

Supporting Information

***N*⁶-Isopentenyladenosine in RNA Determines the Cleavage Site of
Endonuclease Deoxyribozymes**

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Experimental Procedures

1. Materials and Methods

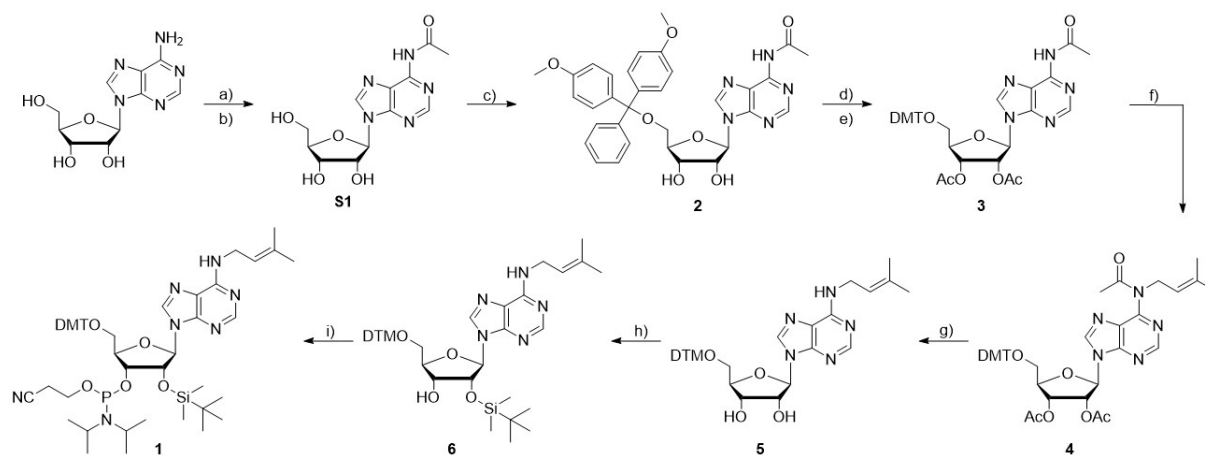
Unmodified DNA oligonucleotides were purchased from Microsynth and purified by denaturing PAGE (10-20% polyacrylamide) prior to use. Modified DNA (including primers) and RNA oligonucleotides were prepared in house by solid phase synthesis by using standard phosphoramidite chemistry. Solid supports for solid phase synthesis and unmodified DNA and 2'-O-TOM-protected RNA phosphoramidites were purchased from Sigma Proligo, ChemGenes, or Glen Research. Fluorescein-thiosemicarbazide and 6-carboxyfluorescein succinimidyl ester (NHS-fluorescein) were purchased from Sigma. 6-FAM-azide and deoxyribonucleotide triphosphate (dNTPs) were from Jena Bioscience. T4 Polynucleotide Kinase (PNK), T4 DNA Ligase and DreamTaq polymerase were purchased from Thermo Fisher Scientific.

2. Synthetic procedures

General Information

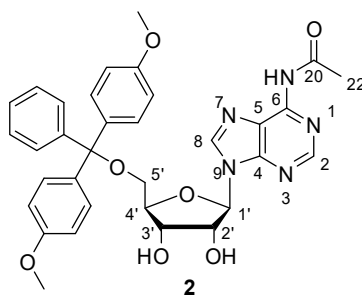
All reaction were performed under nitrogen atmosphere. The obtained products were stored at 4 °C. The chemicals used for the reactions were purchased 'Pro analysis'- or 'For synthesis' quality and used without additional purification. Solvents for column chromatography were used in technical quality and distilled prior to use. Dry solvents (dichloromethane, acetonitrile, THF, DMF) were obtained from solvent purification system (SPS). Methanol was pre-dried over molecular sieves and further dried with magnesium turnings. Column chromatography was performed on silica gel (Kieselgel 60, Merck, 0.063 – 0.200 mm). TLC was performed on Alugram DIL G/UV254 (Machery-Nagel, UV visualization, 254 nm). NMR spectra were recorded on a Bruker Avance III HD 400 spectrometer. Chemical shifts were measured relative to the residual solvent signals as internal standards (in ppm; CDCl₃: ¹H = 7.26, ¹³C = 77.16, DMSO: ¹H = 2.50, ¹³C = 39.52). ESI-MS of the products and the oligonucleotides were recorded on a Bruker micrOTOF-Q III spectrometer.

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Synthesis of i⁶A phosphoramidite

a) TMS-Cl, pyridine, 18 h, 0 °C - r.t. b) MeCN, AcCl, 1 h 0 °C - r.t. c) DMT-Cl, pyridine, 2 h, r.t. d) Ac₂O, pyridine, 1.5 h, r.t. e) imidazole, MeOH, 2 h, r.t. f) K₂CO₃, isopentenyl bromide, DMF, 20 h, 50 °C g) MeNH₂ in EtOH, 16 h, r.t. h) TBDMS-Cl, AgNO₃, THF/pyridine, 19 h, r.t. i) Me₂NET, CEP-Cl, 2.5 h, r.t.

The synthesis of i⁶A-phosphoramidite building block **1** started with acetyl protection of the N⁶ amino group of adenosine, followed by protection of the 5'-OH group with 4,4'-dimethoxytrityl chloride to produce compound **2**, following published procedures.^[1] Compound **2** was treated with Ac₂O to give N⁶-acetyl-2',3'-O-diacetyl-5'-O-(4,4'-dimethoxytrityl)adenosine (**3**).^[2] The isopentenyl group was regioselectively introduced at N⁶ using isopentenyl bromide and K₂CO₃ to produce key intermediate **4**, following a procedure previously reported for N⁶-alkylation of tetraacetyladenosine.^[2] The acetyl groups were then removed under basic conditions to give **5**,^[3] followed by treatment with TBDMS-Cl in the presence of silver nitrate.^[4] The 2'-silylated compound **6** was then converted to the phosphoramidite **1**.

N⁶-Acetyl-5'-O-(4,4'-dimethoxytrityl)adenosine (**2**)

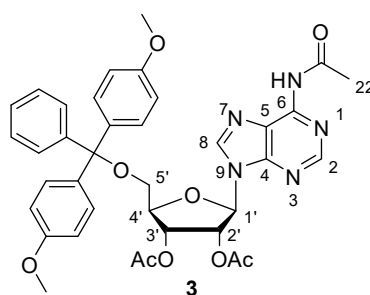
Starting from adenosine, N⁶-acetyl-5'-O-DMT-adenosine (**2**) was produced following the published procedure.^[1] The analytical parameters coincided with the ones reported by Pitsch et al., but the assignments of H2 and H8 need to be revised, as described here.

HR-MS (ESI⁺): Exact mass calculated for C₃₃H₃₃N₅NaO₇ [M+Na]⁺: 634.22722, found: 634.22888.

¹H NMR (400 MHz, CDCl₃) δ (ppm) = 8.63 (s, 1H, **2-H**), 8.22 (s, 1H, **8-H**), 7.30 – 7.21 (m, 2H, DMT), 7.20 – 7.14 (m, 7H, DMT), 6.77 – 6.69 (m, 4H, DMT), 6.03 (d, *J* = 5.7 Hz, 1H, 1'-H), 4.86 (dd, *J* = 5.7, 5.1 Hz, 1H, 2'-H), 4.46 (dd, *J* = 5.1, 2.3 Hz, 1H, 3'-H), 4.44 – 4.40 (m, 1H, 4'-H), 3.76 (d, *J* = 0.8 Hz, 6H, 2x CH₃), 3.44 (dd, *J* = 10.6, 3.5 Hz, 1H, 5'-H), 3.30 (dd, *J* = 10.6, 3.4 Hz, 1H, 5'-H), 2.61 (s, 3H, Acetyl-CH₃).

¹³C NMR (101 MHz, CDCl₃) δ (ppm) = 170.38 (C_q, Acetyl), 158.68, 158.65 (C_q, C-OCH₃, DMT), 152.11 (C-2), 150.60 (C-4), 149.45 (C-6), 144.41 (C_q, DMT), 141.40 (C-8), 135.54, 135.53 (C_q, DMT), 130.11 (DMT), 130.06 (DMT), 128.10 (DMT), 127.99 (DMT), 127.08 (DMT), 122.14 (C-5), 113.27 (DMT), 90.73 (C-1'), 86.76 (C_q, C-O, DMT), 86.01 (C-4'), 75.88 (C-2'), 72.41 (C-3'), 63.55 (C-5'), 55.35 (2xCH₃, DMT), 25.83 (C-22, Acetyl-CH₃).

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*N*⁶-Acetyl-2',3'-O-diacetyl-5'-O-(4,4'-dimethoxytrityl)adenosin (**3**)

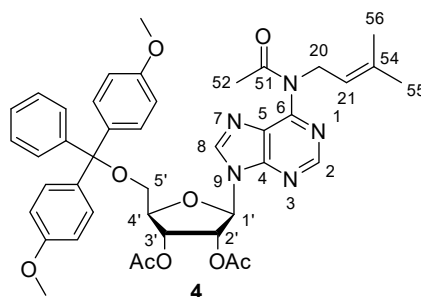
*N*⁶-acetyl-5'-O-(4,4'-dimethoxytrityl)adenosine (**2**, 230 mg, 376 μ mol, 1 eq) was dissolved in dry pyridine (1.5 mL), treated with Ac₂O (190 μ L, 1.90 mmol, 5.05 eq) and stirred for 1.5 h at room temperature. After addition of EtOH (2.5 mL) the solvent was removed under reduced pressure. The reaction mixture was diluted with ethylacetate (20 mL) and washed with brine and the organic phase was dried over Na₂SO₄. After evaporation of the solvent under reduced pressure, the product (**3**, 208 mg, 299 μ mol, 79%) was obtained as a colorless foam.

Chemical formula: C₃₇H₃₇N₅O₉, Molecular weight: 695.73 g·mol⁻¹

HR-MS (ESI⁺): Exact mass calculated for C₃₇H₃₇N₅NaO₉ [M+Na]⁺: 718.24835, found: 718.24876.

¹H NMR (400 MHz, CDCl₃) δ (ppm) = 8.64 (s, 1H, 2-H), 8.13 (s, 1H, 8-H), 7.43 – 7.39 (m, 2H, DMT), 7.34 – 7.23 (m, 6H, DMT), 7.24 – 7.19 (m, 1H, DMT), 6.84 – 6.75 (m, 4H, DMT), 6.32 (d, *J* = 6.6 Hz, 1H, 1'-H), 6.11 (dd, *J* = 6.6, 5.3 Hz, 1H, 2'-H), 5.69 (dd, *J* = 5.3, 3.0 Hz, 1H, 3'-H), 4.36 (q, *J* = 3.2 Hz, 1H, 4'-H), 3.78 (t, *J* = 0.7 Hz, 6H, 2 x CH₃), 3.51 (dd, *J* = 10.7, 3.1 Hz, 1H, 5'-H), 3.46 (dd, *J* = 10.7, 3.5 Hz, 1H, 5'-H), 2.59 (d, *J* = 1.5 Hz, 3H, Acetyl-CH₃), 2.13 (s, 3H, Acetyl-CH₃), 2.05 (s, 3H, Acetyl-CH₃).

¹³C NMR (101 MHz, CDCl₃) δ (ppm) = 170.28 (C_q, Acetyl), 169.87 (C_q, Acetyl), 169.54 (C_q, Acetyl), 158.81 (C_q, C-OCH₃, DMT), 152.83 (C-2), 151.43 (C-4), 149.36 (C-6), 144.19 (C_q, DMT), 141.08 (C-8), 135.34, 135.30 (C_q, DMT), 130.29, 130.27 (DMT), 128.34 (DMT), 128.16 (DMT), 127.27 (DMT), 121.97 (C-5), 113.42 (DMT), 87.27 (C_q, C-O, DMT), 85.16 (C-1'), 82.79 (C-4'), 73.41 (C-2'), 71.83 (C-3'), 63.12 (C-5'), 55.39 (2x CH₃, DMT), 25.81 (C-22, CH₃, Acetyl), 20.82 (CH₃, Acetyl), 20.56 (CH₃, Acetyl).

*N*⁶-Acetyl-2',3'-O-diacetyl-5'-O-(4,4'-dimethoxytrityl)-*N*⁶-isopentenyladenosine (**4**)

*N*⁶-Acetyl-2',3'-O-diacetyl-5'-O-(4,4'-dimethoxytrityl)adenosine (**3**, 1.0 g, 1.44 mmol, 1 eq) in dry DMF (15 mL) was treated with K₂CO₃ (1.39 g, 10.1 mmol, 7 eq) and isopentenyl bromide (1.29 g, 996 μ L, 8.62 mmol, 6 eq) and stirred for 20 h at 50 °C. The solution was allowed to cool down to room temperature, diluted with ethyl acetate (42 mL) and washed with brine. The organic phase was dried over Na₂SO₄ and the solvent was removed under reduced pressure. The residual was purified by column chromatography (DCM + 1% NEt₃ → DCM:EtOH = 99:1 + 1% NEt₃) to yield the product (**4**, 590 mg, 54%) as a slightly yellow foam. A significant fraction of unreacted starting material was recovered (**3**, 225 mg, 22%).

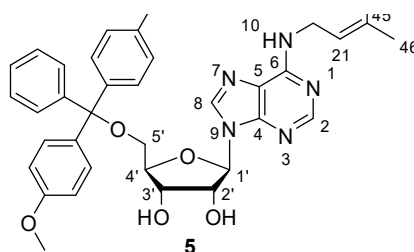
Chemical formula: C₄₂H₄₅N₅O₉, Molecular weight: 763.9 g·mol⁻¹

HR-MS (ESI⁺): Exact mass calculated for C₄₂H₄₅N₅NaO₉ [M+Na]⁺: 786.31095, found: 786.31438.

¹H NMR (400 MHz, CDCl₃) δ (ppm) = 8.72 (s, 1H, 2-H), 8.20 (s, 1H, 8-H), 7.43 – 7.39 (m, 2H, DMT), 7.33 – 7.29 (m, 4H, DMT), 7.26 (s, 2H, DMT), 7.24 – 7.18 (m, 1H, DMT), 6.84 – 6.78 (m, 4H, DMT), 6.35 (d, *J* = 6.5 Hz, 1H, 1'-H), 6.11 (dd, *J* = 6.4, 5.3 Hz, 1H, 2'-H), 5.71 (dd, *J* = 5.3, 3.2 Hz, 1H, 3'-H), 5.23 (ddt, *J* = 6.6, 5.3, 1.4 Hz, 1H, 21-H), 4.84 (d, *J* = 6.7 Hz, 2H, CH₂, 20-H), 4.37 (q, *J* = 3.3 Hz, 1H, 4'-H), 3.78 (d, *J* = 1.6 Hz, 6H, 2xCH₃, DMT), 3.52 (dd, *J* = 10.7, 3.1 Hz, 1H, 5'-H), 3.46 (dd, *J* = 10.8, 3.6 Hz, 1H, 5'-H), 2.27 (s, 3H, CH₃, H-52), 2.14 (s, 3H, CH₃, Acetyl), 2.07 (s, 3H, CH₃, Acetyl), 1.60 (d, *J* = 1.3 Hz, 3H), 1.59 (d, *J* = 1.3 Hz, 3H, H-55, H-56).

¹³C NMR (101 MHz, CDCl₃) δ (ppm) = 171.41 (C_q C-51), 169.84 (C_q C-46), 169.52 (C_q C-45), 158.80 (C_q, C-OCH₃, DMT), 153.85 (C-6), 153.08 (C-4), 152.35 (C-2), 144.20 (C_q, DMT), 141.89 (C-8), 135.96 (C_q, C-54), 135.37, 135.33 (C_q, DMT), 130.26 (DMT), 128.30 (DMT), 128.13 (DMT), 127.40 (C-5), 127.23 (DMT), 120.28 (C-21), 113.41 (DMT), 87.23 (C-22), 85.37 (C-1'), 82.65 (C-4'), 73.30 (C-2'), 71.68 (C-3'), 63.03 (C-5'), 55.37 (2xCH₃, DMT), 45.79 (C-20), 25.78 (C-55, CH₃), 24.46 (C-52, CH₃), 20.81, 20.59 (C-48, C-49, 2xCH₃, Acetyl), 18.07 (C-56, CH₃).

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*N*⁶-Isopentenyl-5'-O-(4,4'-dimethoxytrityl)adenosine (5)

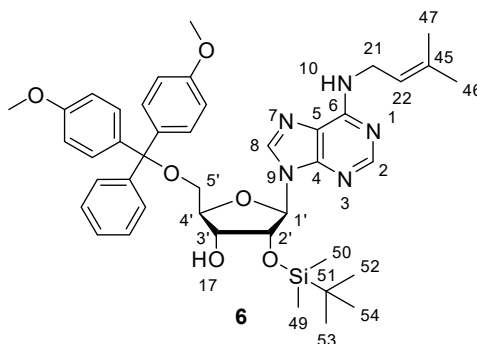
*N*⁶-Acetyl-2',3'-O-diacetyl-5'-O-(4,4'-dimethoxytrityl)-*N*⁶-isopentyladenosine (**4**, 500 mg, 655 μmol, 1 eq) in MeOH (6 mL) was treated with methylamine in EtOH (33%, 5.7 mL, 45.8 mmol, 70 eq) and stirred at room temperature overnight. After evaporation of the solvent, the residue was purified by column chromatography (DCM + 1% NEt₃ → DCM:EtOH = 99:1 + 1% NEt₃ → DCM:EtOH = 97:3 + 1% NEt₃) to yield the product (293 mg, 459 μmol, 70%) as a colorless foam.

Chemical formula: C₃₆H₃₉N₅O₆, Molecular weight: 637.7 g·mol⁻¹

HR-MS (ESI)⁺: Exact mass calculated for C₃₆H₄₀N₅O₆ [M+H]⁺: 638.29731, found: 638.29719

¹H NMR (400 MHz, CDCl₃) δ (ppm) = 8.34 (s, 1H, 2-H), 8.02 (s, 1H, 8-H), 7.28 – 7.24 (m, 2H, DMT), 7.20 – 7.14 (m, 7H, DMT), 6.76 – 6.70 (m, 4H, DMT), 5.94 (d, *J* = 6.0 Hz, 1H, 1'-H), 5.79 (s, 1H, NH), 5.41 – 5.34 (m, 1H, 21-H), 4.76 (dd, *J* = 6.1, 5.1 Hz, 1H, 2'-H), 4.42 (td, *J* = 3.3, 1.8 Hz, 1H, 4'-H), 4.37 (dd, *J* = 5.1, 1.9 Hz, 1H, 3'-H), 4.22 (s, 2H, CH₂, 20-H), 3.76 (d, *J* = 1.4 Hz, 6H, 2xCH₃, DMT), 3.43 (dd, *J* = 10.5, 3.5 Hz, 1H, 5'-H), 3.22 (dd, *J* = 10.5, 3.4 Hz, 1H, 5'-H), 1.74 (dd, *J* = 4.0, 1.3 Hz, 6H, 2xCH₃, 46-H, 47-H).

