Supporting Information

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RNA-cleaving deoxyribozymes differentiate methylated cytidine isomers in RNA

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Abstract: Deoxyribozymes are emerging as modification-specific endonucleases for the analysis of epigenetic RNA modifications. Here, we report RNA-cleaving deoxyribozymes that differentially respond to the presence of natural methylated cytidines, 3-methylcytidine (m^3C), N^4-methylcytidine (m^4C), and 5-methylcytidine (m^5C), respectively. Using *in vitro* selection, we found several DNA catalysts, which are selectively activated by only one of the three cytidine isomers, and display 10- to 30-fold accelerated cleavage of their target m^3C-, m^4C- or m^5C-modified RNA. An additional deoxyribozyme is strongly inhibited by any of the three methylcytidines, but effectively cleaves unmodified RNA. The m^XC-detecting deoxyribozymes are programable for the interrogation of natural RNAs of interest, as demonstrated for human mitochondrial tRNAs containing known m^3C and m^5C sites. The results underline the potential of synthetic functional DNA to shape highly selective active sites.

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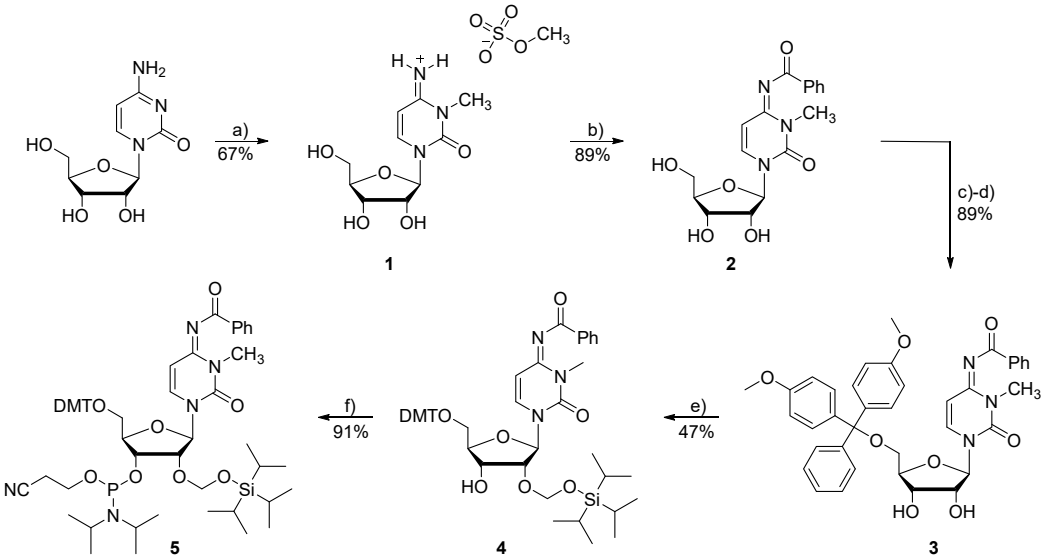
General information

DNA oligonucleotides were purchased from Microsynth. RNA oligonucleotides R1−R26 (Table S1) were prepared by solid-phase synthesis using 5'-*O*-DMT-2'-*O*-TOM-protected 3'-β-cyanoethyl phosphoramidites of N^6-acetyladenosine, N^4-acetylcytidine, N^2-acetylguanosine and uridine (purchased from Chemgenes), N^4-methylcytidine (synthesized according to the known procedure[1]), N^3-methylcytosine (prepared using the procedure described below), 5'-*O*-DMT-2'-*O*-TBDMS-N^4-benzoyl-5-methylcytidine 3'-β-cyanoethyl phosphoramidite (from Chemgenes), and (2-cyanoethoxy)-5-hexyn-1-yl-*N*,*N*-diisopropylaminophosphine (prepared according to the known procedure[2]). N^4,N^4-Dimethylcytidine-modified RNA oligonucleotide R27 was prepared by solid-phase synthesis using 2'-*O*-TOM-protected ribonucleoside phosphoramidites for unmodified positions and "convertible" 5'-*O*-(4,4'-Dimethoxytrityl)-2'-*O*-(triisopropylsilyl)oxymethyl-O^4-(4-chlorophenyl)uridine 3'-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite) (prepared according to the known procedure[3]) for the modified position followed by conversion of the resulting O^4-chlorophenyluridine-modified RNA with *N,N*-dimethylamine (see below for the conditions). RNA oligonucleotides R28−R34 were prepared by in vitro transcription from the corresponding DNA templates. Fluorescein thiosemicarbazide was purchased from Sigma-Aldrich. 6-FAM-azide, N^6-(6-aminohexyl)-ATP-6-FAM, N^6-(6-aminohexyl)-ATP-Cy3, N^6-(6-aminohexyl)-ATP-ATTO-647N, nucleotide triphosphates (NTPs) and deoxyribonucleotide triphosphates (dNTPs) were purchased from Jena Bioscience. m^5CTP, hm^5CTP and f^5CTP were purchased from TriLink Biotechnologies. T4 PNK, T4 DNA Ligase, and DreamTaq polymerase were purchased from Thermo Fisher Scientific. γ-^{32}P-Adenosine 5'-triphosphate (γ-^{32}P-ATP) was purchased from Hartmann Analytic. All DNA and RNA oligonucleotides were purified by denaturing PAGE (10–20% acrylamide/bis-acrylamide 19:1, 7 M urea) with running buffer 1x TBE (89 mM Tris, 89 mM boric acid and 2 mM EDTA, pH 8.3), 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. For preparative gels, oligonucleotides were visualized by UV shadowing on a TLC plate. Fluorescence gel images were recorded with a Biorad ChemiDoc MP using epi illumination. Analytical anion-exchange HPLC was carried out on a GE Healthcare Life Sciences ÄKTA™ micro system using DNAPac™ PA200 columns (4 x 250 mm, Thermo Scientific) at 60 °C. Solvent A: 25 mM Tris-HCl (pH 8.0), 6 M Urea. Solvent B: 25 mM Tris-HCl (pH 8.0), 6 M Urea, 0.5 M $NaClO_4$. Gradient: linear, 0–54% solvent B, 4% solvent B per 1 CV. The solvents were filtered through 0.2 µm cellulose acetate filters (Sartorius, Germany) prior to usage. HR-ESI-MS spectra of the synthetic products and oligonucleotides were recorded on a Bruker micrOTOF-Q III spectrometer.

Synthesis of m^3C phosphoramidite

All synthetic procedures were performed under nitrogen atmosphere. The chemicals were purchased 'Pro analysis'- or 'For synthesis' grade and used without additional purification. Dry solvents (DCM, MeCN, DMF, THF) were obtained in a solvent purification system (SPS). DCE was dried over molecular sieves. Solvents for column chromatography (technical quality) were distilled prior to use. The obtained compounds were stored at 4 °C (-20 °C in case of TOM-protected compounds and the final phosphoramidite). Column chromatography was performed on silica gel (Kieselgel 60, Merck, 0.040-0.063 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 are reported relative to the residual solvent signals. $CDCl_3$: 7.26 ppm (^{1}H), 77.16 ppm (^{13}C). DMSO: 2.50 ppm (^{1}H), 39.52 ppm (^{13}C).



Scheme S1. Synthesis of m^3C phosphoramidite. a) DMF, dimethyl sulfate, 37 °C, 30 min. b) TMSCl, pyridine, 1 h, r.t., then NEt_3, BzCl, 4 h, r.t. c) DMF-DMA, pyridine, o.n., r.t. d) DMT-Cl, pyridine, r.t., 2 h. e) *i*Pr_2NEt, *t*Bu_2SnCl_2, DCE, r.t., 1 h, then TOM-Cl, 80 °C, 20 min. f) Me_2NEt, CEP-Cl, DCM, r.t.

SUPPORTING INFORMATION

The synthesis of m^{3}C-phosphoramidite building block **5** was performed in five steps. The methyl group was introduced at position N3 of cytosine with dimethyl sulfate,[4] resulting in formation of salt **1**. The transient protection of the ribose hydroxy groups with TMSCl, allowed selective protection of the amino group with benzoyl chloride to obtain compound **2**.[5] In the next step, the 2'- and 3'-hydroxy groups of compound **2** were transiently protected as acetal with DMF-DMA, and the 5'-hydroxy group was selectively protected upon reaction with DMT-Cl. Compound **3** was then activated with *t*Bu_2SnCl_2 and treated with TOM-Cl to yield compound **4**. Finally, compound **4** was treated with CEP-Cl to afford m^{3}C phosphoramidite **5**.

N^3-Methylcytidinum methyl sulfate (**1**)

As described earlier,[4] cytidine (500 mg, 2.06 mmol, 1.00 eq.) was suspended in anhydrous dimethylformamide (5 mL) and warmed to 37 °C under nitrogen atmosphere. Afterwards, dimethyl sulfate (2.00 mL, 21.1 mmol, 10.2 eq.) was added. After 30 min stirring, the resulting clear solution was diluted to 20 mL with methanol, and ethyl acetate was added until a precipitate formed. The title compound was obtained by filtration as a colorless solid (510 mg, 1.38 mmol, 67%).
^{1}H NMR (MeOD, 400 MHz): δ (ppm) = 3.50 (s, 3H, NCH_3), 3.68 (s, 3H, $^-OS(O)_2OCH_3$), 3.78 (dd, J = 12.4, 2.5 Hz; 1H, H^1-5'), 3.94 (dd, J = 12.4, 2.5 Hz; 1H, H^2-5'), 4.08 (dt, J = 6.1, 2.5 Hz; 1H, H-4'), 4.15 (dd, J = 6.1, 4.9 Hz; 1H, H-3'), 4.18 (dd, J = 4.9, 2.7 Hz; 1H, H-2'), 5.87 (d, J = 2.7 Hz; 1H, H-1'), 6.16 (d, J =7.9 Hz; 1H, H-5), 8.50 (d, J = 7.9 Hz; 1H, H-6).
^{13}C NMR (MeOD): δ (ppm) = 30.93 (N-CH_3), 55.11 ($^-OS(O)_2OCH_3$), 61.15 (C5'), 70.09 (C3'), 76.28 (C2'), 86.22 (C4'), 93.05 (C1'), 94.75 (C5), 143.57 (C6), 149.13 (C=O), 161.29 (C=NH_2^+).
HR-MS (ESI$^+$): Exact mass calculated for $C_{10}H_{16}N_3O_5$ [M+H]$^+$: 258.1084, found: 258.1086.

N^4-Benzoyl-*N*^{3}-methylcytidine (**2**)

N^3-Methylcytidinium methyl sulfate **1** (395 mg, 1.07 mmol, 1.00 eq.) was treated with trimethylsilyl chloride (TMSCl, 528 µL, 4.17 mmol, 3.90 eq.) in anhydrous pyridine (7 mL) under nitrogen atmosphere. After stirring for 1 h, triethylamine (297 µL, 2.14 mmol, 2.00 eq.) was added, and the reaction mixture was stirred for further 5 min at room temperature. Afterwards, benzoyl chloride (149 µL, 1.28 mmol, 1.20 eq.) was added dropwise. After stirring for 4 h at room temperature, the reaction mixture was quenched with water (1.5 mL). The resulting mixture was stirred for 5 min at room temperature followed by addition of aqueous ammonia (2.6 mL). After further 15 min stirring, the volatiles were removed under reduced pressure. The crude residue was purified by column chromatography (DCM/MeOH, 20:1) to give the title compound as a colorless foam (344 mg, 952 µmol, 89%).
^{1}H NMR (MeOD, 400 MHz): δ (ppm) = 3.54 (s, 3H, N-CH_3), 3.74 (dd, J = 12.3, 2.9 Hz; 1H, H^1-5'), 3.86 (dd, J = 12.3, 2.9 Hz; H^2-5'), 4.02 (dt, J = 5.2, 2.9 Hz; 1H, H-4'), 4.15 (t, J = 5.2 Hz; 1H, H-3'), 4.19 (dd, J = 5.2, 4.0 Hz; 1H, H-2'), 5.94 (d, J = 4.0 Hz; 1H, H-1'), 6.41 (d, J = 8.2 Hz; 1H, H-5), 7.42–7.48 (m, 2H, bz), 7.53–7.58 (m, 1H, bz), 7.93 (d, J = 8.2 Hz; 1H, H-6), 8.09-8.13 (m, 2H, bz).
^{13}C NMR (MeOD, 100 MHz): δ (ppm) = 30.48 (N-CH_3), 61.99 (C5'), 70.96 (C3'), 75.94 (C2'), 86.19 (C4'), 91.79 (C1'), 98.54 (C5), 129.34 (bz), 130.72 (bz), 133.71 (bz), 137.22 (C_q-bz), 138.80 (C6), 151.94 (C2), 158.18 (C4), 179.35 (C=O).
HR-MS (ESI$^+$): Exact mass calculated for $C_{17}H_{19}N_3NaO_6$ [M+Na]$^+$: 384.1166, found: 384.1171.

N^4-Benzoyl-5'-*O*-(4,4'-dimethoxytrityl)-*N*^{3}-methylcytidine (**3**)

Compound **2** (265 mg, 734 µmol, 1.00 eq.) was co-evaporated with pyridine (2 mL) and re-dissolved in anhydrous pyridine (2.5 mL) under nitrogen atmosphere. *N*,*N*-dimethylformamide dimethyl acetal (DMF-DMA, 175 mg, 196 µL, 1.47 mmol, 2.00 eq.) was added. The resulting mixture was stirred for 18 h at room temperature and then evaporated to dryness. The residue was re-dissolved in dry pyridine (2.5 mL) and 4,4'-dimethoxytrityl chloride (298 mg, 881 µmol, 1.20 eq.) was added in one portion. After stirring for 2 h at room temperature, the reaction mixture was quenched with methanol (3 mL). The reaction mixture was evaporated to dryness and then co-evaporated with toluene (3 mL). The residue was diluted with dichloromethane (30 mL) and then washed with aqueous citric acid (5%), water and saturated aqueous $NaHCO_3$. The organic layer was dried with Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by column chromatography (DCM/MeOH, 80:1 with 2% v/v NEt_3) to give the title compound as a colorless foam (433 mg, 653 µmol, 89%).
^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) = 3.38 (dd, J = 11.0, 3.0 Hz; 1H, H^1-5'), 3.47 (dd, J = 11.0, 3.0 Hz; 1H, H^2-5'), 3.56 (s, 3H, N-CH_3), 3.77 (d, J = 2.2 Hz; 6H, OCH_3), 4.23 (dt, J = 5.0, 3.0 Hz; 1H, H-4'), 4.28 (dd, J = 5.0, 3.6 Hz; 1H, H-2'), 4.35 (t, J = 5.0 Hz; 1H, H-3'), 5.85 (d, J = 3.6 Hz; 1H, H-1'), 6.21 (d, J = 8.2 Hz; H-5), 6.78-6.83 (m, 4H, trityl), 7.16-7.21 (m, 1H, trityl), 7.21-7.28 (m, 6H, trityl), 7.32-7.35 (m, 2H, trityl), 7.41-7.46 (m, 2H, bz), 7.50-7.55 (m, 1H, bz), 7.66 (d, J = 8.2 Hz; H-6), 8.11-8.14 (m, 2H, bz).
^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) = 30.17 (N-CH_3), 55.37 (OCH_3), 62.48 (C5'), 70.86 (C3'), 76.41 (C2'), 84.73 (C4'), 87.14 (C_q-trityl), 91.85 (C1'), 98.24 (C5), 113.40, 113.51, 113.53 (trityl), 127.24 (trityl), 128.11 (trityl), 128.22 (trityl), 128.32 (bz), 129.83 (bz), 130.12, 130.16 (trityl), 132.55 (bz), 135.30 (C_q-trityl), 135.74 (C6), 136.02 (C_q-bz), 144.18 (C_q-trityl), 151.27 (C2), 155.86 (C4), 158.77 (C_q-OCH_3, trityl), 177.43 (*C*=O, bz).
HR-MS (ESI$^+$): Exact mass calculated for $C_{38}H_{37}N_3NaO_8$ [M+Na]$^+$: 686.2473, found: 686.2467.

