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Supporting Information

DNA-Catalyzed Introduction of Azide at Tyrosine for Peptide Modification

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Oligonucleotides, peptides, conjugates, and reagents

DNA oligonucleotides and oligonucleotide conjugates were obtained from Integrated DNA Technologies (Coralville, IA) or prepared by solid-phase synthesis on an ABI 394 instrument using reagents from Glen Research. All oligonucleotides and conjugates were purified by 7 M urea denaturing PAGE with running buffer 1× TBE (89 mM each Tris and boric acid and 2 mM EDTA, pH 8.3), extracted from the polyacrylamide with TEN buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 300 mM NaCl), and precipitated with ethanol as described previously.[1] PEG_{5k}-alkyne was obtained from Laysan Bio (Arab, AL). Fluorescein-alkyne was obtained from Tenova Pharmaceuticals (San Diego, CA). 2′-Az-dATP was obtained from TriLink BioTechnologies (San Diego, CA). The peptides sCT and ANP were obtained from Anaspec (Fremont, CA).

Solid-phase peptide synthesis. All amino acid monomers were obtained from Chem-Impex (Wood Dale, IL). Peptides were prepared by solid-phase synthesis using Fmoc Rink amide MBHA resin from Chem-Impex, with *N,N,N′,N′*-tetramethyl-*O*-(7-azabenzotriazol-1-yl)uronium hexafluorophosphate (HATU) as coupling agent. Each synthesis was performed at 0.2 mmol scale, initiated using 0.260 mg of Rink amide resin with a loading capacity of 0.77 mmol/g. All peptide synthesis operations were performed as previously described.[2]

Preparation of DNA-anchored peptide substrates. DNA-anchored peptide conjugates were synthesized by disulfide formation between a DNA HEG-tethered 3′-thiol and the N-terminal cysteine side chain of the peptide, as previously reported [HEG = hexa(ethylene glycol)].[3] The DNA anchor oligonucleotide was 5′-X-HEG-p-C_3-SS-C_3-OH-3′, where X represents the specific oligonucleotide sequence (Table S1). The 3′-disulfide linker was introduced via standard solid-phase DNA synthesis and unmasked to a 3′-thiol by DTT treatment. The DNA-HEG-p-C_3-SH was activated as the pyridyl disulfide by treatment with 2,2′-dipyridyl disulfide in DMF. The DNA-HEG-p-C_3-SSPy was coupled with the free thiol of the peptide N-terminal Cys, forming the DNA-anchored peptide conjugate. All steps were performed as previously described.[3]

Preparation of azido-modified oligonucleotide for capture control reaction. At the outset of these selection experiments, we did not have any way to prepare an exact standard with an azido-adenylylated peptide moiety. Therefore, for the capture control reaction (all data shown with grey bars in Figure S2), we used an azido-modified substrate that was prepared by conjugating a 3′-C_3-NH_2 oligonucleotide with 6-azidohexanoic acid as described in the next paragraph. Once the initial selection with the CAAYAA substrate was completed, we verified that CuAAC capture with PEG_{5k}-alkyne proceeded with similar yields and similar product PAGE migration positions for the control oligonucleotide and for the azido-adenylylated CAAYAA product, as prepared by the new DzAz1 deoxyribozyme (data not shown).

The azido-modified substrate was prepared as follows. A sample containing 5 nmol of 3′-C_3-NH_2 oligonucleotide was brought to 100 μL total volume containing 100 mM MES, pH 6.0, 6 mM 6-azidohexanoic acid (Chem-Impex), 6 mM *N*-hydroxysuccinimide (NHS), 50 mM *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide (EDC), and 10% (v/v) DMF. The sample was incubated at room temperature for 12 h and purified by HPLC [Shimadzu Prominence instrument; Phenomenex Gemini-NX C_{18} column, 5 μm, 10 × 250 mm; gradient of 10% solvent A (20 mM triethylammonium acetate in 50% acetonitrile/50% water, pH 7.0) and 90% solvent B (20 mM triethylammonium acetate in water, pH 7.0) at 0 min to 70% solvent A and 30% solvent B at 40 min with flow rate of 3.5 mL/min].

