DNAzymes for Amine and Peptide Lysine Acylation

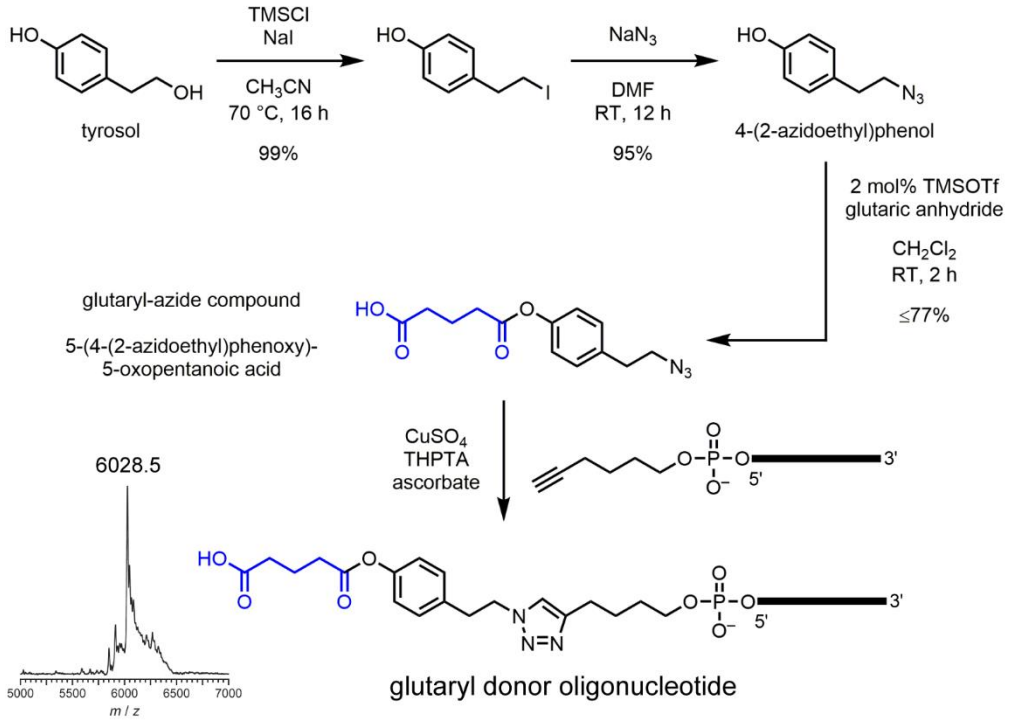
Tianjiong Yao, Jack J. Przybyla, Peter Yeh, Austin M. Woodard, Hannah J. Nilsson, Benjamin M. Brandsen, and Scott K. Silverman*

Department of Chemistry, University of Illinois at Urbana-Champaign,
600 South Mathews Avenue, Urbana, IL 61801, USA

Table of Contents

Additional preparative procedures for oligonucleotides and conjugates

The 5′-aryl ester oligonucleotide (PE and 4FPE) substrates were each prepared by EDC activation and reaction with phenol and 4-fluorophenol. A 40 µL sample containing 3 nmol of 5′-CO_2H oligonucleotide, 50 mM EDC, and 50 mM phenol or 4-fluorophenol was incubated in 100 mM MES, pH 6.0 and 150 mM NaCl at RT for 12 h. The PE or 4FPE substrate was purified by HPLC, using a Shimadzu Prominence instrument with a Phenomenex Gemini-NX C_{18} column (5 µm, 10 × 250 mm), solvent A (20 mM triethylammonium acetate in 50% acetonitrile/50% water, pH 7.0), solvent B (20 mM triethylammonium acetate in water, pH 7.0), and flow rate of 3.5 mL/min. For PE, the gradient was 20% solvent A/80% solvent B at 0 min to 60% solvent A/40% solvent B at 60 min. For 4FPE, the gradient was 25% solvent A/75% solvent B at 0 min to 55% solvent A/45% solvent B at 60 min. The separated sample (see Figure S5 for the HPLC chromatograms) was lyophilized, precipitated with ethanol, and quantified by UV absorbance (A_{260}). The typical isolated yield of PE/4FPE oligonucleotide substrate was 500 pmol (17%).



The glutaryl donor oligonucleotide (Figure 7) was prepared by synthesis of the illustrated glutaryl-azide compound and subsequent CuAAC reaction with 5′-alkyne-C_6-GAAGAGATGGCGACTTCG-3′ (5′-alkyne modifier from Glen Research). 4-(2-Azidoethyl)phenol was prepared from tyrosol in two steps as described.[2] Then, 4-(2-azidoethyl)phenol was esterified using glutaric anhydride and TMSOTf by adaptation of a reported method,[3] forming the glutaryl-azide compound, formally, 5-(4-(2-azidoethyl)phenoxy)-5-oxopentanoic acid. A mixture of 4-(2-azidoethyl)phenol (166 mg, 1.02 mmol) and glutaric anhydride (125 mg, 1.10 mmol) was dissolved in CH_2Cl_2 (2 mL). Neat TMSOTf (3.6 µL, 0.02 mmol) was added, and the sample was stirred at room temperature for 2 h. The reaction was monitored by TLC (CH_2Cl_2:MeOH 6:1); the R_f of 4-(2-azidoethyl)phenol is 0.65, and the R_f of the glutaryl-azide compound is 0.56. Additional CH_2Cl_2 (3 mL) and saturated aqueous $NaHCO_3$ (5 mL) were added. The organic layer was separated, extracted twice with saturated aqueous NaCl (5 mL), and dried over $MgSO_4$. The solvent was evaporated to give the crude glutaryl-azide compound as a yellow oil (216 mg, ≤77% yield; ESI HRMS *m*/*z* calcd. for $[M+Na]^+$ 300.0960, found 300.0957), which was used directly in the CuAAC conjugation reaction with 5′-alkyne DNA to form the glutaryl donor oligonucleotide. For this purpose, 2.2 mg of the glutaryl-azide compound was dissolved in 30 µL of DMSO to make a 250 mM stock solution. The CuAAC conjugation was performed as followed. A 20 µL sample containing 1.37 nmol of 5′-alkyne DNA oligonucleotide, 250

nmol of glutaryl-azide compound (12.5 mM), 40 mM THPTA, 5 mM $CuCl_2$, and 10 mM sodium ascorbate was incubated in 100 mM HEPES, pH 7.0 at room temperature for 30 min. The sample was extracted with phenol/chloroform, precipitated with ethanol, and quantified by UV absorbance (A_{260}). The isolated yield of glutaryl donor oligonucleotide was 1.15 nmol (84%).

Mass spectrometry of oligonucleotides, conjugates, and DNAzyme products

Data were acquired on a Bruker UltrafleXtreme MALDI-TOF mass spectrometer with matrix 3-hydroxypicolinic acid in positive ion mode at the UIUC School of Chemical Sciences Mass Spectrometry Laboratory. All *m*/*z* values are for $[M+H]^+$. Samples were desalted by Millipore C_{18} ZipTip before analysis.

Data for oligonucleotides and conjugates were as follows. For those that bind to the left-hand DNAzyme binding arm, the DNA sequence was 5′-GGATAATACGACTCACTAT-3′. For those that bind to the right-hand DNA binding arm, the DNA sequence was 5′-GAAGAGATGGCGACTTCG-3′.

Oligonucleotides and conjugates that bind to the left-hand DNAzyme binding arm

DNA-C_3-NH_2	*m*/*z* calcd. 5934.9, found 5931.1, Δ = –0.06%
DNA-HEG-AAAKAA	*m*/*z* calcd. 6934.6, found 6938.9, Δ = +0.06%

Oligonucleotides and conjugates that bind to the right-hand DNAzyme binding arm

5′-CO_2H-C_9-DNA	*m*/*z* calcd. 5839.9, found 5840.3, Δ = +0.007%
PE substrate	*m*/*z* calcd. 5916.0, found 5916.8, Δ = +0.01%
4FPE substrate	*m*/*z* calcd. 5934.0, found 5932.5, Δ = –0.03%
5′-alkyne-C_6-DNA	*m*/*z* calcd. 5750.8, found 5747.6, Δ = –0.04%
glutaryl donor oligo	*m*/*z* calcd. 6028.1, found 6028.5, Δ = +0.007% (see image on page S2)

The products of several representative individual DNAzymes were analyzed by MALDI mass spectrometry. Each product was prepared from a 21 μL sample containing 200 pmol of DNA-C_3-NH_2 or DNA-HEG-AAAKAA substrate, 220 pmol of DNAzyme, and 240 pmol of acyl donor substrate. The sample was annealed in 5 mM HEPES, pH 7.5 or CHES, pH 9.0, 15 mM NaCl, and 0.1 mM EDTA by heating at 95 °C for 3 min and cooling on ice for 5 min. The DNAzyme-catalyzed reaction was initiated by bringing the sample to 30 μL total volume containing 70 mM HEPES, pH 7.5, 40 mM $MgCl_2$, 20 mM $MnCl_2$, 1 mM $ZnCl_2$, and 150 mM NaCl or 50 mM CHES, pH 9.0, 40 mM $MgCl_2$, and 150 mM NaCl. The sample was incubated at 37 °C for 48 h, precipitated with ethanol, and purified by 20% PAGE.