¹³C NMR (101 MHz, CDCl₃) δ (ppm) = 158.62 (C_q, C-OCH₃), 154.87 (C-6), 152.70 (C-2), 147.89 (C-4), 144.40 (C_q, DMT), 137.92 (C-8), 137.36 (C-45), 135.70, 135.50 (C_q, DMT), 130.09 (DMT), 130.04 (DMT), 128.11 (DMT), 127.96 (DMT), 127.00 (DMT), 120.29 (C-5), 119.96 (C-22), 113.24 (DMT), 91.23 (C-1'), 86.71, 86.61 (C-4', C_q, C-O), 76.48 (C-2'), 73.15 (C-3'), 63.73 (C-5'), 55.32 (CH₃, DMT), 38.77 (C-21), 25.83 (CH₃, C-46), 18.17 (CH₃, C-47).

*N*⁶-Isopentenyl-5'-O-(4,4'-dimethoxytrityl)-2'-O-(tert-butyldimethylsilyl)adenosine (6)

To *N*⁶-isopentenyl-5'-O-(4,4'-dimethoxytrityl)adenosine (**5**, 335 mg, 525 μmol, 1 eq) in dry THF/pyridine (1:1, 4 mL) AgNO₃ (134 mg, 788 μmol, 1.5 eq) and TBDMS-Cl (87.1 mg, 578 μmol, 1.1 eq) were added and stirred for 19 h at room temperature. The reaction was stopped with MeOH (4 mL) and filtered over celite. The filtrate was evaporated to dryness under reduced pressure. The mixture of 2' and 3' silylated isomers was repeatedly separated by column chromatography (hexane:ethylacetate = 4:1 + 1% NEt₃ → 3:1 → 2:1) to yield the pure isomer compound **6** (182 mg, 242 μmol, 46%) as a colorless foam.

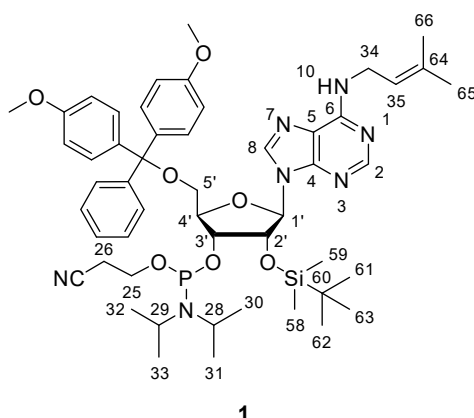
Chemical formula: C₄₂H₅₃N₅O₆Si, Molecular weight: 752.0 g·mol⁻¹

HR-MS (ESI)⁺: Exact mass calculated for C₄₂H₅₄N₅O₆Si [M+H]⁺: 752.38379, found: 752.38394

¹H NMR (400 MHz, CDCl₃) δ (ppm) = 8.31 (s, 1H, 2-H), 7.94 (s, 1H, 8-H), 7.47 – 7.43 (m, 2H, DMT), 7.36 – 7.31 (m, 4H, DMT), 7.27 (s, 2H, DMT), 7.23 – 7.18 (m, 1H, DMT), 6.84 – 6.77 (m, 4H, DMT), 6.01 (d, *J* = 5.5 Hz, 1H, 1'-H), 5.61 (s, 1H, NH), 5.38 (tdt, *J* = 7.1, 2.9, 1.4 Hz, 1H, 22-H), 4.99 (t, *J* = 5.3 Hz, 1H, 2'-H), 4.33 (dt, *J* = 5.2, 3.7 Hz, 1H, 3'-H), 4.24 (q, *J* = 3.5 Hz, 1H, 4'-H), 4.22 (s, 2H, CH₂, 21-H), 3.78 (d, *J* = 1.2 Hz, 6H, 2xCH₃, DMT), 3.51 (dd, *J* = 10.6, 3.3 Hz, 1H, 5'-H), 3.37 (dd, *J* = 10.6, 4.0 Hz, 1H, 5'-H), 2.72 (d, *J* = 4.0 Hz, 1H, OH), 1.78 – 1.73 (m, 6H, 2xCH₃, 46-H, 47-H), 1.26 (t, *J* = 7.1 Hz, 1H), 0.84 (s, 9H, 3xCH₃, 52-H, 53-H, 54-H), -0.02 (s, 3H, CH₃, H-49), -0.14 (s, 3H, CH₃, 50-H).

¹³C NMR (101 MHz, CDCl₃) δ (ppm) = 158.66 (C_q, C-OCH₃), 154.73 (C-6), 153.43 (C-2), 149.28 (C-4), 144.75 (C_q, DMT), 138.56 (C-8), 137.13 (C_q, C-45), 135.83 (C_q, DMT), 130.23 (DMT), 128.30 (DMT), 128.03 (DMT), 127.05 (DMT), 120.32 (C-5), 120.21 (C-22), 113.32 (DMT), 88.23 (C-1'), 86.71 (C_q, DMT), 84.18 (C-4'), 75.73 (C-2'), 71.72 (C-3'), 63.58 (C-5'), 55.36 (2xCH₃, DMT), 38.85 (C-21), 25.85 (C-46), 25.73 (C-52, C-53, C-54), 18.17 (C-47), -4.82, -5.11 (C-49, C-50).

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***N*⁶-Isopentenyl-5'-O-(4,4'-dimethoxytrityl)-2'-O-(*tert*-butyldimethylsilyl)adenosine 3' cyanoethyl *N,N*-diisopropyl phosphoramidite (**1**)**

*N*⁶-Isopentenyl-5'-O-DMT-2'-O-TBDMS-adenosine (**6**, 70 mg, 93.1 μmol, 1 eq) was dissolved in dry DCM (1 mL) and Me₂NET (101 μL, 931 μmol, 10 eq) and CEP-Cl (33.0 mg, 140 μmol, 1.5 eq) were added. After stirring for 2.5 h at room temperature, the solvent was removed under reduced pressure. The residue was purified by column chromatography (ethylacetate:hexane = 2:1 + 1% NEt₃) to yield compound **1** (79 mg, 83.0 μmol, 89%).

Chemical formula: C₅₁H₇₀N₇O₇PSi, Molecular weight: 952.22 g·mol⁻¹

HR-MS (ESI)*: Exact mass calculated for C₅₁H₇₁N₇O₇PSi [M+H]⁺: 952.49164, found: 952.49298

¹H NMR (400 MHz, CDCl₃) δ (ppm) = 8.29 (s, 1H, 2-H), 8.27 (s, 1H, 2-H diast.), 7.97 (s, 1H, 8-H), 7.94 (s, 1H, 8-H diast.), 7.49 – 7.42 (m, 4H, DMT), 7.38 – 7.31 (m, 7H, DMT), 7.31 – 7.21 (m, 6H, DMT), 7.24 – 7.17 (m, 2H, DMT), 6.84 – 6.77 (m, 8H, DMT), 6.00 (d, *J* = 6.5 Hz, 1H, 1'-H), 5.96 (d, *J* = 6.1 Hz, 1H, 1'-H diast.), 5.58 (s, 2H, NH), 5.38 (dddd, *J* = 6.8, 5.4, 3.2, 1.5 Hz, 2H, 35-H), 5.07 (ddd, *J* = 8.6, 6.3, 4.6 Hz, 2H, 2'-H), 4.39 (m, 2H, 3'-H, 4'-H), 4.32 (q, *J* = 4.0 Hz, 1H, 4'-H), 4.21 (s, 4H, CH₂, 34-H), 4.01 – 3.92 (m, 1H, CH₂, 25-H), 3.88 (ddt, *J* = 10.3, 8.3, 6.7 Hz, 1H, CH₂, 25-H), 3.78 (d, *J* = 1.3 Hz, 7H, 2xCH₃, DMT), 3.78 (d, *J* = 0.6 Hz, 6H, 2xCH₃, DMT diast.), 3.71 – 3.62 (m, 1H, CH₂, 25-H diast.), 3.59 (dq, *J* = 10.2, 3.2 Hz, 4H, 25-H, 5'-H, 28-H, 29-H), 3.54 (dd, *J* = 10.7, 4.0 Hz, 1H, 5'-H diast.), 3.32 (dd, *J* = 9.8, 3.4 Hz, 1H, 5'-H), 3.28 (dd, *J* = 9.9, 3.4 Hz, 1H, 5'-H), 2.65 (td, *J* = 6.5, 4.4 Hz, 2H, CH₂, 26-H), 2.30 (td, *J* = 6.7, 1.9 Hz, 1H, CH₂, 26-H diast.), 1.76 (d, *J* = 1.3 Hz, 6H, 2xCH₃, 65-H, 66-H), 1.74 (d, *J* = 1.3 Hz, 5H, 2xCH₃, 65-H, 66-H diast.), 1.20 – 1.02 (m, 21H, 4xCH₃, 30-H, 31-H, 32-H, 33-H), 0.77 (s, 8H, 3xCH₃, 61-H, 62-H, 63-H), 0.76 (s, 8H, 3xCH₃, 61-H, 62-H, 63-H diast.), -0.03 (s, 2H, CH₃, 58-H), -0.06 (s, 3H, CH₃, 58-H diast.), -0.19 (s, 2H, CH₃, 59-H), -0.21 (s, 3H, CH₃, 59-H diast.).

¹³C NMR (101 MHz, CDCl₃) δ (ppm) = 158.64 (C_q-OMe, DMT), 154.72 (C-6), 153.30 (C-2), 149.20 (C-4), 144.79, 144.69 (C_q, DMT), 139.02 (C-8), 137.10 (C.64), 135.97, 135.93, 135.80, 135.76 (C_q, DMT), 130.32, 130.27, 130.23 (C-DMT), 128.41, 128.29 (C-DMT), 128.02, 128.00 (C-DMT), 127.03 (C-DMT), 120.47 (C-5), 120.22 (C-35), 117.80, 117.46 (CN), 113.30, 113.26 (C-DMT), 88.40, 88.13 (C-1'), 86.77, 86.63 (C_q-O, DMT), 84.06, 83.81 (C-4'), 75.13, 75.11, 74.59, 74.54 (C-2'), 73.56, 73.46, 73.01, 72.86 (C-3'), 63.55, 63.38 (C-5'), 59.08, 58.91, 57.93, 57.72 (C-25), 55.37, 55.35 (O-CH₃, DMT), 43.58, 43.45, 43.12, 43.00 (C-28, C-29), 38.76 (C-34), 25.84, 25.80, 25.76 (C-61, C-62, C-63), 24.92, 24.85, 24.78, 24.67 (C-30, C-31, C-32, C-33), 20.62, 20.56, 20.22, 20.15 (C-26), 18.16, 18.10, 18.04 (C-65, C-66), -4.58, -4.60, -5.00 (C58, C-59).

³¹P NMR (162 MHz, CDCl₃) δ (ppm) = 150.81, 148.84.

3. Synthesis and Labeling of RNA

The following solutions were used for automated solid-phase synthesis: 100 mM solutions in dry acetonitrile of the phosphoramidites, 0.25 M ethylthiotetrazole (ETT, activator) in dry acetonitrile, 3% dichloro acetic acid in 1,2-dichloroethane (deprotection), 0.5 M DMAP in acetonitrile (Cap A), acetic anhydride/sym-collidine/acetonitrile = 20/30/50 (Cap B), 10 mM I₂ in acetonitrile/sym-collidine/H₂O = 10/1/5 (oxidation). Oligonucleotide synthesis was carried out at 0.6 μmol scale. Forward primer for PCR was coupled with hexynyl group at 5' end and reverse primer was linked with a 12-nt tail via a non-extendable hexaethyleneglycol spacer. The coupling efficiencies were determined using absorbance of released dimethoxytrityl (DMT) cation and exceeded 98% for all syntheses. DNA oligonucleotides were deprotected using NH₄OH and MeNH₂ at 55°C for 5 hours. For RNA oligonucleotides deprotection was performed in two steps using MeNH₂ in aqueous ethanol, followed by 1 M TBAF in THF. 3'-Amino modified RNA was synthesized on amino-on-solid support and deprotected using NH₄OH and MeNH₂ in aqueous solution for 6 hours at 55°C, followed by TBAF in THF. Deprotected oligonucleotide samples were first desalted by size exclusion chromatography on 3×5 ml HiTrap columns (GE Healthcare), then purified by denaturing PAGE and analyzed via anion exchange HPLC and ESI-MS.

Anion exchange chromatography was performed on Dionex DNAPAc PA200 column, 2x250 mm, with 25 mM Tris.HCl pH 8.0, 6 M urea (buffer A) and 0.5 M NaClO₄ in 25 mM Tris.HCl pH 8.0, 6 M urea (buffer B), gradient: 0-48% B in 12 column volumes, flow rate 0.5 mL/min, 60°C. UV-detection at 260 nm.

PAGE purification was performed on 320x160x0.7mm denaturing polyacrylamide gels (10-20% acrylamide/ bisacrylamide 19:1, 7 M urea) with 1× TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH=8.3) and run at 35 W power for 2 hours. Oligonucleotides were

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visualized using UV shadowing on TLC plate, excised from the gel, extracted by crush and soak into TEN buffer (10 mM Tris-HCl, pH = 8.0, 1 mM EDTA, 300 mM NaCl) and recovered by precipitation with ethanol.

Labeling of 5'-alkyne functionalized oligonucleotides

Oligonucleotides with 5'-alkyne group were labeled with 6-FAM-azide using copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC). Oligonucleotide (5 nmol in 5 μ L of H₂O) was mixed with 3 μ L of DMSO/tBuOH (3:1 v/v) and 0.5 μ L of 6-FAM-azide (50 mM in DMSO/tBuOH 3:1). The solution of CuBr (100 mM) was freshly prepared in DMSO/tBuOH 3:1 and mixed with a solution of TBTA (100 mM) in a 1:2 ratio. From this solution, 1.5 μ L was added to the reaction mixture and incubated at 37°C for 3 hours in the dark. Afterwards, the sample was ethanol precipitated to remove excess fluorophore and labeled oligonucleotides were purified by PAGE.

Labeling of 3'-amino functionalized oligonucleotides

Oligonucleotides with 3'-amino group were labeled with 6-carboxyfluorescein succinimidyl ester (NHS-fluorescein). Amino-modified RNA (2.5 nmol in 2.5 μ L of H₂O) was dissolved in carbonate buffer (20 μ L, 100 mM, pH = 9.0) and solution of NHS-fluorescein in DMF (2.5 μ L, 30 mM) was added. The reaction mixture was incubated at 37°C for 2 hours in the dark. Labeled RNA was purified by denaturing PAGE.

Labeling of native RNA 3'-ends

Unfunctionalized RNA was labeled at 3'-end with fluorescein-5-thiosemicarbazide by periodate oxidation method. RNA (5 nmol in 10 μ L of H₂O) was mixed with sodium phosphate buffer (10 μ L, 100 mM, pH = 7.4) and freshly prepared solution of NaIO₄ (2.5 μ L, 100 mM). The reaction mixture was incubated at 37°C for 10 minutes. The reaction was quenched by Na₂SO₃ (5 μ L, 100 mM) and incubated at 37°C for 5 minutes. To this reaction mixture, a solution of fluorescein-5-thiosemicarbazide (5 μ L, 10 mM) in DMF was added and the reaction was incubated at 37°C for 3 hours in the dark. Labeled product was purified by denaturing PAGE.

5'-Phosphorylation of RNA substrates

Phosphorylation at 5'end of RNA substrates was carried with T4 PNK enzyme. RNA (2-5 nmol) incubated with 10 \times PNK buffer A (5 μ L, 500 mM Tris-HCl, 10 mM MgCl₂, 50 mM DTT, 1 mM spermidine), ATP (5 μ L, 10 mM) and T4 PNK enzyme (5 μ L, 10 U/ μ L) at 37°C for 5 hours. The total volume of the reaction mixture was 50 μ L. Afterwards, RNA was extracted with phenol/ chloroform/isoamylalcohol mixture followed by ethanol precipitation.

4. In vitro selection

Splint ligation of RNA substrate to deoxyribozyme selection pool

The RNA substrate was ligated with ssDNA pool using T4 DNA ligase in presence of complementary DNA splint. Deoxyribozyme pool (1.6 nmol), 5'-phosphorylated RNA and complementary splint were used in a molar proportion of 1: 1.5: 1.25 respectively in total volume of 12 μ L. To this mixture, 10 \times annealing buffer (2 μ L, 40 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH = 8.0) was added and heated at 95°C for 4 minutes. The mixture was allowed to cool down to 25°C for 10 minutes. Afterwards, 10 \times ligase buffer (2 μ L, 400 mM Tris-HCl, 100 mM MgCl₂, 100 mM DTT, 5 mM ATP, pH = 7.8) and T4 DNA ligase (2 μ L, 5 U/ μ L) was added to the solution and incubated at 37°C for 2-3 hours. Ligated product (DNA-RNA hybrid) was resolved and purified by denaturing PAGE.

Selection Step

The DNA-RNA hybrid (250 pmol in the first round and 10-30 pmol in further rounds) was incubated with 10 \times selection buffer (1 μ L, 500 mM Tris-HCl, 1.5 M NaCl), pH = 7.5) in a total volume of 9.5 μ L. To initiate the reaction, MgCl₂ (0.5 μ L, 100 mM) with a final concentration of 5 mM was added and reaction mixture was incubated at 37°C for 16 hours. The active fraction of DNA enzymes was separated by denaturing PAGE and the areas corresponding to cleaved products (determined by comparison to size marker) were cut and extracted. Extracted products were ethanol precipitated and subjected to PCR amplification.