oligonucleotide purpose	oligonucleotide sequence
Selection with DNA-HEG-CAAYAA (leading to DzAz1)	
DNA-HEG-CAAYAA substrate	GGACTATTGAAAGACATAT–HEG-CAAYAA
forward primer for selection	CGAAATGATGGCTATTTC
random pool for selection	CGAAATGATGGCTATTTC-N_{40}-ATATGTCTTTCAATAGAGCTGATCCTGATGG
splint for ligation step during selection	ATATGTCTTTCAATAGTCCCCATCAGGATCAGCTCTATTGAAAGACATAT
Reselection of DzAz1 deoxyribozyme (leading to DzAz1b and DzAz1c)	
DNA-HEG-CAAYAA substrate	GGATCCTGGATACAAATAT–HEG-CAAYAA
forward primer for selection	CGAAGTATAAACCTGTTC
partially randomized pool for selection [a]	CGAAGTATAAACCTGTTC-N_{40}-ATATTTGTATCCAGGAAGCTGATCCTGATGG
splint for ligation step during selection	ATATTTGTATCCAGGATCCCCATCAGGATCAGCTTCCTGGATACAAATAT
Selection with DNA HEG-CLQTYPRT (leading to DzAz2–DzAz6)	
DNA-HEG-CLQTYPRT substrate	GGACTACCTTTATGCGTAT–HEG-CLQTYPRT
forward primer for selection	CGAACGAAAGCCTCCTTC
random pool for selection	CGAACGAAAGCCTCCTTC-N_{40}-ATACGCATAAAGGTAGAGCTGATCCTGATGG
splint for ligation step during selection	ATACGCATAAAGGTAGTCCCCATCAGGATCAGCTCTACCTTTATGCGTAT
Selection with DNA HEG-CQQPYITN (leading to DzAz7)	
DNA-HEG-CQQPYITN substrate	GGAATATCTCGTTTCTTAT–HEG-CQQPYITN
forward primer for selection	CGAATTAAGACTGAATTC
random pool for selection	CGAATTAAGACTGAATTC-N_{40}-ATAAGAAACGAGATATAGCTGATCCTGATGG
splint for ligation step during selection	ATAAGAAACGAGATATTCCCCATCAGGATCAGCTATATCTCGTTTCTTAT
Selection with DNA HEG-CERSYLMK (leading to DzAz8)	
DNA-HEG-CERSYLMK substrate	GGAATGGCTTGATTGGTAT–HEG-CERSYLMK
forward primer for selection	CGAATTGAGTAAATATTC
random pool for selection	CGAATTGAGTAAATATTC-N_{40}-ATACCAATCAAGCCATAGCTGATCCTGATGG
splint for ligation step during selection	ATACCAATCAAGCCATTCCCCATCAGGATCAGCTATGGCTTGATTGGTAT
Selection with DNA HEG-CFQPYMQE (leading to DzAz9)	
DNA-HEG-CFQPYMQE substrate	GGATCAGGTTACTAATTAT–HEG-CFQPYMQE
forward primer for selection	CGAAATAGATTATCATTC
random pool for selection	CGAAATAGATTATCATTC-N_{40}-ATAATTAGTAACCTGAAGCTGATCCTGATGG
splint for ligation step during selection	ATAATTAGTAACCTGATCCCCATCAGGATCAGCTTCAGGTTACTAATTAT

Table S1. Oligonucleotide sequences used in this study. All sequences are written 5′ to 3′. For all selections, the reverse PCR primer was $(AAC)_4$XCCATCAGGATCAGCT, where X denotes the hexa(ethylene glycol) [HEG] spacer to stop Taq polymerase. The underlined sequence segments are explained in Figure S3.

[a] The partially randomized pool was prepared by solid-phase synthesis such that each nucleotide of the initially random (N_{40}) region is the parent nucleotide of DzAz1 (Figure S3) with 75% probability and one of the other three nucleotides with 25% probability.

In vitro selection procedure

The selection procedure, cloning, and initial screening of individual clones were performed essentially as described previously.[2a] An overview of the key selection and capture steps of each round is shown in Figure 1; nucleotide details for the initial selection with CAAYAA are shown in Figure S1. All oligonucleotide sequences are listed in Table S1.