DNAzyme products

8FL205 with DNA-C_3-NH_2, PE, pH 7.5	*m*/*z* calcd. 11756.8, found 11751.8, Δ = –0.04%
7FN216 with DNA-C_3-NH_2, 4FPE, pH 7.5	*m*/*z* calcd. 11756.8, found 11753.1, Δ = –0.03%
11HB201 with DNA-HEG-AAAKAA, PE, pH 7.5	*m*/*z* calcd. 12756.5, found 12758.5, Δ = +0.02%
11HC206 with DNA-HEG-AAAKAA, PE, pH 9.0	*m*/*z* calcd. 12756.5, found 12761.5, Δ = +0.04%
11HF210 with DNA-HEG-AAAKAA, 4FPE, pH 9.0	*m*/*z* calcd.12756.5, found 12757.7, Δ = +0.01%
8FL205 with DNA-C_3-NH_2, glutaryl donor, pH 7.5	*m*/*z* calcd. 6049.0, found 6047.2, Δ = –0.03%
8FL219 with DNA-C_3-NH_2, glutaryl donor, pH 7.5	*m*/*z* calcd. 6049.0, found 6048.5, Δ = –0.008%
7FN221 with DNA-C_3-NH_2, glutaryl donor, pH 7.5	*m*/*z* calcd. 6049.0, found 6047.9, Δ = –0.02%

In vitro selection with thioester acyl donor substrate

We performed several sets of in vitro selection experiments using the DNA-C_3-NH_2 or DNA-HEG-AAAKAA nucleophile and a thioester acyl donor electrophile (Figure S1). The 5′-thioester oligonucleotide (5′-glutaryl-S-C_6-DNA-3′) was synthesized by reaction of 5′-thiol DNA with glutaric anhydride (100 mM sodium phosphate, pH 7.4, 60 mM glutaric anhydride, room temperature, 2 h), followed by removal of salts using a 3 kDa centrifugation filter (Amicon) and HPLC purification.[4] Using either the four standard DNA nucleotides or replacing dT with one of several modified nucleotides as we reported for DNAzyme-catalyzed amide hydrolysis,[5] no activity was observed after 10 rounds. These selection experiments used a capture reaction in which the carboxyl group of the glutaryl moiety transferred to the amine in the selection step was subsequently captured using a 5′-amino oligonucleotide, DMT-MM, and a DNA splint.[4]

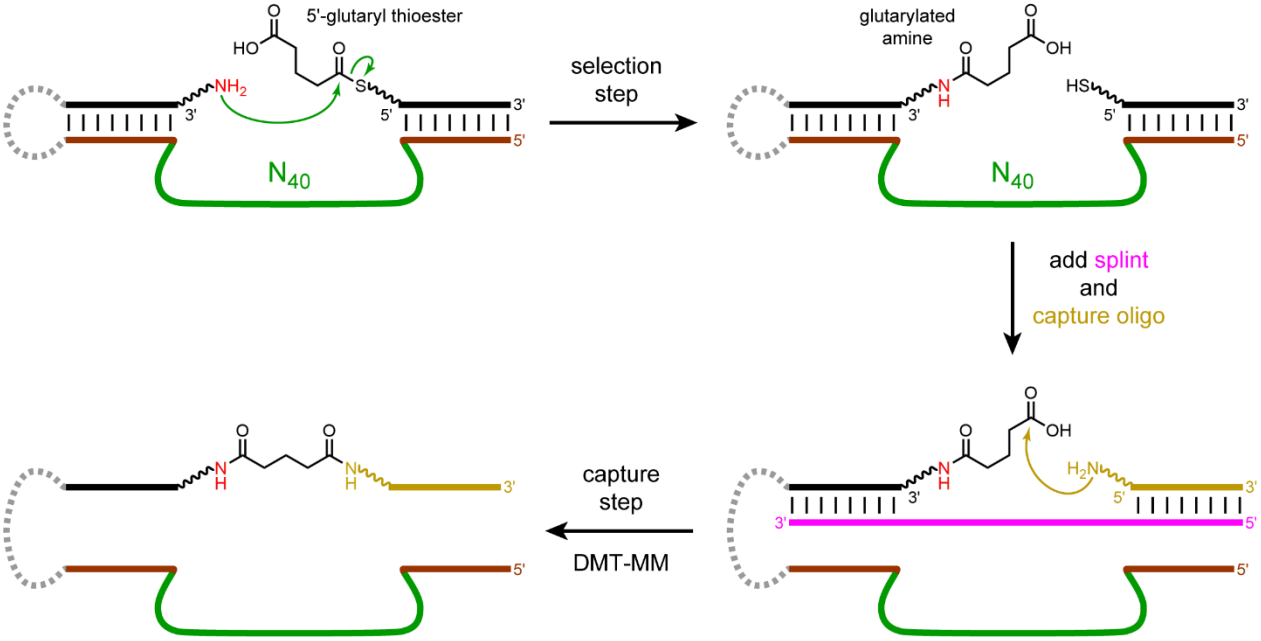


Figure S1. Key selection and capture steps for in vitro selection using a thioester acyl donor.

Procedures for in vitro selection and cloning

The key selection step of each round using a 5′-aryl ester oligonucleotide substrate is shown in Figure 2C and Figure S2. The DNA anchor oligonucleotide sequence was 5′-GGATAATACGACTCACTAT-3′. The 5′-aryl ester oligonucleotide sequence was 5′-GAAGAGATGGCGACTTCG-3′. The random DNAzyme pool was 5′-CGAAGTCGCCATCTCTTC-N_{40}-ATAGTGAGTCGTATTAAGCTGATCCTGATGG-3′. PCR primers were 5′-CGAAGT-CGCCATCTCTTC-3′ (forward primer) and 5′-$(AAC)_{18}$XCCATCAGGATCAGCT-3′, where X is the HEG spacer to stop Taq polymerase (reverse primer). In each round, the ligation step to attach the DNAzyme pool at its 3′-end with the 5′-end of the amine-containing substrate was performed using a DNA splint and T4 DNA ligase. The splint sequence was 5′-ATAGTGAGTCGTATTATCCCCATCAGGATCAGCTTAATACGACTCACTAT-3′.

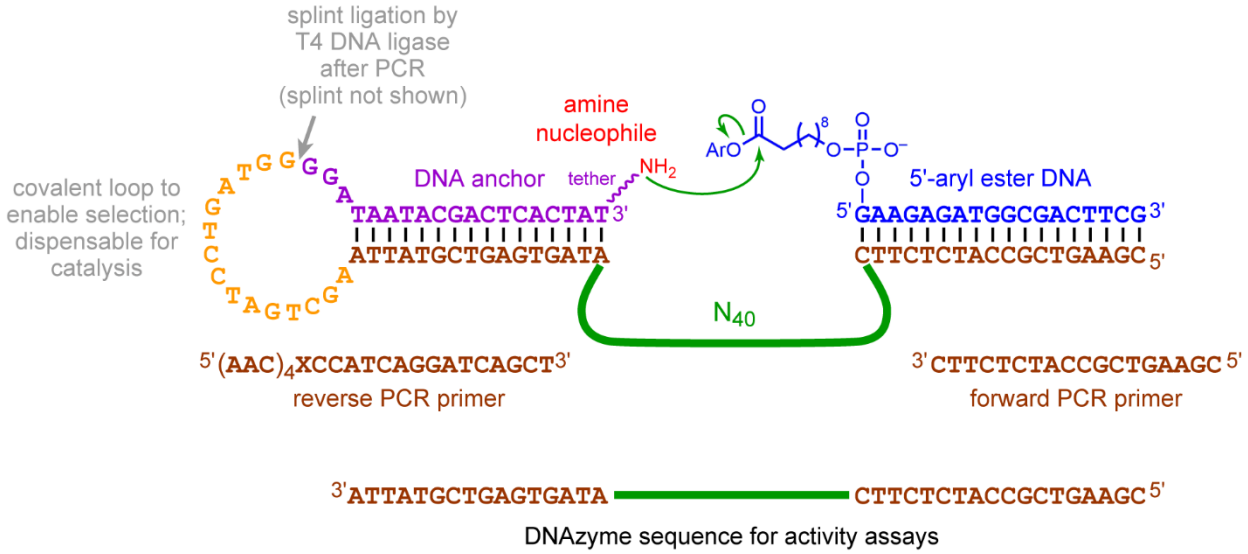


Figure S2. Nucleotide details of the in vitro selection experiments with a 5′-aryl ester substrate. The complete structures of the DNA-anchored amine substrates are in Figure 2A.

Procedure for ligation step in round 1. A 25 µL sample containing 600 pmol of DNA pool, 750 pmol of DNA splint, and 900 pmol of DNA-C_3-NH_2 or DNA-HEG-AAAKAA substrate was annealed in 5 mM Tris, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA by heating at 95 °C for 3 min and cooling on ice for 5 min. To this solution was added 3 µL of 10× T4 DNA ligase buffer (400 mM Tris, pH 7.8, 100 mM $MgCl_2$, 100 mM DTT, and 5 mM ATP) and 2 µL of 5 U/µL T4 DNA ligase (Thermo Fisher). The sample was incubated at 37 °C for 12 h and separated by 8% PAGE.

Procedure for ligation step in subsequent rounds. A 17 µL sample containing the PCR-amplified DNA pool (~5–10 pmol), 30 pmol of DNA splint, and 50 pmol of DNA-C_3-NH_2 or DNA-HEG-AAAKAA substrate was annealed in 5 mM Tris, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA by heating at 95 °C for 3 min and cooling on ice for 5 min. To this solution was added 2 µL of 10× T4 DNA ligase buffer and 1 µL of 1 U/µL T4 DNA ligase. The sample was incubated at 37 °C for 12 h and separated by 8% PAGE.

Procedure for selection step in round 1. Each selection experiment was initiated with 200 pmol of the ligated pool. A 20 µL sample containing 200 pmol of ligated pool and 300 pmol of 5′-aryl ester DNA was annealed in 5 mM HEPES, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA or 5 mM CHES, pH 9.0, 15 mM NaCl, and 0.1 mM EDTA by heating at 95 °C for 3 min and cooling on ice for 5 min. The selection reaction was initiated by bringing the sample to 40 µL total volume containing 70 mM HEPES, pH 7.5, 40 mM $MgCl_2$, 20 mM $MnCl_2$, 1 mM $ZnCl_2$, and 150 mM NaCl or 50 mM CHES, pH 9.0, 40 mM $MgCl_2$, and 150 mM NaCl. The Mn^{2+} was added from a 10× stock solution containing 200 mM $MnCl_2$. The Zn^{2+} was added from a 10× stock solution containing 10 mM $ZnCl_2$, 20 mM HNO_3, and 200 mM HEPES at pH 7.5; this stock solution was freshly prepared from a 100× stock of 100 mM $ZnCl_2$ in 200 mM HNO_3. The metal ion stocks were added last to the final sample. The sample was incubated at 37 °C for 16 h and separated by 8% PAGE.

Procedure for selection step in subsequent rounds. A 14 µL sample containing the ligated pool and 30 pmol of 5′-aryl ester DNA was annealed in 5 mM HEPES, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA or 5 mM CHES, pH 9.0, 15 mM NaCl, and 0.1 mM EDTA by heating at 95 °C for 3 min and cooling on ice for 5 min. The selection reaction was initiated by bringing the sample to 20 µL total volume containing 70 mM HEPES, pH 7.5, 40 mM $MgCl_2$, 20 mM $MnCl_2$, 1 mM $ZnCl_2$, and 150 mM NaCl or 50 mM CHES, pH 9.0, 40 mM $MgCl_2$, and 150 mM NaCl. The sample was incubated at 37 °C for 16 h and separated by 8% PAGE.