N^4-Benzoyl-5'-*O*-(4,4'-dimethoxytrityl)-N^3-methyl-2'-*O*-{[(triisopropylsilyl)oxy]methyl}cytidine (**4**)

To a solution of compound **3** (345 mg, 520 µmol, 1.00 eq.) in anhydrous 1,2-dichloroethane (4 mL), *N,N*-diisopropylethylamine (311 µL, 1.82 mmol, 3.50 eq.) and di-*tert*-butyltin dichloride (174 mg, 572 µmol, 1.1 eq.) were added. After stirring at room temperature for 1 h, the reaction mixture was heated to 80 °C, and TOM-Cl (150 mg, 676 µmol, 1.30 eq.) was added. After 20 min, the reaction mixture was cooled down to room temperature and diluted with dichloromethane (40 mL). The organic layer was separated, washed with saturated aqueous $NaHCO_3$ (3 x 20 mL) and dried over Na_2SO_4. The crude compound was purified by column chromatography (hexane/EtOAc, 5:1 with 2% v/v NEt_3), to give the title compound as a colorless foam (207 mg, 243 µmol, 47%).
^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) = 1.05-1.13 (m, 21H, $Si(CH(CH_3)_2)_3$), 3.20 (d, J = 6.4 Hz; 1H, C3'-O*H*), 3.48-3.50 (m; 2H, H-5'), 3.54 (s, 3H, N-CH_3), 3.78 (d, J = 1.0 Hz; 6H, OCH_3), 4.09 (dt, J = 6.6, 2.5 Hz; 1H, H-4'), 4.21 (dd, J = 5.0, 2.9 Hz; 1H, H-2'), 4.39-4.48 (m, 1H, H-3'), 5.06 (d, J = 4.7 Hz; 1H, OCH_2O), 5.25 (d, J = 4.7 Hz; 1H, OCH_2O), 6.04 (d, J = 2.9 Hz; 1 H, H-1'), 6.06 (d, J = 8.2 Hz; H-5), 6.80-6.84 (m, 4H, trityl), 7.17-7.22 (m, 1H, trityl), 7.23-7.29 (m, 6H, trityl, bz), 7.34-7.38 (trityl), 7.41-7.46 (bz), 7.50-7.55 (m, 1H, bz), 7.79 (d, J = 8.2 Hz; H-6), 8.11-8.14 (m, 1H, bz).
^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) = 11.99, 17.94 ($Si(CH(CH_3)_2)_3$), 30.02 (N-CH_3), 55.36 (OCH_3), 61.98 (C5'), 69.10 (C3'), 83.27 (C2'), 83.66 (C4'), 87.15 (C_q-OCH_2), 88.72 (C1'), 90.80 (OCH_2O), 98.29 (C5), 113.40 (trityl), 127.24 (trityl), 128.13 (trityl), 128.30, 128.35 (trityl, bz), 129.82 (bz), 130.15, 130.24 (trityl), 132.50 (bz), 135.34, 135.64 (trityl, C_q), 135.88 (C6), 136.07 (bz, C_q), 144.18 (trityl, C_q), 150.40 (NC(=O)N), 155.83 (C=N), 158.77 (trityl, C_q-OCH_3), 177.44 (C=O, bz).
HR-MS (ESI$^+$): Exact mass calculated for $C_{48}H_{59}N_3NaO_9Si$ $[M+Na]^+$: 872.3913, found: 872.3895.

N^4-Benzoyl-5'-*O*-(4,4'-dimethoxytrityl)-N^3-methyl-2'-*O*-{[(triisopropylsilyl)oxy]methyl}cytidine 3'-*O*-(2-cyanoethyl diisopropylphosphoramidite) (**5**)

To a solution of compound **4** (188 mg, 221 µmol, 1.00 eq.) in anhydrous dichloromethane (4 mL), *N,N*-dimethylethylamine (240 µL, 2.21 mmol, 10.0 eq.) and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (78.6 mg, 332 µmol, 1.50 eq.) were added, and the reaction mixture was stirred for 2.5 h at room temperature. Afterwards, the reaction mixture was evaporated to dryness under reduced pressure. The crude product was purified by column chromatography (hexane/EtOAc, 3:1 with 2% v/v NEt_3) to yield the title compound as a colorless foam (211 mg, 201 µmol, 91%).
^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) = 0.99-1.08 (m, 42H, CH_3 (isopropyl-N); $Si(CH(CH_3)_2)_3$, diast.), 1.14-1.19 (m, 18H, CH_3 (isopropyl-N), diast.) 2.37 (td, 2H, J = 6.5, 2.0 Hz; $POCH_2CH_2$, diast.), 2.64 (td, 2H, J = 6.1, 4.0 Hz; $POCH_2CH_2$, diast.), 3.38 (td, 2H, J = 10.6, 2.90 Hz; H-5', diast.), 3.53 (s, 3H, diast., N-CH_3), 3.53 (s, 3H, N-CH_3), 3.53-3.60 (m, 4H, diast., H-5', N($CH(CH_3)_2)_2$ and H^2, $POCH_2$), 3.61-3.68 (m, 1H, H^2, $POCH_2$), 3.78 (d, 5H, diast., OCH_3), 3.79 (d, 6H, J = 0.8 Hz; OCH_3), 3.81-3.88 (m, 1H, H^1, $POCH_2$), 3.94 (ddt, 1H, J = 10.5, 7.5, 6.1 Hz; diast., H^1, $POCH_2$), 4.20 (dt, 1H, J = 4.7, 2.7 Hz; diast., H-4'), 4.27 (dd, 1H, J = 5.3, 2.5 Hz; H-4'), 4.39-4.42 (m, diast. H-3', H-2'), 4.35-4.38 (m, 1H, H-2'), 4.45 (dt, 1H, J = 10.3, 5.4 Hz; H-3'), 5.03 (d, 1H, J = 4.8 Hz; H^2, OCH_2O), 5.05 (d, 1H, diast. H^2, OCH_2O), 5.09 (d, 2H, J = 4.8 Hz, H^1, OCH_2O, diast.), 6.08 (d, 1H, diast., H-5), 6.13 (d, 1H, J = 8.2 Hz, H-5), 6.16 (d, 1H, J = 4.2 Hz; H-1'), 6.18 (d, 1H, diast., H-1'), 6.79-6.85 (m, 8H, trityl, diast.), 7.17-7.23 (m, 2H, trityl, diast.), 7.24-7-30 (m, 7H, trityl), 7.33-7.40 (m, 4H, trityl, diast.), 7.41-7.46 (m, 4H, bz, diast.), 7.50-7.55 (m, 2H, bz, diast.), 7.66 (d, 1H, diast., J = 8.2 Hz; H-6), 7.74 (d, 1H, J = 8.2 Hz; H-6), 8.11-8.15 (m, 4H, bz, diast.).
^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) = 12.1, 18.0 ($Si(CH(CH_3)_2)_3$), 20.3, 20.5 ($POCH_2CH_2$), 24.7, 24.8 (CH_3, isopropyl-N), 30.1 (N-CH_3), 43.2, 43.4 (N($CH(CH_3)_2)_2$), 55.4 (OCH_3), 57.4, 58.1, 58.9, 59.1 ($POCH_2$), 61.9 (C5'), 70.2, 70.7 (C3'), 77.9, 78.5 (C2'), 83.2, 83.3 (C4'), 87.1, 87.3 (trityl, C_qOCH_2), 88.1, 88.3 (C1'), 89.2, 89.5 (OCH_2O), 113.4 (trityl), 117.5, 117.8 (CN), 127.3 (trityl), 128.1, 128.3, 128.4 (bz, trityl), 128.5 (trityl), 129.8 (bz), 130.2, 130.3 (trityl), 132.4 (bz), 135.3, 135.4, 135.6 (trityl), 136.1, 136.2 (bz, C_q), 144.1, 144.2 (trityl), 150.4 (C2), 155.8, 155.9 (C4), 158.8 (C_q-OCH_3), 177.4 (C=O, bz).
^{31}P-NMR ($CDCl_3$, 162 MHz): 150.12. 150.50.
HR-MS (ESI$^+$): Exact mass calculated for $C_{57}H_{76}N_5NaO_{10}PSi$ $[M+Na]^+$: 1072.4991, found: 1072.4968.

Solid-phase synthesis of oligoribonucleotides

RNA oligonucleotide syntheses were carried out at 0.7–1 µmol scale on ABI 394 DNA/RNA synthesizer using standard β-cyanoethyl phosphoramidite chemistry with the building blocks mentioned above. Deprotection of m^3C-modified oligoribonucleotides was performed with a mixture of concentrated aqueous NH_3 and EtOH (3:1) at 55 °C for 6 h followed by incubation with 1 M TBAF in THF under standard conditions. Conversion of the O^4-chlorophenyluridine-modified oligoribonucleotide into the $m^{4,4}$C-modified RNA (Table S1, R27) was performed by incubation of the solid support with 0.7 ml of 40% aqueous Me_2NH at 37°C for 5 h followed by addition of 0.5 ml of aqueous NH_3 and further incubation at 55 °C for 2 h to ensure complete deprotection of cyanoethyl and acetyl groups. Deprotection of TOM groups was achieved with TBAF in THF under standard conditions. Deprotection of other oligonucleotides was performed under standard conditions with $MeNH_2$ in aqueous ethanol and TBAF in THF. The crude oligonucleotides were desalted by size exclusion chromatography on GE Healthcare Life Sciences ÄKTA™ start system (3x5 ml HiTrap columns), purified by PAGE, and analyzed by anion exchange HPLC and HR-ESI-MS.

SUPPORTING INFORMATION

Labeling of oligonucleotides

Labeling of 5'-alkyne functionalized oligonucleotides

Oligonucleotides with 5'-alkynes were labeled with 6-FAM-azide using copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC). Oligonucleotide (5 nmol in 5 µl of H_2O) was mixed with 3 µl of DMSO/tBuOH (3:1 v/v) and 0.5 µl 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 1:2 ratio. From this solution, 1.5 µl was added to the reaction mixture and incubated at 37°C for 3 hours in the dark. Afterwards, the reaction mixture was precipitated with ethanol to remove excess fluorophore, and the labeled oligonucleotide was purified using PAGE.

Labeling of RNA at 3'-end

Unfunctionalized oligoribonucleotides were labeled by periodate oxidation followed by a reaction with fluorescein-5-thiosemicarbazide. In a typical procedure, RNA (0.3–1 nmol) was dissolved in 7.5 µl water, followed by addition of 5x sodium phosphate buffer (2 µl, 250 mM, pH 7.4) and a freshly prepared aqueous solution of $NaIO_4$ (0.5 µl, 400 mM). The resulting mixture was incubated for 10–15 min at 37 °C. The excess of $NaIO_4$ was quenched by treatment with aqueous Na_2SO_3 (1 µl, 1 M) for 5–10 min at 37 °C. Afterwards, a solution of fluorescein-5-thiosemicarbazide (1 µl, 10 mM) in DMF was added, and the reaction mixture was incubated in the dark for additional 1 h at 37 °C. The labeled product was then purified by PAGE (10–20% polyacrylamide) and recovered by extraction and ethanol precipitation.

Radioactive labeling of RNA at 5'-end

Radioactive labeling of RNA was performed by phosphorylation using γ-^{32}P-ATP. In a typical procedure, RNA (200–500 pmol) was incubated with 10x PNK buffer A (5 µl, 500 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM DTT, 1 mM spermidine), γ-^{32}P-ATP (0.5 µl, 10 µCi/µl) and T4 PNK enzyme (0.5 µl, 10 U/µl) at 37 °C for 1 hours. The labeled RNA was recovered by ethanol precipitation.

Ribozyme-catalyzed fluorescent labeling of RNA

Oligoribonucleotides R12, R16 and R20 were labeled at 2'-OH of internal adenosine (A9) with N^6-(6-aminohexyl)-ATP-6-FAM, N^6-(6-aminohexyl)-ATP-Cy3, N^6-(6-aminohexyl)-ATP-ATTO-647N, respectively, according to the known procedure.[6] In a typical procedure, RNA (500 pmol) and ribozyme FH14 (700 pmol) were incubated in total volume of 20 µl of the reaction buffer (5 mM NaCl, 120 mM KCl, 50 mM HEPES, pH 7.5) including 40 mM $MgCl_2$ and 200 µM of the N^6-modified ATP. The annealing step (95 ºC for 3 min, then r.t. for 10 min) was performed prior to addition of $MgCl_2$ and ATP. The reaction mixture was incubated overnight at 37 ºC and then quenched with 20 µl of stop solution. The labeled RNA was purified by PAGE (20%).

In-vitro selection

Phosphorylation of RNA substrates for selection

Phosphorylation at 5'-end of RNA substrates was carried out with T4 PNK as follows: RNA (2–5 nmol) was incubated with 10x PNK buffer A (5 µl, 500 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM DTT, 1 mM spermidine), ATP (5 µl, 10 mM) and T4 PNK (5 µl, 10 U/µl) at 37 °C for 5 hours. Total volume of the reaction mixture was 50 µl. The phosphorylated RNA was extracted with phenol/ chloroform/isoamylalcohol mixture and ethanol precipitated.

Splinted ligation of RNA substrate and deoxyribozyme library

Splinted ligation was carried out with T4 DNA ligase as described earlier.[7] In a typical procedure, the deoxyribozyme pool, 5'-phosphorylated RNA and complementary DNA splint (1.8 nmol, 2.4 nmol and 2 nmol, respectively) were dissolved in water (9 µl), and 10x annealing buffer (1 µl; 40 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 8.0) was added. After 2 min incubation at 95 °C, the solution was allowed to cool down to r.t. for 15 min and then further cooled down to 0 °C for 10 min. 10x DNA Ligase buffer (2 µl; 400 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP, pH 7.8), T4 DNA Ligase (2 µl; 5 U/µl) and water to a total volume of 20 µl were added, and the resulting mixture incubated for 2 h at 37 °C to yield a DNA-RNA hybrid product which was purified by PAGE.

Intramolecular cleavage of RNA-DNA hybrids (key selection step)

The DNA-RNA hybrid (250 pmol in first round and 10–30 pmol in further rounds) was incubated with 10x selection buffer (1 µl, 500 mM Tris-HCl, 1.5 M NaCl, pH 7.5) in a total volume of 9 µl. To initiate the reaction, $MgCl_2$ (1 µl, 100 mM) was added to the final concentration of 10 mM and reaction mixture was incubated at 37 °C for 16 hours. Active fraction of DNA enzymes was separated using denaturing PAGE (10%) and the gel areas corresponding to the cleaved products (determined by comparison with a size marker) were cut and extracted. Extracted products were ethanol precipitated and amplified with PCR.

PCR amplification of the enriched DNA library

The enriched DNA library was amplified via two subsequent asymmetric PCR reactions.[7] In the first PCR, the extracted product from previous step was used as a template. Template (30 µl), forward primer D2 (200 pmol), reverse primer D3 (50 pmol), dNTP mixture (0.625 µl, 20 mM each dNTP), 10x DreamTaq buffer (5 µl) and DreamTaq DNA polymerase (0.25 µl, 5 U/µl) were mixed, and the total volume was adjusted to 50 µl with water. The resulting mixture was subjected to a 10-cycle PCR reaction (95 °C for 4 minutes, 10× [95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min] and 72 °C for 5 minutes) using a Bio-Rad thermal cycler. For the second PCR reaction, an aliquot (1–2 µl) from PCR-I was used as template. This aliquot was mixed with fluorescently labeled forward primer D2.1 (200 pmol), reverse primer D3 (50 pmol), dNTP mixture (0.6 µl, 20 mM), 10x DreamTaq buffer (5 µl), DreamTaq DNA polymerase (0.5 µl, 5 U/µl) and water to a final volume of 50 µl. The resulting solution was subjected to a 30-cycle PCR reaction (95 °C for 4 minutes, 30x [95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min], and 72 °C for 5 min). The PCR product was subjected to denaturing PAGE and the short strand with the fluorescent label was isolated. The resulting ssDNA was used for ligation to RNA substrate to initiate the next round of selection.