PCR amplification of active DNA library fraction

The active fraction of the DNA library recovered in the selection step was amplified through two subsequent asymmetrical PCRs. For the first PCR, the extracted product from previous step was used as template. Template (dissolved in 30 μ L ultra pure water), forward primer (D2, 200 pmol), reverse primer (D3, 50 pmol), dNTP mixture (0.63 μ L, 20 mM), 10 \times DreamTaq buffer (5 μ L) and DreamTaq DNA polymerase (0.25 μ L, 5 U/ μ L) were mixed and volume was adjusted to 50 μ L with water. The reaction mixture was subjected to PCR reaction with the following conditions: [95°C for 4 minutes, 10 \times (95°C for 30 sec, 60°C for 30 sec, 72°C for 1 min) and 72°C for 5 minutes]. PCR-I product was stored at -20°C as backup sample (in case to repeat the selection round). For the second PCR, an aliquot (2 μ L) from PCR-I was used as template and mixed with fluorescently labeled forward primer (D2-flu, 200 pmol), reverse primer (D3, 50 pmol), dNTPs mixture (0.63 μ L, 20 mM), 10 \times Dream Taq buffer (5 μ L) and Dream Taq DNA polymerase (0.5 μ L, 5 U/ μ L) and water to a final volume of 50 μ L. PCR conditions were: [95°C for 4 minutes, 30 \times (95°C for 30 sec, 60°C for 30 sec, 72°C for 1 min) and 72°C for 5 minutes]. The PCR product was resolved on denaturing PAGE and the fluorescently labeled shorter strand was isolated. The purified PCR product then ligated to the RNA substrate to initiate the next round of selection.

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5. Deep sequencing analysis of enriched selection libraries

A schematic summary of sample preparation and sample nomenclature is shown in Figure S2a. In total, five Illumina libraries were prepared. The enriched DNA library from round 15 (AB_R15) was subjected to NGS and named ab1. It was also used for additional 16th round (AB_R16) with unmodified RNA (active: ab2, inactive: ab3), and with modified RNA (active: ab4, inactive: ab5). The isolated active and inactive fractions were amplified in a 2-step PCR protocol, in which Illumina adaptors and index sequences were added (Figure S2b). 1st PCR: forward primer (D6, 30 pmol), reverse primer (D7, 30 pmol), dNTPs mixture (0.63 μ L, 20 mM), 10 \times DreamTaq buffer (5 μ L) and DreamTaq DNA polymerase (0.25 μ L, 5 U/ μ L) were mixed and volume was adjusted to 50 μ L with water. PCR conditions were: [95°C for 4 minutes, 25 \times (95°C for 30 sec, 60°C for 30 sec, 72°C for 1 min) and 72°C for 5 minutes]. 2nd PCR was done with primers having index sequences. For each sample there was a unique index sequence in the reverse primer (D9-D13). An aliquot (2 μ L) from 1st PCR was used as template and mixed with forward primer_D8 (100pmol), reverse primers_D9-D13 (100 pmol), dNTPs mixture (0.63 μ L, 20 mM), 10 \times Dream Taq buffer (5 μ L), DreamTaq DNA polymerase (0.25 μ L, 5 U/ μ L) and water to a final volume of 200 μ L. PCR conditions were: [95°C for 4 minutes, 30 \times (95°C for 30 sec, 60°C for 30 sec, 72°C for 1 min) and 72°C for 5 minutes]. The PCR products were purified on a 2% agarose gel and submitted to the Core Unit Systems Medicine (University of Würzburg) for amplicon sequencing. After quality control on a Bioanalyzer, the libraries were pooled, and sequenced as a fraction of a NextSeq-500 HighOutput 75nt single end run. After trimming and demultiplexing, the fastq and fastqc files were obtained from the Core unit and further processed. The raw fastq files were deduplicated of PCR duplicates with PRINSEQ^[5] (prinseq-lite.pl -fastq INPUT_FILE -out_format 3 -derep 1 -out_good OUTPUT_FILE). Afterwards, the reads were trimmed of UMIs, and constant regions and filtered to the length of 18–21 nt with cutadapt^[6] (cutadapt -g "CGACTAGTTACGGAAG;e=0.2" -a "CTTCATTGAGTTGGCC;e=0.3" -m 18 -M 21 --overlap 10 --times 2 -o OUTPUT_FILE INPUT_FILE). The processed reads were further analyzed using FASTAptamer^[7] and custom R scripts. Multiple sequence alignment was carried out with msa^[8] and sequence logos were generated using ggseqlogo.^[9]

6. Single turnover kinetics characterization of deoxyribozymes

In a typical experiment for determining the trans-activity of individual deoxyribozymes, the synthetic deoxyribozyme (100 pmol) was mixed with 3' fluorescently labeled RNA substrate (10 pmol) in a final volume of 8.5 μ L. Mixture was heated to 95°C for 4 minutes followed by cooling at 25°C for 10 minutes. To initiate the reaction, 10 \times kinetic assay buffer (1 μ L, 500 mM Tris-HCl, 1.5 M NaCl), pH = 7.5 and MgCl₂ (0.5 μ L, 400 mM) were added and reaction mixture was incubated at 37°C. Aliquots (1 μ L) were taken at different time points (0, 10, 30, 60, 120, 180, and 360 min, sometimes 22h), quenched with loading buffer and analyzed by denaturing PAGE. The gels were imaged on a Chemidoc device and the cleavage yield was determined from band intensities quantified by ImageLab software. Values of k_{obs} (observed cleavage rate) and Y_{max} (maximum yield) were obtained by fitting cleavage yield *versus* time (min) with first order kinetics equation; $Y = Y_{\text{max}} * (1 - e^{-k_{\text{obs}} * t})$. Three independent replicates were performed for each experiment.

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7. Supporting Tables

Table S1. Sequences and ESI-MS of RNA oligonucleotides

No	Description	5'-Sequence-3'	mol.wt. calc. amu	mol.wt. found m/z
R1	Unmodified RNA	AUAGACUGAAUGAAGGACUUCGUAACU	8966.24	8966.31
R1a	Unmod. with 3'-C ₆ -NH ₂	AUAGACUGAAUGAAGGACUUCGUAACU-NH ₂	9145.31	9145.49
R2	i ⁶ A RNA	AUAGACUGAAUGAAGGi ⁶ ACUUCGUAACU	9034.30	9034.35
R2a	i ⁶ A with 3'-C ₆ -NH ₂	AUAGACUGAAUGAAGGi ⁶ ACUUCGUAACU-NH ₂	9213.37	9213.48
R3	m ⁶ A RNA	AUAGACUGAAUGAAGGm ⁶ ACUUCGUAACU	8980.34	8980.45
R3a	m ⁶ A with 3'-C ₆ -NH ₂	AUAGACUGAAUGAAGGm ⁶ ACUUCGUAACU-NH ₂	9159.41	9159.33

Table S2. Sequences of DNA oligonucleotides

No	Description	5'-Sequence-3'
D1	Selection pool	GTGACGCGACTAGTTACGGGAAGN ₂₀ CTTCATTCAGTTGGCGCCTCC
D2	Selection forward primer	Hexynyl-GTGACGCGACTAGTTAC
D3	Selection reverse primer (tailed)	(CAA) ₄ E ₃ GGAGGCGCCAACCTGAATGAA
D4	Splint for selection pool (round 1)	TTCATTCAGTCTATGGAGGCGCCAACCTG
D5	Splint for other selection rounds	TTCATTCAGTCTATGGAGGCGCCAACCTG
D6	1 st _PCR_forward_NGS_UMI	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNNNNNNCGACTAGTTACGGGAAG
D7	1 st _PCR_reverse_NGS	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCCAACTGAATGAAG
D8	2 nd _PCR_forward_NGS_i5_S502	AATGATACGGCGACCACCGAGATCTACACCTCTCTATTCGTCGGCAGCGTC
D9	2 nd _PCR_reverse_NGS_i7_N706	CAAGCAGAAGACGGCATAACGAGATCATGCTTAGTCTCGTGGGCTCGG
D10	2 nd _PCR_reverse_NGS_i7_N707	CAAGCAGAAGACGGCATAACGAGATGTAGAGAGGTCTCGTGGGCTCGG
D11	2 nd _PCR_reverse_NGS_i7_N710	CAAGCAGAAGACGGCATAACGAGATCAGCCTCGGTCTCGTGGGCTCGG
D12	2 nd _PCR_reverse_NGS_i7_N711	CAAGCAGAAGACGGCATAACGAGATTCCTCTTGTCTCGTGGGCTCGG
D13	2 nd _PCR_reverse_NGS_i7_N712	CAAGCAGAAGACGGCATAACGAGATTCCTCTACGTCTCGTGGGCTCGG
DNA enzymes		
D14	AA07	TAGTTACGGAAGGGGTTCGGTGGAGCGGCGGATTTCATTCAGT
D15	AA14	TAGTTACGGAAGTGGTCTCGCGGTTCCTGGTTATTCATTCAGT
D16	AA17	TAGTTACGGAAGCCTGCAAGGAGGTTTACCGGGTTCATTCAGT
D17	AB08	TAGTTACGGAAGGGGAAGCCAGTGGTACGTTCTTCATTCAGT
D18	AB08_A4G	TAGTTACGGAAGGGGGAGCCAGTGGTACGTTCTTCATTCAGT
D19	AB08_A4C	TAGTTACGGAAGGGGGCAGCCAGTGGTACGTTCTTCATTCAGT
D20	AB08_A4T	TAGTTACGGAAGGGGGTAGCCAGTGGTACGTTCTTCATTCAGT
D21	AB08_ΔA4	TAGTTACGGAAGGGGG_AGCCAGTGGTACGTTCTTCATTCAGT
D22	AB08_T19A	TAGTTACGGAAGGGGAAGCCAGTGGTACGTACTTCATTCAGT
D23	AB08_T19C	TAGTTACGGAAGGGGAAGCCAGTGGTACGTCCTTCATTCAGT
D24	AB08_T19G	TAGTTACGGAAGGGGAAGCCAGTGGTACGTCCTTCATTCAGT
D25	AB08* (AB08_□T14,T19G)	TAGTTACGGAAGGGGAAGCCAGTGGTACGTT_ACGTCTTCATTCAGT
D26	AB08_restored binding arm	TAGTTACGGAAGGGGAAGCCAGTGGTACGTTCTTCATTCAGT
D27	AC17	TAGTTACGGAAGGGGTCTCCAGCCGGACGTTATTCATTCAGT
D28	AC17_ΔA20	TAGTTACGGAAGGGGTCTCCAGCCGGACGTT_TTCATTCAGT
D29	AC17_ΔT19	TAGTTACGGAAGGGGTCTCCAGCCGGACGTT_ATTCATTCAGT
D30	AC17_A20C	TAGTTACGGAAGGGGTCTCCAGCCGGACGTTCTTCATTCAGT
D31	AC17_A20G	TAGTTACGGAAGGGGTCTCCAGCCGGACGTTCTTCATTCAGT
D32	AC17_C12T (VMC10)	TAGTTACGGAAGGGGTCTCCAGCTGGACGTTATTCATTCAGT

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Table S3. Summary of NGS analysis data

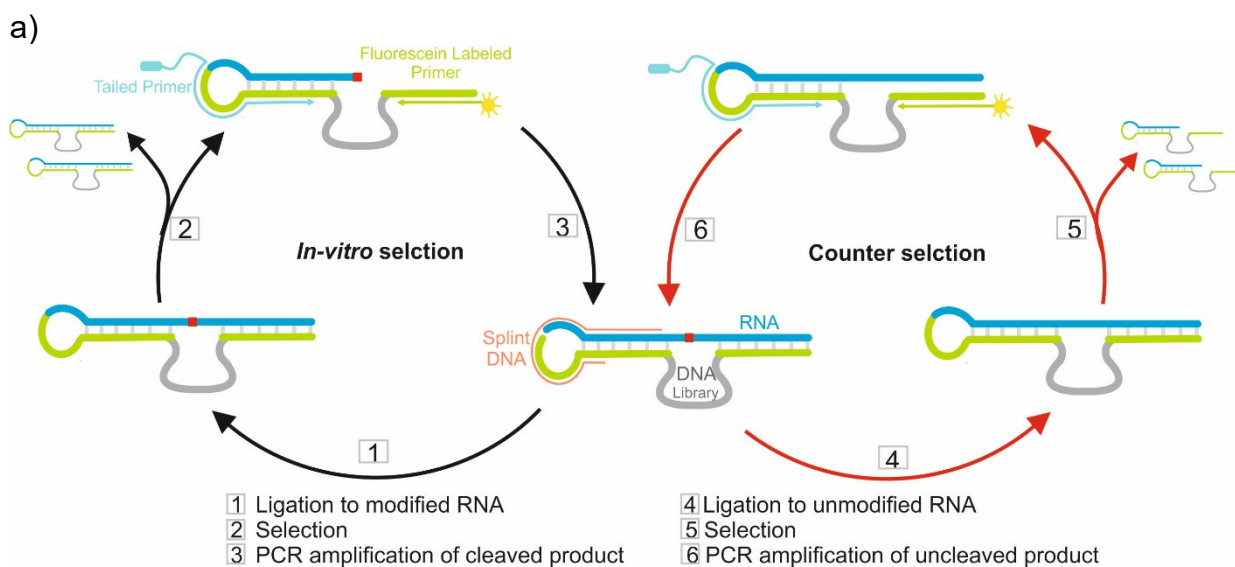
library	ab1	ab4	ab5	sum I (ab1+4+5)	ab2	ab3	sum II (ab2+3)
Round selection	15 reference	16 positive	16 positive		16 negative	16 negative	
RNA activity	- reference	i ⁶ A (R2) cleaved active	i ⁶ A (R2) uncleaved inactive		A (R1) cleaved active	A (R1) uncleaved inactive	
total reads (deduplicated)	750.106	345.138	252.618	1.347.862	773.935	616.504	1.390.439
unique sequences	115.993	16.248	43.496	175.737	62.876	104.136	167.012
seqs (> 50 reads)	796	340	275	1411	909	663	1572
AB08 (-d4 -f50)							
cluster size	73	54	27	154	48	60	108
reads	47.794	37.702	11.663	97.159	21.968	36.872	58.840
% of total	6.4	10.9	4.6	21.9	2.8	6.0	8.8
AC17							
# variants	538	125	225	888	1.177	426	1.603
AC17_ΔA20							
# variants	1.277	420	524	2.221	2.172	1.025	3.197
reads	15.930	6.491	3.626	26.047	26.979	10.047	37.026
% of total	2.1	1.9	1.4	5.4	3.5	1.6	5.1

Table S4. Catalytic activities of DNA enzymes reported in this study

DNA	Unmodified RNA (R1)			i ⁶ A-modified RNA (R2)		
	site	k_{obs} [$\cdot 10^{-2} \text{min}^{-1}$]	yield% @ 6h	site	k_{obs} [$\cdot 10^{-2} \text{min}^{-1}$]	yield% @ 6h
AA07	G15	0.11	42	-	no cleavage	-
AA14	G15	0.66	74	-	no cleavage	-
AA17	G16	0.65	77	-	no cleavage	-
AB08	-	no cleavage	-	G16	0.86	75
AB08-A4G	-	no cleavage	-	G16	0.27	65
AB08-A4C	-	no cleavage	-	G16	0.64	77
AB08-A4T	-	no cleavage	-	G16	0.30	67
AB08-ΔA4	-	no cleavage	-	-	no cleavage	-
AB08-T19G	-	no cleavage	-	G16	0.28	58
AB08-T19A	-	no cleavage	-	G16	0.26	54
AB08-T19C	-	no cleavage	-	-	no cleavage	-
AB08*Δ14T19G	-	no cleavage	-	G16	0.97	75
AC17	G16	1.8	82	G15	0.16	51
AC17-ΔT19	G16	0.41	60	G15	0.70	73
AC17-A20G	G16	1.1	84	G15	0.12	48
AC17-A20C	G16	1.7	87	G15	0.05	18
AC17-ΔA20	G16	0.04	21	G15	1.3	81
AC17-C12T	G16	0.77	81	G15	0.08	25

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8. Supporting Figures



b)

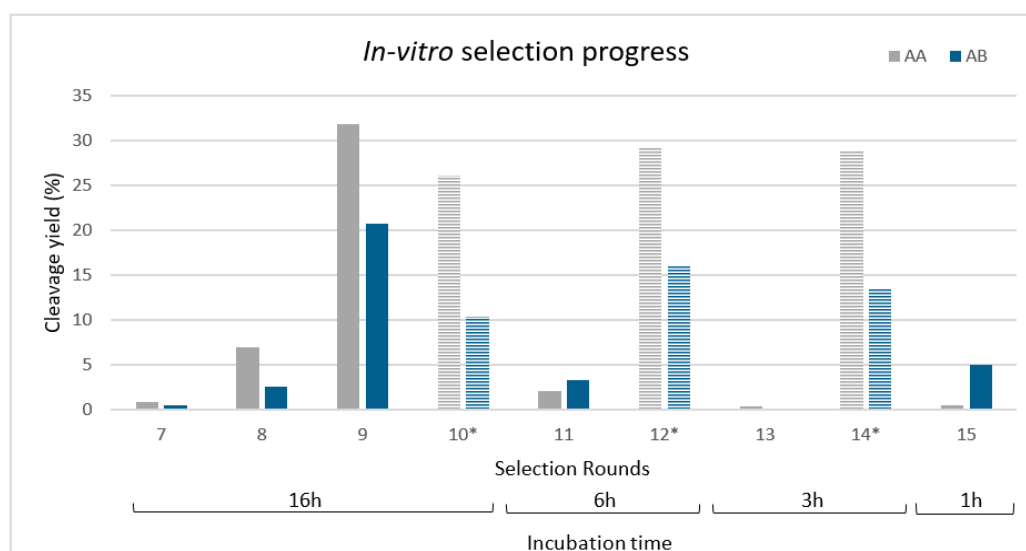
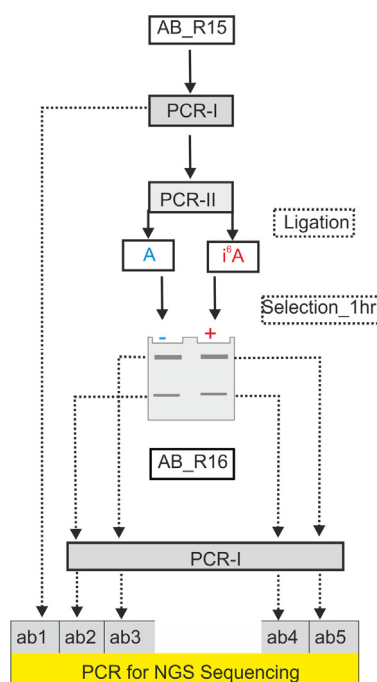


Figure S1. a) Schematic presentation of in vitro selection experiment to evolve i^6 A-sensitive RNA cleaving deoxyribozymes (AB selection). In-Vitro selection: 1. Ligation of DNA library with modified RNA (R2). 2. Selection step in presence of $MgCl_2$ (5 mM, pH 7.5, 37°C). Cleaved fraction is isolated by PAGE. 3. PCR amplification of active fraction. Counter selection to enhance selectivity of the DNA enzymes: 4. Ligation of DNA library to unmodified RNA (R1). 5. Selection in presence of $MgCl_2$ (5 mM, pH 7.5, 37°C). The uncleaved fraction is isolated from PAGE. 6. PCR amplification of uncleaved fraction. For the AA selection, the procedure started with ligation of unmodified RNA, and the counter selection rounds were performed with i^6 A-RNA. b) Progress of in-vitro selection plotted as % cleaved in each selection round. From round 1-6 fluorescence was below the detection limit. From round 7, the DNA pool was enriched enough to determine cleavage yield by measuring fluorescent band intensity during selection step by imaging the gel on a ChemiDoc imager. In round 11, the incubation time for selection was decreased to 6 h, and then further to 3 h in round 13 and finally to 1 h in round 15. The counter selection rounds are marked with asterisks and incubation time was 16 hour for each counter selection step.