Procedure for PCR. In each selection round, two PCR reactions were performed, 10-cycle PCR followed by 30-cycle PCR. First, a 100 µL sample was prepared containing the PAGE-purified selection product, 200 pmol of forward primer, 50 pmol of reverse primer, 20 nmol of each dNTP, and 10 µL of 10× Taq polymerase buffer [1× = 20 mM Tris-HCl, pH 8.8, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, and 0.1% Triton X-100]. This sample was cycled 10 times according to the following PCR program: 94 °C for 2 min, 10× (94 °C for 30 s, 47 °C for 30 s, 72 °C for 30 s), 72 °C for 5 min. Taq polymerase was removed by phenol/chloroform extraction. Second, a 50 µL sample was prepared containing 1 µL of the 10-cycle PCR product, 100 pmol of forward primer, 25 pmol of reverse primer, 10 nmol of each dNTP, 20 µCi of α-^{32}P-dCTP (800 Ci/mmol), and 5 µL of 10× Taq polymerase buffer. This sample was cycled 30 times according to the following PCR program: 94 °C for 2 min, 30× (94 °C for 30 s, 47 °C for 30 s, 72 °C for 30 s), 72 °C for 5 min. Samples were separated by 8% PAGE.

Cloning and screening of individual DNAzymes. The PCR primers used for cloning were 5′-CGAA-GTCGCCATCTCTTC-3′ (forward primer; same as in selection) and 5′-TAATTAATTAATTACCCATCAGGATCA-GCT-3′ (reverse primer). The 10-cycle PCR product from the appropriate selection round was diluted 10^3-fold. A 50 µL sample was prepared containing 1 µL of the diluted 10-cycle PCR product from the appropriate selection round, 100 pmol of forward cloning primer, 25 pmol of reverse cloning primer, 10 nmol of each dNTP, and 5 µL of 10× Taq polymerase buffer. This sample was cycled 30 times according to the following PCR program: 94 °C for 2 min, 30× (94 °C for 30 s, 47 °C for 30 s, 72 °C for 30 s), 72 °C for 5 min. The sample was separated by 1% agarose gel and extracted using a GeneJET Gel Extraction Kit (Fermentas). The extracted product was quantified by absorbance (A_{260}) and diluted to 5–10 ng/µL. A 4 µL portion of the diluted PCR product was inserted into the pCR2.1-TOPO vector using a TOPO TA cloning kit (Life Technologies). Individual *E. coli* colonies harboring plasmids with inserts were identified by blue-white screening and grown in LB/amp media. Miniprep DNA was prepared using a GeneJET Plasmid Miniprep Kit (Fermentas) and screened for properly sized inserts by EcoRI digestion and agarose gel analysis. Before sequencing, assays of individual DNAzyme clones were performed with PAGE-purified DNA strands prepared by PCR from the miniprep DNA, using the single-turnover assay procedure described in the main text.

In vitro selection progressions with DMTE and TFPE acyl donor substrates

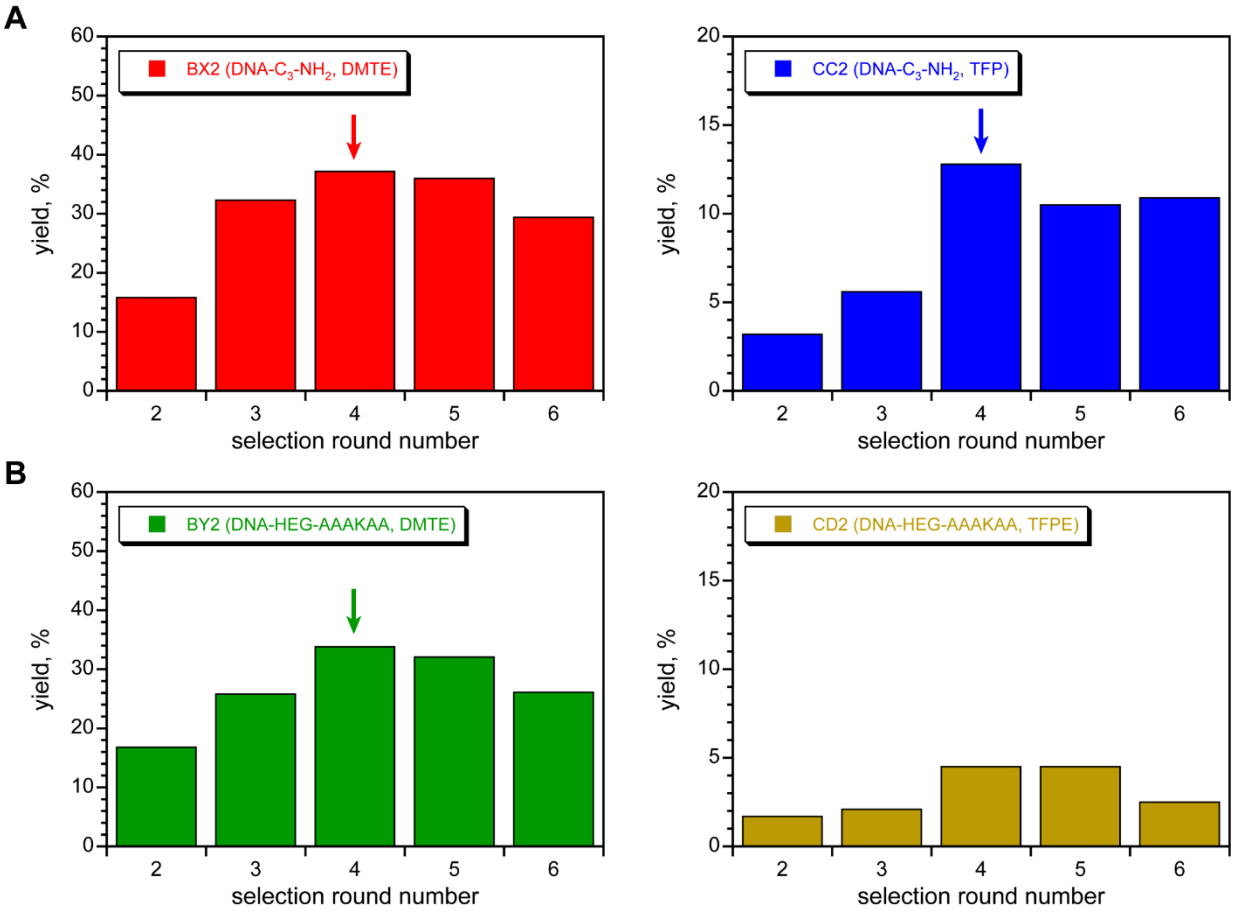


Figure S3. Progressions of the in vitro selection experiments using the DMTE and TFPE acyl donors. (A) The BX2 and CC2 selections, with DNA-C_3-NH_2 and DMTE or TFPE acyl donor, respectively. Incubation conditions: 100 mM MOPS, pH 7.0, and 150 mM Na^+ at 37 °C for 2 h with 1 mM Zn^{2+} alone (DMTE; BX2 selection) or 40 mM Mg^{2+}, 20 mM Mn^{2+}, and 1 mM Zn^{2+} (TFPE; CC2 selection). (B) The BY2 and CD2 selections, with DNA-HEG-AAAKAA and DMTE or TFPE acyl donor, respectively. Incubation conditions as in panel A. All metal ions were provided as chloride salts. Arrows mark the cloned rounds. The second selection in panel B, with DNA-HEG-AAAKAA and TFPE acyl donor, was not cloned because of the relatively low yield. In all cases, the round 1 yield could not be quantified because nonradiolabeled pool was used.

Sequences of individual DNAzymes

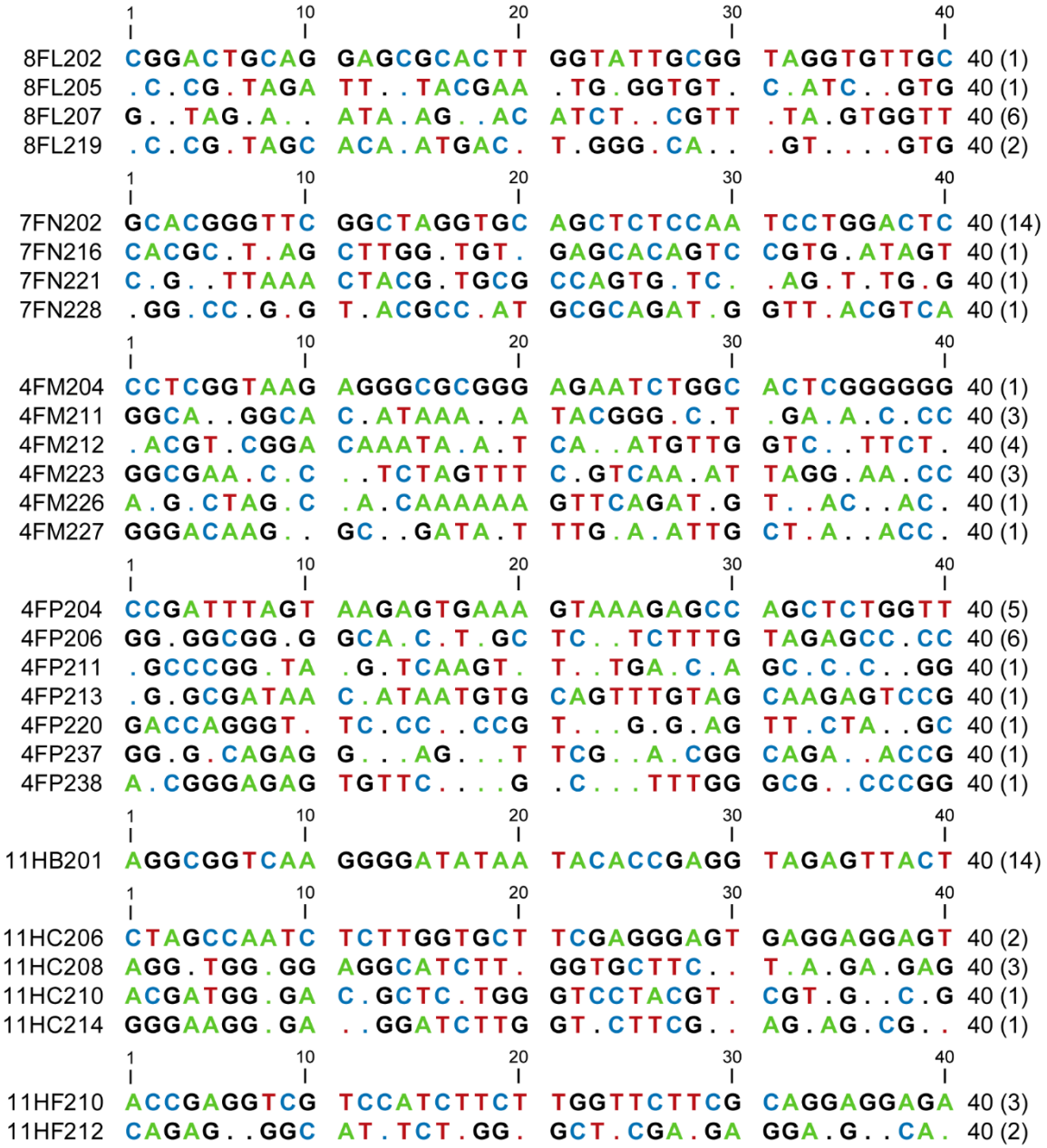


Figure S4. Sequences of the DNAzymes identified in this study. In all cases, see main text for details of the selection process, including incubation conditions. Only the initially random (N_{40}) sequences are shown. All DNAzymes were used as 5′-CGAAGTCGCCATCTCTTC-N_{40}-ATAGTGAGTCGTATTA-3′. In each alignment, a dot denotes conservation, i.e., the same nucleotide as in the uppermost sequence; a dash denotes a gap. On the far right is shown the sequence length and (in parentheses) the number of times that the particular sequence was found during cloning. No common or conserved motifs were apparent either within or between sequence families. See Table S2 and Figure S7 for predicted secondary structures.