Cloning and sequencing

After 18 rounds of selection, the enriched libraries AK, AL, AM and AN were cloned using TOPO-TA cloning and transformed into *E.Coli.* The plasmids of 40 randomly picked colonies were isolated and sequenced using Sanger sequencing. Enriched libraries from rounds 7 and 18 were also subjected to deep sequencing. For this purpose, total eight selection libraries were amplified in a 2-step PCR protocol, in which Illumina adaptors and unique indices were introduced. The PCR products were purified on PAGE (10%) and with ZYMO Oligo Clean & Concentrator kit. After quality control on a Bioanalyzer, the libraries were pooled, and sequenced as a fraction of a NextSeq 500 High Output 75 nt single-end run in the Core Unit Systems Medicine (University of Würzburg). After demultiplexing, the resulting fastq files were further processed using custom bash, R and Python scripts.

Kinetics characterization of deoxyribozyme-catalyzed RNA cleavage

Kinetics characterization was carried out as described previously[8] to analyze trans-activity of individual deoxyribozymes or active deoxyribozyme pools under single turnover conditions. In a typical procedure, a deoxyribozyme (100 pmol) was mixed with a fluorescently labeled RNA substrate (10 pmol) in a final volume of 8 µl. The resulting mixture was heated to 95 °C for 4 min and then cooled to 25 °C for 10 min. To initiate the reaction, 10x kinetic assay buffer (1 µl, 500 mM Tris-HCl, 1.5 M NaCl), pH 7.5) and $MgCl_2$ (1 µl, 200 mM) were added and the resulting mixture was incubated at 37 °C. Aliquots (1 µl) were taken at different time points (e.g. 0, 15 min, 30 min, 60 min, 180 min, and 360 min), quenched with loading buffer and analyzed by denaturing PAGE. Cleavage yields were determined by measuring fluorescent intensities of the corresponding bands. Values of k_{obs} (observed cleavage rate) and Y_{max} (maximum fraction cleaved) were obtained by fitting fraction cleaved versus time with the first-order kinetics equation $Y = Y_{max} * (1 - e^{-kobs*t})$. Each experiment was repeated at least trice, and values are reported as mean of three individual experiments.

Synthesis and analysis of mt-tRNAs

Unmodified mt-tRNA^{Glu} was prepared by *in vitro* transcription from a synthetic DNA template using T7 RNA polymerase. Reactions were performed in aqueous solution containing 40 mM Tris-HCl, pH 8.0, 10mM DTT, 4 mM each NTP, 30 mM $MgCl_2$ and 2 mM spermidine and 20 µg of T7 RNA polymerase (prepared in house) at 37 °C for 5 h. The transcription products were purified by denaturing PAGE (10% polyacrylamide) and dephosphorylated by calf intestinal alkaline phosphatase. The m^{5}C-modified mt-tRNA^{Glu} was assembled by splint ligation of two RNA fragments (R25 and R26). The m^{3}C-modified mt-tRNA^{Thr} and unmodified mt-tRNA^{Thr} were constructed by splint ligation of three synthetic RNA fragments (R21, R22, R23) and (R21, R22, R24) respectively. In a typical procedure, RNA fragments and complementary splint (D52/D53) in equimolar ratio were annealed in 10x annealing buffer (40 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 8.0) and ligation reactions were then performed with T4 DNA ligase for 16 h at 37 °C. Full length products were separated by PAGE (10% polyacrylamide), and labeled with γ-^{32}P-ATP at their 5'end.Aliquots of the full-length tRNA samples were then subjected to DNAzyme-mediated cleavage using the corresponding DNAzymes (D54/D55/D57/D58) as described above.

SUPPORTING INFORMATION

To disrupt the secondary structure of tRNAs close to DNAzyme binding site, disruptor oligonucleotides (D56/D59) were added in the cleavage reactions. Labeled tRNAs (approx. 10 pmol) were annealed with corresponding DNAzyme (100 pmol) and disruptor oligonucleotides (100 pmol). The reactions were incubated in a final volume of 10 µl of 1x selection buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) including 40 mM $MgCl_2$ at 37°C for 18 h. Aliquots (2 µl) were taken at 0, 6 h and 18 h, quenched with loading buffer and analyzed by denaturing PAGE. The gels were dried under vacuum for 30 min at 80 °C, exposed overnight to a phosphor storage screen and imaged on a Typhoon phosphorimager.

SUPPORTING INFORMATION

Supporting Tables

Table S1. Sequences of RNA oligonucleotides

No	Description/motif	5'-sequence-3'	mol. wt. calc., amu	mol. wt. found. m/z
R1	unmodified/NC	AUAGACUGAAUGAANCGUAACUAGUC	-	-
R2	Nm^{3}C	AUAGACUGAAUGAANm^{3}CGUAACUAGUC	-	-
R3	Nm^{4}C	AUAGACUGAAUGAANm^{4}CGUAACUAGUC	-	-
R4	Nm^{5}C	AUAGACUGAAUGAANm^{5}CGUAACUAGUC	-	-
R5	unmodified/AC	AUAGACUGAAUGAACGUAACUAGUC	8339.17	8339.34
R6	unmodified/GC	AUAGACUGAAUGAAGCGUAACUAGUC	8355.17	8355.25
R7	unmodified/CC	AUAGACUGAAUGAACCGUAACUAGUC	8315.16	8315.19
R8	unmodified/UC	AUAGACUGAAUGAAUCGUAACUAGUC	8316.14	8316.21
R9	Am^{3}C	AUAGACUGAAUGAAAm^{3}CGUAACUAGUC	8353.19	8353.00
R10	Gm^{3}C	AUAGACUGAAUGAAGm^{3}CGUAACUAGUC	8369.18	8369.27
R11	Cm^{3}C	AUAGACUGAAUGAACm^{3}CGUAACUAGUC	8329.18	8329.31
R12	Um^{3}C	AUAGACUGAAUGAAUm^{3}CGUAACUAGUC	8330.16	8330.27
R13	Am^{4}C	AUAGACUGAAUGAAAm^{4}CGUAACUAGUC	8353.19	8253.20
R14	Gm^{4}C	AUAGACUGAAUGAAGm^{4}CGUAACUAGUC	8369.18	8369.19
R15	Cm^{4}C	AUAGACUGAAUGAACm^{4}CGUAACUAGUC	8329.18	8329.20
R16	Um^{4}C	AUAGACUGAAUGAAUm^{4}CGUAACUAGUC	8330.16	8330.18
R17	Am^{5}C	AUAGACUGAAUGAAAm^{5}CGUAACUAGUC	8353.19	8353.25
R18	Gm^{5}C	AUAGACUGAAUGAAGm^{5}CGUAACUAGUC	8369.18	8369.24
R19	Cm^{5}C	AUAGACUGAAUGAACm^{5}CGUAACUAGUC	8329.18	8329.25
R20	Um^{5}C	AUAGACUGAAUGAAUm^{5}CGUAACUAGUC	8330.16	8330.20
R21	mt-tRNA^{Thr} 5' fragment	GUCCUUGUAGUAUAAACUAAUACA		
R22	mt-tRNA^{Thr} 3' fragment	pGAUGAAAACCUUUUUCCAAGGACAp		
R23	mt-tRNA^{Thr} m^{3}C ASL	CCAGUm^{3}CUUGUAAACCGGA		
R24	mt-tRNA^{Thr} unmod. ASL	CCAGUCUUGUAAACCGGA		
R25	mt-tRNA^{Glu} 5'-fragment	GUUCUUGUAGUUGAAAUACAACGAUGGUUUUUCAUA		
R26	mt-tRNA^{Glu} m^{5}C 3'-fragment	UCAUUGGUm^{5}CGUGGUUGUAGUCCGUGCGAGAAUA		
R27	Um^{4,4}C	AUAGACUGAAUGAAUm^{4,4}CGUAACUAGUC	8344.14	8344.24
R28	unmodified/UC transcript	GGAUAGACUGAAUGAAUCGUAACUAGUCA[a]	9575.14	9575.18
R29	unmodified/UU transcript	GGAUAGACUGAAUGAAUUGUAACUAGUCAA[a]	9905.14	9905.28
R30	unmodified/UA transcript	GGAUAGACUGAAUGAAUAGUAACUAGUCAA[a]	9928.15	9928.30
R31	unmodified/UA transcript	GGAUAGACUGAAUGAAUGGUAACUAGUCAA[a]	9944.14	9944.30
R32	Um^{5}C transcript	GGAUAGAm^{5}CUGAAUGAAUm^{5}CGUAAm^{5}CUAGUm^{5}CA[a]	9630.19	9630.31
R33	Uhm^{5}C transcript	GGAUAGAhm^{5}CUGAAUGAAUhm^{5}CGUAAhm^{5}CUAGUhm^{5}CA[a]	9694.17	9694.27
R34	Uf^{5}C transcript	GGAUAGAf^{5}CUGAAUGAAUf^{5}CGUAAf^{5}CUAGUf^{5}CA[a]	9686.11	9686.19

[a] non-templated A added by T7 RNA polymerase in vitro transcription

Table S2. Sequences of DNA oligonucleotides

No	Description	5'-Sequence-3'[a]
D1	Selection pool	GTGACGCGACTAGTTACN$_{20}$TTCATTCAGTTGGCGACTCC
D2	Selection forward primer	GTGACGCGACTAGTTAC
D2.1	Selection forward primer (for 5'-labeling)	Alk-GTGACGCGACTAGTTAC
D3	Selection reverse primer (tailed)	(CAA)$_4$E$_3$GGAGGCGCCAACTGAATGAA
D4	Splint for selection pool (round1)	TTCATTCAGTCTATGGAGTCGCCAACTG
D5	Splint for other selection rounds	TTACGTTTCATTCAGTCTATTGGAGTCGCCAACT
D6	AK02	GACTAGTTAC CAAGGAGCACGGAACTTTC TTCATTCAGT
D7	AK03	GACTAGTTAC GGCAGGAGCAGTCACATCG TTCATTCAGT
D8	AK04	GACTAGTTAC CCAACCAGGCCGGCACATA TTCATTCAGT
D9	AK11	GACTAGTTAC CAACCAGGATGCGGAACCA TTCATTCAGT
D10	AK12	GACTAGTTAC GGGGTATACAGTGGGGCGA TTCATTCAGT

D11	AK13	GACTAGTTAC GGGGTATACAGTGGCGCGAA TTCATTCAGT
D12	AK24	GACTAGTTAC CCAACGGTTGAGTCACCCC TTCATTCAGT
D13	AL01	GACTAGTTAC CGCAGGTGTTTGTGGGGTAA TTCATTCAGT
D14	AL03	GACTAGTTAC GGTTCGGGACGGCCTGGTAA TTCATTCAGT
D15	AL04	GACTAGTTAC GGGGTAGCCAGTGGACGTT TTCATTCAGT
D16	AL07	GACTAGTTAC GGGGCAGGTGGTGGGAAGGG TTCATTCAGT
D17	AL10	GACTAGTTAC CGAGGCGCGGAACCCCCAC TTCATTCAGT
D18	AL12	GACTAGTTAC CGTTACGGAGTGCGTGGTAA TTCATTCAGT
D19	AL13	GACTAGTTAC GGGGGTAAGACTGGGGTAG TTCATTCAGT
D20	AL15	GACTAGTTAC GGTTACGTAGTGCCTGGTTG TTCATTCAGT
D21	AL18	GACTAGTTAC CCGATCGTGGACTCCGGAGA TTCATTCAGT
D22	AL21	GACTAGTTAC GGCACCAACTGGAGTCTGGG TTCATTCAGT
D23	AM05	GACTAGTTAC GGGGTACCGGTGGAGACTA TTCATTCAGT
D24	AM06	GACTAGTTAC CCAACAAGGAGGGTCACCC TTCATTCAGT
D25	AM11	GACTAGTTAC CAAACGATCGCCGGCACCG TTCATTCAGT
D26	AM12	GACTAGTTAC CACCACAATCGCGGAACCG TTCATTCAGT
D27	AM13	GACTAGTTAC GGGAGCGACACAACCATCA TTCATTCAGT
D28	AM17	GACTAGTTAC CACGTGGTGAGCACTCTAAC TTCATTCAGT
D29	AM18	GACTAGTTAC CACGTGGTGAGCACTTCTAC TTCATTCAGT
D30	AM20	GACTAGTTAC GGCACCCACTGGAGTCTGGT TTCATTCAGT
D31	AM21	GACTAGTTAC CGCTGGGTTGGGTTGCGCGG TTCATTCAGT
D32	AN01	GACTAGTTAC CCAACAGACAGCGGCACTC TTCATTCAGT
D33	AN02	GACTAGTTAC GGGGCGGAACGTAATCTTA TTCATTCAGT
D34	AN03	GACTAGTTAC CCACAGGCACGAGGATGTAA TTCATTCAGT
D35	AN04	GACTAGTTAC GGGGTATACAGTGGCGCGA TTCATTCAGT
D36	AN05	GACTAGTTAC GGTAGTAGGATGGCGATCCG TTCATTCAGT
D37	AN07	GACTAGTTAC GGACACAGCTGGTACCGGT TTCATTCAGT
D38	AN12	GACTAGTTAC CACCAACTAGGAGCACCCG TTCATTCAGT
D39	AN14	GACTAGTTAC CCCATACTAGCGTCGTCCG TTCATTCAGT
D40	AN19	GACTAGTTAC CGGCTGGACGTTGACGCGGT TTCATTCAGT
D41	AN24	GACTAGTTAC CCAGTAACGTGTCCGCCAT TTCATTCAGT
D42	AK104	GACTAGTTAC GGGTGCCGGGTGAGCGTTAT TTCATTCAGT
D43	AK118	GACTAGTTAC GGGGTAGCCAGTGGGACGTG TTCATTCAGT
D44	AL107	GACTAGTTAC GGGGTAGGTGGTGGGAAGGG TTCATTCAGT
D45	AL112	GACTAGTTAC GGTTGCGGTAGCGCCTGGTG TTCATTCAGT
D46	AM101	GACTAGTTAC CGGGCCGGGGGTTAATCG TTCATTCAGT
D47	AM102	GACTAGTTAC TCAACAGCCGAAACGACCA TTCATTCAGT
D48	AM106	GACTAGTTAC GGGGTATACAGTGGTGCGA TTCATTCAGT
D49	AM108	GACTAGTTAC CGGCTGGACGTTGACGCGA TTCATTCAGT
D50	AN109	GACTAGTTAC TCAACAGCCGAAACGACAC TTCATTCAGT
D51	AL112_G20A	GACTAGTTAC GGTTGCGGTAGCGCCTGGTA TTCATTCAGT
D52	Splint mt-tRNA^{Thr}	AAGGTTTTCATCTCCGGTTTACAAGACTGGTGTATTAGTTT
D53	Splint mt-tRNA^{Glu}	GACCAATGATATGAAAAACCATCG
D54	AK104 for mt-tRNA^{Thr}	TCCGGTTTACAA GGGTGCCGGGTGAGCGTTAT CTGGTGTATT
D55	AL112 for mt-tRNA^{Thr}	TCCGGTTTACAA GGTTGCGGTAGCGCCTGGTG CTGGTGTATT
D56	Disruptor for mt-tRNA^{Thr}	TTTATACTACAAGGAC
D57	AK104 for mt-tRNA^{Glu}	GACTACAACCAC GGGTGCCGGGTGAGCGTTAT CCAATGATAT
D58	AN05 for mt-tRNA^{Glu}	GACTACAACCAC GGTAGTAGGATGGCGATCCG CCAATGATAT
D59	Disruptor for mt-tRNA^{Glu}	TGGTATTCTCGCACGGAC

[a] Alk = 5-hexynyl, E_3 = triethylene glycol

SUPPORTING INFORMATION

Table S3. Summary of enrichment data and cleavage activity for deoxyribozymes reported in this study.