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a)



b)

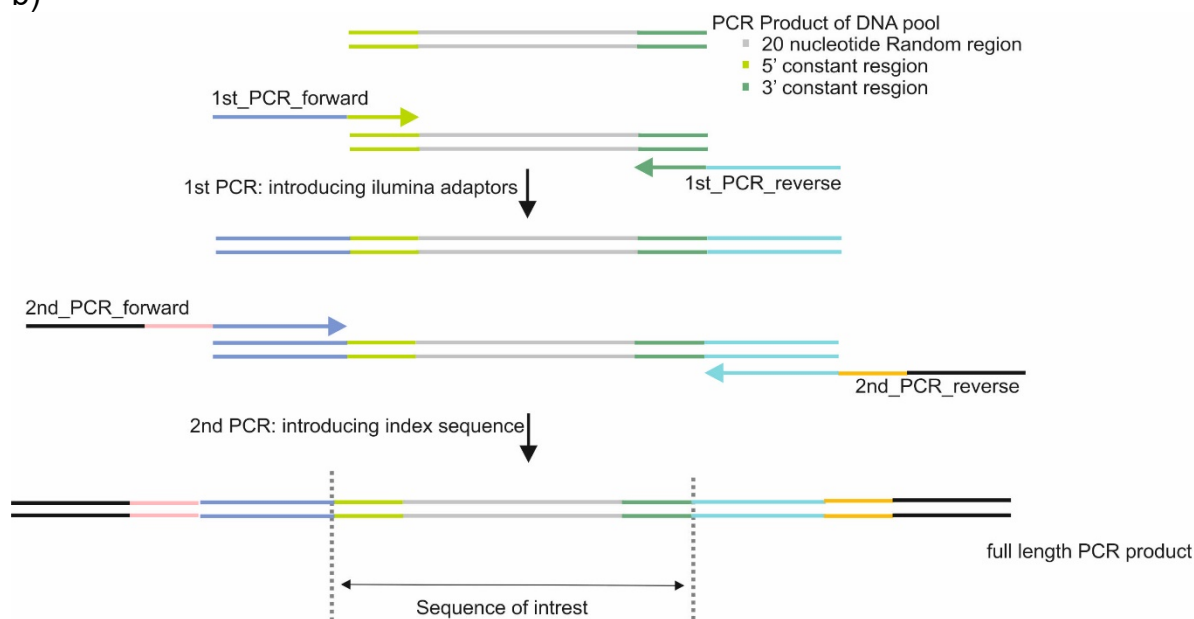


Figure S2. a) Schematic presentation of NGS libraries from round 15 and round 16. b) Schematic presentation of two-step-PCR for NGS library preparation to include Illumina adaptors and barcodes. The catalytic core (from 20 nt random region) is shown in grey, the 5' primer binding site in light green and 3' primer binding site in dark green. The 1st PCR introduces adaptor sequences (presented in blue and cyan, with primers D6 and D7 in Table S1), and the 2nd PCR introduces index sequences (pink and orange) from primers D8-D13. A unique index primer (2nd_PCR_reverse i7_primer, D9-D13) is used for each sample ab1-ab5.

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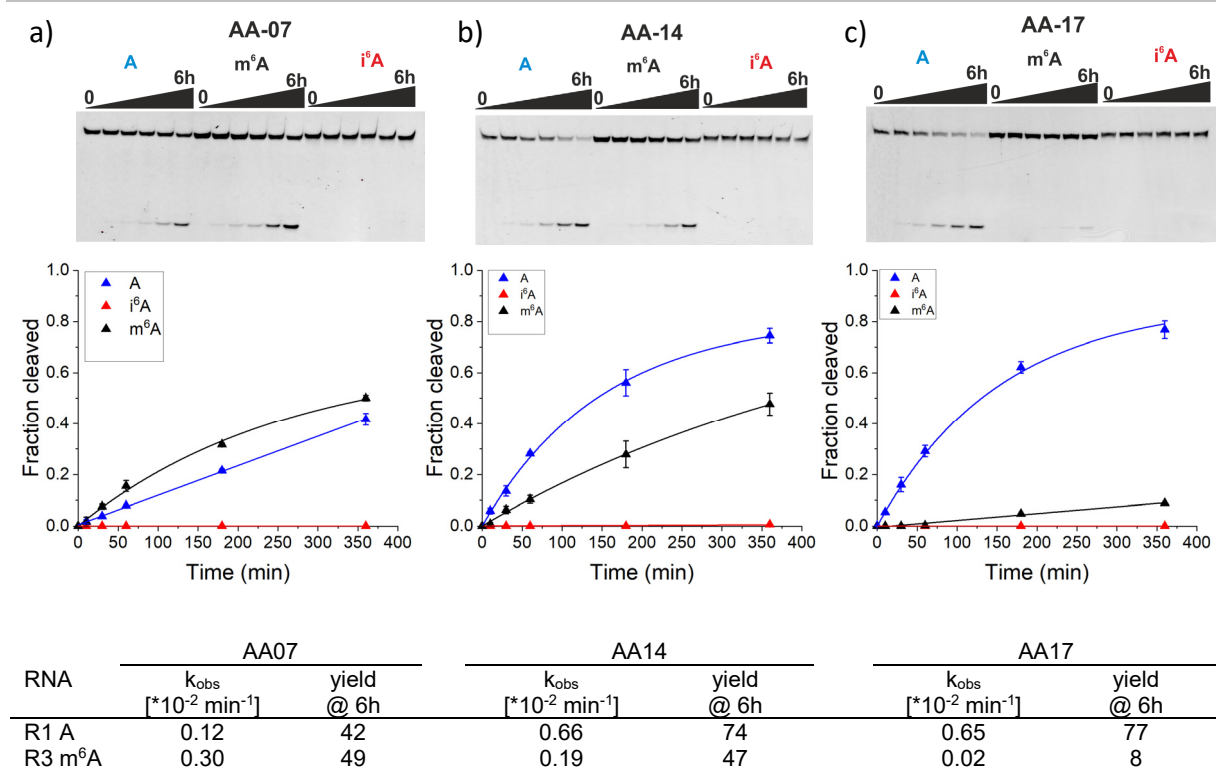


Figure S3. Gel images, kinetic plots, rates and yields for AA DNA enzymes tested with unmodified RNA (R1), i⁶A-RNA (R2) and m⁶A-RNA (R3). Conditions: 1 μM RNA incubated with 10 μM DNA enzyme, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20 mM MgCl₂, 37°C. time points: 0, 10, 30, 60, 180, 360 min.

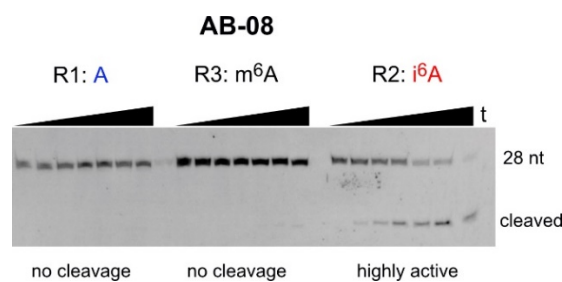
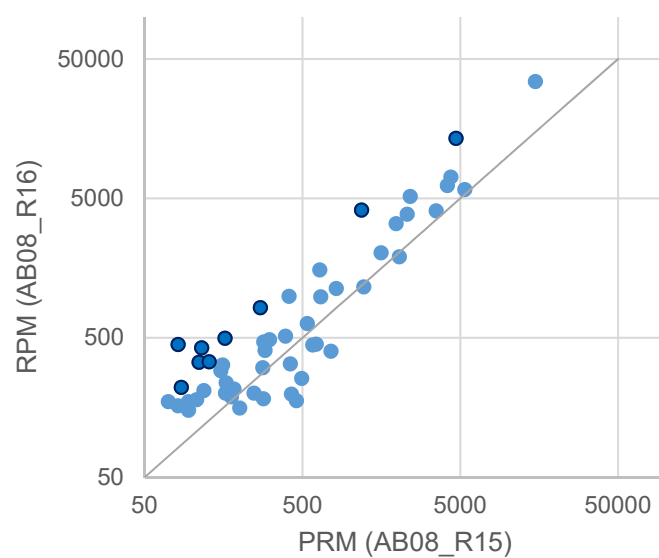


Figure S4. Gel image of AB08 tested on m⁶A-RNA (R3), in comparison to R1 and R2. No cleavage was observed for m⁶A-modified RNA, indicating that AB08 is specific for i⁶A RNA. Conditions: 1 μM RNA incubated with 10 μM DNA enzyme in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20 mM MgCl₂, 37°C. Time points: 0, 10, 30, 60, 120, 180, 360 min.

SUPPORTING INFORMATION



	Sequence	RPM (x) R15(ab1)	RPM (y) R16(ab4)	enrichment (y/x)
AB08	GGGAAGCCAGTGGTACGTT	14981.9	34351.5	2.29
	GGG T AGCCAGTGG_A C GT G	81.3	446.2	5.49
	GGG T AGCCAGTGG_A C GT T	114.7	423.0	3.69
AB08*	GGGAAGCCAGTGG_A C GT G	1185.2	4111.4	3.47
	GGGAAGCCAGTGG_A C GT T	161.3	495.5	3.07
	GGG T AGCCAGTGG_A C GT A	270.6	820.0	3.03
	GGG C AGCCAGTGG_A C GT T	110.7	333.2	3.01
AB08_A4C	GGG C AGCCAGTGGTACGTT	4695.3	13461.3	2.87
	GG A AAGCCAGTGGTACGTT	128.0	336.1	2.63
	GGGAAGCC G GTGGTACGTT	85.3	220.2	2.58

Figure S5. Abundance of AB08 variants in NGS data from round 15 and round 16. Only Sequences with >50 RPM in round 16 are plotted. The most enriched variants are highlighted in dark blue, their sequences, RPM and enrichment factors are given, and mutations in comparison to AB08 are highlighted in red.

SUPPORTING INFORMATION

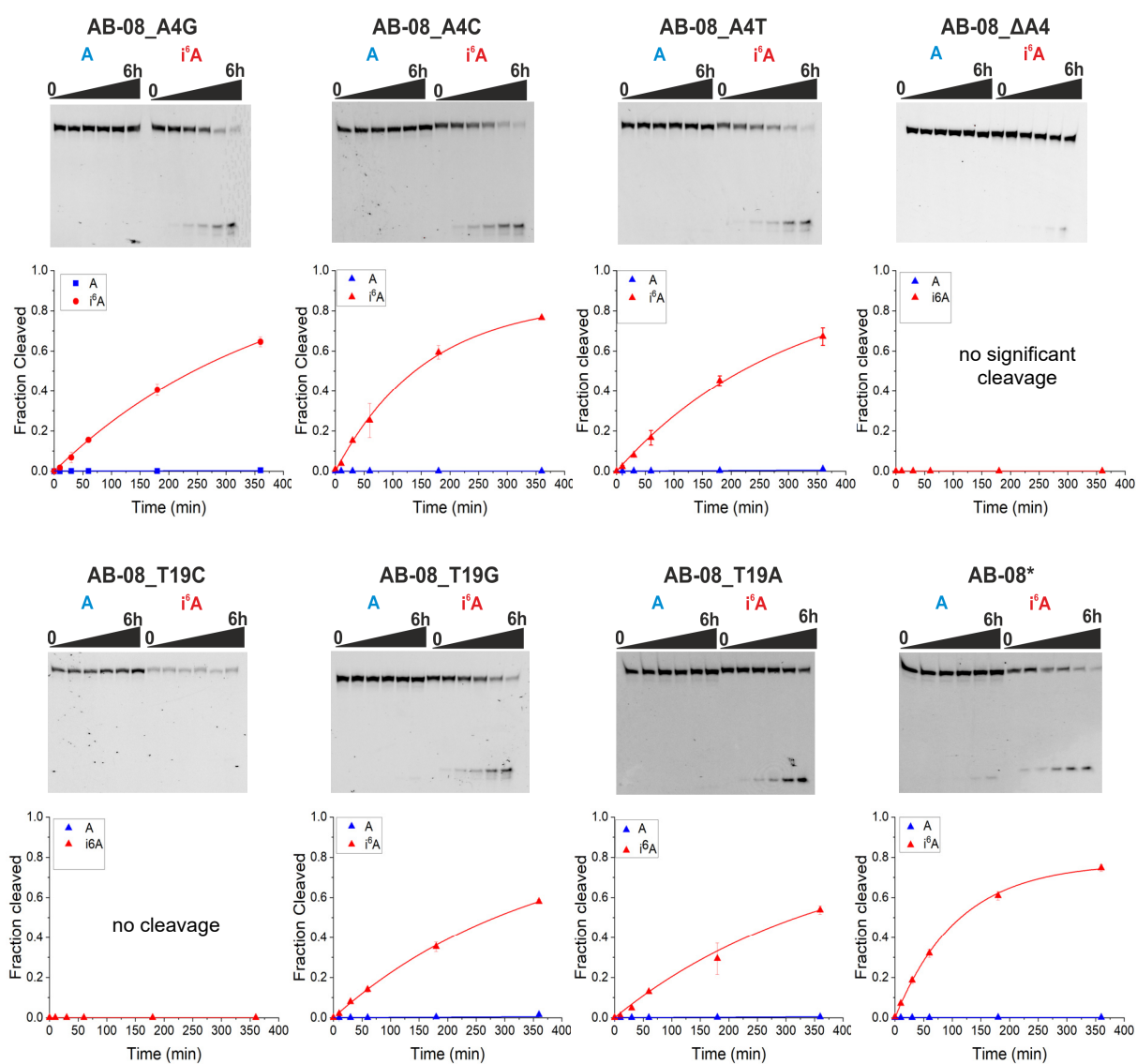


Figure S6. Gel images and kinetic plots for AB08 variants given in Table 2 in the manuscript. Conditions: 1 μM RNA incubated with 10 μM DNA enzyme in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20 mM MgCl₂, 37°C. Time points: 0, 10, 30, 60, 180, 360 min.

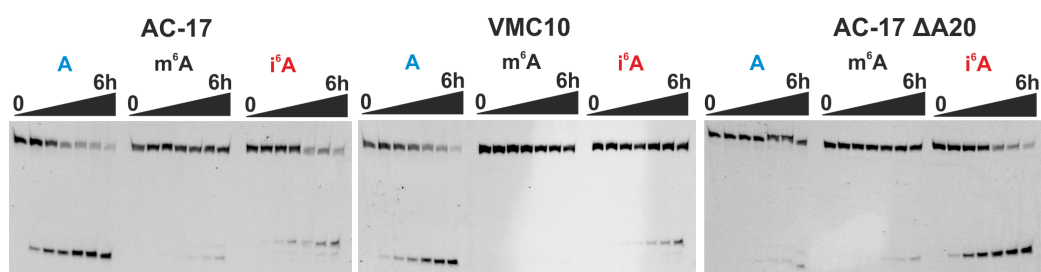


Figure S7. Gel images for plots in Figure 4. Conditions: 1 μM RNA incubated with 10 μM DNA enzyme in 50 mM Tris-HCl, pH 7.5, 150 mm NaCl, 20 mM MgCl₂, 37°C. Timepoints: 0, 10, 30, 60, 120, 180, 360 min.

SUPPORTING INFORMATION

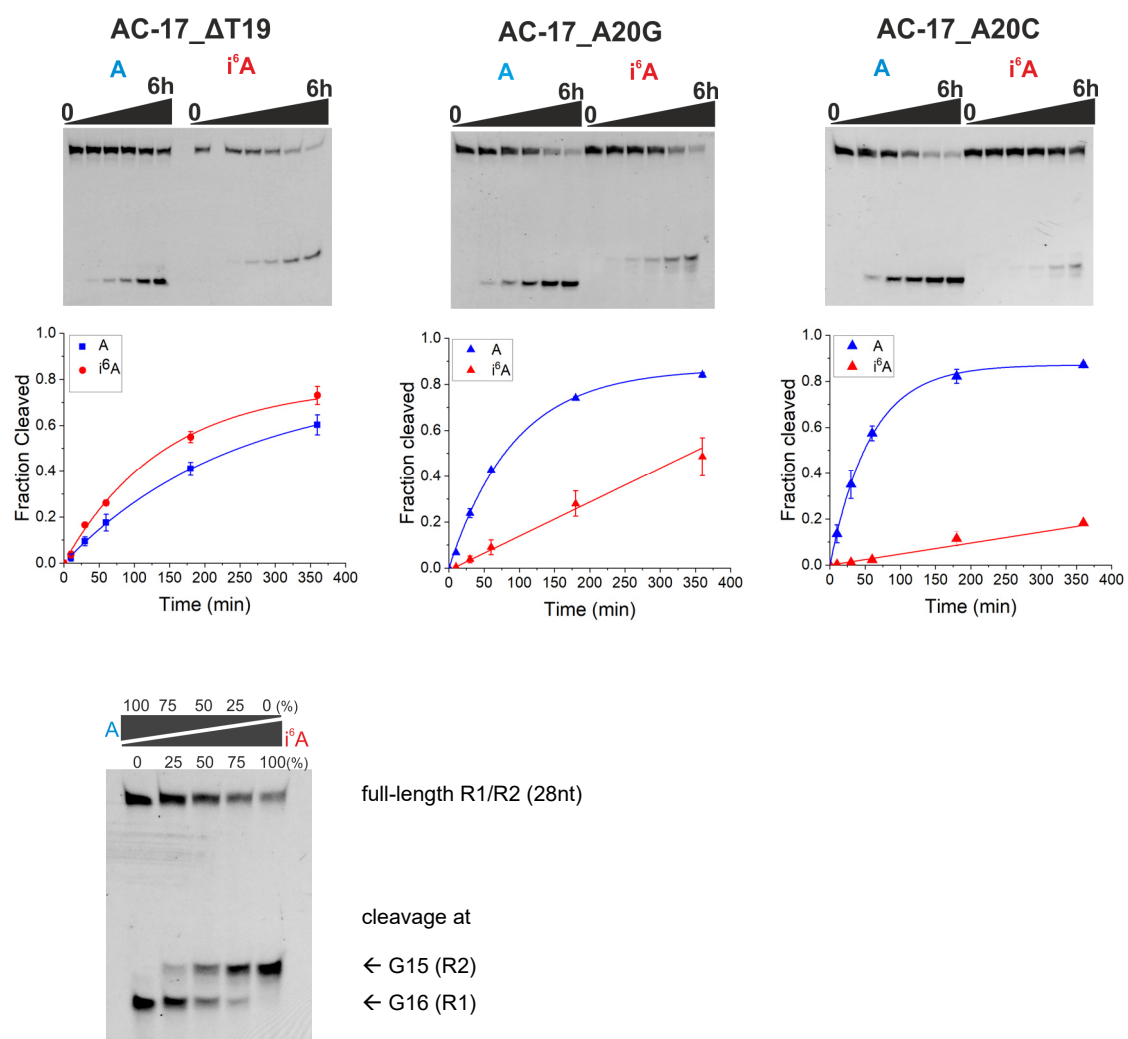


Figure S8. Top: Gel images and kinetic plots of AC17 variants given in Table 2 in the manuscript. Conditions: 1 μ M RNA incubated with 10 μ M DNA enzyme in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20 mM MgCl₂, 37°C. Timepoints: 0, 10, 30, 60, 180, 360 min. Bottom: Samples containing both R1 and R2 - with increasing amount of i⁶A-RNA from left to right - were cleaved with AC17-ΔT19 for 6 h. The ratio of both cleavage products correlates to the i⁶A content.