HPLC chromatograms for purification of the PE and 4FPE oligonucleotide substrates

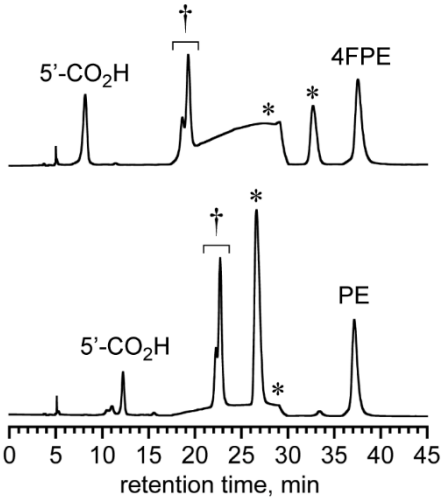


Figure S5. HPLC chromatograms (260 nm detection) for purification of the PE and 4FPE oligonucleotide substrates. The HPLC procedures are provided at the start of the ESI. Note that the gradients were different for the two purifications. The peaks assigned as the PE and 4FPE substrates were validated by MALDI mass spectrometry, as were the peaks assigned to the 5′-CO_2H DNA oligonucleotide (precursor to PE or 4FPE substrate). The peaks marked with daggers (†) are oligonucleotides, as assessed from their full UV spectra (PDA detector), and are likely the *N*-acylurea dead-end products formed by rearrangement after EDC activation to form the transient *O*-acylisourea products. The peaks marked with asterisks (*) are small-molecule compounds rather than oligonucleotides, as assessed from their full UV spectra (PDA detector).

Assays for PE and 4FPE acyl donor stability and background reactivity

We sought to identify incubation conditions in which each acyl donor substrate satisfies two key criteria: (a) stability to the extent of at least 25% acyl donor remaining intact (i.e., unhydrolyzed) at the end of the incubation period in the absence of amine nucleophile, and ideally higher than 25% remaining intact; and (b) observable but modest background amine acylation reactivity (amide formation) in the presence of the amine nucleophile. We chose criterion (a) because this extent of stability, under the inherently single-turnover arrangement of in vitro selection (i.e., each candidate DNAzyme molecule is persistently Watson-Crick bound to a single acyl donor oligonucleotide molecule for the duration of the reaction; Figure 2C), means that a substantial fraction of the population's DNA sequences have the opportunity to catalyze amine acylation at some point during the incubation and therefore survive the selection round. We chose criterion (b) because the negative outcome with the thioester selections implies that beginning with absolutely no observable background reactivity could result in the inability of any DNAzyme sequences to emerge from the selection process. That is, even appreciable rate enhancement relative to a very low background reactivity could still result in undetectable DNAzyme-catalyzed reactivity of the population after enrichment of catalytically active sequences through many selection rounds.

For each of the PE and 4FPE acyl donor substrates, we identified suitable incubation conditions that satisfy both criteria (a) and (b), as shown in Table S1. We did not yet know whether the most appropriate incubation conditions for each substrate would involve relatively high or low background reactivity within the explored range. Instead, the optimum background reactivity for identifying DNAzymes, as dictated by choice of incubation conditions (pH, metal ions, and time), would have to be determined empirically through the in vitro selection process itself. Based on the tabulated data, we chose two particular incubation conditions for each acyl donor substrate: lower pH of 7.5 (70 mM HEPES, pH 7.5, 40 mM $MgCl_2$, 20 mM $MnCl_2$, 1 mM $ZnCl_2$, and 150 mM NaCl at 37 °C for 16 h) and higher pH of 9.0 (50 mM CHES, pH 9.0, 40 mM $MgCl_2$, and 150 mM NaCl at 37 °C for 16 h). Each of Mg^{2+}, Mn^{2+}, and Zn^{2+} was included at pH 7.5 on the basis of our many prior successful DNAzyme selection efforts using these metal ions at pH 7.5.

However, at pH 9.0 only Mg^{2+} can be included, because Mn^{2+} oxidizes and Zn^{2+} precipitates at this higher pH.

Per Table S1 the evaluated incubation conditions lead to background yields up to 3.5%, where these values are uncorrected for partial hydrolysis of the acyl donor during the incubation period. For example, considering entry 8, in the limiting case that only 74% of intact acyl donor is present for the entire duration of the incubation period (i.e., all of the 26% of hydrolysis occurs immediately at the start of the incubation time), the observed background yield of 3.5% would be relative to a maximum possible background yield of 74%, for a corrected background yield of 4.7%. However, it is unlikely that most of the loss of acyl donor occurs immediately upon starting the incubation period. Instead, as indicated by comparing entries 7 and 8, the loss of acyl donor is more gradual, and the properly corrected background yield would lie somewhere between 3.5% and 4.7%. Because, for example, a background yield of 5% (meaning that 5% of all sequences survive the selection round regardless of their catalytic ability) limits the per-round enrichment to 20-fold, which is still substantial, we decided that the range of conditions represented by the four even-numbered entries in Table S1 (all with incubation time of 16 h rather than 2 h) were suitable for performing in vitro selection.

For the intact acyl donor assays of Table S1, a 14 µL sample containing 1 pmol of 3′-^{32}P-radiolabeled acyl donor oligonucleotide (PE or 4FPE substrate) was annealed in 5 mM HEPES, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA or 5 mM CHES, pH 9.0, 15 mM NaCl, and 0.1 mM EDTA by heating at 95 °C for 3 min and cooling on ice for 5 min. The assay was initiated by bringing the sample to 20 µL total volume containing 70 mM HEPES, pH 7.5, 40 mM $MgCl_2$, 20 mM $MnCl_2$, 1 mM $ZnCl_2$, and 150 mM NaCl or 50 mM CHES, pH 9.0, 40 mM $MgCl_2$, and 150 mM NaCl. The sample was incubated at 37 °C for 2 h or 16 h and separated by 20% PAGE. The extent of PE/4FPE hydrolysis was calculated from the the bands corresponding to PE/4FPE and 5′-CO_2H oligonucleotides.

For the background yield assays of Table S1, a 14 µL sample containing 0.5 pmol of 5′-^{32}P-radiolabeled DNA-C_3-NH_2, 10 pmol of splint oligonucleotide (5′-CGAAGTCGCCATCTCTTCATAGTGAGTCGTATTATCC-3′), and 20 pmol of nonradiolabeled PE or 4FPE substrate was annealed in 5 mM HEPES, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA or 5 mM CHES, pH 9.0, 15 mM NaCl, and 0.1 mM EDTA by heating at 95 °C for 3 min and cooling on ice for 5 min. The background reaction was initiated by bringing the sample to 20 µL total volume containing 70 mM HEPES, pH 7.5, 40 mM $MgCl_2$, 20 mM $MnCl_2$, 1 mM $ZnCl_2$, and 150 mM NaCl or 50 mM CHES, pH 9.0, 40 mM $MgCl_2$, and 150 mM NaCl. The sample was incubated at 37 °C for 2 h or 16 h and separated by 20% PAGE. The background yield was calculated from the bands corresponding to DNA-C_3-NH_2 and acylation product.

Table S1. Determining the optimal incubation conditions for the PE and 4FPE acyl donors, considering both the stability toward hydrolysis and the uncatalyzed, splinted background reactivity with the DNA-C_3-NH_2 substrate

entry[a]	substrate	pH	metal ions	time	intact acyl donor, %[b]	uncorrected background yield, %[c]
1	PE	7.5	Mg^{2+}/$Mn^{2+}$$Zn^{2+}$	2 h	92.4 ± 1.5	0.1
2	PE	7.5	Mg^{2+}/$Mn^{2+}$$Zn^{2+}$	16 h	81.5 ± 0.2	0.3
3	PE	9.0	Mg^{2+}	2 h	94.2 ± 0.7	0.7
4	PE	9.0	Mg^{2+}	16 h	80.4 ± 5.1	2.4
5	4FPE	7.5	Mg^{2+}/$Mn^{2+}$$Zn^{2+}$	2 h	93.0 ± 0.3	0.02
6	4FPE	7.5	Mg^{2+}/$Mn^{2+}$$Zn^{2+}$	16 h	77.7 ± 0.3	0.6
7	4FPE	9.0	Mg^{2+}	2 h	93.3 ± 0.2	0.5
8	4FPE	9.0	Mg^{2+}	16 h	74.1 ± 4.5	3.5

[a] Incubation conditions: 70 mM HEPES, pH 7.5, 40 mM $MgCl_2$, 20 mM $MnCl_2$, 1 mM $ZnCl_2$, and 150 mM NaCl at 37 °C, or 50 mM CHES, pH 9.0, 40 mM $MgCl_2$, and 150 mM NaCl at 37 °C. In vitro selection experiments were subsequently performed using each of the two acyl donors at each of the two pH values, with incubation time of 16 h (i.e., all four even-numbered entries). [b] Intact acyl donor values were determined by PAGE. Each value is the mean of two or three independent assays, with the error as standard deviation (n = 3; entries 4 and 8) or half of the range (n = 2; all other entries). [c] Uncorrected background yield % values were determined by PAGE using the general assay procedure and a complementary DNA splint rather than a DNAzyme. Each value is the mean of n = 3–6 independent assays (n = 2 for entries 5 and 7), with standard deviation (or half of range) ≤0.2%.