Deoxyribozyme	log_2f_{CA}[a]	$log_2f_{CA,neg}$[b]	Cleavage site	N[c]	Activity, FC[d](k_{obs}* 10^{-3} min^{-1})[e]			
					C	m^3C	m^4C	m^5C
AK12	4.76	-2.94 (AL)	AN | CG	A	12	0	2	2
				G	89	0	69	49
				C	0	0	0	0
				U	6	0	0	0
AK13	1.32	-4.36 (AL)	AN | CG	A	12	0	0	0
				G	77	0	48	51
				C	0	0	0	0
				U	3	0	0	0
AK104[f]	10.64	-5.15 (AM) -12.91 (AN)	AN | CG A | NCG AN | CG	A	95	0	0	0
				G	94	0	0	0
				C	0	29	0	0
				U	85(3.2)	0	0	0
AK118	4.73	-8.94 (AN) -6.28 (AL)	AN | CG	A	0	0	0	0
				G	62	0	30	53
				C	0	0	0	0
				U	23	10	3	12
AL01	-0.49	-1.45	AN | CG	A	12	27	14	9
				G	12	29	14	8
				C	0	0	0	0
				U	0	0	0	0
AL04	13.04	2.39	AN | CG	A	0	0	0	0
				G	13	58	1	4
				C	0	24	0	0
				U	0	0	0	0
AL07	3.00	-1.30	AN | CG	A	0	15	0	0
				G	0	11	1	0
				C	2	60	7	0
				U	27	87	53	21
AL12	-3.18	-4.17	AN | CG	A	0	13	6	0
				G	18	28	23	13
				C	0	0	0	0
				U	0	0	0	0
AL13	11.32	4.07	ANC | G	A	<1	68	0	0
				G	6	73	14	8
				C	0	3	0	0
				U	<1	12	0	0
AL15	-0.86	-3.19	AN | CG	A	0	31	3	0
				G	25	63	47	17
				C	0	7	1	0
				U	1	11	4	2
AL18	1.41	0.53	AN | CG	A	2	3	3	2
				G	40	26	45	26
				C	0	0	0	0
				U	0	0	0	0
AL21	7.90	4.17	AN | CG	A	0	5	0	0
				G	0	4	0	0
				C	<1	30	2	0
				U	6	55	15	5
AL107	2.93	-1.37	AN | CG	A	0	14	0	0
				G	0	9	0	0
				C	0	54	2	0
				U	21	93	45	18
AL112[f]	-2.94	-	AN | CG	A	0	45	0	0
				G	0	40	0	0
				C	0	57	0	0
				U	3(0.06)	70(1.8)	10(0.2)	4(0.08)
AM05	12.82	1.64	ANC | G	A	0	0	0	0
				G	0	0	3	0
				C	0	0	0	0
				U	66	8	81	56
AM17	5.48	2.21	AN | CG	A	0	0	0	0
				G	47	49	18	39
				C	0	0	0	0
				U	0	0	0	0

SUPPORTING INFORMATION

Deoxyribozyme	log_2f_{CA}[a]	$log_2f_{CA,neg}$[b]	Cleavage site	N[c]	Activity, FC[d] (k_{obs}* 10^{-3} min^{-1})[e]			
					C	m^3C	m^4C	m^5C
AM18	4.28	1.56	AN | CG	A	0	0	0	0
				G	21	24	17	12
				C	0	0	0	0
				U	0	0	0	0
AM21	0.59	-6.63	AN | CG	A	29	71	72	35
				G	6	14	3	4
				C	0	0	0	0
				U	9	8	9	8
AM101[f]	10.02	1.41	ANC | G	A	0	0	0	0
				G	0	0	0	0
				C	0	0	22	0
				U	18(0.5)	0	73(5.3)	0
AM102	6.38	-2.90	ANC | G	A	27	44	52	25
				G	71	74	82	64
				C	49	47	84	57
				U	88	71	91	88
AM106	7.78	4.61	AN | CG	A	9	0	0	0
				G	81	0	55	31
				C	0	0	0	0
				U	0	0	0	0
AM108	5.86	0.21	AN | CG	A	10	19	15	7
				G	42	41	50	29
				C	0	0	0	0
				U	0	0	0	0
AN03	2.07	-0.01	AN | CG	A	0	10	5	6
				G	17	13	12	13
				C	0	1	0	0
				U	0	0	0	0
AN04	3.15	2.28	AN | CG	A	16	0	6	4
				G	78	0	66	73
				C	0	0	0	0
				U	5	0	2	12
AN05[f]	12.35	-4.68	A | NCG	A	0	0	0	0
				G	0	0	0	0
				C	0	0	0	0
				U	33(0.7)	0	0	90(6.7)
AN07	9.29	3.01	AN | CG	A	0	0	0	0
				G	0	0	0	0
				C	0	0	0	0
				U	47	0	28	39
AN19	3.81	-3.05	AN | CG	A	0	22	0	0
				G	0	9	2	0
				C	0	2	0	0
				U	67	60	82	62
AN109	8.96	-4.81	ANC | G	A	25	42	49	28
				G	86	71	87	67
				C	43	47	83	62
				U	88	79	93	93

Color code	Activity	FC[d] range
	Low	0 ≤ FC < 10
	Moderate	10 ≤ FC < 50
	High	50 ≤ FC ≤ 100

[a] log_2 fold change in abundance between rounds 18 and 7 of the modification-specific selection (AK for C, AL for m^3C, AM for m^4C and AN for m^5C) log_2f_{CA} is defined as $log_2(CPM_{R18} / CPM_{R7})$, where CPM_{R18} is the read counts per million for the corresponding deoxyribozyme in round 18, and CPM_{R7} is read counts per million in round 7. [b] log_2 fold abundance change between rounds 18 and 7 of the negative selection (AK for m^3C, m^4C, m^5C). [c] Nucleotide preceding the target cytosine site in the RNA substrate. [d] Fraction cleaved (%) after 6 h incubation (100 pmol deoxyribozyme, 10 pmol RNA substrate, 20 mM $MgCl_2$, 50 mM Tris-HCl, 150 mM NaCl, pH 7.5). [e] Observed kinetic constants for single-turnover cleavage. [f] The activity assays were performed at least twice to check for reproducibility. Mean FC values are presented.

SUPPORTING INFORMATION

Supporting figures

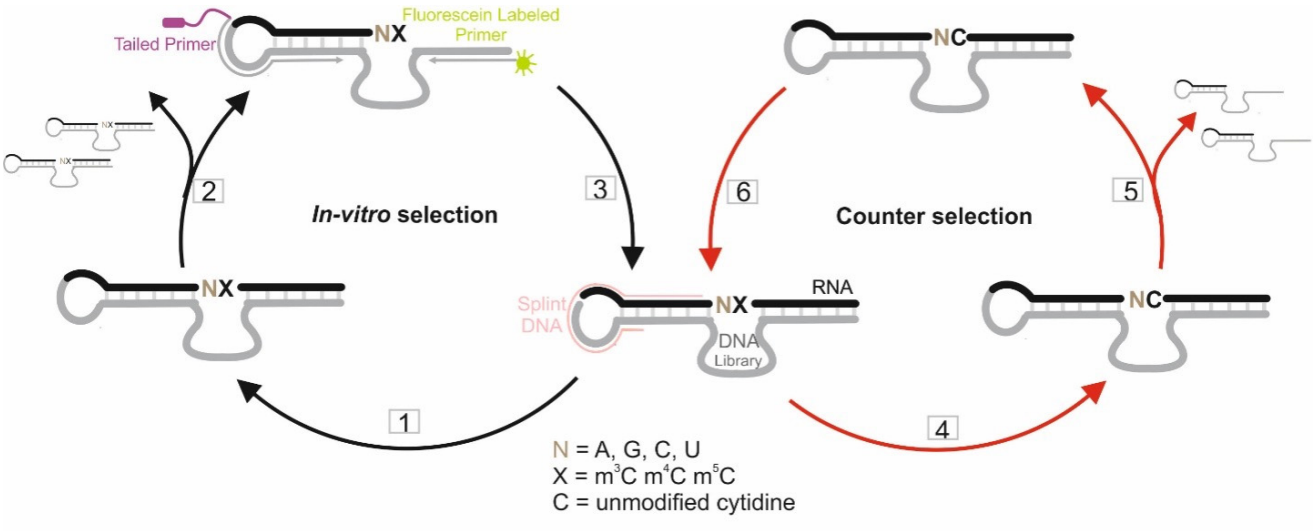


Figure S1. Schematic of *in vitro* selection of RNA-cleaving deoxyribozymes sensitive to m^3C, m^4C or m^5C modifications (AL, AM and AN selections, respectively): 1) Ligation of DNA library to modified RNA (R2, R3, R4). 2) Incubation in presence of $MgCl_2$. The cleaved fraction is isolated by PAGE. 3) PCR amplification of active fraction. 4) Ligation of DNA library to unmodified RNA (R1). 5) Incubation in presence of $MgCl_2$. The uncleaved fraction is isolated by PAGE. 6) PCR amplification of uncleaved fraction.

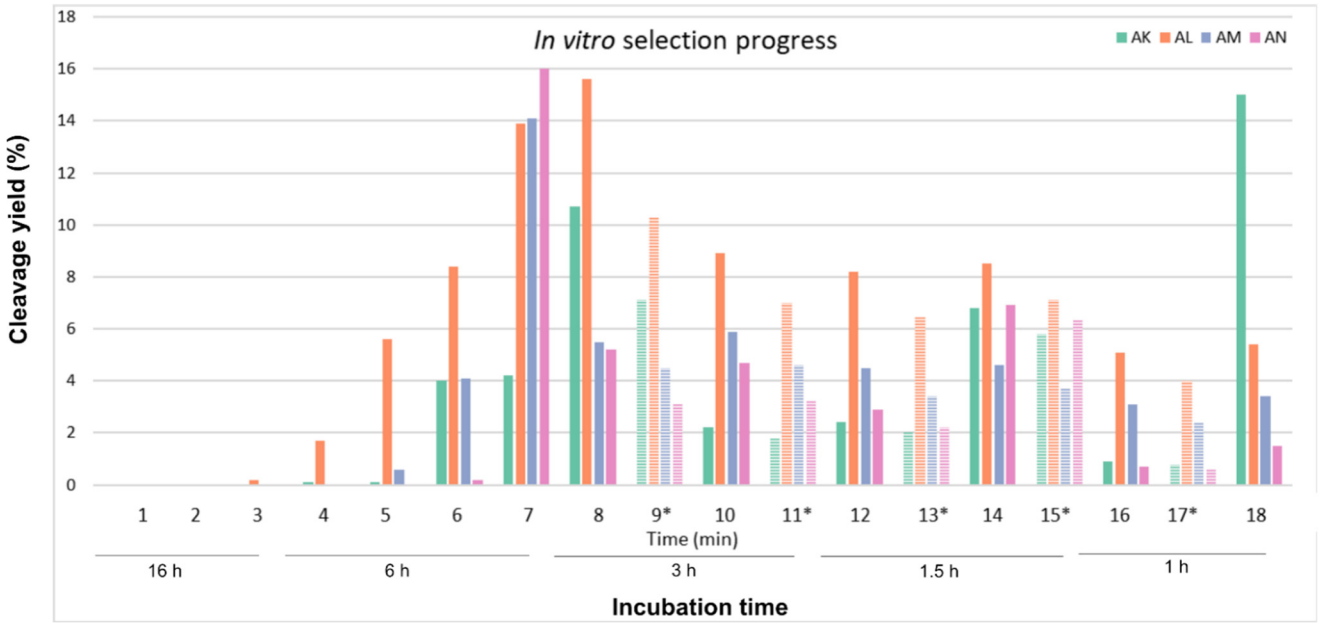


Figure S2. Progress of in-vitro selection plotted as cleavage yield in every selection round. From round 4, cleavage activity was visible and cleavage yield was determined by quantification of fluorescent bands in the selection step. The counter selection rounds are marked with asterisks and incubation time was 16 h for every counter selection round.

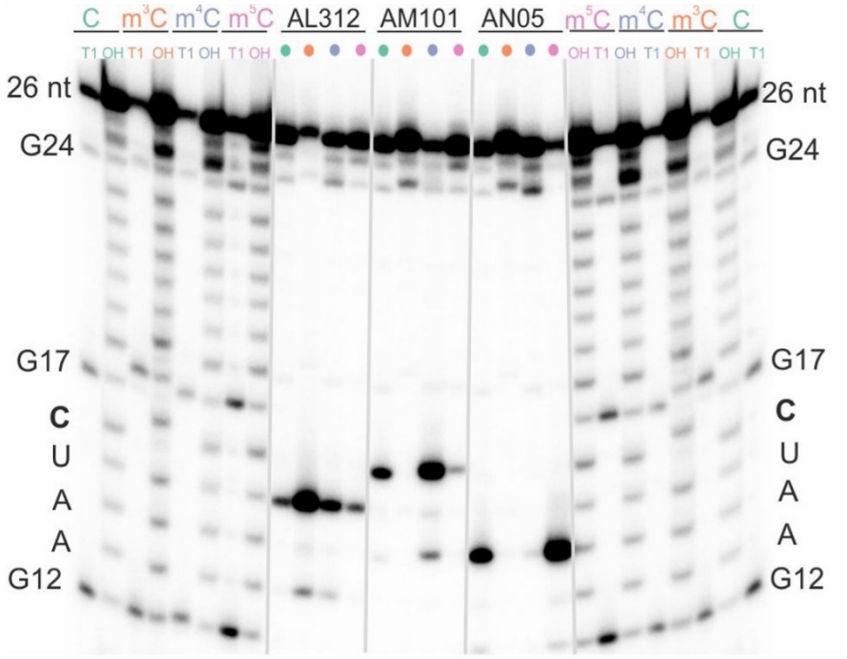


Figure S3. PAGE analysis of RNA cleavage mediated by deoxyribozymes AL112, AM101 and AN05 and comparison of cleavage activity for unmodified (green), m^3C- (orange), m^4C- (blue), and m^5C-modified (magenta) RNA. 5'-Radioactively labeled RNA was incubated with DNA enzymes in presence of 20 mM $MgCl_2$ at 37 °C for 6 h. Lanes OH: alkaline hydrolysis of RNA in presence of 25 mM NaOH at 95 °C for 3 min. Lanes T1: RNase T1 ladder showing positions of Gs in the RNA substrate.

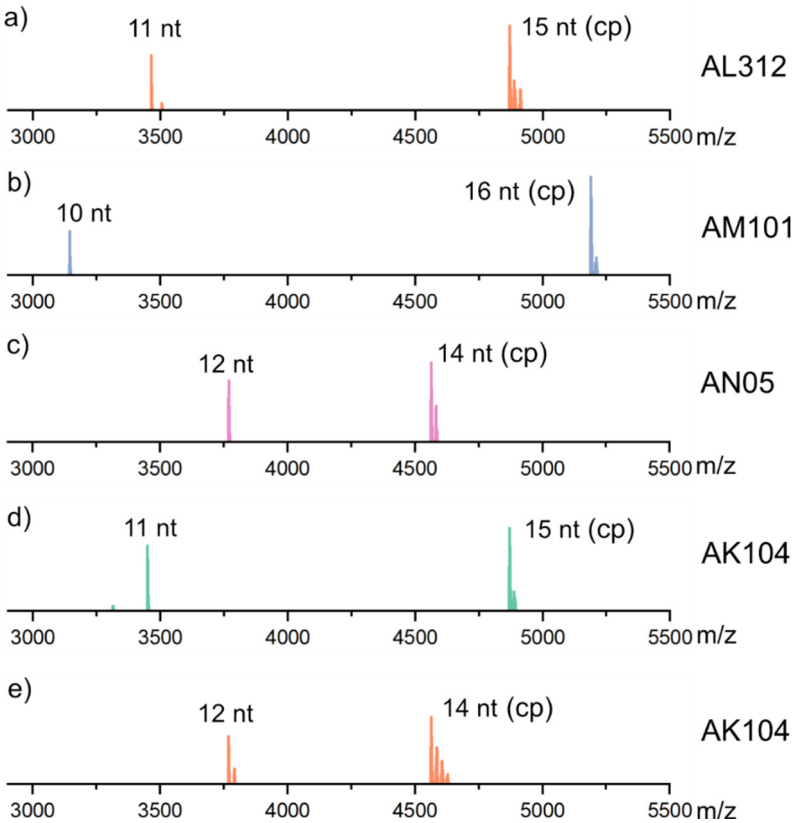


Figure S4. High-resolution electrospray ionization mass spectrometry (HR-ESI-MS) analysis of cleaved products for AL112, AM101, AN05 and AK104. a) RNA fragments formed by cleavage of R12 (Um^3C) with AL312. b) RNA fragments formed by cleavage of R16 (Um^4C) with AM101. c) RNA fragments formed by cleavage of R20 (Um^5C). d) AK104 cleaved R8 (UC motif) with formation of 15-nt fragment which contained 2',3'-cyclic phosphate (cp) and 11-nt fragment which contained 5'-OH. e) AK104 shifted its cleavage site one nucleotide upstream in reaction with R11 (Cm^3C) as confirmed by formation of 14-nt and 12-nt fragments.