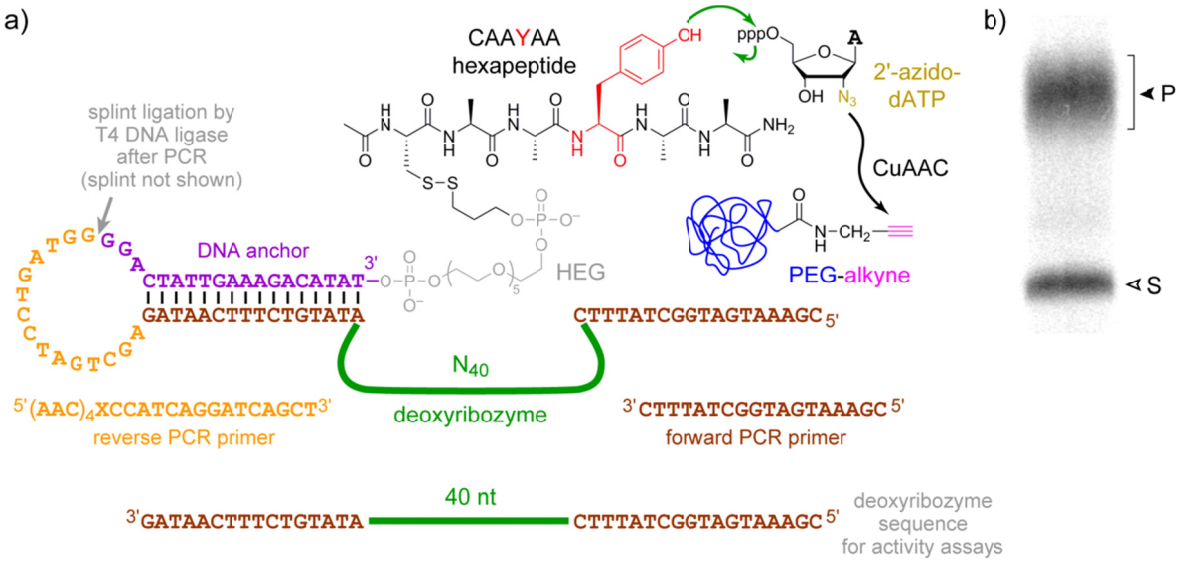

Figure S1. Nucleotide details of the in vitro selection experiments. a) Sequences and structures for the selection with the CAAYAA hexapeptide. Note that the specific nucleotide sequences for the other selections are different (Table S1). Sequences of the initially random (N_{40}) regions are in Figure S3. b) PAGE image of capture control reaction using PEG_{5k}-alkyne (S = substrate, P = product).

Procedure for ligation step in round 1. A 25 μL sample containing 500 pmol of DNA pool, 630 pmol of DNA splint, and 750 pmol of 5′-phosphorylated DNA-anchored peptide 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 that lacks DTT (400 mM Tris, pH 7.8, 100 mM $MgCl_2$, 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 purified by 8% PAGE.

Procedure for ligation step in subsequent rounds. A 17 μL sample containing the PCR-amplified DNA pool (~5–10 pmol), 40 pmol of DNA splint, and 60 pmol of 5′-phosphorylated DNA-anchored peptide 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 that lacks DTT (400 mM Tris, pH 7.8, 100 mM $MgCl_2$, and 5 mM ATP) and 1 μL of 1 U/μL T4 DNA ligase (Thermo Fisher). The sample was incubated at 37 °C for 12 h and purified by 8% PAGE.

Procedure for selection step in round 1. Each selection experiment was initiated with 200 pmol of the ligated pool. A 16 μL sample containing 200 pmol of ligated pool was annealed in 5 mM HEPES, 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. 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 $ZnCl_2$, 150 mM NaCl, and 100 μM 2′-Az-dATP. 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 14 h and precipitated with ethanol.

Procedure for selection step in subsequent rounds. An 8 μL sample containing the ligated pool was annealed in 5 mM HEPES, 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. 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 $ZnCl_2$, 150 mM NaCl, and 100 μM 2′-Az-dATP. The sample was incubated at 37 °C for 14 h and precipitated with ethanol.