SUPPORTING INFORMATION

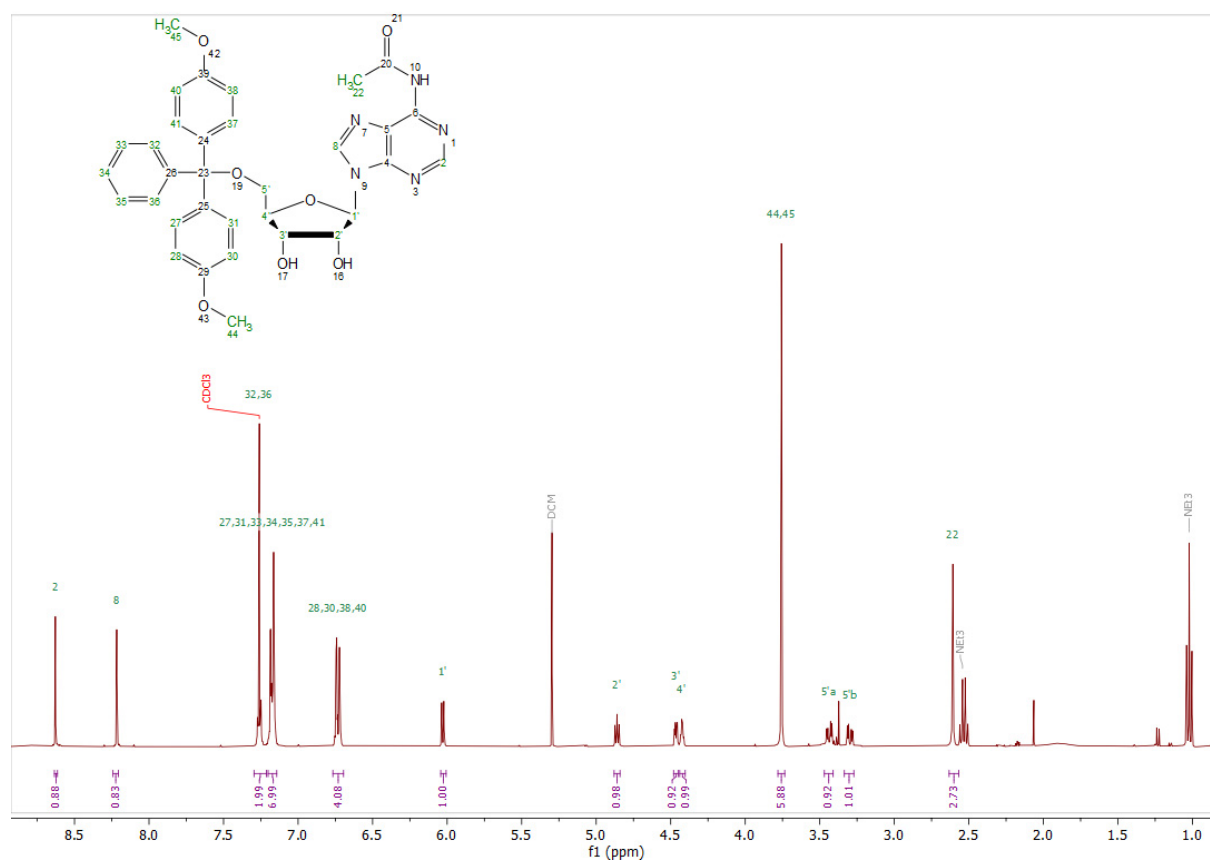


Figure S9. $^1\text{H-NMR}$ spectrum for **2**.

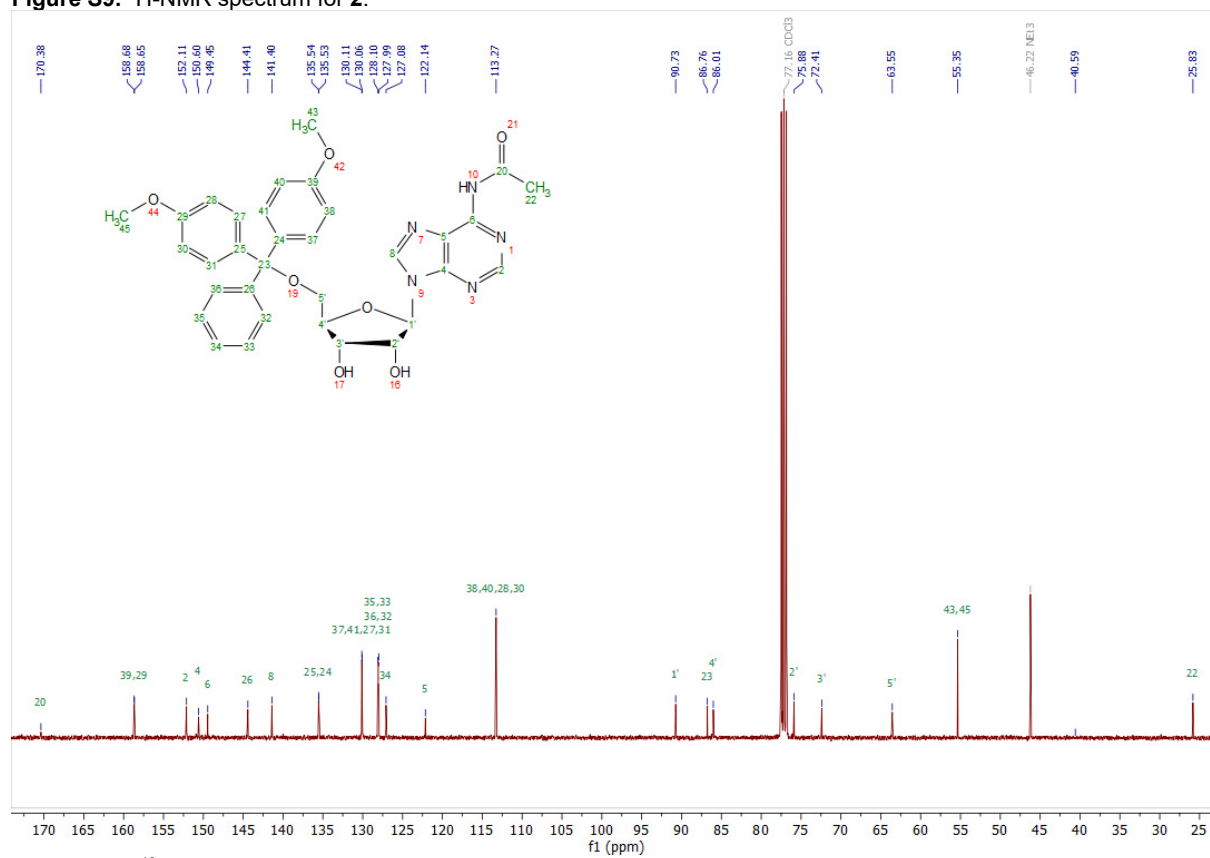
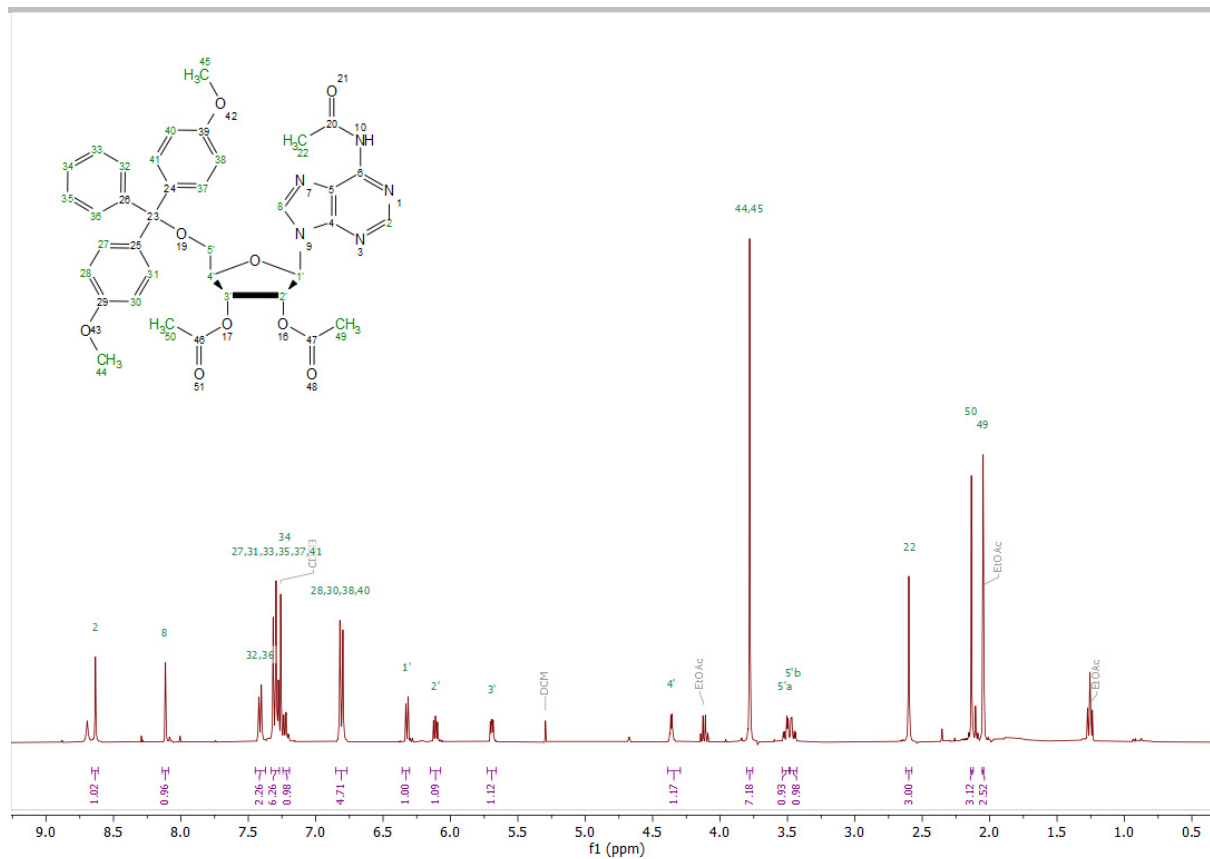
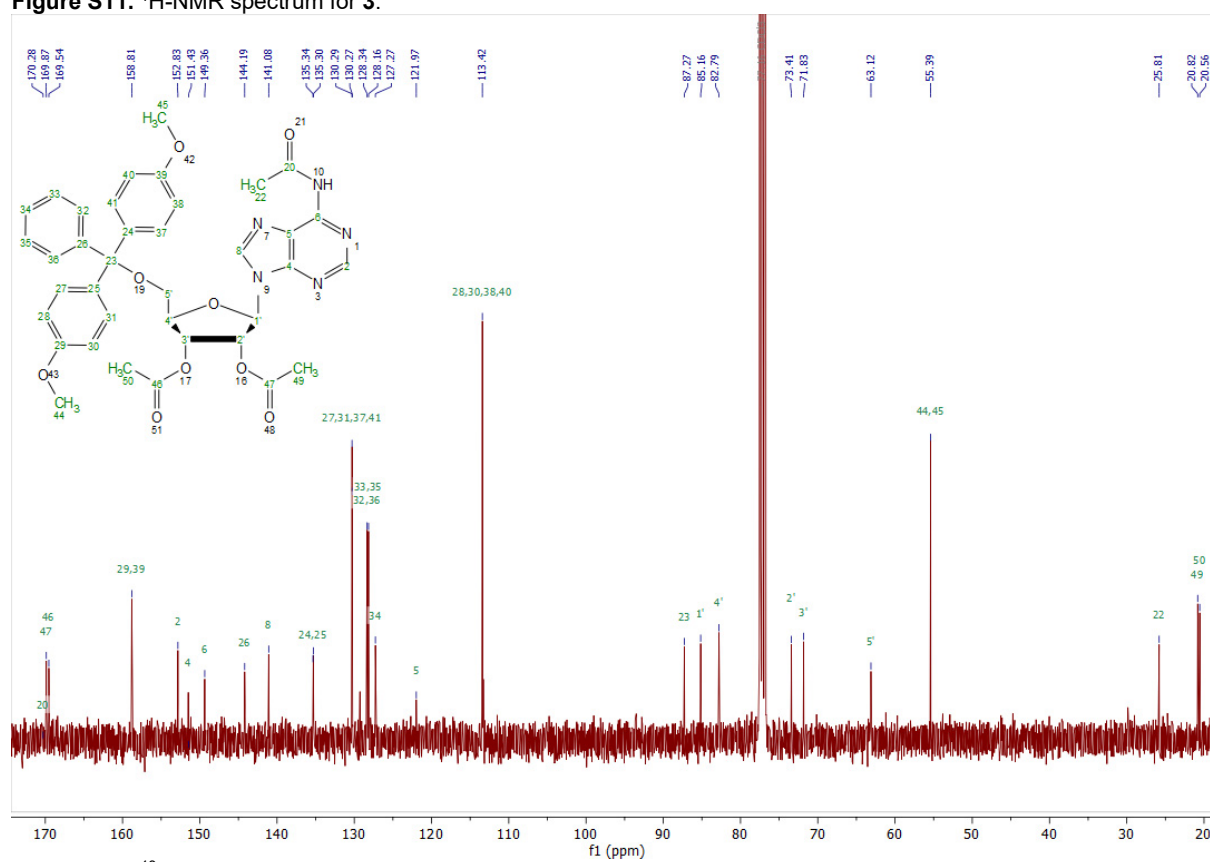


Figure S10. $^{13}\text{C-NMR}$ spectrum for **2**.

SUPPORTING INFORMATION

Figure S11. ¹H-NMR spectrum for 3.Figure S12. ¹³C-NMR spectrum for 3.

SUPPORTING INFORMATION

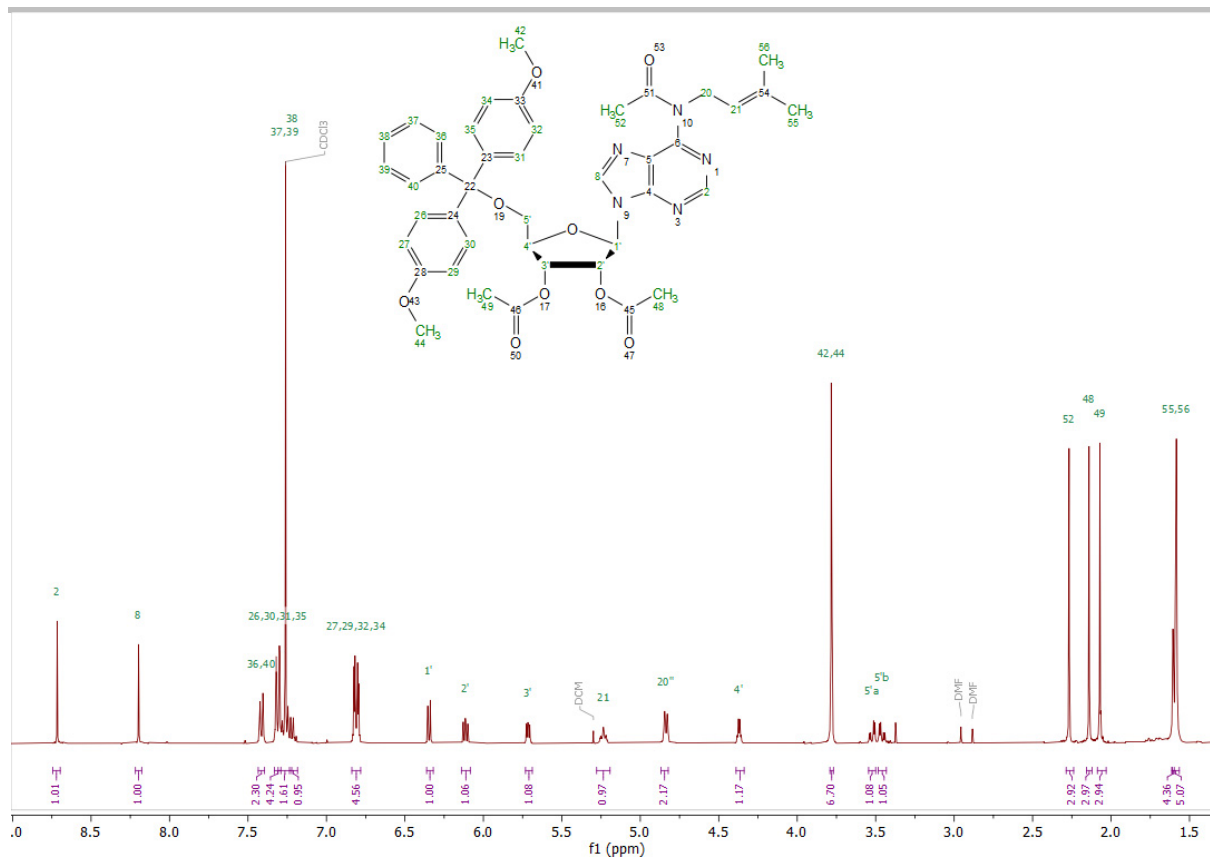


Figure S13. $^1\text{H-NMR}$ spectrum for 4.