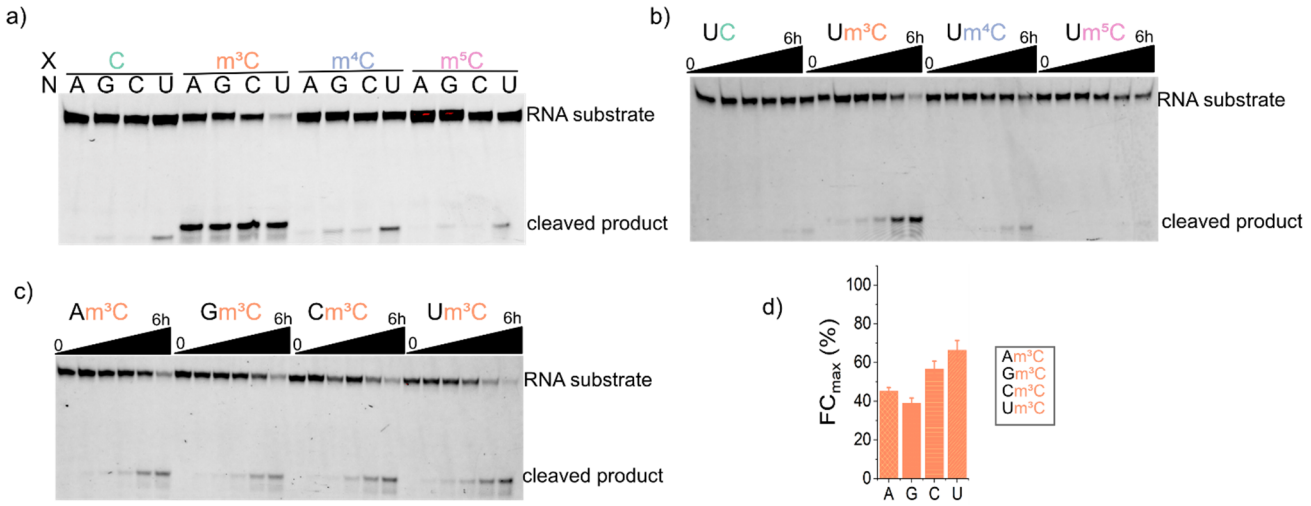


Figure S5. Activity assays for deoxyribozyme AL112. Cleavage yields (FC) and k_{obs} values are presented in Table S3. a) Gel image for cleavage of R5–R20. X – modification at the target cytosine site. N – nucleotide preceding the target cytosine site. Conditions: 100 pmol deoxyribozyme, 10 pmol RNA substrate, 20 mM $MgCl_2$, 50 mM Tris-HCl, 150 mM NaCl, pH 7.5, 37 °C, 6 h. b) Gel-based kinetics assays for cleavage of R8 (UC), R12 (Um^3C), R16 (Um^4C) and R20 (Um^5C). Time points: 0, 10, 30, 60, 180, 360 min. Kinetics plots are presented in Figure 2b. c) Gel-based kinetics assay for cleavage of R9 (Am^3C), R10 (Gm^3C), R11 (Cm^3C) and R12 (Um^3C). d) Comparison of FC values obtained after 6 h cleavage of R9–R12. Each bar represents the mean from three independent experiments with error bars showing the ± SD.

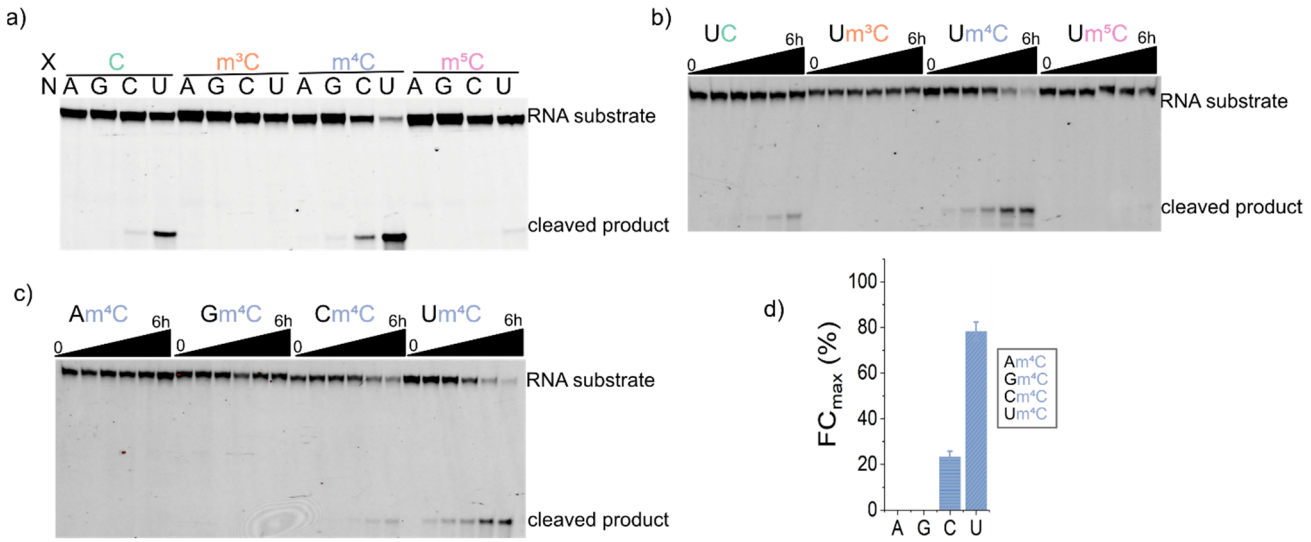


Figure S6. Activity assays for deoxyribozyme AM101. Cleavage yields (FC) and k_{obs} values are presented in Table S3. a) Gel image for cleavage of R5–R20. X – modification at the target cytosine site. N – nucleotide preceding the target cytosine site. Conditions: 100 pmol deoxyribozyme, 10 pmol RNA substrate, 20 mM $MgCl_2$, 50 mM Tris-HCl, 150 mM NaCl, pH 7.5, 37 °C, 6 h. b) Gel-based kinetics assays for cleavage of R8 (UC), R12 (Um^3C), R16 (Um^4C) and R20 (Um^5C). Time points: 0, 10, 30, 60, 180, 360 min. Kinetics plots are presented in Figure 2b. c) Gel-based kinetics assay for cleavage of R13 (Am^4C), R14 (Gm^4C), R15 (Cm^4C) and R16 (Um^4C). d) Comparison of FC values obtained after 6 h cleavage of R13–R16. Each bar represents the mean from three independent experiments with error bars showing the ± SD.

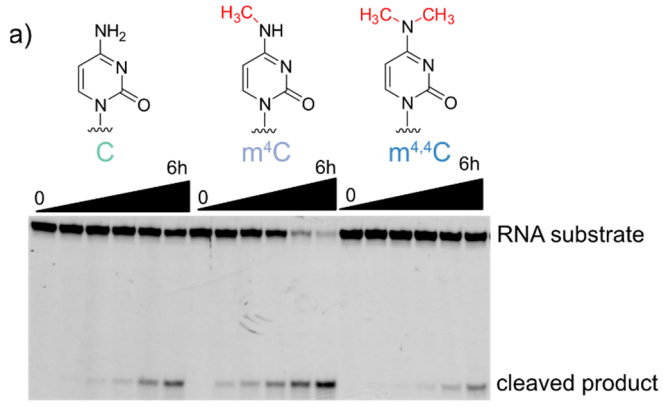


Figure S7. Single-turnover cleavage of unmodified, m^4C-modified and $m^{4,4}C$-modified RNA substrates with m^4C-specific deoxyribozyme AM101. a) Gel images for cleavage of R8, R20 and R27. Conditions: 100 pmol deoxyribozyme, 10 pmol RNA substrate, 20 mM $MgCl_2$, 50 mM Tris-HCl, 150 mM NaCl, pH 7.5, 37 °C, 6 h. b) Kinetic plots. d) Comparison of FC values obtained after 6 h cleavage.

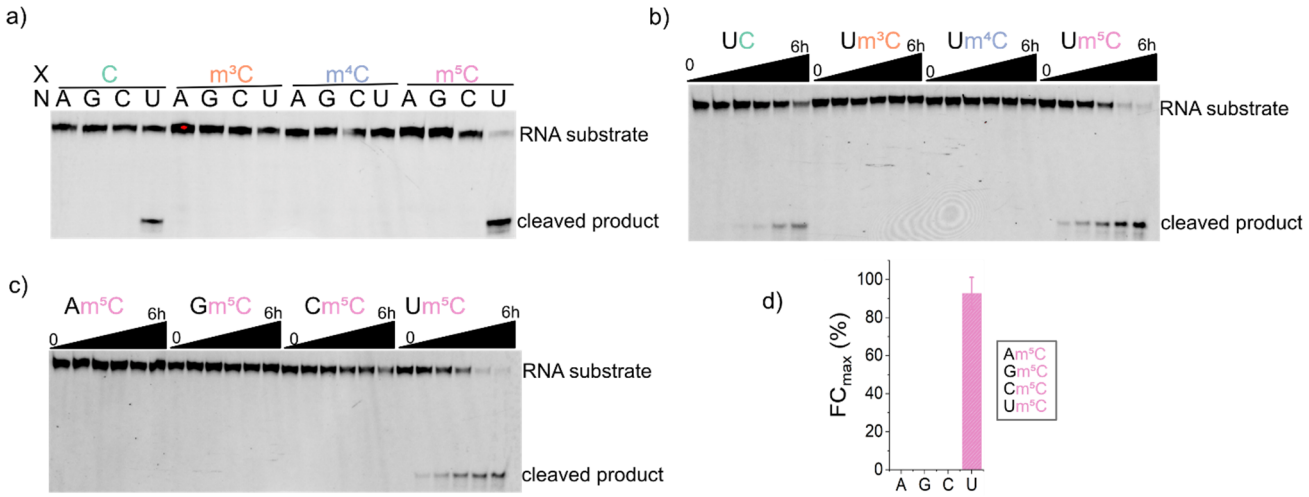


Figure S8. Activity assays for deoxyribozyme AN05. Cleavage yields (FC) and k_{obs} values are presented in Table S3. a) Gel image for cleavage of R5–R20. X – modification at the target cytosine site. N – nucleotide preceding the target cytosine site. Conditions: 100 pmol deoxyribozyme, 10 pmol RNA substrate, 20 mM $MgCl_2$, 50 mM Tris-HCl, 150 mM NaCl, pH 7.5, 37 °C, 6 h. b) Gel-based kinetics assays for cleavage of R8 (UC), R12 (Um^3C), R16 (Um^4C) and R20 (Um^5C). Time points: 0, 10, 30, 60, 180, 360 min. Kinetics plots are presented in Figure 2b. c) Gel-based kinetics assay for cleavage of R17 (Am^5C), R18 (Gm^5C), R19 (Cm^5C) and R20 (Um^5C). d) Comparison of FC values obtained after 6 h cleavage of R17–R20. Each bar represents the mean from three independent experiments with error bars showing the ± SD.

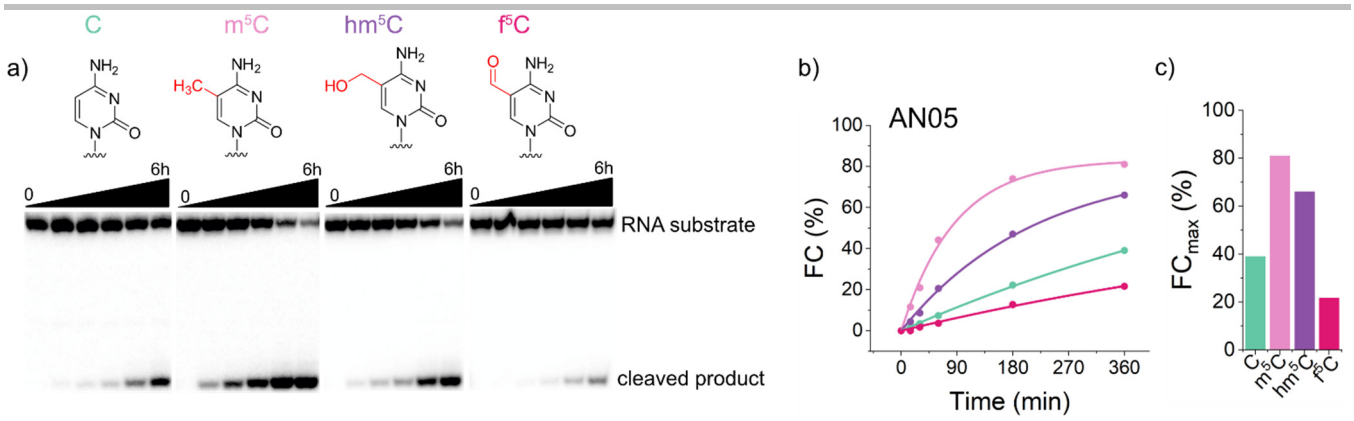


Figure S9. Single-turnover cleavage of unmodified, m^{5}C-, hm^{5}C- and f^{5}C-modified RNA substrates with m^{5}C-specific deoxyribozyme AN05. a) Gel images for cleavage of R28 and R32−R34. Conditions: 100 pmol deoxyribozyme, 10 pmol RNA substrate, 20 mM $MgCl_2$, 50 mM Tris-HCl, 150 mM NaCl, pH 7.5, 37 °C, 6 h. b) Kinetic plots. d) Comparison of FC values obtained after 6 h cleavage.

