambda Exo Cleanup of 68
68 was loaded into a PCR tube (5 µg) in 30 uL water. To each tube CutSmart (5 µL at 10x), ATP (5µL at 5 mM) and DTT (5µL at 50 mM) was added. Finally Lambda exonuclease (2.5 µL) and T4PNK (2.5 µL) were added. These tubes were incubated for 90 minutes at 37°C at which point the enzymes were thermally deactivated at 75°C for 10 minutes.

68→SI-42→69
was added to a PCR tube in 38 µL water. To this tube DMSO (8 µL), Tris buffer (2 µL, 1M at pH 8.5) and DBCO Fluorescein (2 µL, 10 mM, in DMSO) were added. This reaction was allowed to incubate at 37°C for 120 minutes and excess regents were removed by zymo spin column. Resulting in FAM labeled probe \textbf{SI-42}. To \textbf{SI-42} in water (30 µL), Tris Buffer (1 uL, 1 M pH 8.5), BTTP (2 µL, 20 mM in water), CuSO₄ (1 µL, 20 mM, in water), DMSO (14 µL), MgCl₂ (1 µL, 1 M in water), and BHQ-Azide \textbf{SI-61} (1 µL, 5 mM in DMSO) were added. Finally, NaAsc (1 µL 100mM) was added and the reaction was incubated at 37°C for 60 minutes. The final dual labeled probe \textbf{67} was added and the reaction was incubated at 37°C for 60 minutes. The final dual labeled probe \textbf{67} was isolated by ethanol precipitation and analyzed by HPLC MS.

\textbf{68}→\textbf{SI-42}

\begin{align*}
\text{Molecular Weight: 8261.46} \\
\text{Molecular Weight: 665.72} \\
\text{Molecular Weight: 8927.18}
\end{align*}
SI-42→69

Molecular Weight: 704.79

SI-42

100 μM BHiQ Azide
800 μM BTTP
400 μM CuSO₄
20 mM Tris pH 8.5
20 mM MgCl₂
2 mM Nacac
15% DMSO

Molecular Weight: 9631.97

(69)
LCMS Characterization of SI-41

Produced from 66 by General Procedure 3:
Using Ψ-5
Expected Mass: 7996.2
Expected (M-7)/7 Ion: 1141.3
Found Deconvoluted Mass: 7996.0
Found (M-7)/7 Ion: 1140.93

\[ \text{Molecular Weight: 7996.17} \]

A260 Chromatogram

Absorbance (260 nm)

Time (min)
Mass Spectrum (RT= 2.10 min)
Deconvoluted Mass Spectrum
LCMS Characterization of 67

Produced from SI-41 by rSAP and ExoIII:
Expected Mass: 7916.2
Expected (M-7)/7 Ion: 1129.88
Found Deconvoluted Mass: 7917.0
Found (M-7)/7 Ion: 1129.65

Molecular Weight: 7916.19

A260 Chromatogram
Mass Spectrum (RT= 2.07 min)
Deconvoluted Mass Spectrum
LCMS Characterization of 68

Produced from 61 by General Procedure 2:
Using Ψ-17
Expected Mass: 8261.5
Expected (M-7)/7 Ion: 1179.2
Found Deconvoluted Mass: 8262.0
Found (M-7)/7 Ion: 11790.1

Molecular Weight: 8261.46

A260 Chromatogram
Mass Spectrum (RT = 2.2 min)
Deconvoluted Mass Spectrum
LCMS Characterization of 68 After Enzymatic Cleanup

Produced from 68:
Expected Mass: 8261.5
Expected (M-7)/7 Ion: 1179.2
Found Deconvoluted Mass: 8262.0
Found (M-7)/7 Ion: 11790.1

Molecular Weight: 8261.46

A260 Chromatogram
Mass Spectrum (RT = 2.2 min)
Deconvoluted Mass Spectrum
LCMS Characterization of SI-42

Produced from 68 by General Procedure SPAAC:

Expected Mass: 8927.2
Expected (M-8)/8 Ion: 1114.9
Found Deconvoluted Mass: 8927.00
Found (M-7)/7 Ion: 1114.51

A260 Chromatogram
LCMS Characterization of 69

Produced from SI-42 by General Procedure CuAAC

Expected Mass: 9631.97
Expected (M-8)/8 Ion: 1202.98
Found Deconvoluted Mass: 9631.0
Found (M-7)/7 Ion: 1202.7