Kinetic plots for DNAzymes with DNA-C_3-NH_2 and PE or 4FPE substrates

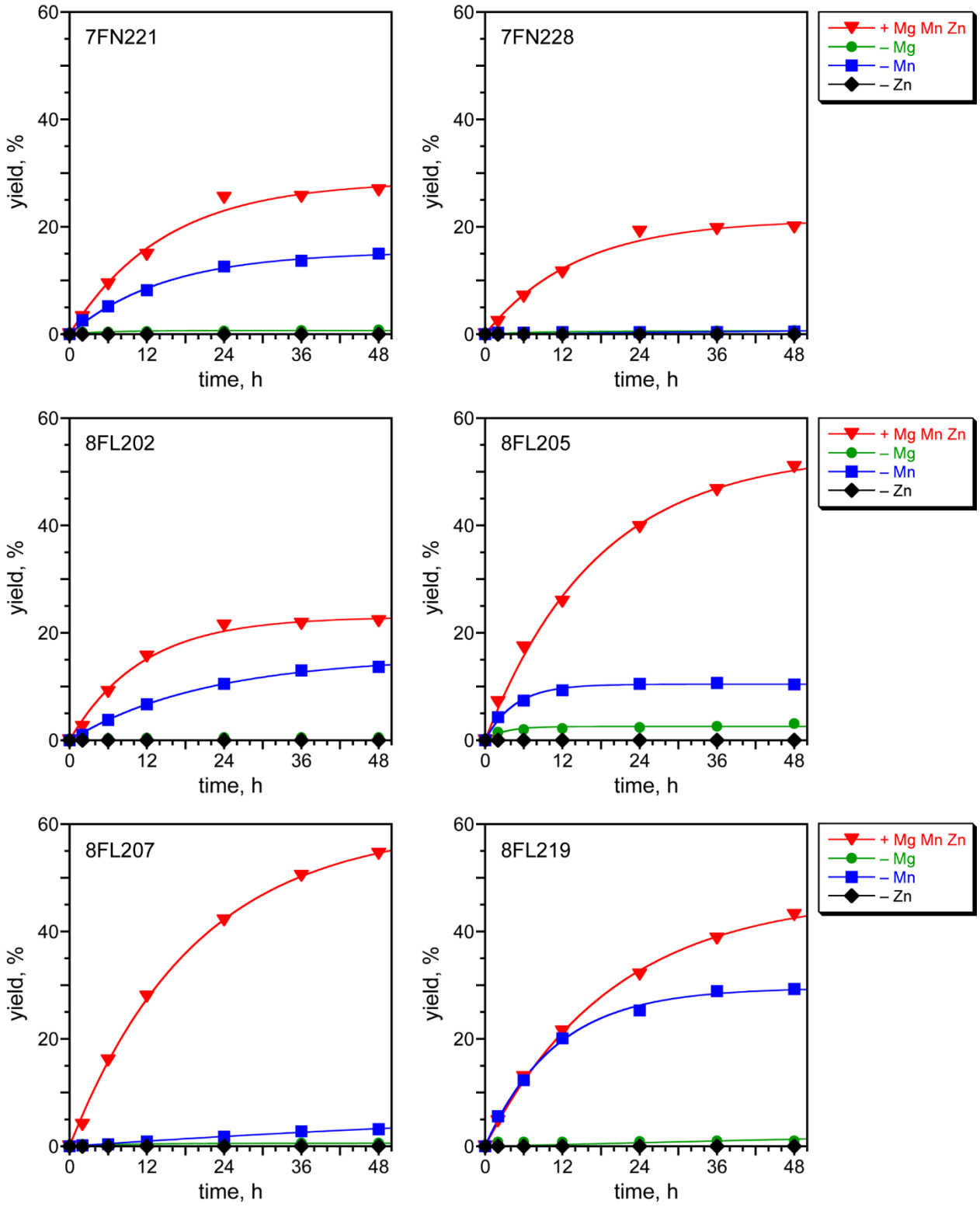


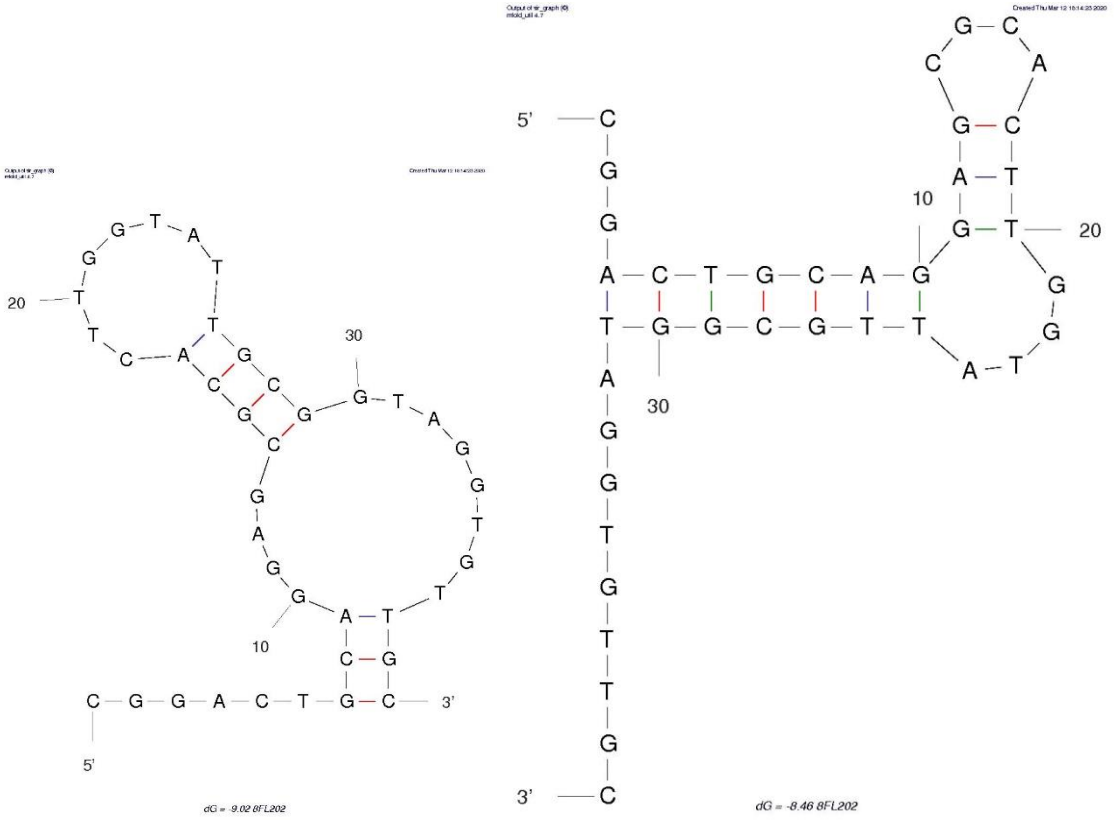
Figure S6. Kinetic plots for DNAzymes with DNA-C_3-NH_2 and PE or 4FPE substrates. See Figure 4 for details and plots for 7FN216 and 7FN202. k_{obs} values (h^{-1}, n = 1, with Mg^{2+}/Mn^{2+}/Zn^{2+}): 7FN221, 0.069; 7FN228, 0.071; 8FL202, 0.090; 8FL205, 0.058; 8FL207, 0.051; 8FL219, 0.050; background with complementary DNA splint and PE ester (k_{bkgd} for 8FL2 DNAzymes),1.3×10^{-4} h^{-1} Rate enhancements (k_{obs}/k_{bkgd}), in same DNAzyme order: 410, 420, 690, 450, 390, 380.

DNAzyme secondary structure predictions using mfold

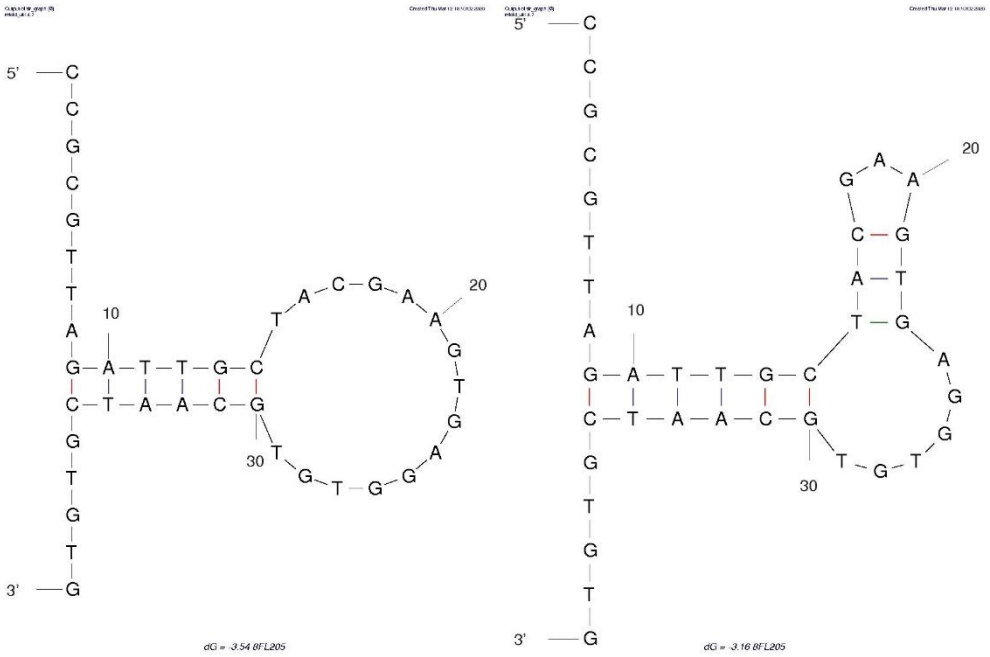
DNAzyme	number of structures	lowest ΔG, kcal/mol	DNAzyme	number of structures	lowest ΔG, kcal/mol
8FL202	**2**	**–9.0**	4FP204	2	–2.2
8FL205	**3**	**–3.5**	4FP206	6	–4.0
8FL207	**2**	**–5.5**	4FP211	1	–4.6
8FL219	**1**	**–8.4**	4FP213	6	–1.0
7FN202	**3**	**–3.8**	4FP220	3	–3.1
7FN216	**4**	**–3.0**	4FP237	1	–2.7
7FN221	**7**	**–2.2**	4FP238	4	–3.3
7FN228	**1**	**–3.9**	**11HB201**	**2**	**–2.5**
4FM204	1	–4.1	**11HC206**	**4**	**–2.3**
4FM211	1	–4.2	11HC208	1	–2.9
4FM212	2	–3.9	11HC210	4	–3.6
4FM223	3	–1.5	11HC214	1	–2.2
4FM226	5	–1.1	**11HF210**	**5**	**–1.1**
4FM227	4	–0.3	11HF212	5	–2.0