Procedure for capture step in each round. The sample from the selection step was brought to 20 μL total volume containing 100 mM HEPES, pH 7.5, 10 mM PEG_{5k}-alkyne, 40 mM tris(3-hydroxypropyltriazolylmethyl)amine (THPTA), 10 mM sodium ascorbate, and 5 mM $CuSO_4$. The concentrations of all reagents were optimized (not shown). The four reagents were added from 100 mM (10×), 1 M (25×), freshly prepared 100 mM (10×), and 100 mM (20×) stock solutions, respectively; the four reagents were mixed together and then added at once to the other components. The sample was incubated at 4 °C for 1 h and separated by 8% PAGE.

Procedure for DTT reduction in each round. The sample from the capture step was brought to 20 μL total volume containing 100 mM HEPES, pH 7.5, and 50 mM DTT. The sample was incubated at 37 °C for 2 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 DTT reduction product, 200 pmol of forward primer, 50 pmol of reverse primer, 20 nmol of each dNTP, 10 μL of 10× 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], and Pfu polymerase. 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), 5 μL of 10× polymerase buffer, and Pfu polymerase. 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 deoxyribozymes. The PCR primers used for cloning were the forward primer used in selection and the reverse primer as 5′-`TAATTAATTAATTACCCATCAGGATCAGCT`-3′. 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, 25 pmol of forward cloning primer, 25 pmol of reverse cloning primer, 10 nmol of each dNTP, 5 μL of 10× Taq polymerase buffer, and Taq polymerase. 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 2% agarose gel and extracted using a GeneJET Gel Extraction Kit (Thermo Fisher). The extracted product was quantified by absorbance (A_{260}) and diluted to 5–10 ng/μL. A 1 μL portion of the diluted PCR product was inserted into the pCR2.1-TOPO vector using a TOPO TA cloning kit (Thermo Fisher). 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 (Thermo Fisher) and screened for properly sized inserts by EcoRI digestion and agarose gel analysis. Before sequencing, assays of individual deoxyribozyme clones were performed with PAGE-purified DNA strands prepared by PCR from the miniprep DNA, using the single-turnover assay procedure described below.