A260 Chromatogram
Deconvoluted Mass Spectrum
Process for Mass Fragmentation of 68

A crude solution of 68 in water (100 µM) was injected on the Waters I-Class ToF for peak identification. The MS cone voltage was then increased from 5 mV to 30 mV and the same sample was reinjected to fragment the modified oligonucleotide. Diagnostic fragments were then extracted, and their presence confirmed on the total ion current. They are plotted below along with total ion current.
Time (min)

Intensity

Extracted Mass 1076.13

Extracted Mass 860.67

TIC
Probes were produced in an analogous manner to procedure outlined above for the production of 68.
LCMS Characterization of SI-43

Produced from SI-13 by General Procedure 3:
Using Ψ-5
Expected Mass: 7546.89
Expected (M-8)/8 Ion: 942.4
Found Deconvoluted Mass: 7546
Found (M-8)/8 Ion: 942.05

5'-A-C-C-C-G-C-A-T-T-A-G-T-T-T-G-T-G-G-A-C-C-P-O

Molecular Weight: 7546.89

A260 Chromatogram

Oligo03662

2: Diode Array
260.48000Da
Range: 2.503
Mass Spectrum (RT= 2.00 min)
Deconvoluted Mass Spectrum
LCMS Characterization of SI-44

Produced from SI-43 by rSAP and EXOIII:
Expected Mass: 7466.91
Expected (M-8)/8 Ion: 932.4
Found Deconvoluted Mass: 7467.00
Found (M-8)/8 Ion: 932.5

Exact Mass: 7463.23
Molecular Weight: 7466.91

A260 Chromatogram
Mass Spectrum (RT = 1.96 min)
Deconvoluted Mass Spectrum
LCMS Characterization of 70 after SENDR with Ψ-17 and Lambda Exo Cleanup
Produced from SI-44:
Using Ψ-17
Expected Mass: 7812.2
Expected (M-6)/6 Ion: 975.525
Found Deconvoluted Mass: 7813.0
Found (M-6)/6 Ion: 975.4

Molecular Weight: 7812.18

A260 Chromatogram
LCMS Characterization of SI-45

Produced from SI-14:
Using Ψ-5

Expected Mass: 7186.67
Expected (M-8)/8 Ion: 897.33
Found Deconvoluted Mass: 7186.00
Found (M-8)/8 Ion: 897.17

5′-A-C-A-A-T-T-G-C-C-C-C-A-G-C-G-C-T-T-C-A-G-S

Exact Mass: 7183.15
Molecular Weight: 7186.67

A260 Chromatogram
Mass Spectrum (RT= 2.00 min)
Deconvoluted Mass Spectrum
LCMS Characterization of SI-46

Produced from SI-45 by rSAP:
Expected Mass: 7106.69
Expected (M-8)/8 Ion: 887.33
Found Deconvoluted Mass: 886.97
Found (M-8)/8 Ion: 7107.00

Exact Mass: 7103.18
Molecular Weight: 7106.69

A260 Chromatogram
Mass Spectrum (RT = 1.97 min)
LCMS Characterization of 71 After SENDR with Ψ-17 and Lambda Exo Cleanup:
Produced from SI-46:
Using Ψ-17
Expected Mass: 7451.96
Expected (M-6)/6 Ion: 1240.98
Found Deconvoluted Mass: 7452.00
Found (M-6)/6 Ion: 1240.59

A260 Chromatogram
Mass Spectrum
Deconvoluted Mass Spectrum

7452.00

7434.00
LCMS Characterization of SI-47

Produced from **SI-15** by General Procedure 3:

Using **Ψ-5**

Expected Mass: 7273.7

Expected (M-6)/6 Ion: 1211.166

Found Deconvoluted Mass: 7274.00

Found (M-6)/6 Ion: 1211.03

5’—T-T-C-T-G-A-C-C-T-G—A-A-G-G-C-T-C-G-C—G-C-G—P-O

Molecular Weight: 7273.70

A260 Chromatogram
Deconvoluted Mass Spectrum

7274.00

2.68e6
LCMS Characterization of SI-48

Produced from SI-47 by rSAP and EXOIII:
Expected Mass: 7193.72
Expected (M-6)/6 Ion: 1197.95
Found Deconvoluted Mass: 7193.00
Found (M-6)/6Ion: 1197.76