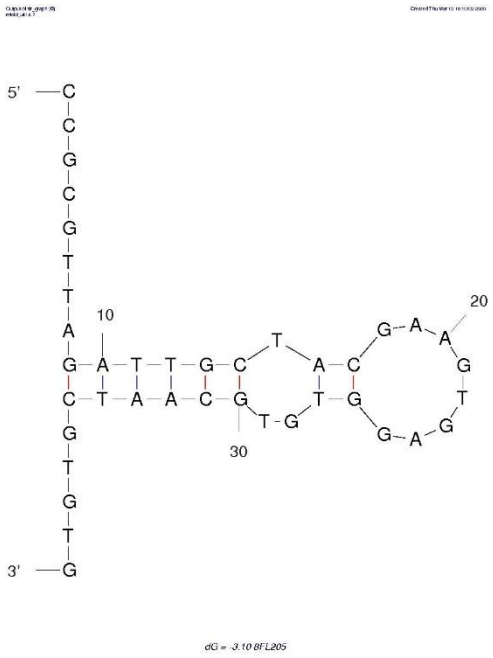
Table S2. Summary of mfold predicted secondary structures of all 28 new DNAzymes reported in this study. The default settings were used for the sequences of the initially random N_{40} regions with the DNA Folding Form at http://unafold.rna.albany.edu/?q=mfold, adjusted to 150 mM Na^+ and 40 mM Mg^{2+}. The boldface entries are those for which all predicted secondary structures are shown in Figure S7.