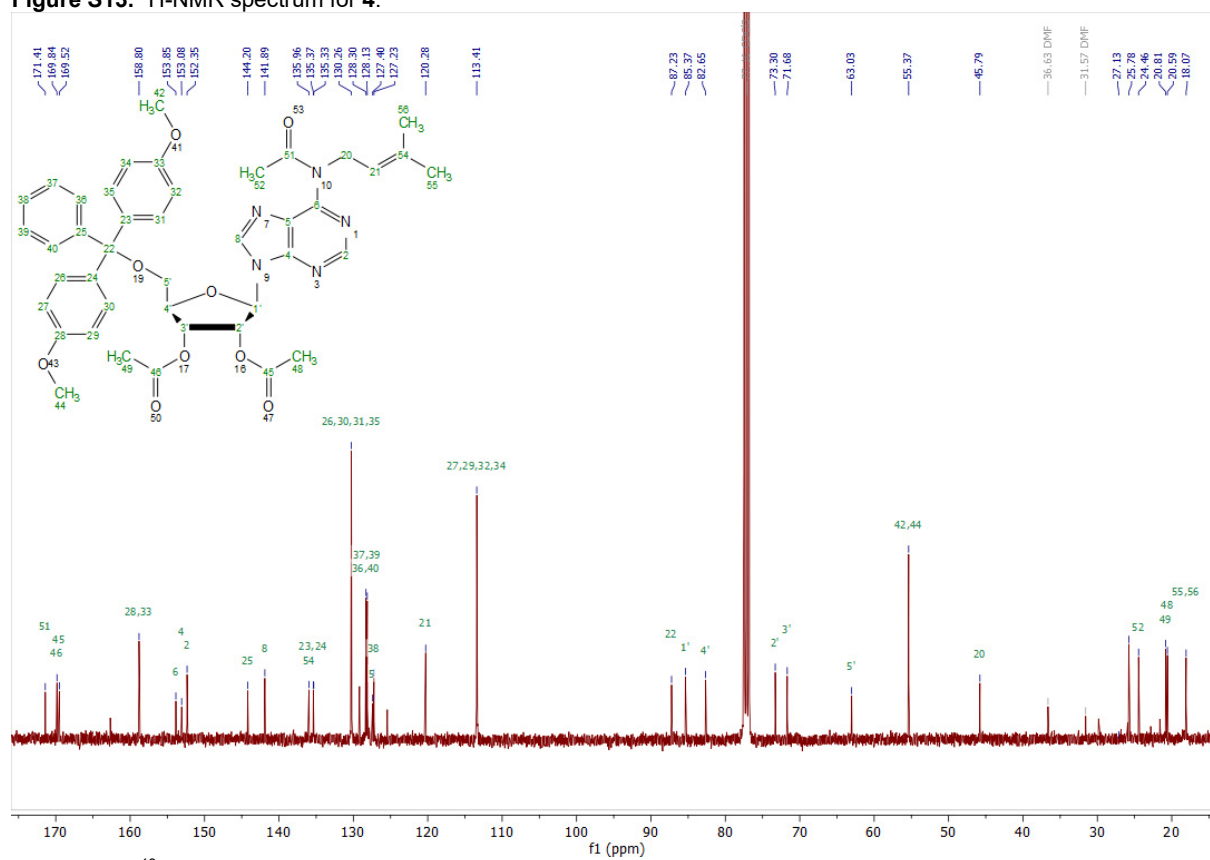
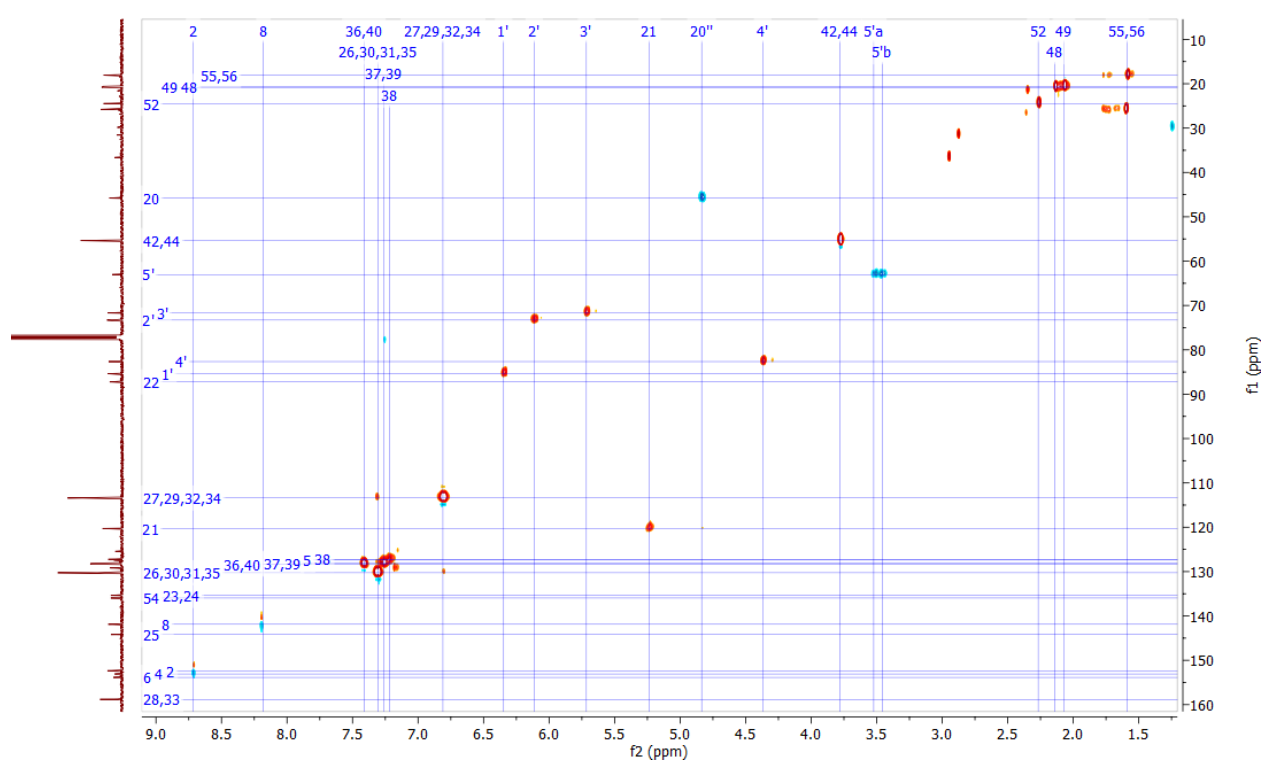


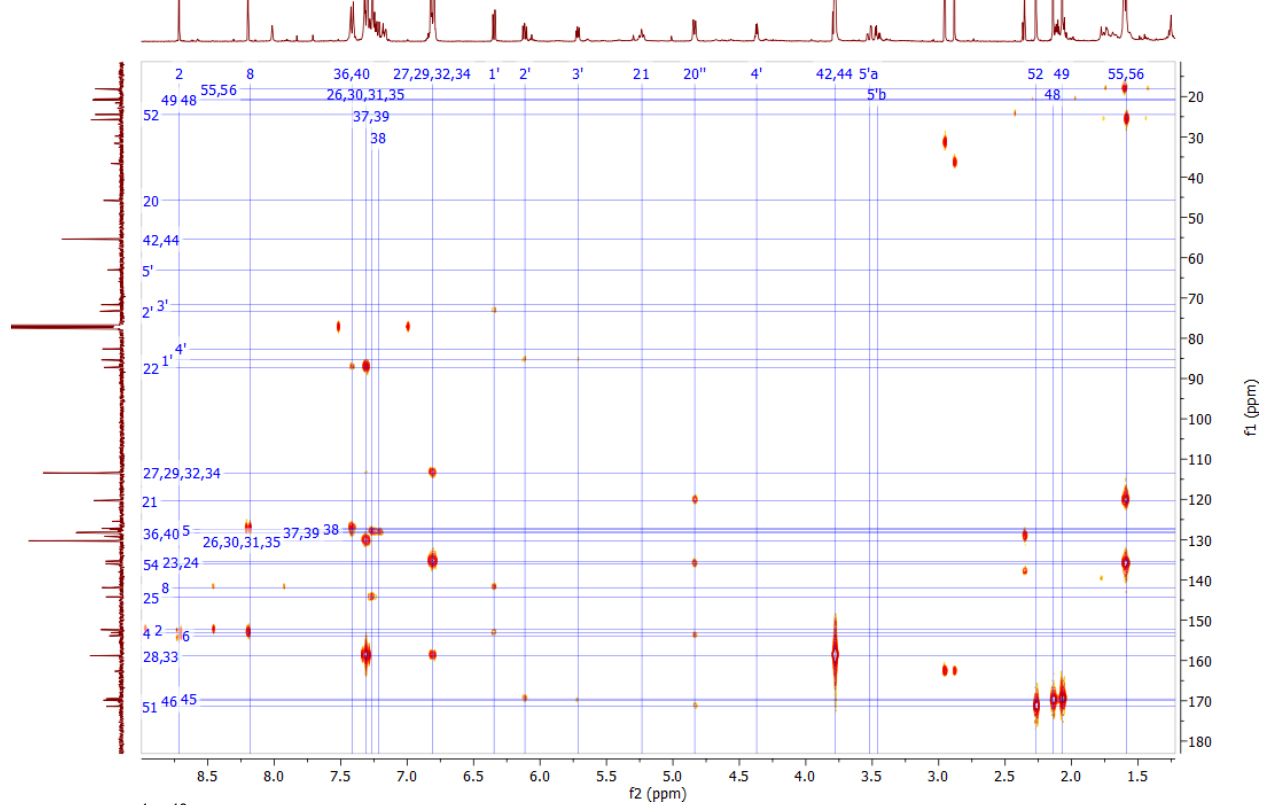
Figure S14. $^{13}\text{C-NMR}$ spectrum for 4.

SUPPORTING INFORMATION

HSQC



HMBC

Figure S15. ^1H , ^{13}C -2D-NMR spectra for **4**.