Molecular Weight: 7193.72

A260 Chromatogram
Deconvoluted Mass Spectrum

7193.00

4.24e5
LCMS Characterization of 74 after SENDR with Ψ-17 and lambda exo cleanup:

Produced from SI-48:
Using Ψ-17
Expected Mass: 7538.99
Expected (M-7)/7 Ion: 1075.5
Found Deconvoluted Mass: 7539.00
Found (M-6)/6 Ion: 1075.6

A260 Chromatogram

Exact Mass: 7535.21
Molecular Weight: 7538.99
Mass Spectrum
Deconvoluted Mass Spectrum
Comparison Between Kit and Microtube Format

Procedure

DNA 3 was synthesized in cartridges according to General Procedure 3. Briefly, the resin (100 μL) was loaded into a fritted cartridge (Bio-Rad, Bio-Spin column, 1.2 mL, Cat. Num. 7326008). PBS (500 μL) was added to the resin bed and allowed to flow through with gravity. The column was capped on the bottom and DNA was loaded (5 nmol in 200 μL PBS) onto the resin bed. The column was then capped (on top), vortexed and agitated for 5 minutes. The load buffer was allowed to flow out with gravity. The Resin bed was washed with DMA (500 μL twice) and THF (500 μL three times), and the solvent was allowed to flow through with gravity each time. The top cap was replaced, and the resin was dried for 2 hours under vacuum (placed on a lyophilizer). A new cap was replaced on the bottom and Ψ-1 was added (300 mM in MeCN, 125 μL) and then DBU was added (900 mM in MeCN, 125 μL). The top cap was replaced, the cartridge was vortexed and the column was incubated at 37 °C for 60 minutes. After the reaction the caps were removed, and the reaction mixture was allowed to flow to waste. The reaction was worked up by washing the resin with PBS and 1:1 PBS:MeCN (500 μL each). The cap washed replaced and elute buffer added (300 μL). The cartridge as vortexed for 30 seconds and agitated for 10 minutes. The cap was removed and the elute buffer was collected. Finally, the DNA was isolated by ethanol precipitation and analyzed by HPLC MS.
LCMS Characterization of 3

Produced from 1 by General Procedure 3:
Using Ψ-1
Yield (A260): >95%
Expected Mass: 7023.6
Expected (M-4)/4 Ion: 1169.02
Found Deconvoluted Mass: 7024.00
Found (M-6)/6 Ion: 1169.2

A260 Chromatogram
Mass Spectrum (RT= 5.28 min)
Deconvoluted Mass Spectrum
Synthesis of Small Molecule Alcohols for use in Ψ-Module Synthesis

Synthesis of SI-49

![SI-49](image)

The synthesis of **SI-49** was carried out according to known literature procedures\(^4\)

Synthesis of SI-50

![SI-50](image)

The synthesis of **SI-50** was carried out according to known literature procedures\(^5\)

Synthesis of SI-51

![SI-51](image)

The synthesis of **SI-51** was carried out according to known literature procedures\(^6\)

Synthesis of SI-52

![SI-52](image)

The synthesis of **SI-52** was carried out according to known literature procedures\(^7\)
Synthesis of SI-53

The synthesis of SI-53 was carried out according to known literature procedures\textsuperscript{8-9}

Synthesis of SI-54

The synthesis of SI-54 was carried out according to known literature procedures\textsuperscript{10}

Synthesis of SI-55

The synthesis of SI-55 was carried out according to known literature procedures\textsuperscript{11}

Synthesis of SI-56

The synthesis of SI-56 was carried out according to known literature procedures\textsuperscript{12}
Synthesis of SI-57

The synthesis of SI-57 was carried out according to known literature procedures\textsuperscript{13}

Synthesis of SI-58

The synthesis of SI-58 was carried out according to known literature procedures\textsuperscript{14}

Synthesis of SI-59

The synthesis of SI-59 was carried out according to known literature procedures\textsuperscript{15}

Synthesis of SI-60

The synthesis of SI-60 was carried out according to known literature procedures\textsuperscript{16}
Synthesis of SI-61

The synthesis of SI-61 was carried out according to known literature procedures\(^9\)

Synthesis of SI-62

The synthesis of SI-62 was carried out according to known literature procedures\(^{17}\)
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