(A) 8FL202

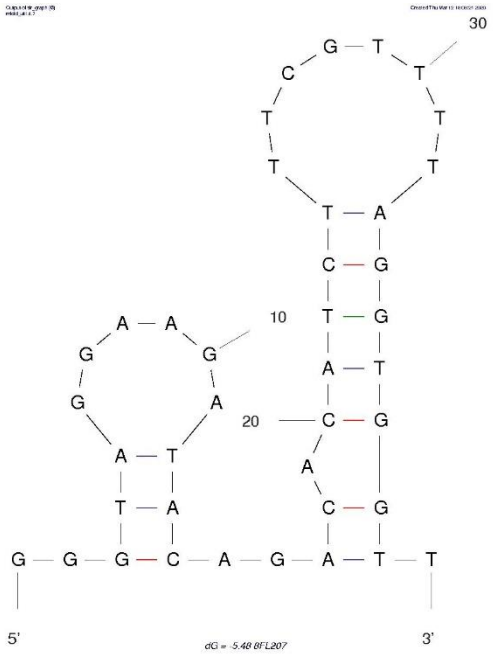


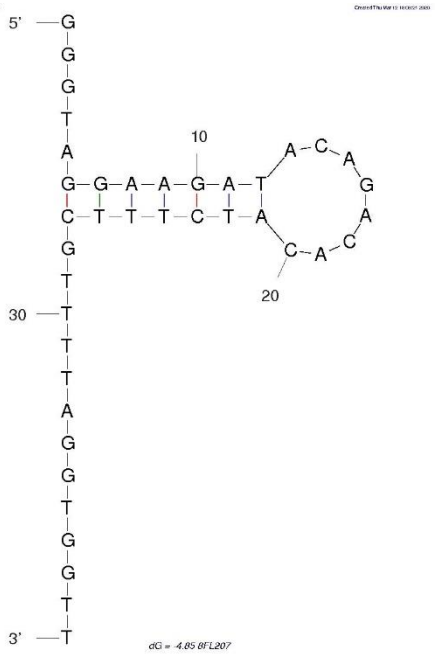
(B) 8FL205



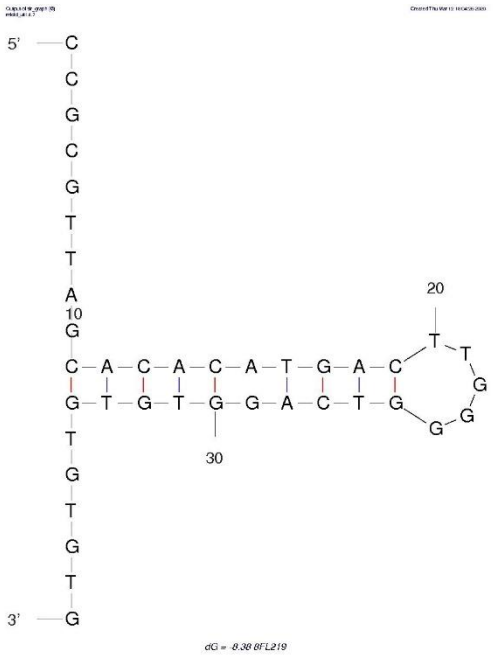

5'
3'
10
20
30
dG = -3.10 8FL205

(C) 8FL207

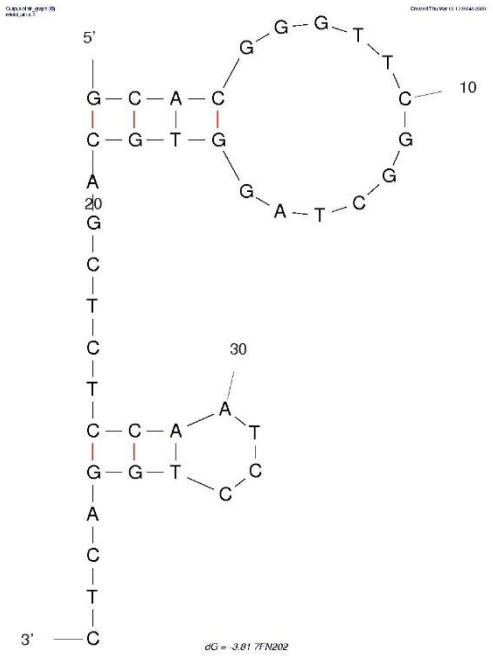

30
10
20
5'
3'
dG = -5.48 8FL207

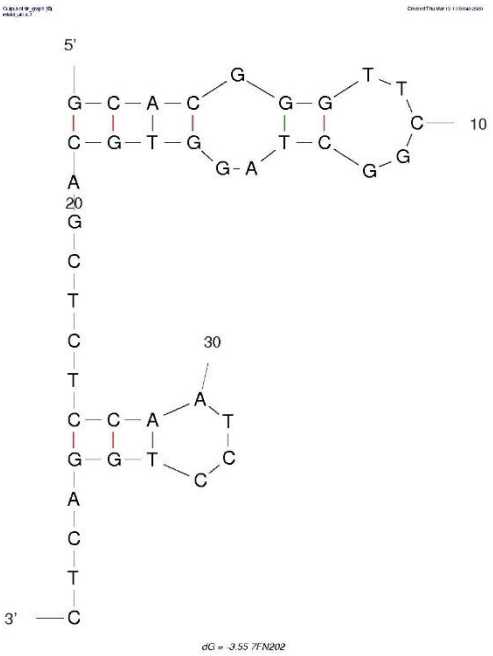

5'
10
20
30
3'
dG = -4.85 8FL207

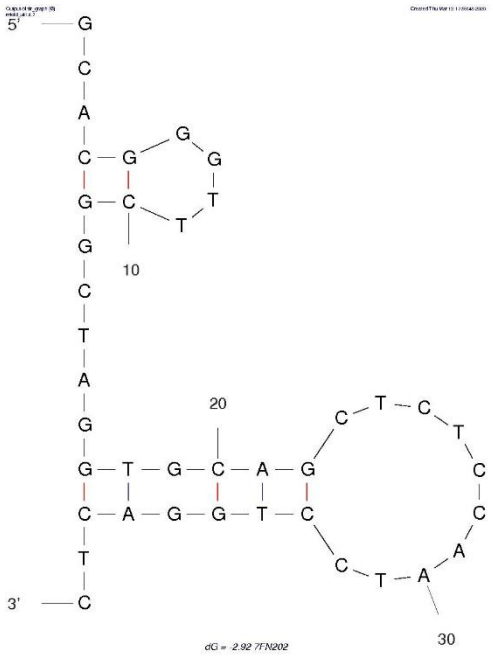
(D) 8FL219


5'
C
C
G
C
G
T
T
A
10
G
C
A
C
A
C
A
T
G
A
C
G
T
G
T
G
G
A
C
T
G
T
T
G
G
G
20
30
T
G
T
G
T
G
3'
dG = -8.38 8FL219

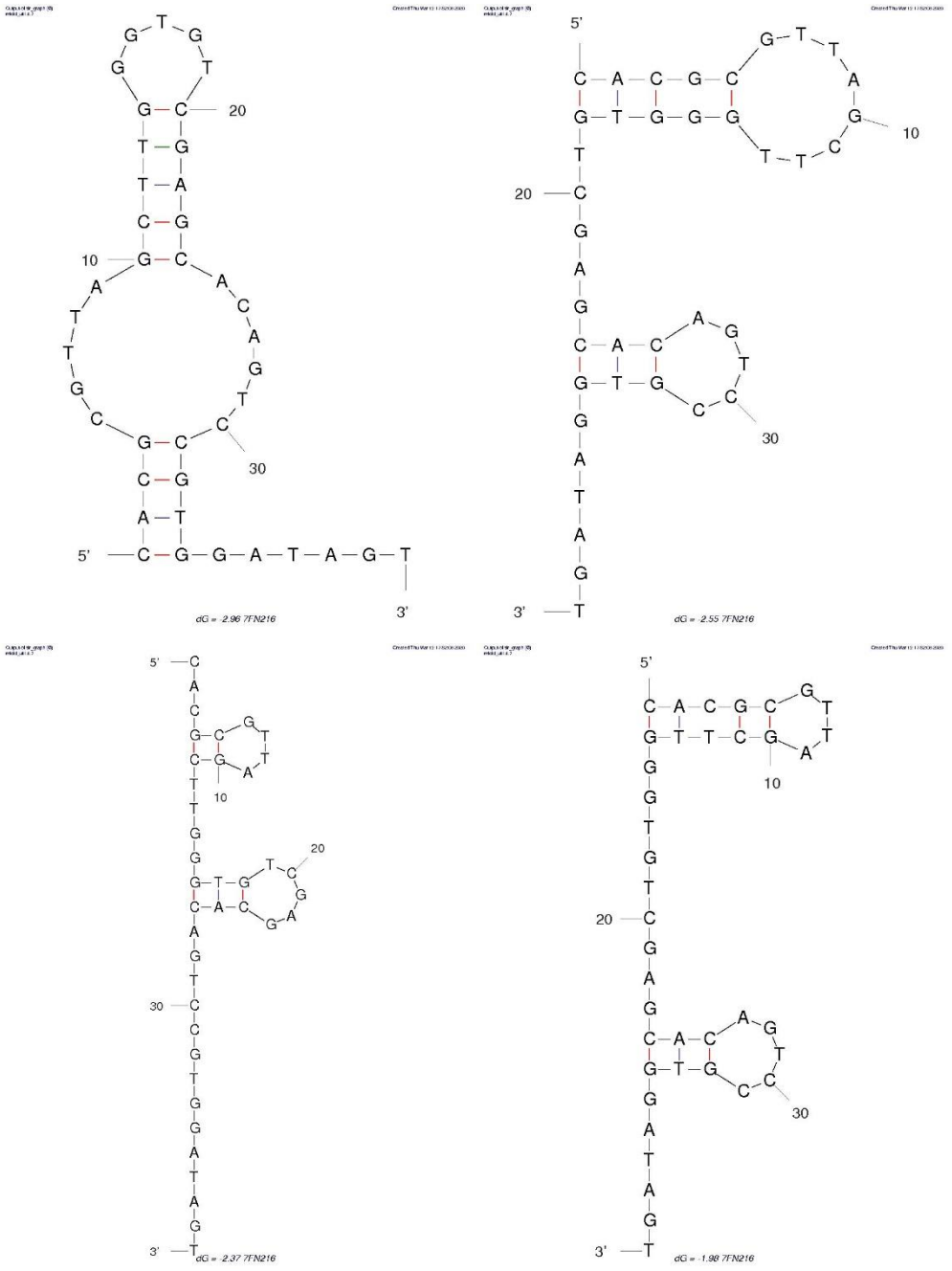
(E) 7FN202


5'
10
20
30
3'
dG = -3.81 7FN202

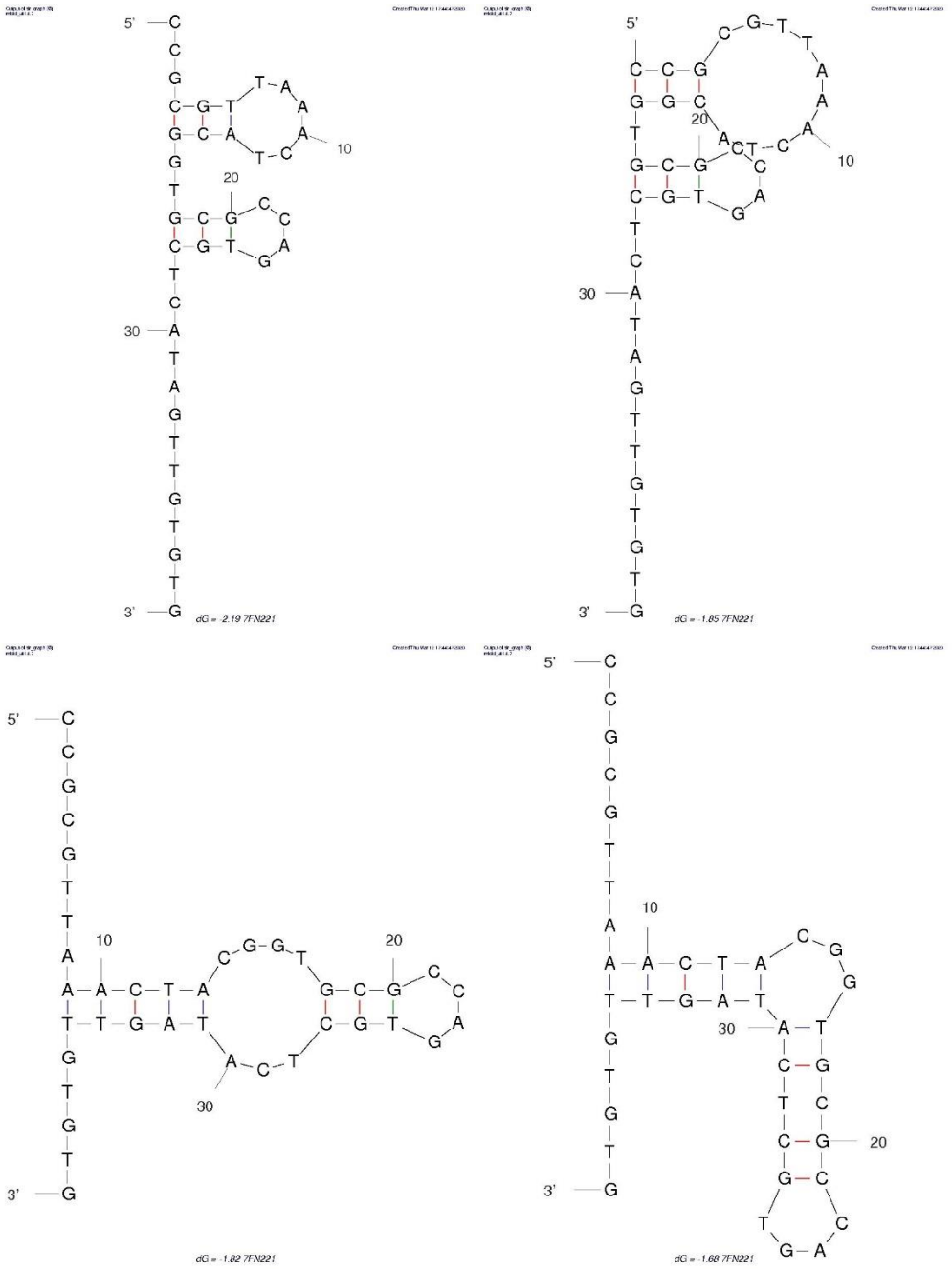

5'
10
20
30
3'
dG = -3.55 7FN202

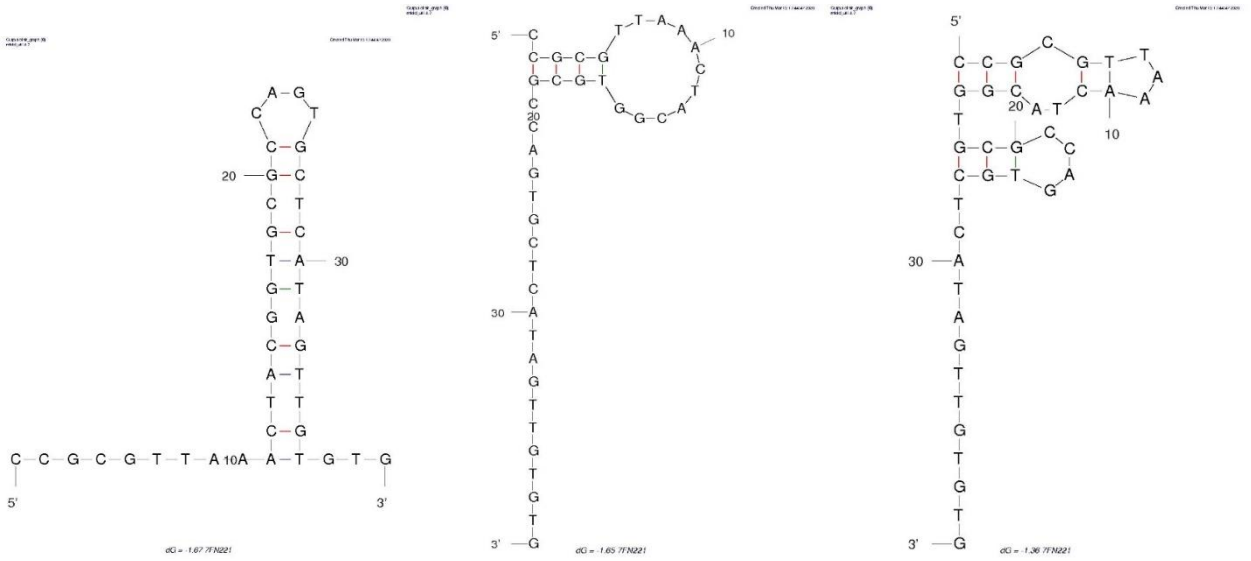

5'
10
20
30
3'
dG = -2.92 7FN202

(F) 7FN216

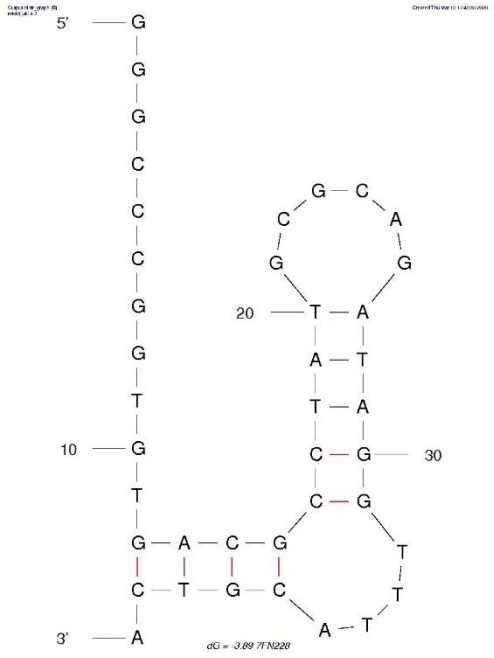


(G) 7FN221

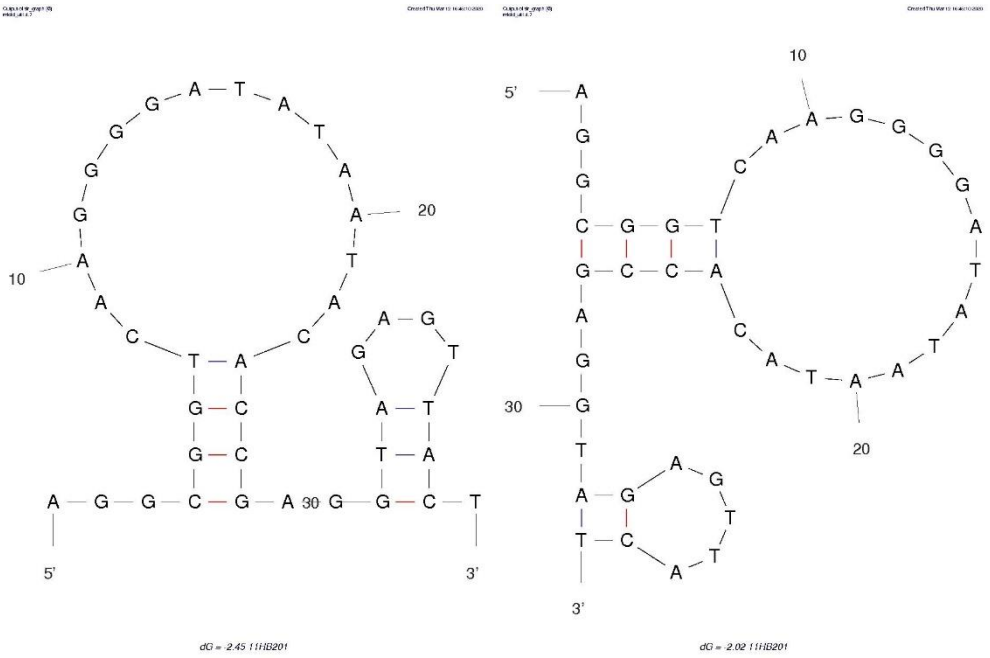




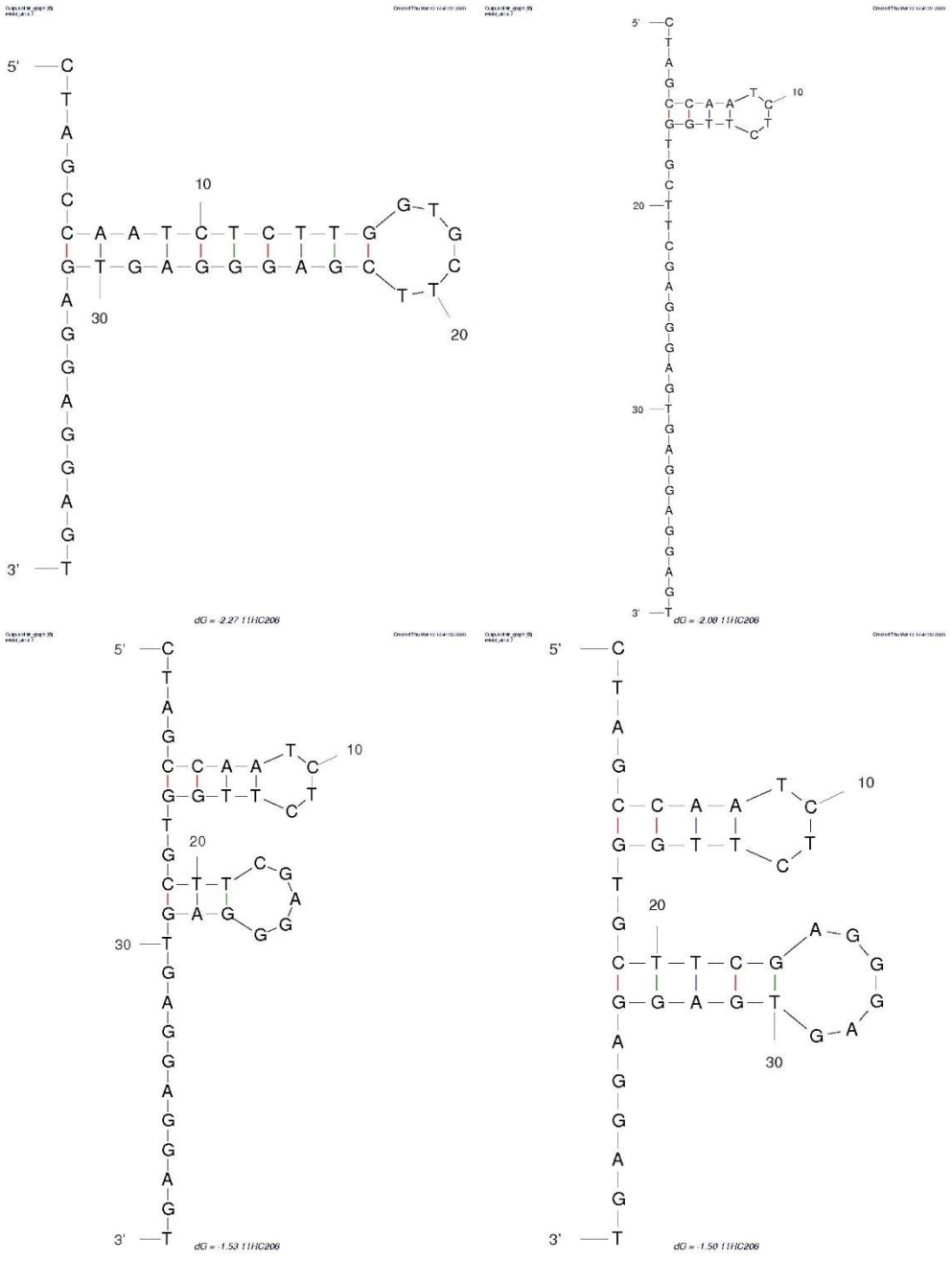
(H) 7FN228



(I) 11HB201



(J) 11HC206



(K) 11HF210

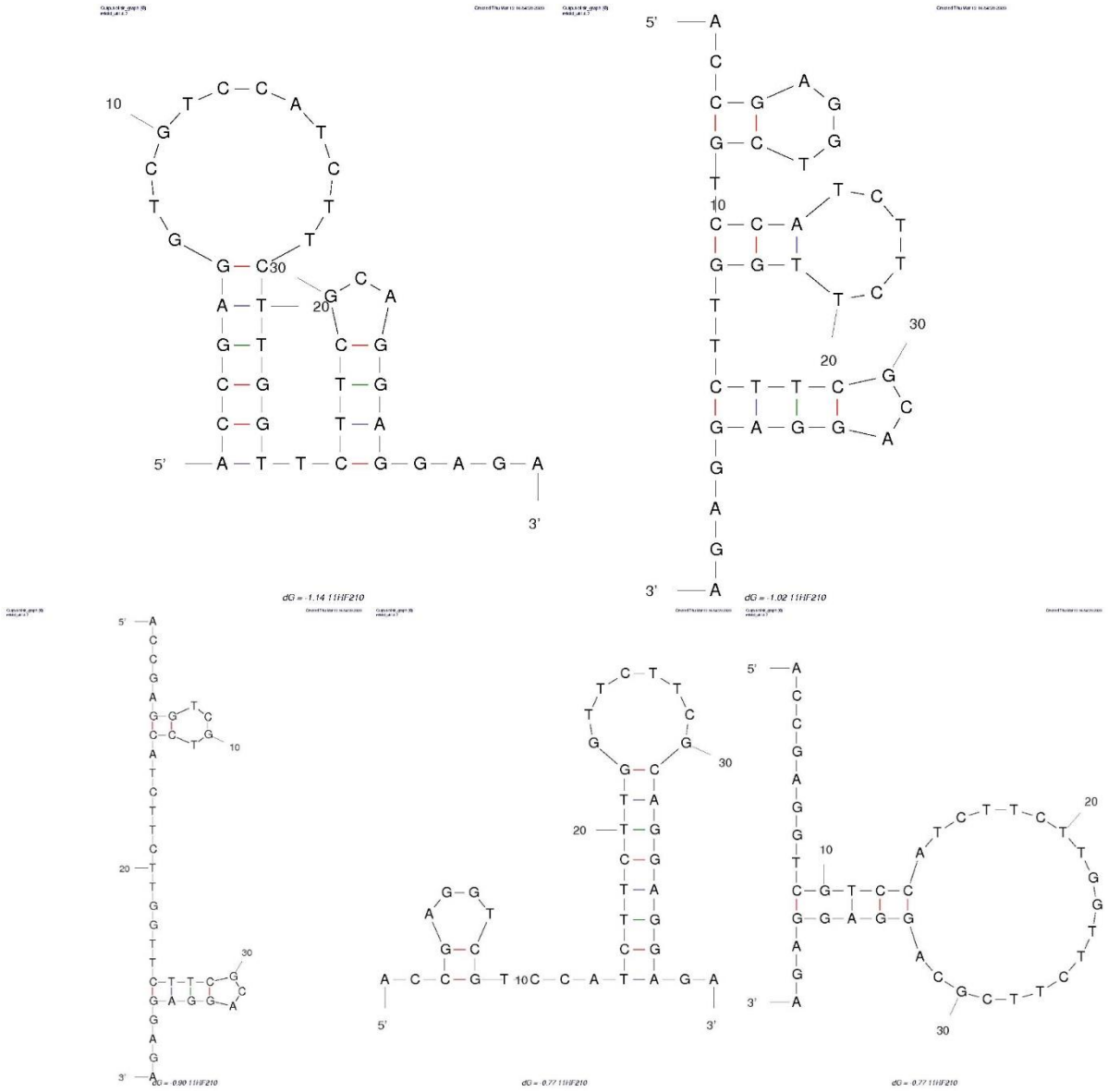


Figure S7. Representative mfold-predicted[53] secondary structures for DNAzymes reported in this study. The default settings were used for the initially random N_{40} regions with the DNA Folding Form at http://unafold.rna.albany.edu/?q=mfold, adjusted to 150 mM Na^+ and 40 mM Mg^{2+}. Shown are structures for 11 of the DNAzymes identified