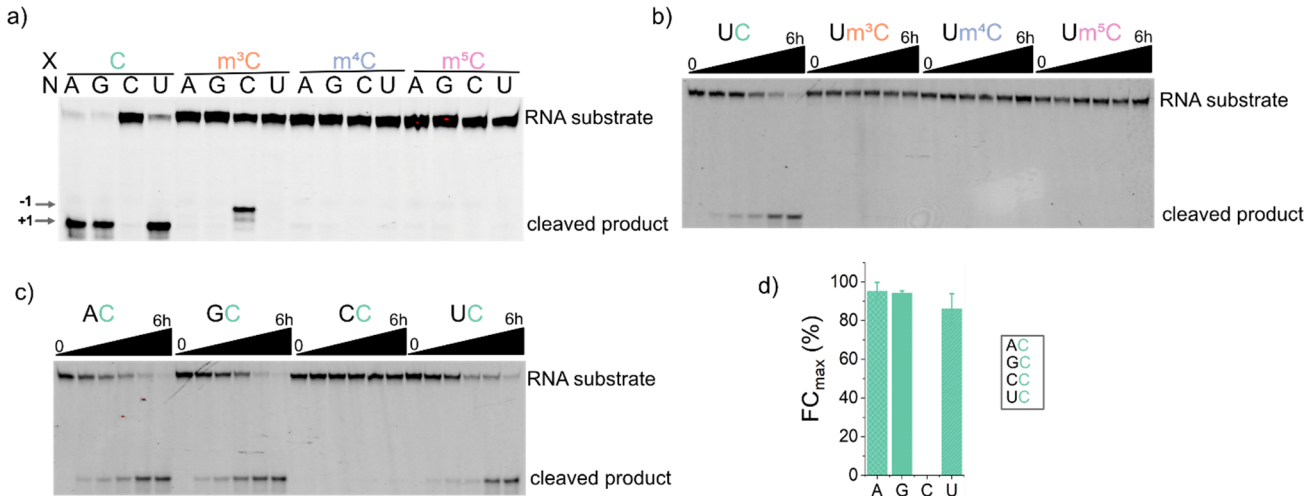


Figure S10. Activity assays for deoxyribozyme AK104. Cleavage yields (FC) and k_{obs} values are presented in Table S3. a) Gel image for cleavage of R5–R20. X – modification at the target cytosine site. N – nucleotide preceding the target cytosine site. Conditions: 100 pmol deoxyribozyme, 10 pmol RNA substrate, 20 mM $MgCl_2$, 50 mM Tris-HCl, 150 mM NaCl, pH 7.5, 37 °C, 6 h. b) Gel-based kinetics assays for cleavage of R8 (UC), R12 (Um^{3}C), R16 (Um^{4}C) and R20 (Um^{5}C). Time points: 0, 10, 30, 60, 180, 360 min. Kinetics plots are presented in Figure 2b. c) Gel-based kinetics assay for cleavage of R5 (AC), R6 (GC), R7 (CC) and R8 (UC). d) Comparison of FC values obtained after 6 h cleavage of R5–R8. Each bar represents the mean from three independent experiments with error bars showing the ± SD.

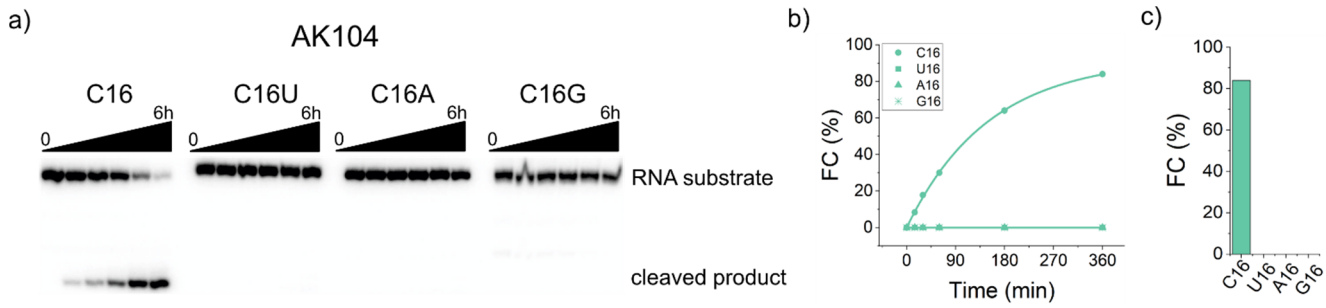


Figure S11. Single-turnover cleavage of the unmodified selection substrate and its C16U, C16A and C16G mutants with deoxyribozyme AK104. a) Gel images for cleavage of R28−R31. Conditions: 100 pmol deoxyribozyme, 10 pmol RNA substrate, 20 mM $MgCl_2$, 50 mM Tris-HCl, 150 mM NaCl, pH 7.5, 37 °C, 6 h. b) Kinetic plots. d) Comparison of FC values obtained after 6 h cleavage.

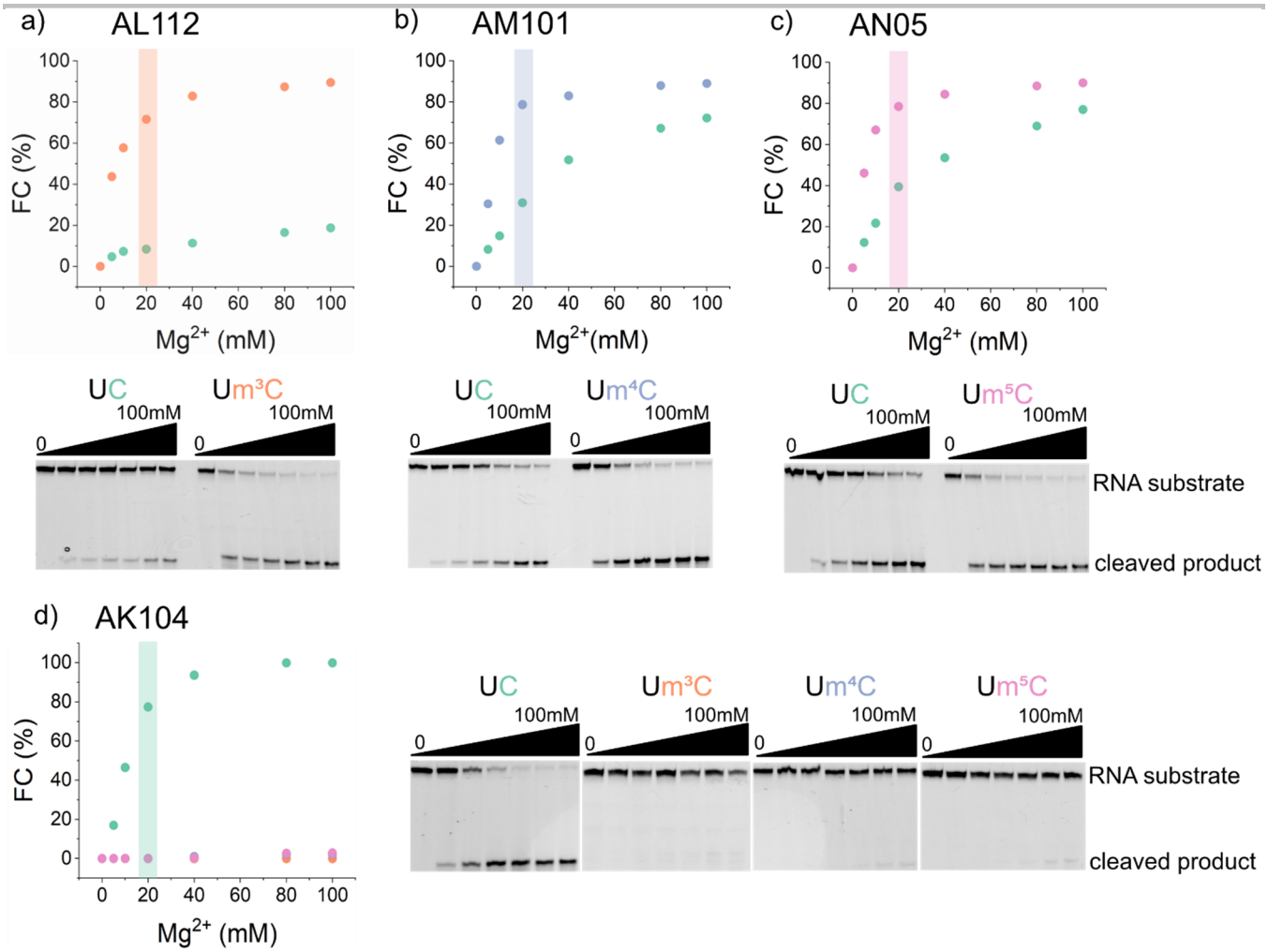


Figure S12. Dependence of cleavage yield on concentrations of Mg^{2+} for the reactions of the selection substrates with deoxyribozymes AL112, AM101, AN05 and AK104. a)−c) Top panel: cleavage yields plotted vs concentration of Mg^{2+} for deoxyribozymes AL112, AM101 and AN05, respectively. Bottom panel: gel images for cleavage of R8 and R12 with AL112 (a), R8 and R16 with AM101 (b), R8 and R20 with AN05 (c). d) Cleavage yields plotted vs concentration of Mg^{2+} for deoxyribozyme AK104 (left). Gel images for cleavage of R8, R12, R16 and R20 with AK104 (right). Conditions: 100 pmol deoxyribozyme, 10 pmol RNA substrate, indicated concentration of $MgCl_2$, 50 mM Tris-HCl, 150 mM NaCl, pH 7.5, 37 °C, 6 h.

SUPPORTING INFORMATION

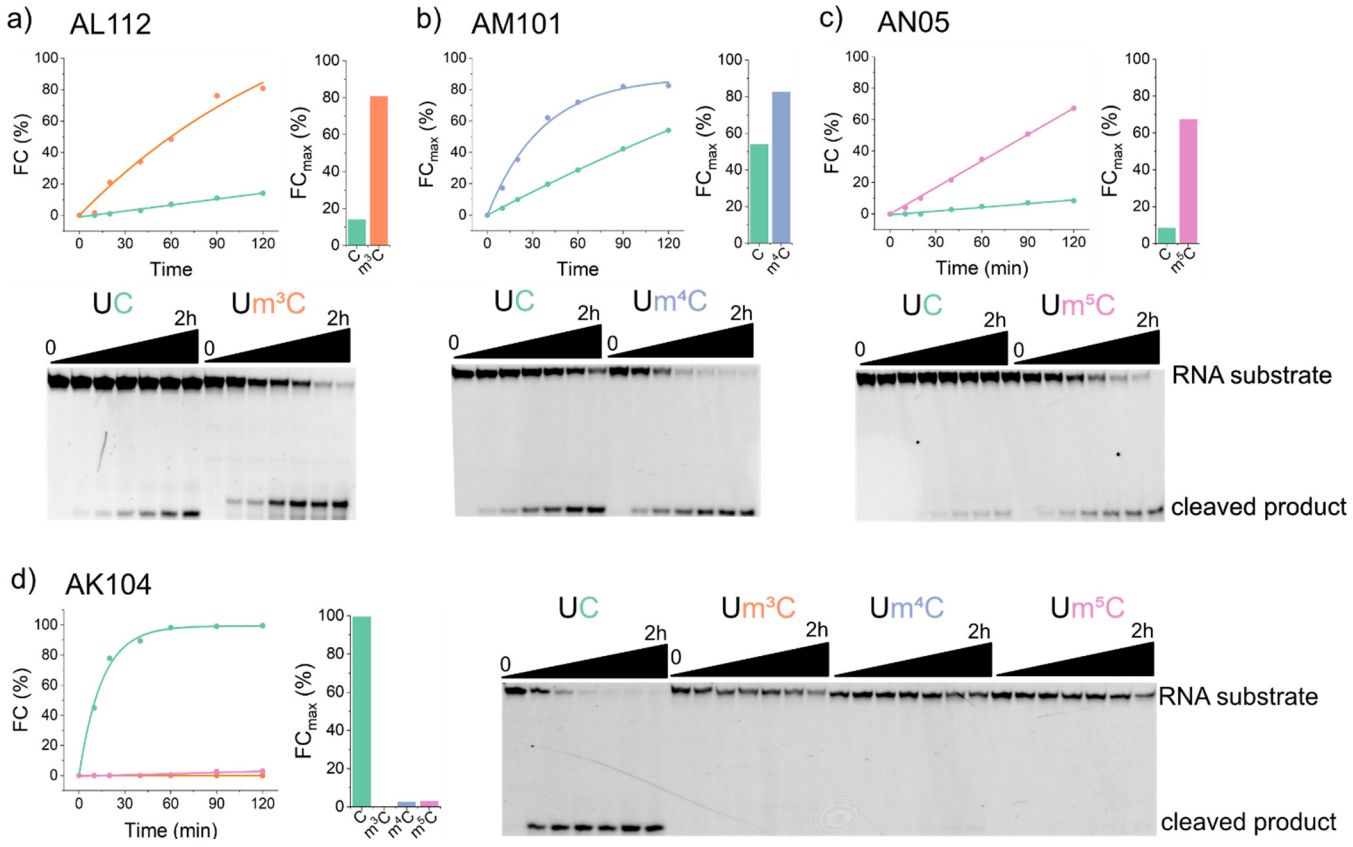
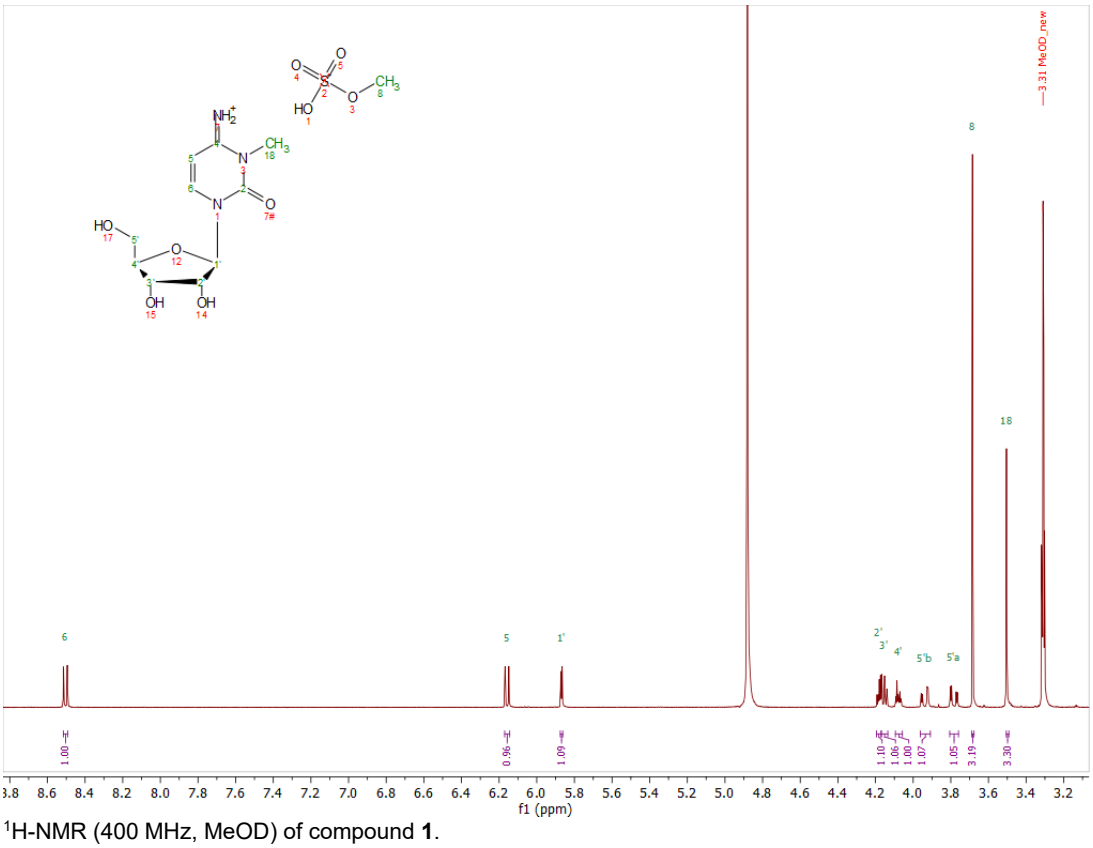
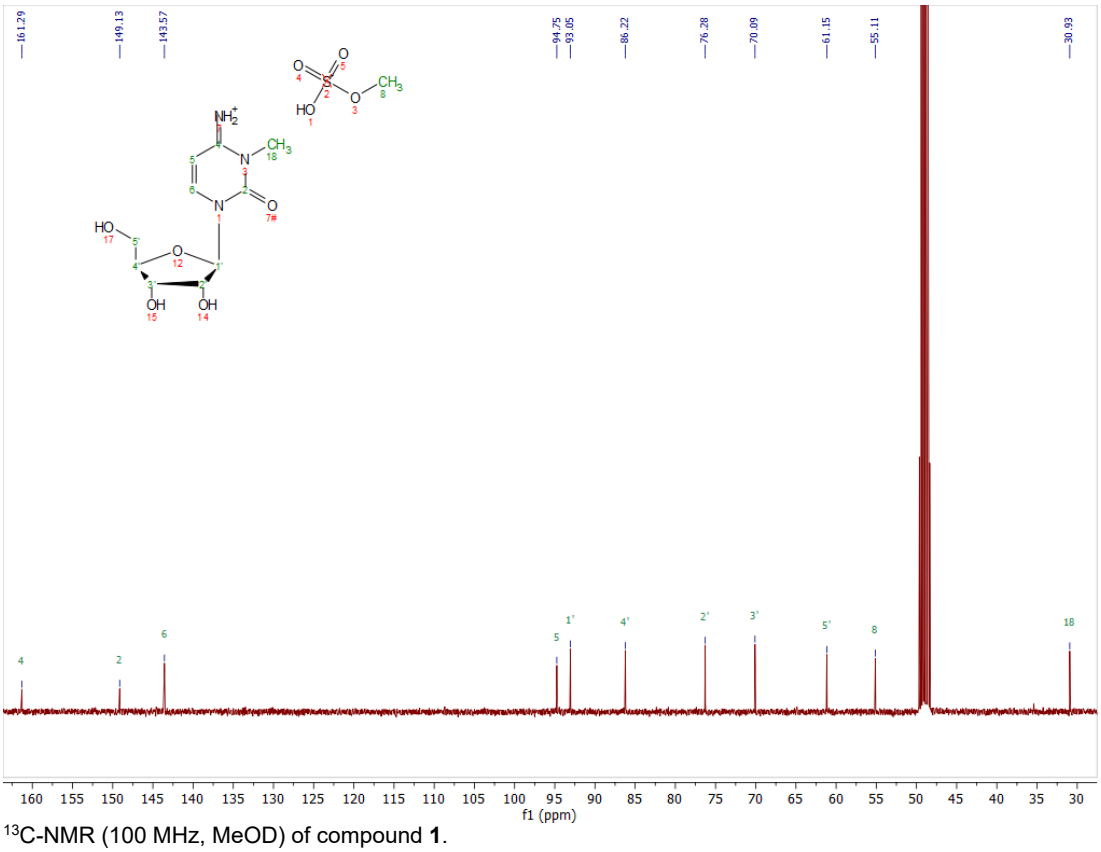