SUPPORTING INFORMATION

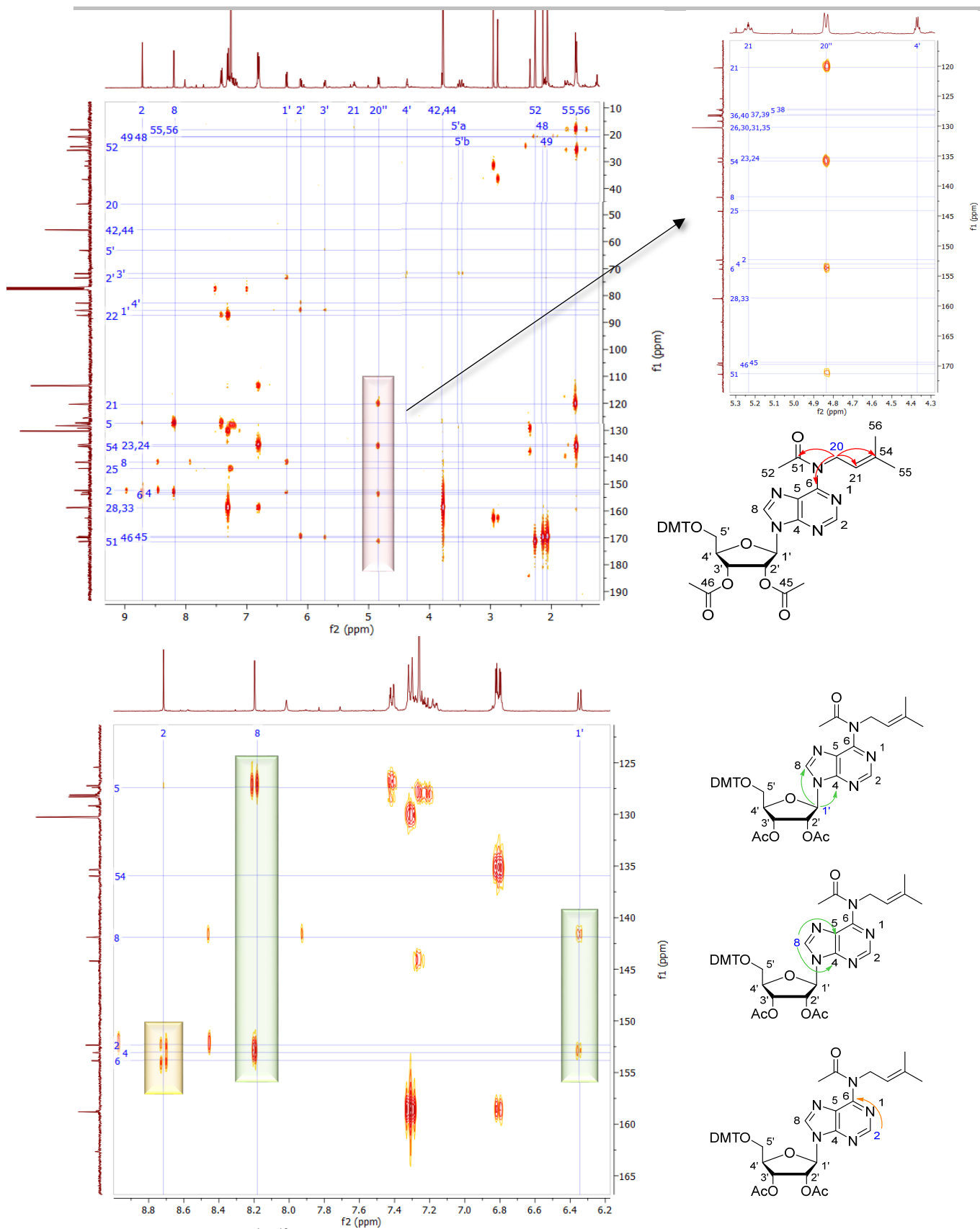
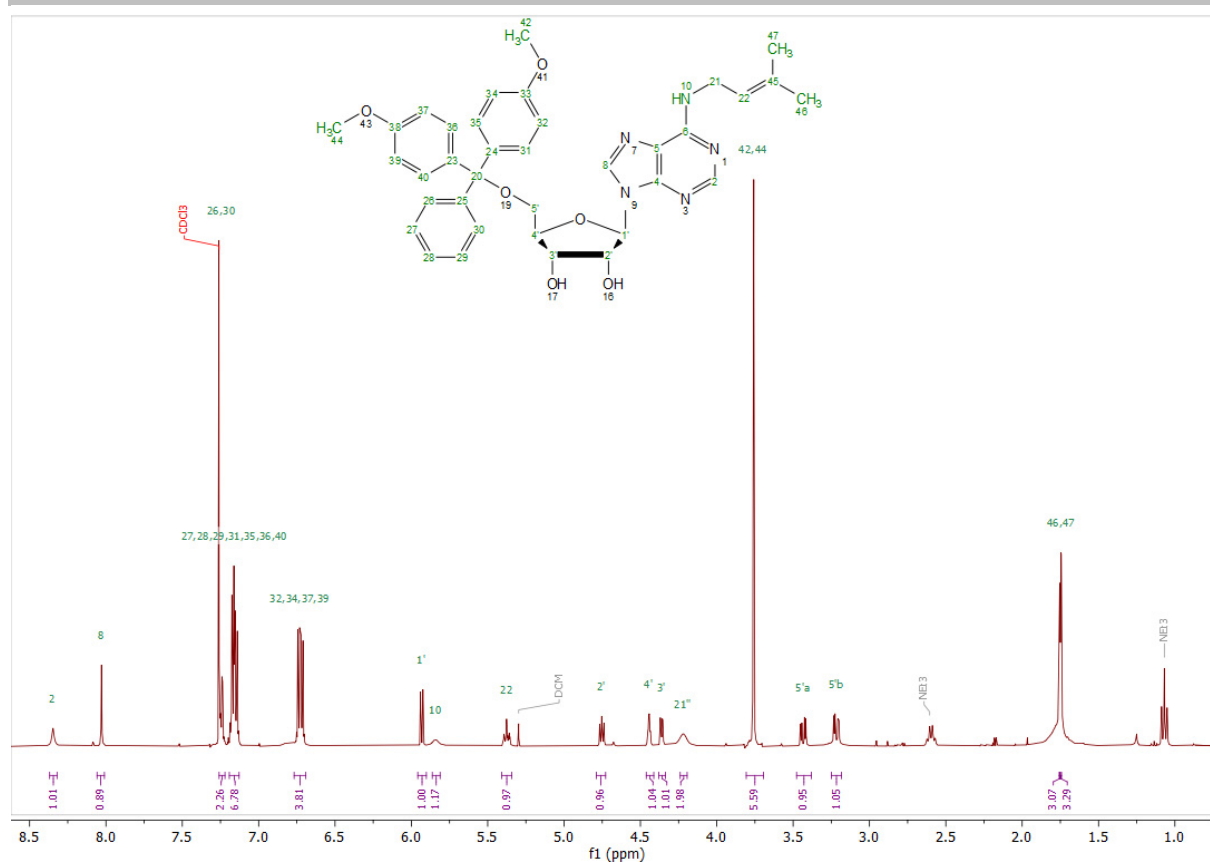
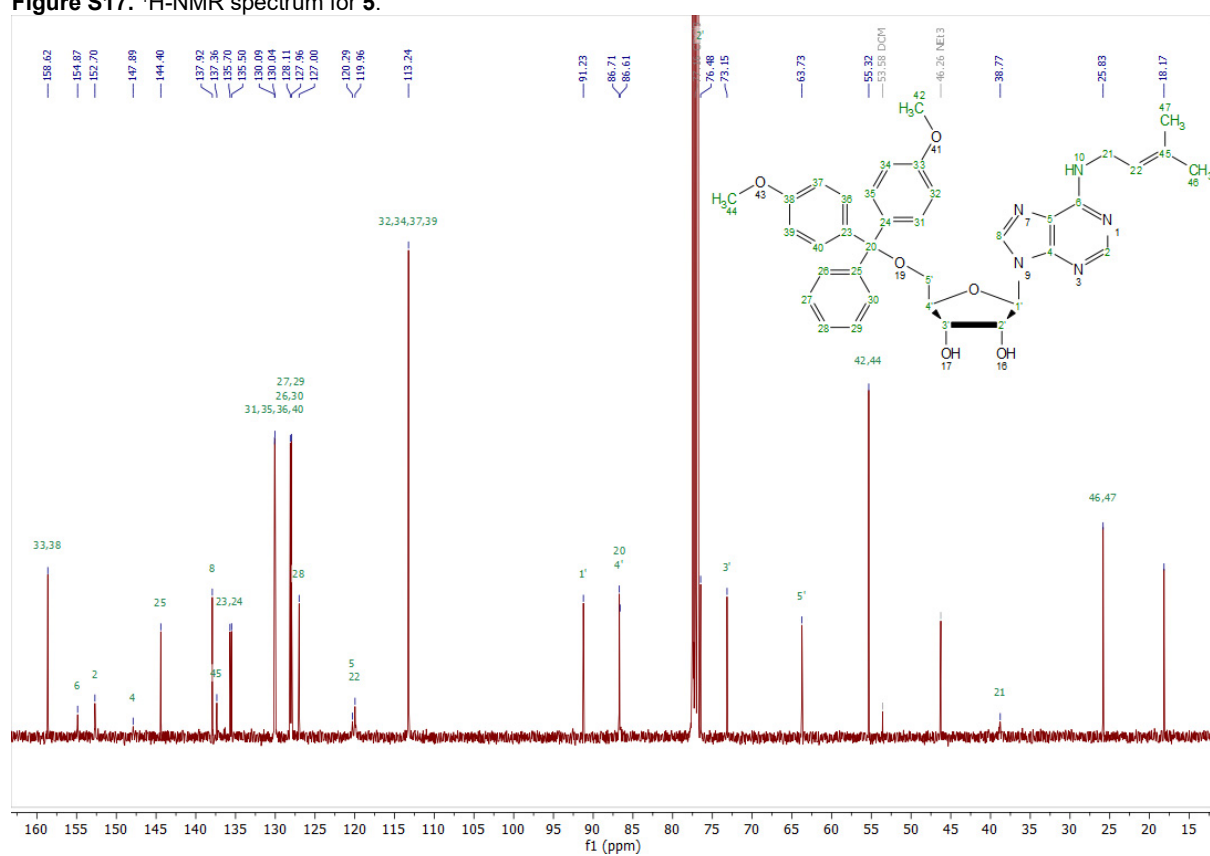
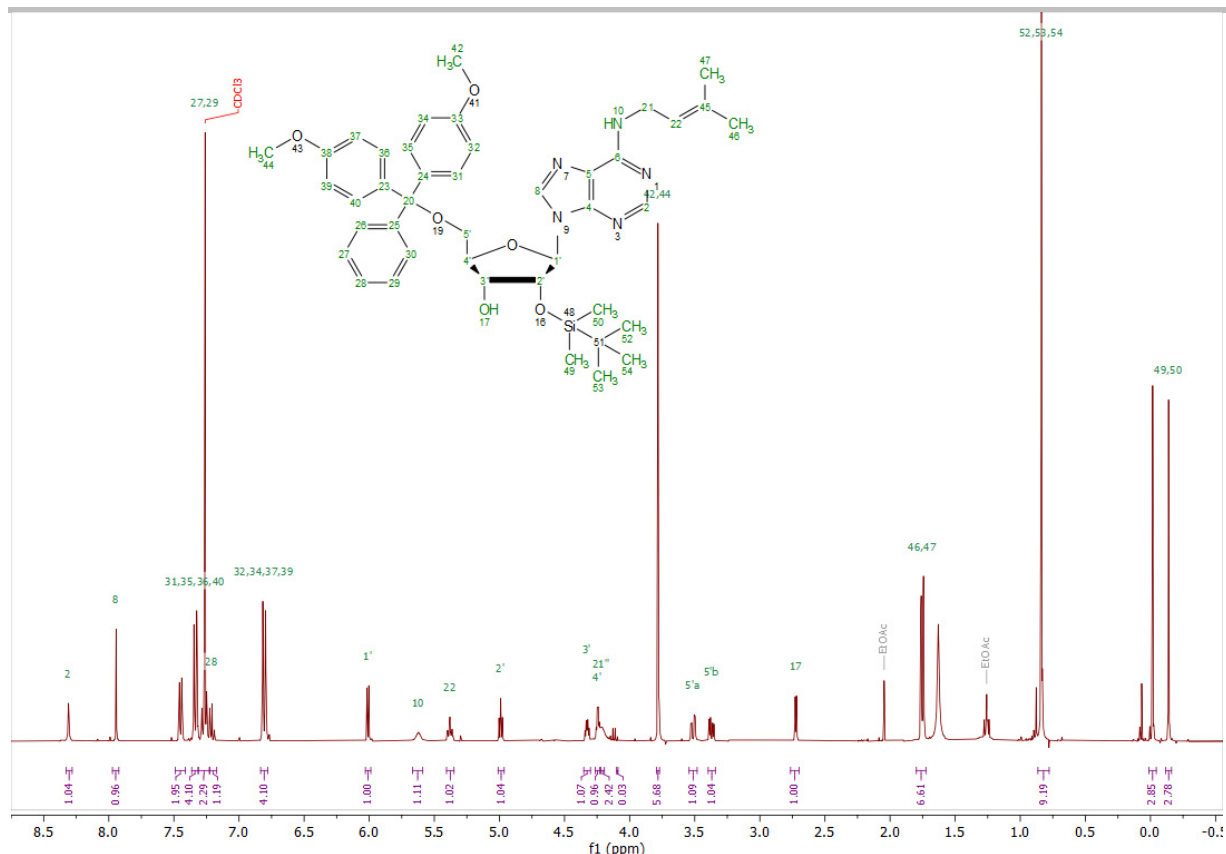
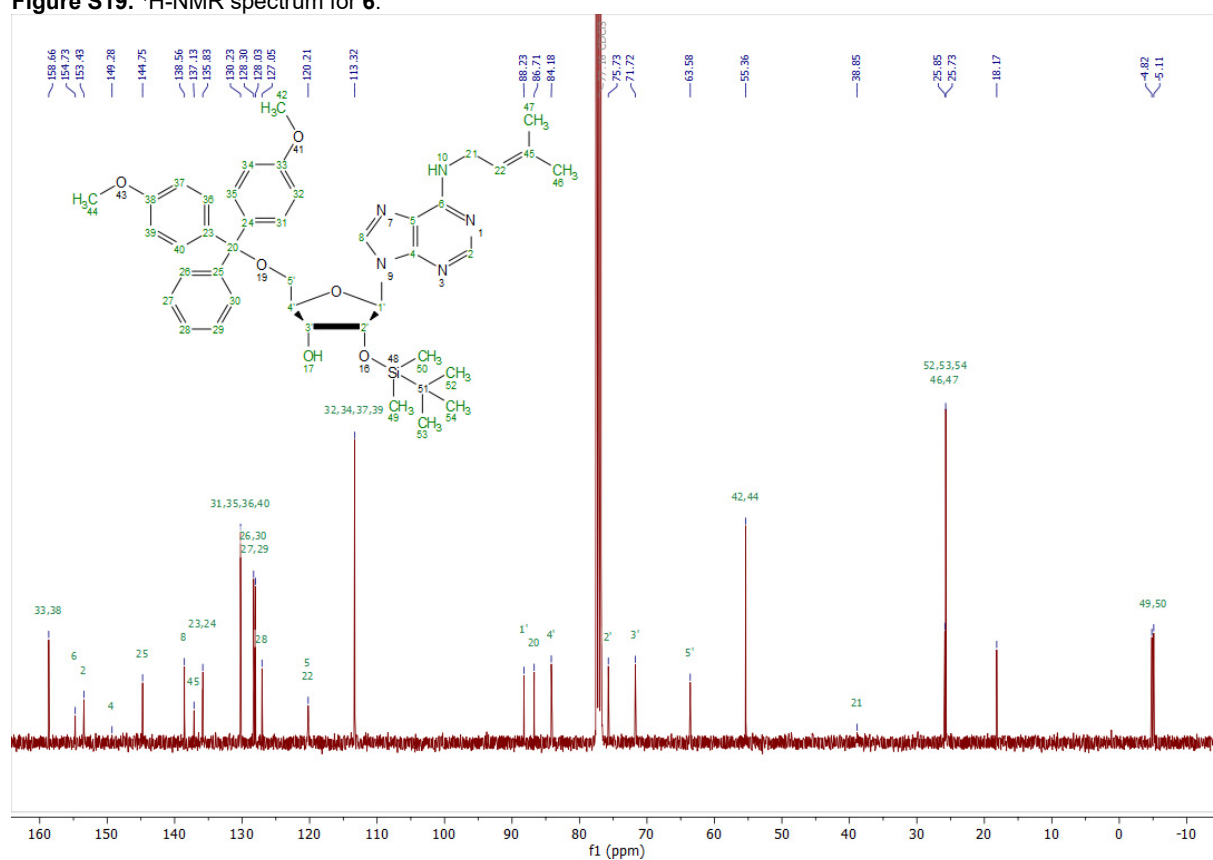


Figure S16. Excerpts of ^1H , ^{13}C -HMBC spectrum for **4** confirming the assignment of N6 alkylation.

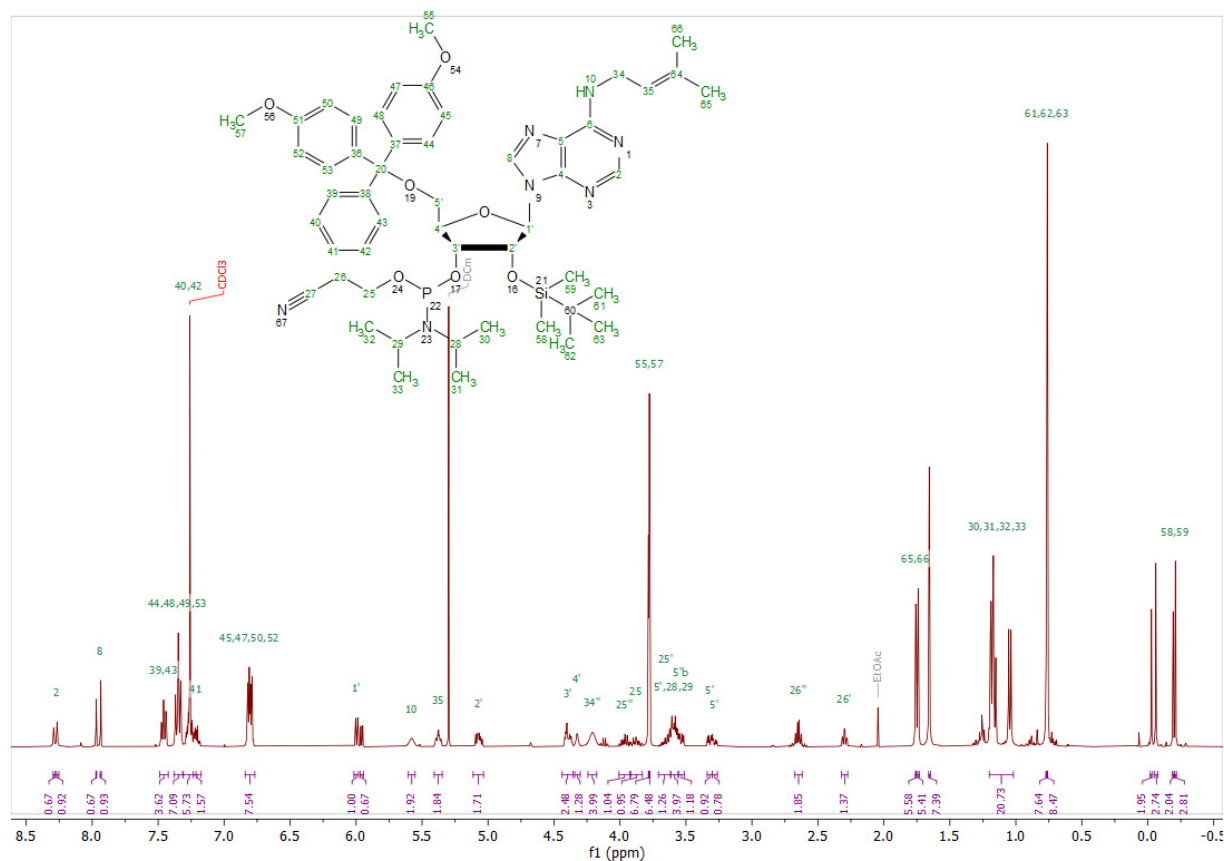
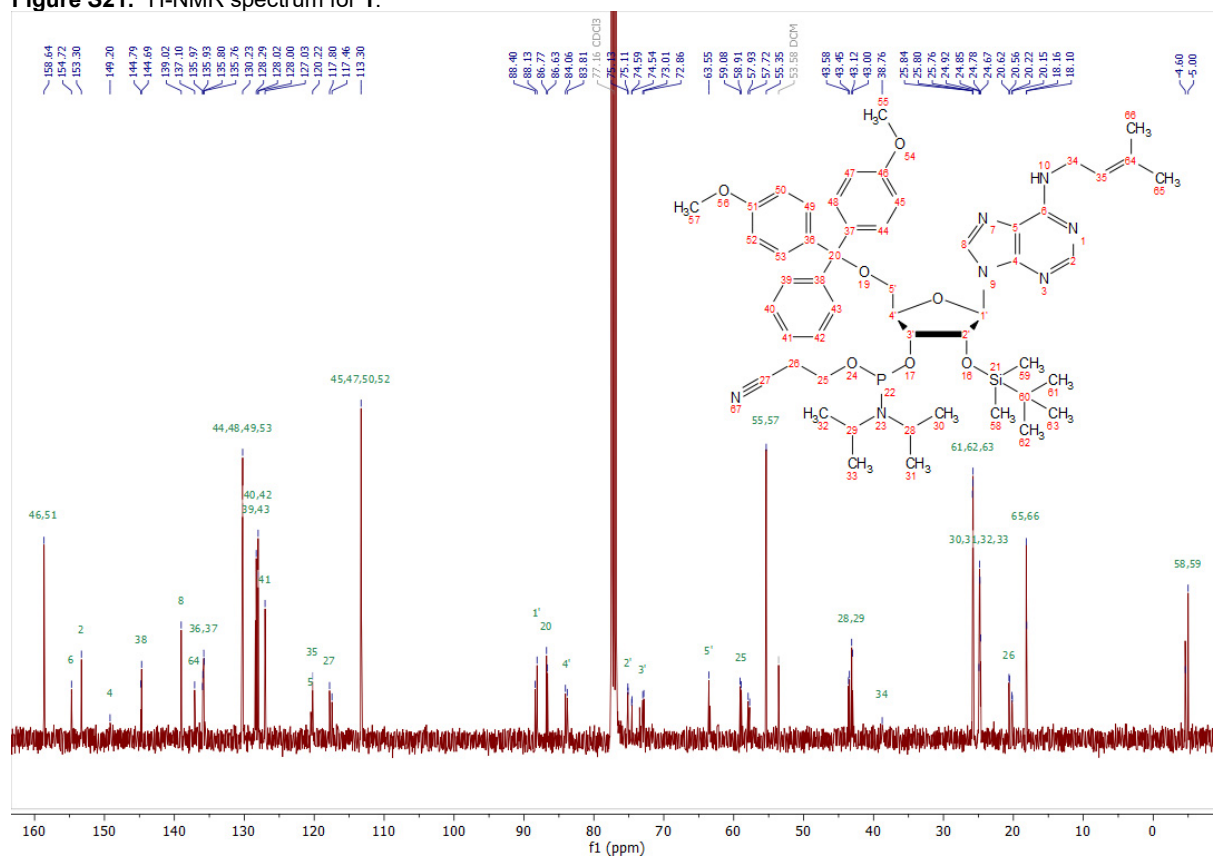
SUPPORTING INFORMATION

Figure S17. $^1\text{H-NMR}$ spectrum for 5.Figure S18. $^{13}\text{C-NMR}$ spectrum for 5.

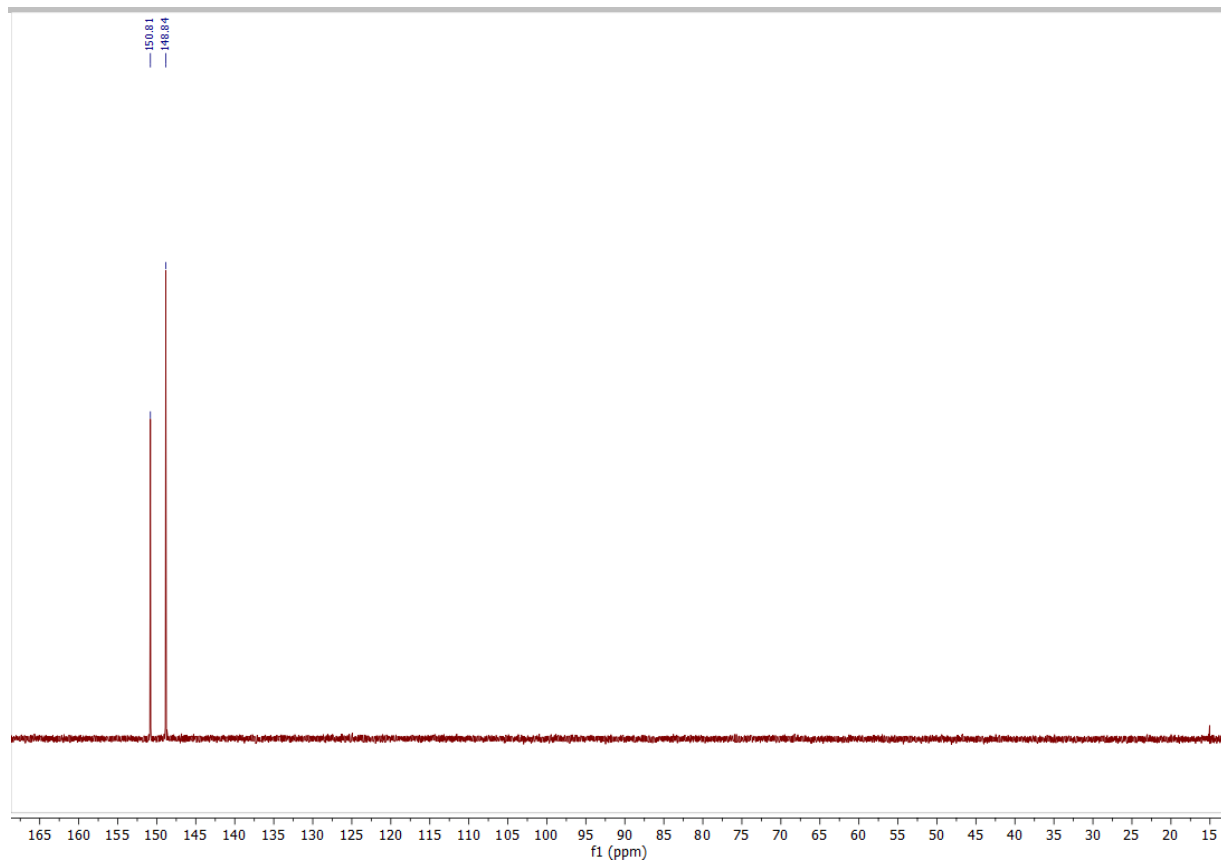
SUPPORTING INFORMATION

Figure S19. $^1\text{H-NMR}$ spectrum for **6**.Figure S20. $^{13}\text{C-NMR}$ spectrum for **6**.