in this study. Where multiple structures are shown for an individual DNAzyme, the lowest-energy structure (with most negative ΔG value) is shown first, followed by the remaining structure(s) in order of increasing energy. See Table S2 for full tabulation of number of structures and lowest ΔG value for each DNAzyme.

References for Electronic Supplementary Information

(1) Chandrasekar, J.; Silverman, S. K. Catalytic DNA with Phosphatase Activity. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 5315-5320.
(2) Rosario-Amorin, D.; Gaboyard, M.; Clérac, R.; Vellutini, L.; Nlate, S.; Heuzé, K. Metallodendritic grafted core-shell γ-Fe_2O_3 nanoparticles used as recoverable catalysts in Suzuki C-C coupling reactions. *Chem. Eur. J.* **2012**, *18*, 3305-3315.
(3) Procopiou, P. A.; Baugh, S. P. D.; Flack, S. S.; Inglis, G. G. A. An Extremely Powerful Acylation Reaction of Alcohols with Acid Anhydrides Catalyzed by Trimethylsilyl Trifluoromethanesulfonate. *J. Org. Chem.* **1998**, *63*, 2342-2347.
(4) Brandsen, B. M. "DNA Catalysts for Amide Bond Cleavage and for Lysine Side Chain Modification". Ph.D. Thesis, University of Illinois at Urbana-Champaign, **2016**.
(5) Zhou, C.; Avins, J. L.; Klauser, P. C.; Brandsen, B. M.; Lee, Y.; Silverman, S. K. DNA-Catalyzed Amide Hydrolysis. *J. Am. Chem. Soc.* **2016**, *138*, 2106-2109.