Figure S13. Cleavage activity of deoxyribozymes AL112, AM101, AN05 and AK104 in the presence of Mn^{2+}. a)−c) Top panel: kinetic plots and comparison of FC values obtained after 2 h cleavage for deoxyribozymes AL112, AM101 and AN05, respectively. Bottom panel: gel images for cleavage of R8 and R12 with AL112 (a), R8 and R16 with AM101 (b), R8 and R20 with AN05 (c). d) kinetic plots and comparison of FC values obtained after 2 h cleavage for deoxyribozyme AK104 (left). Gel image for cleavage of R8, R12, R16 and R20 with AK104 (right). Conditions: 100 pmol deoxyribozyme, 10 pmol RNA substrate, 20 mM $MnCl_2$, 50 mM Tris-HCl, 150 mM NaCl, pH 7.5, 37 °C, 2 h.

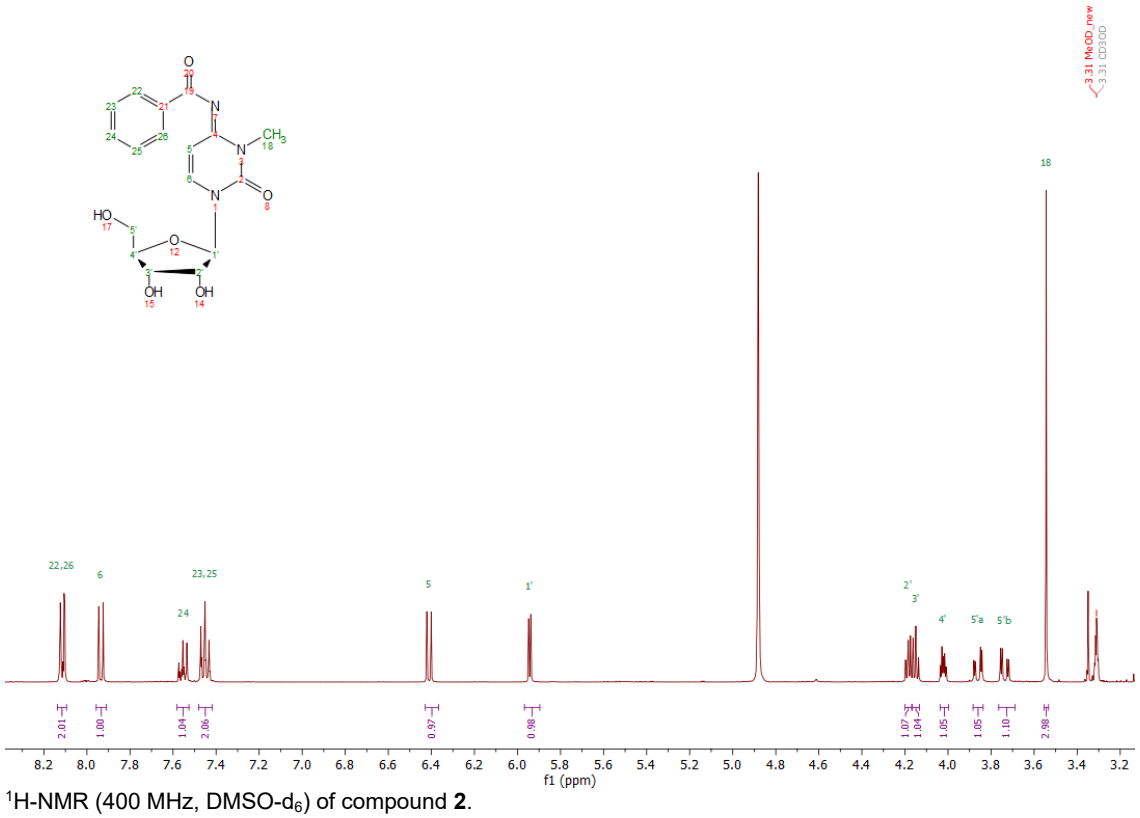
NMR Spectra



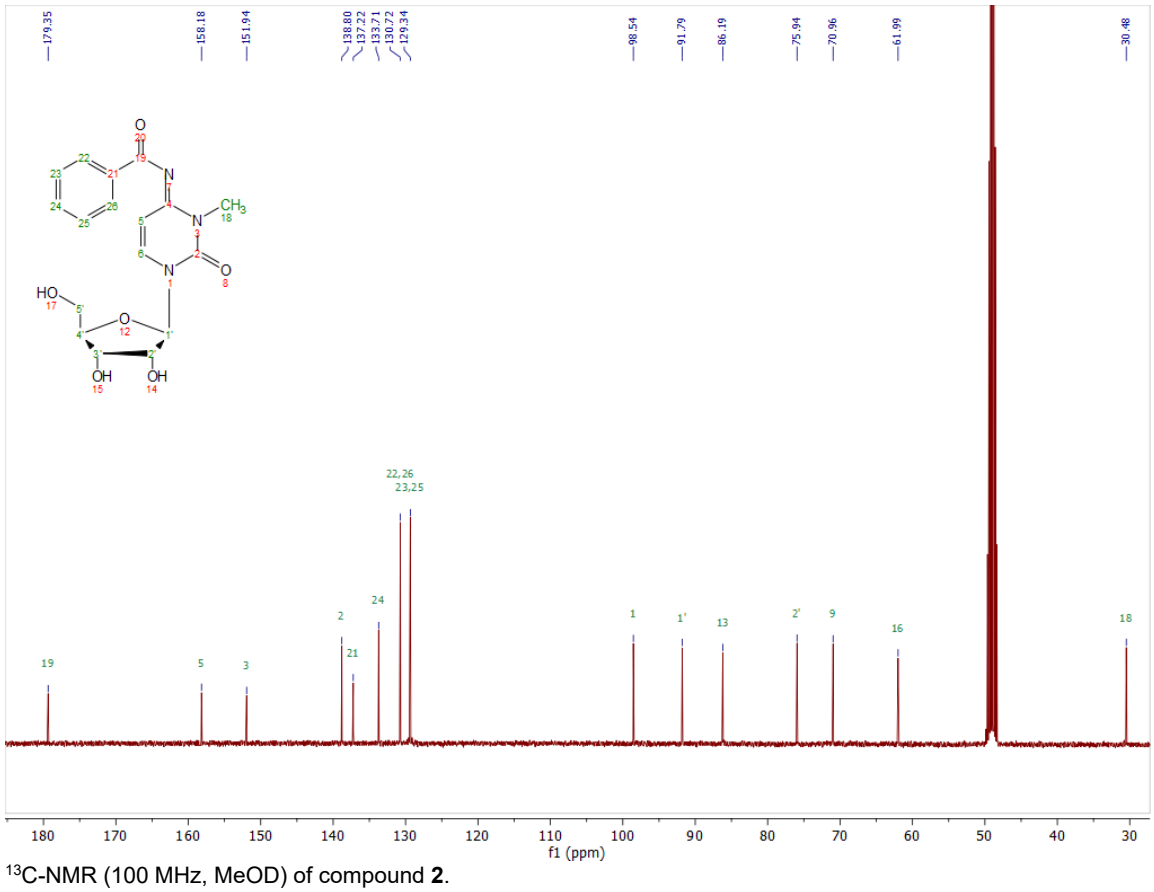
^{1}H-NMR (400 MHz, MeOD) of compound **1**.



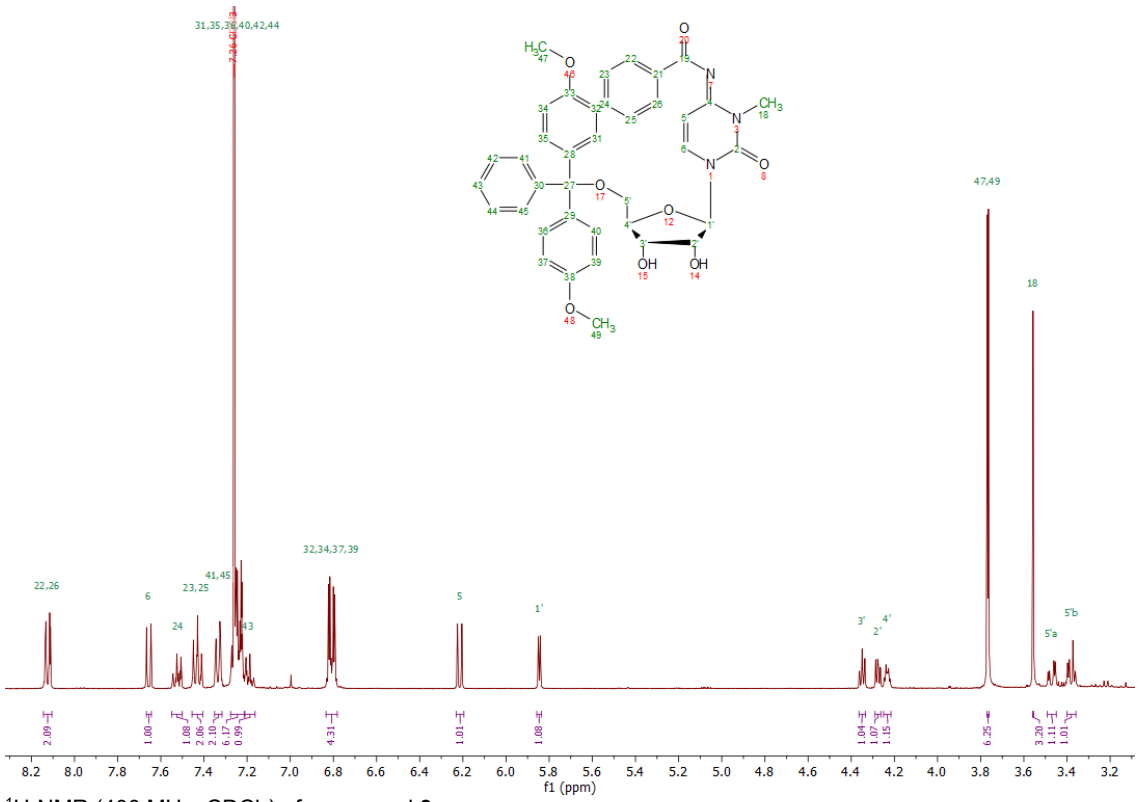
^{13}C-NMR (100 MHz, MeOD) of compound **1**.



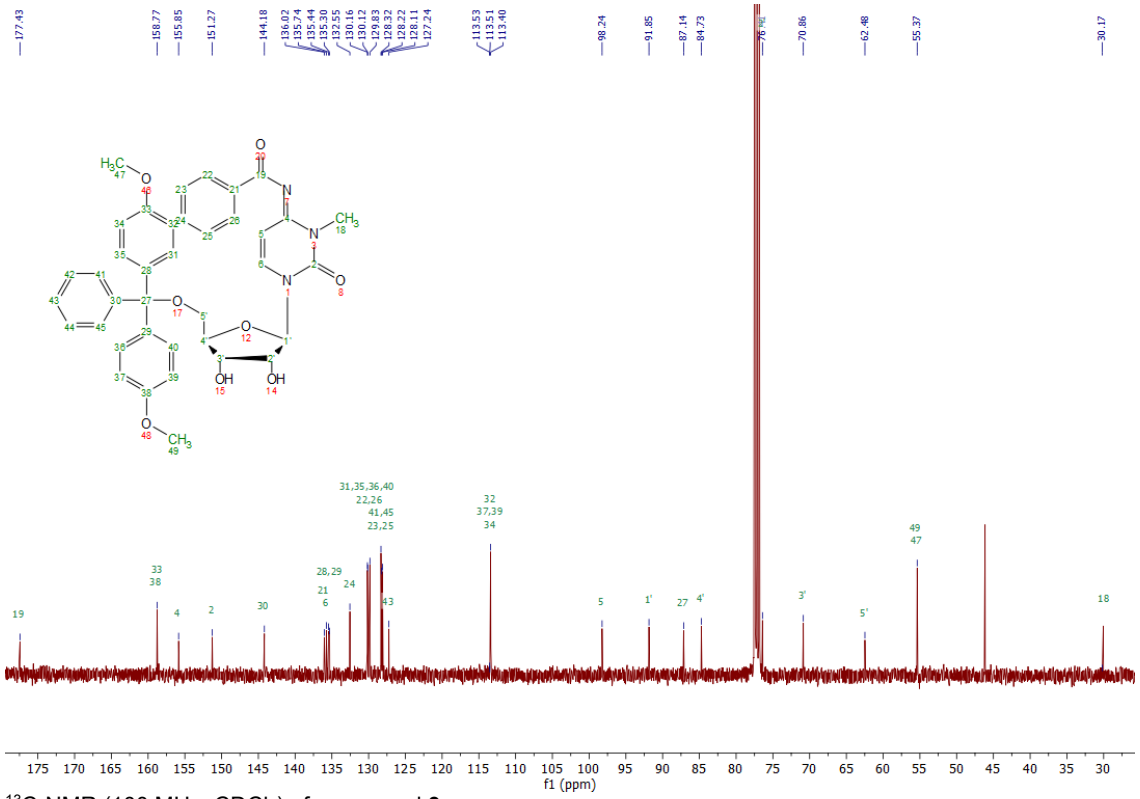
^{1}H-NMR (400 MHz, DMSO-d_6) of compound **2**.