SUPPORTING INFORMATION

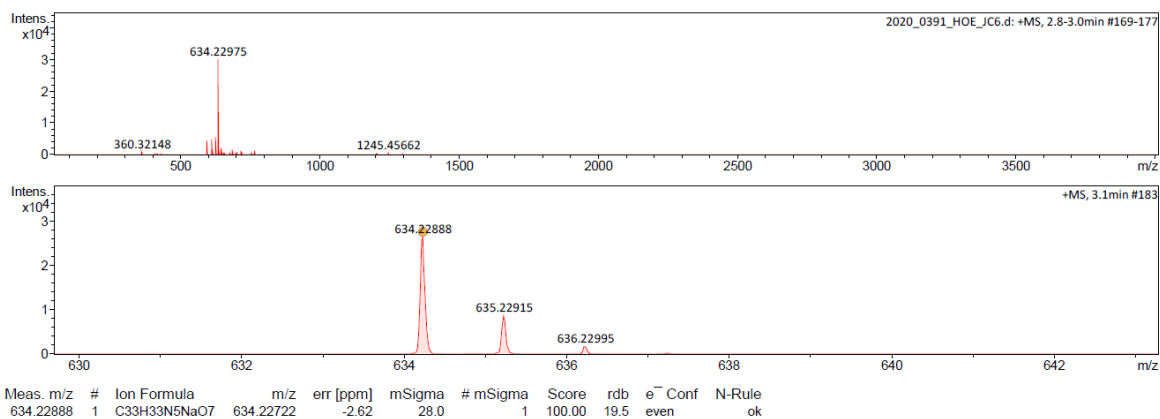
Figure S21. $^1\text{H-NMR}$ spectrum for **1**.Figure S22. $^{13}\text{C-NMR}$ spectrum for **1**.

SUPPORTING INFORMATION

Figure S23. ^{31}P -NMR spectrum for **1**.

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.3 Bar
Focus	Not active	Set Funnel 1 RF	200.0 Vpp	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set Funnel 2 RF	300.0 Vpp	Set Dry Gas	3.5 l/min
Scan End	4000 m/z	Set Hexapole RF	400.0 Vpp	Set Divert Valve	Source

Figure S24. ESI(+)-MS spectrum for **2**.

SUPPORTING INFORMATION

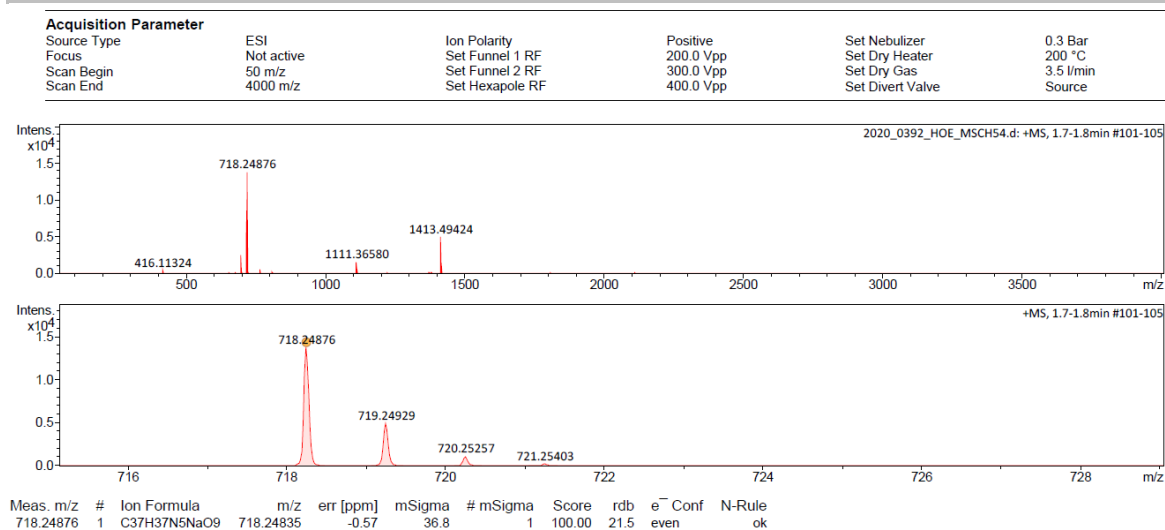


Figure S25. ESI(+)-MS spectrum for 3.

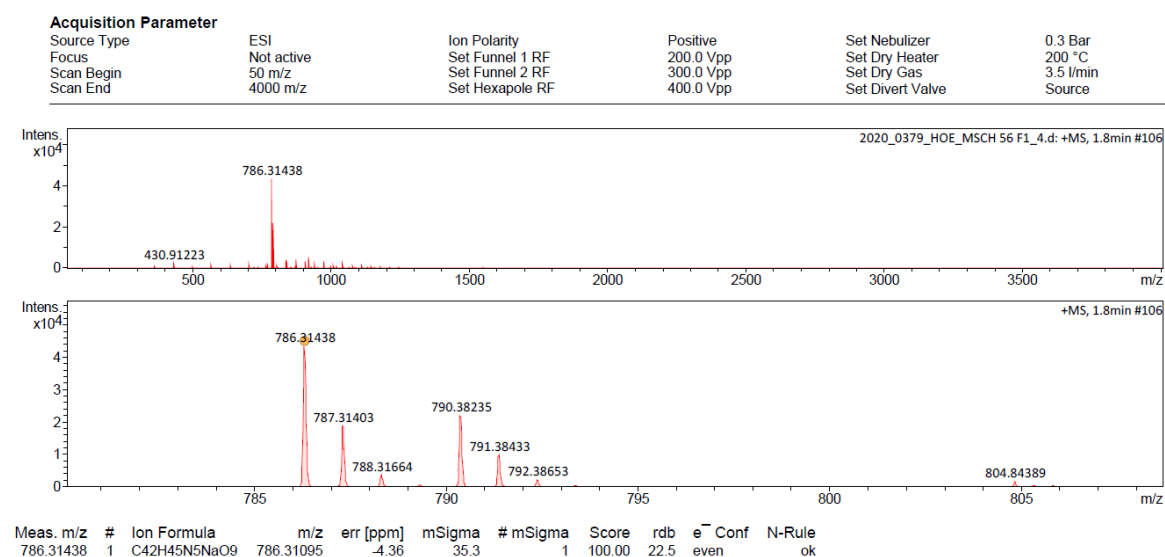


Figure S26. ESI(+)-MS spectrum for 4.

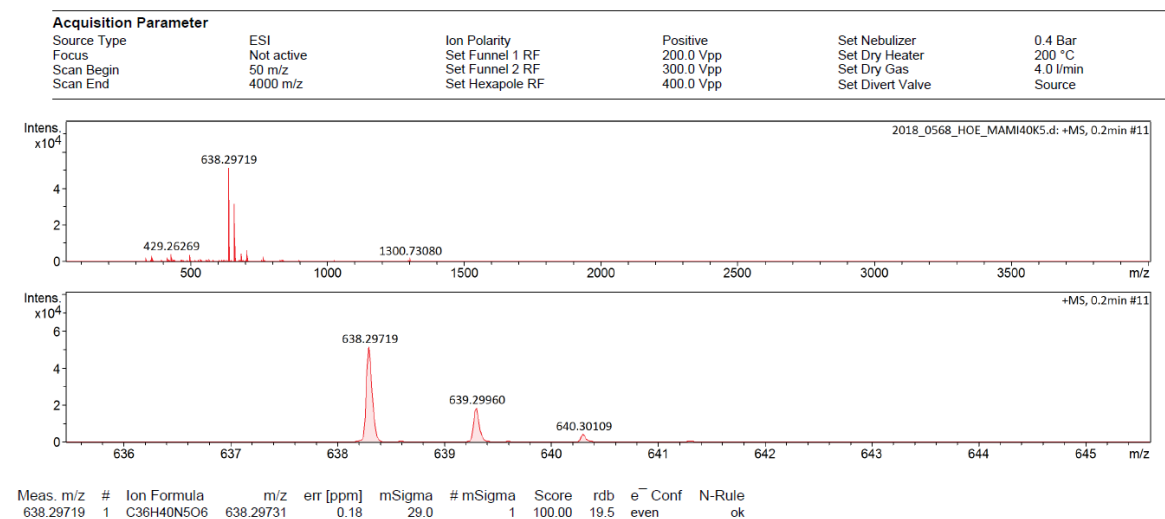


Figure S27. ESI(+)-MS spectrum for 5.

SUPPORTING INFORMATION

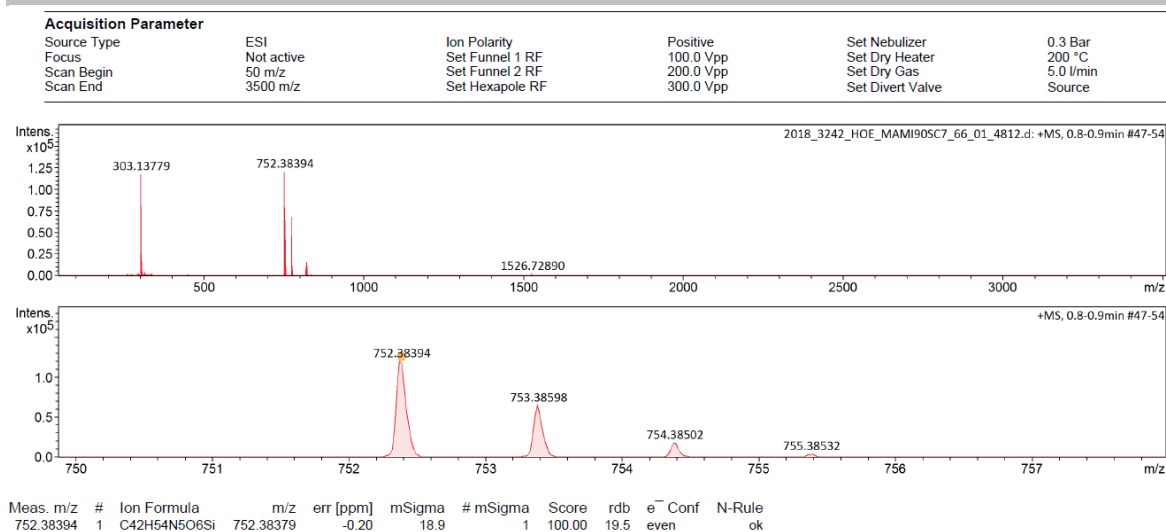


Figure S28. ESI(+)-MS spectrum for 6.

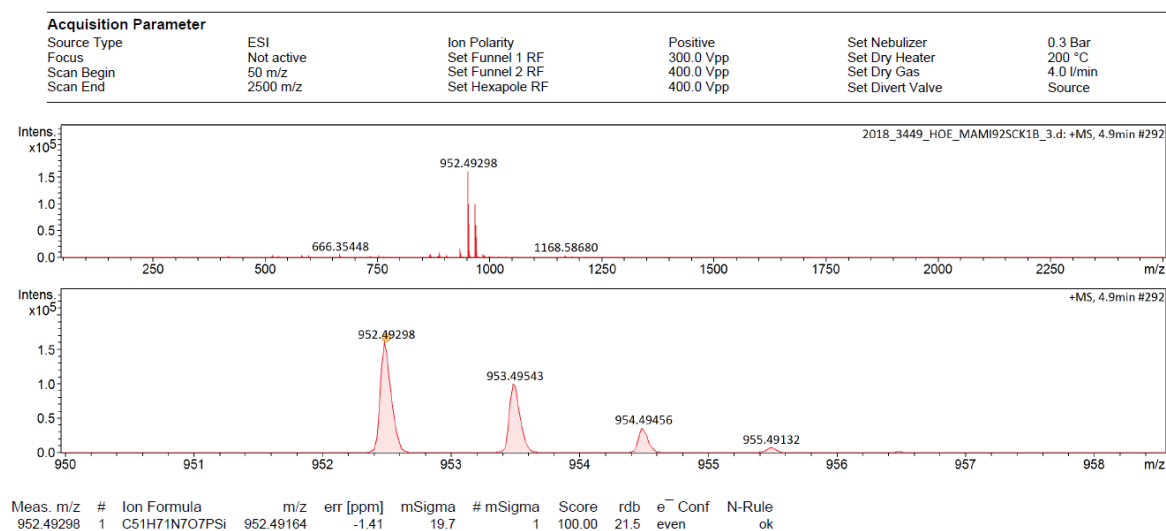


Figure S29. ESI(+)-MS spectrum for 1.

SUPPORTING INFORMATION

9. Alignment of NGS reads for sequence logos

Alignment of 54 AB08 sequence variants used for the sequence logo in Figure 3fusing msa (Bioconductor R package) for multiple sequence alignment^[8]

```

GGGCAG-CCA GTGGTACGTT --
GGGCAG-CCA GTGG-ACGTT --
GGGCAG-CCA GTGGTACGTT T-
GGGCAG-CCA GTGGTACGTG --
GGGCAG-CCA GTGGTACGTA --
GGGTAG-CCA GTGG-ACGTC TT
GGGTAG-CCA GTGG-ACGTC T-
GGGTAG-CCA GTGG-ACGTC TG
GGGTAG-CCA GTGG-ACGTC TA
GGGTAG-CCA GTGG-ACGTG --
GGGTAG-CCA GTGG-ACGTG T-
GGGTATTCCA GTGG-ACGTT --
GGGTATTCCA GTGGTACGTT --
GGGTATACCA GTGG-ACGTT --
GGGTAATCCA GTGG-ACGTT --
GGGTAATCCA GTGG-ACGTG --
GGGTAG-CCA GTGG-ACGTA --
GGGTAG-CCA GTGGTACGTG --
GGGTAG-CCA GTGGATCGTT --
GGGTAG-CCA GTGGATCGTG --
GGGTAG-CCA GTGG-ACGTT T-
GGGTAG-CCA GTGG-ACGTT TT
GGGTAG-CCA GTGG-ACGTT TG
GGGTAG-CCA GTGG-ACGTT TA
GGGTAG-CCA GTGG-ACGTT A-
GGGTAG-CCA GTGG-ACGTT --
GGGTAG-CCA GTGG-ACGTT G-
GGGTAG-CCA GTGGTACGTT --
GGGGAG-CCA GTGGTACGTT --
GGGAAG-CCA GTGGTACGTG --
GGGAAG-CCA GTGG-ACGTG --
GGGAAG-CCA GTGG-ACGTA --
GGGAAG-CCA GTGG-ACGTA T-
GGGAAG-CCA GTGGTACGTA --
GGGAAG-CCA GTGGTACGTC --
GGGAAG-CCA GTGG-ACGTT --
GGGAAG-CCA GTGG-ACGTT G-
GGGAAG-CCA GTGG-ACGTT T-
GGGAAG-CCA GTGGTACGTT T-
-GGGAATCCA GTGGTACGTT --
-GGAAAGCCA GTGGTACGTT --
GGGGAAGCCA GTGGTACGTT --
--GGAAGCCA GTGGTACGTT --
-GGGAAGCCA GTGGTACGTT --
GGGAAG-CCG GTGGTACGTT --
-GGGAGGCCA GTGGTACGTT --
GGGAAG-CCA GAGGTACGTT --
GGGAAG-CCA GTGGTACGCT --
GGGAAG-CCA GTGGTGCGTT --
GGGAAG-CCA GCGGTACGTT --
GGGTTGTCCA GCGGTACGTT --
GGGTTGCCCA GCGGTACGTT --
GGGATGCCCA GCGGTACGTT --
GGGATGTCCA GCGGTACGTT --

```

SUPPORTING INFORMATION

Alignment of 45 AC17 sequence variants used for the sequence logo in Figure 4f

```

-GGGAC-CCA GCTGGACGTT TG
-GGGAC-CCA GCTGGACGTT T-
-GGGAC-CCA GCTGGACGTT TA
-GGGAC-CCA GCTGGACGTT AT
-GGGAC-CCA GCTGGACGTT --
-GGGAC-CCA GCTGGACGTT TT
-GGGAC-CCA GCCGGACGTT TG
-GGGAC-CCA GCCGGACGTT T-
-GGGAC-CCA GCCGGACGTT TA
-GGGAC-CCA GCCGGACGTT TT
-GGGAC-CCA GCCGGACGTT G-
-GGGAC-CCA GCCGGACGTT --
-GGGAC-CCA GCCGGACGTT A-
-GGGACTCCA GCCGGACGTT --
-GGGACTCCA GCCGGTCGTT --
-GGGACTCCA GCCGGACGTG --
-GGGAC-CCA GCCGGACGTG --
-GGGAC-CCA GCCGGACGTC TT
-GGGAC-CCA GCCGGACGTA --
-GGGAC-CCA GCTGGACGTA --
--GGGCTCCA GCTGGTCGTC TA
--GGGCTCCA GCTGGACGTC TT
--GGGCCCCA GCTGGACGTC TA
-GGGACTCCA GCTGGTCGTT --
GGGAT-TCCA GCTGGACGTA --
GGGATATCCA GCTGGACGTA --
GGGAT-TCCA GCTGGACGTT --
GGGAT-TCCA GCCGGACGTA --
GGGATATCCA GCCGGACGTA --
GGGAT-TCCA GCCGGACGTG --
GGGAT-TCCA GCCGGACGTT --
-GGGTCTCCA GCTGGTCGTA --
-GGGTCTCCA GCCGGTCGTA --
-GGGTCTCCA GCTGGTCGTT --
-GGGTCTCCA GCTGGCCGTT --
-GGGTCTCCA GCTGGACGTT --
-GGGTCTCCA GCTGGACGTG --
-GGGTCTCCA GCCGGACGTA --
-GGGTCTCCA GCCGGACGTG --
-GGGTCTCCA GCCGGACGTT --
-GGGCCTCCA GCTGGACGTA --
-GGGTAGCCA G-TGGACGTT A-
-GGGTATCCA G-TGGACGTT --
-GGTTATCCA GCTGGACGTT --
-GGGTATCCA G-TGGACGTA --

```

10. References

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- [2] V. I. Tararov, S. V. Kolyachkina, C. S. Alexeev, S. N. Mikhailov, *Synthesis* **2011**, *2011*, 2483-2489.
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