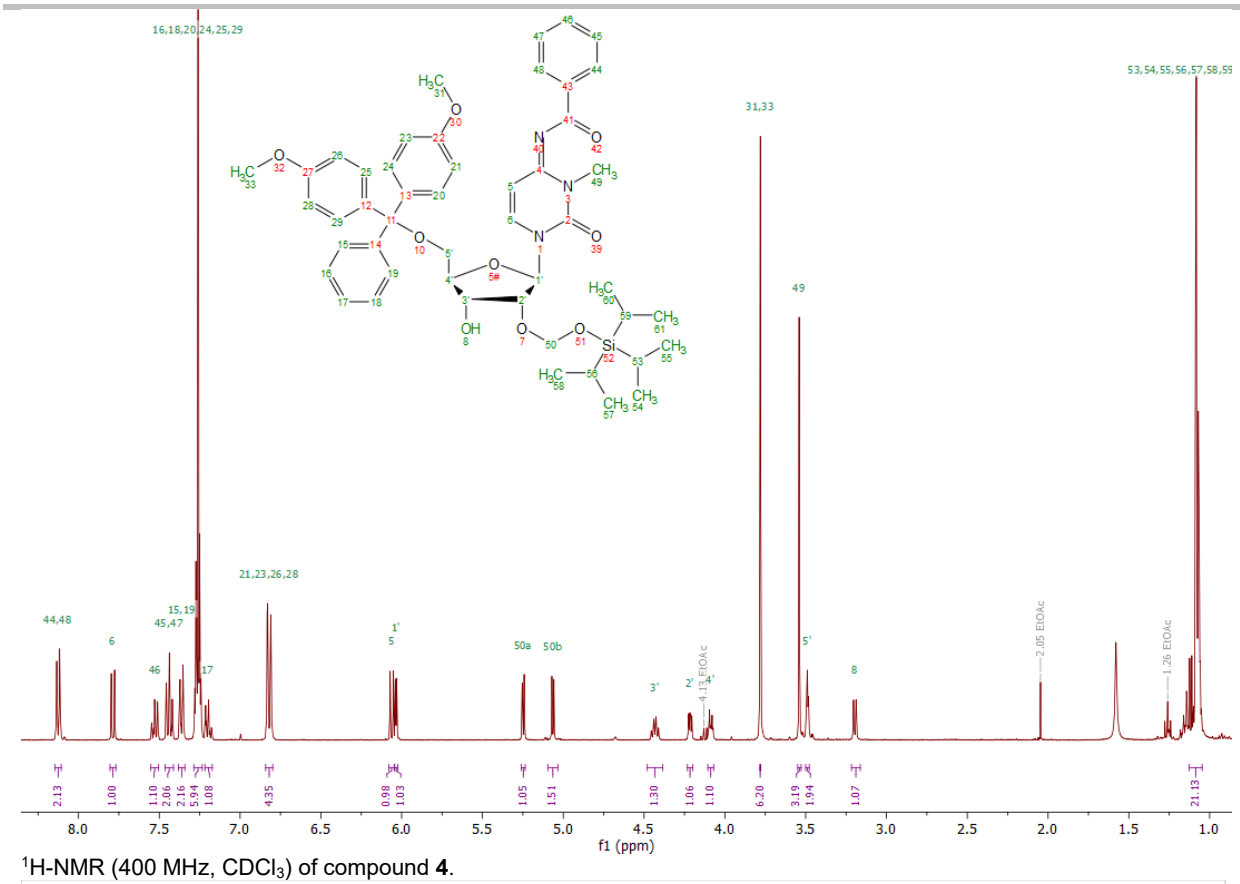
^{13}C-NMR (100 MHz, MeOD) of compound **2**.



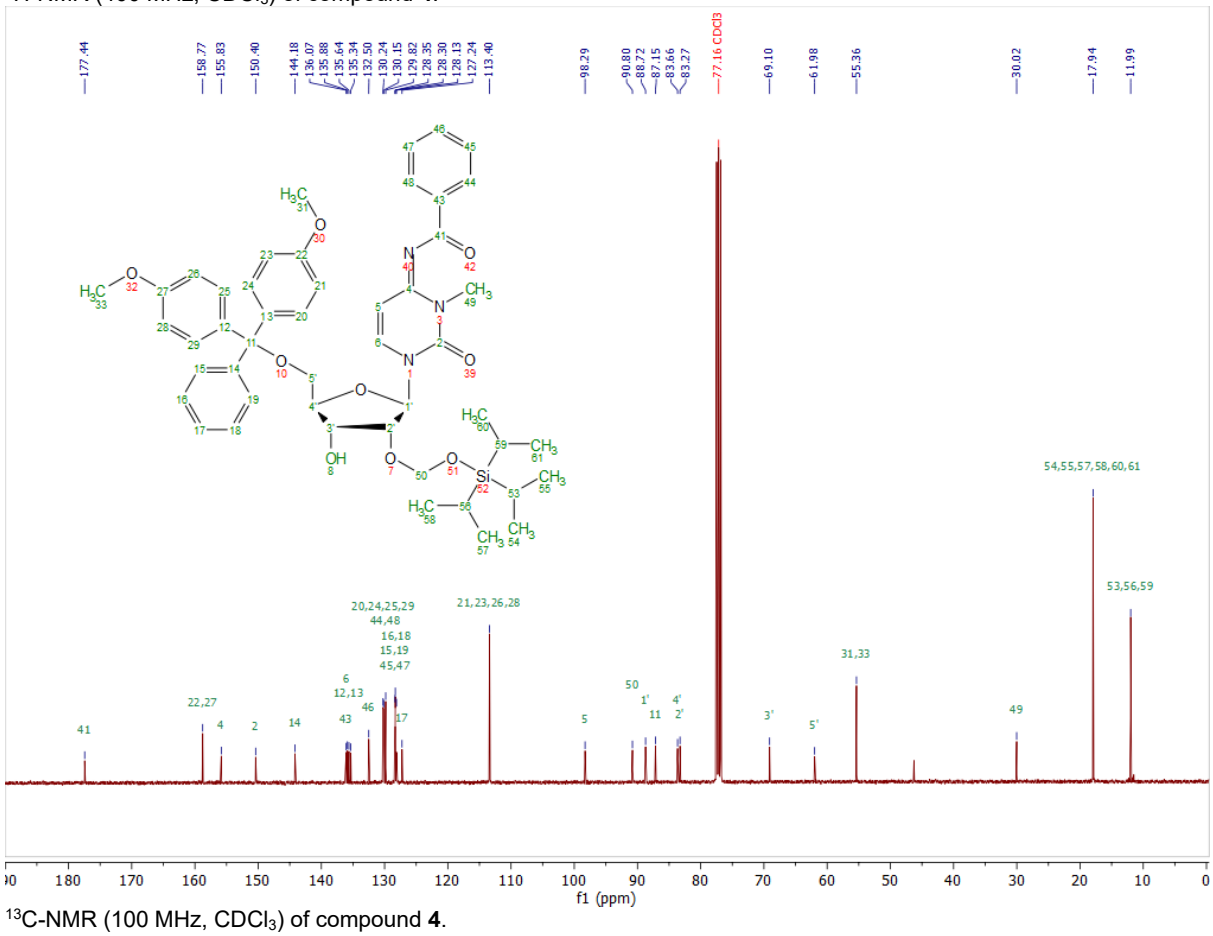
^{1}H-NMR (400 MHz, $CDCl_3$) of compound **3**.



^{13}C-NMR (100 MHz, $CDCl_3$) of compound **3**.



^{1}H-NMR (400 MHz, $CDCl_3$) of compound **4**.



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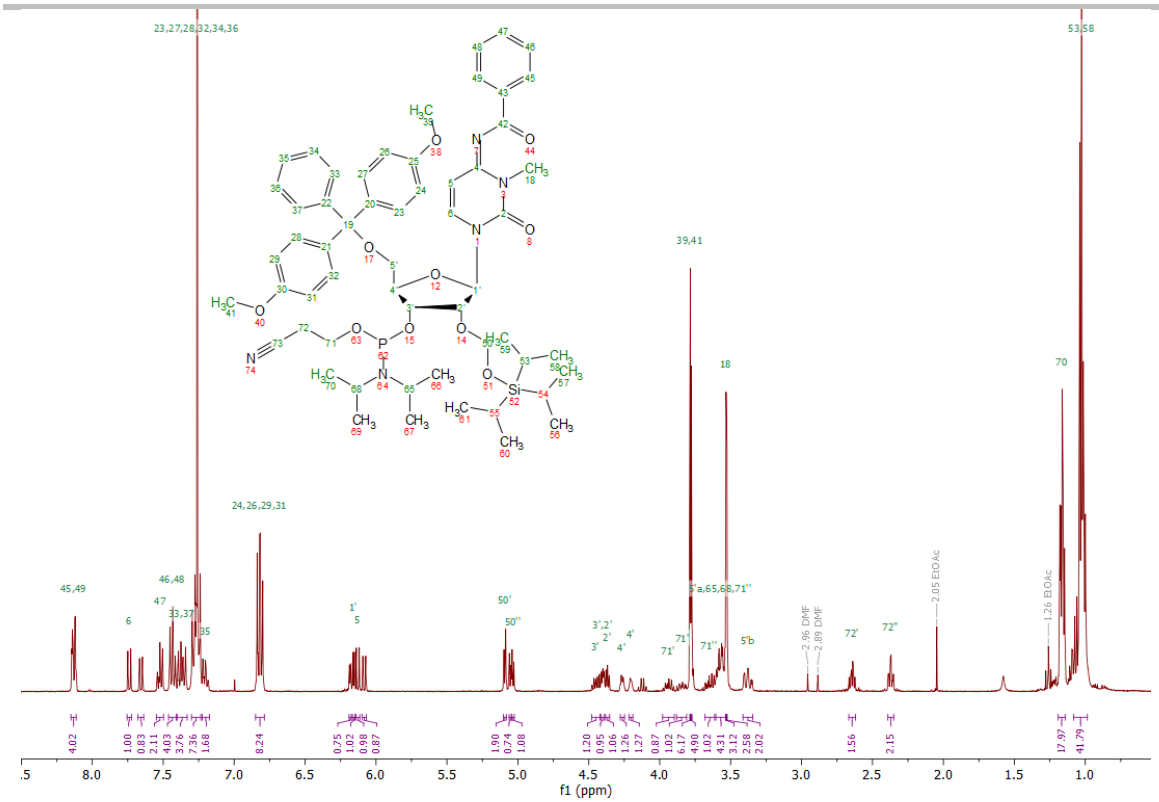
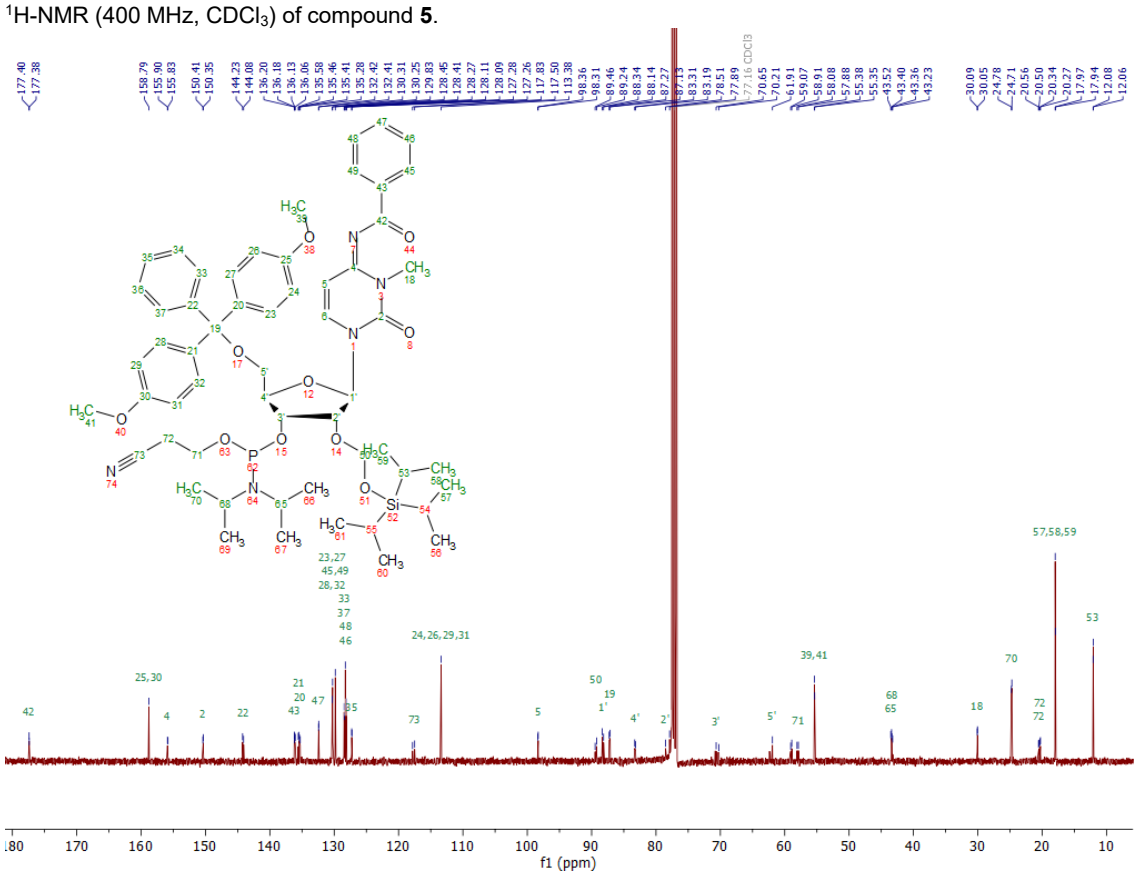
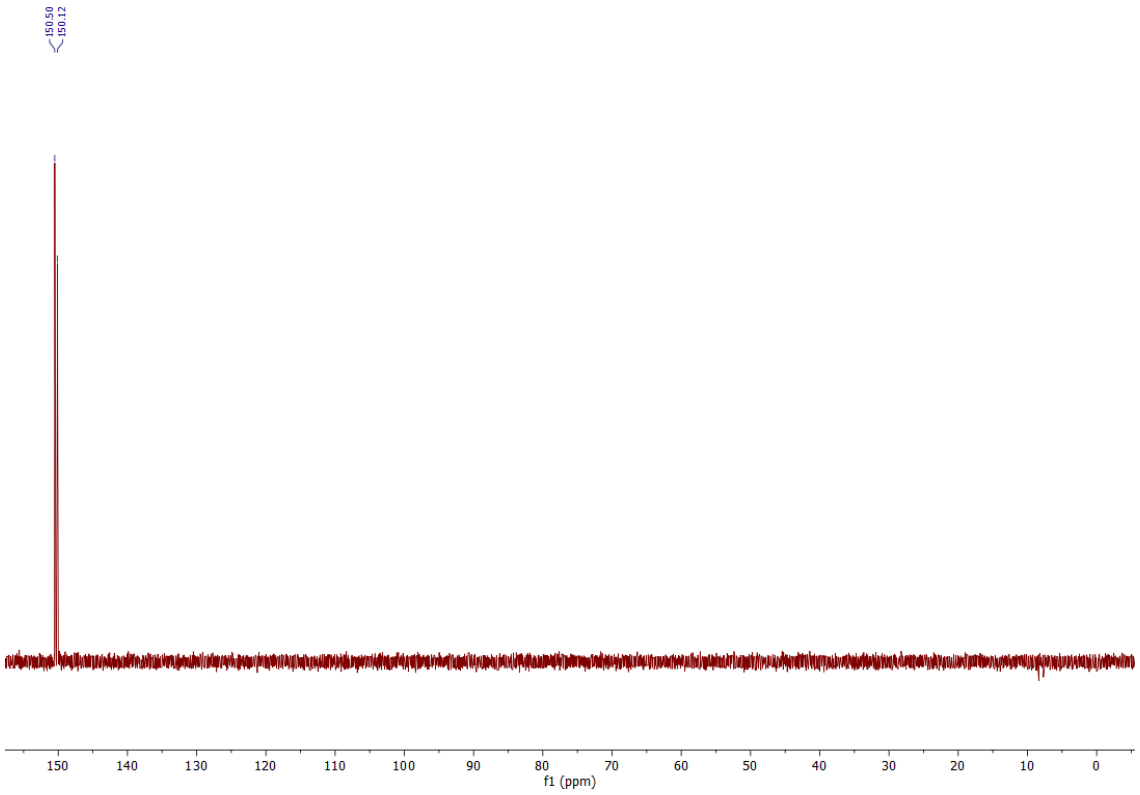


^{1}H-NMR (400 MHz, $CDCl_3$) of compound **5**.



^{13}C-NMR (100 MHz, $CDCl_3$) of compound **5**.



^{31}P-NMR (162 MHz, $CDCl_3$) of compound **5**.

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