Selection progressions

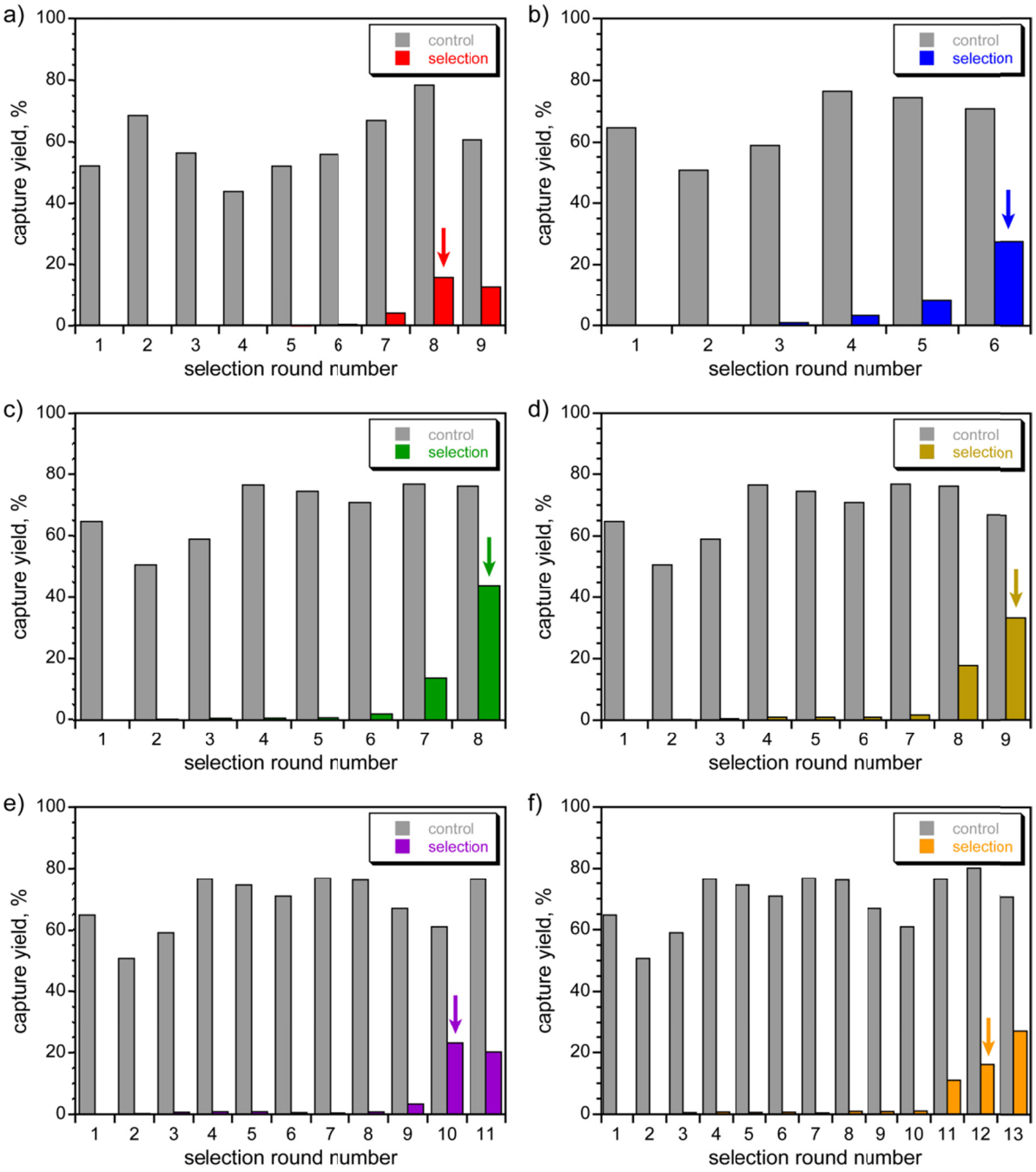

Figure S2. Progressions of the in vitro selection experiments. Arrows mark the cloned rounds. a) Selection with CAAYAA. b) Reselection of DzAz1, which was obtained from selection with CAAYAA. c) Selection with CLQTYPRT. d) Selection with CQQPYITN. e) Selection with CERSYLMK. f) Selection with CFQPYMQE.

Sequences of individual deoxyribozymes

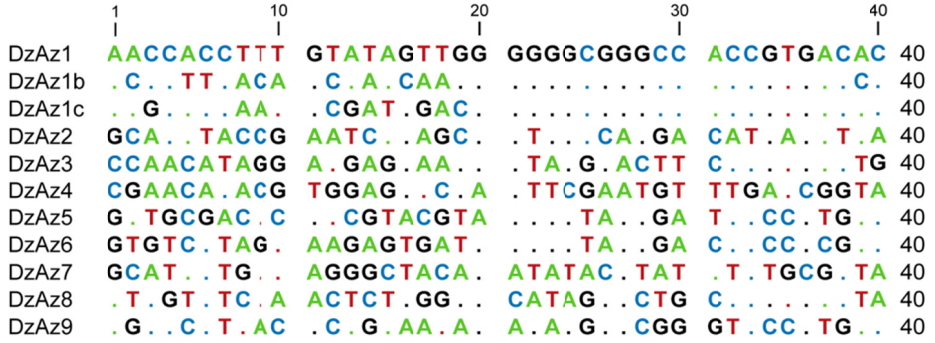

Figure S3. Sequences of the deoxyribozymes described in this study. Dots denote the same nucleotide as in the uppermost sequence of the listing. Only the initially random (N_{40}) sequences are shown. All deoxyribozymes were used as the 74 nt 5′-X-N_{40}-Y-3′, where X is the 18 nt underlined segment on the 5′-side of N_{40} in the relevant random pool in Table S1, and Y is the 16 nt underlined segment on the 3′-side of N_{40} in the relevant random pool in Table S1.

All deoxyribozymes were analyzed by mfold with regard to potential secondary structures; various stem and stem-loop elements were predicted in all cases.[4] Experimentally distinguishing and validating these predicted secondary structures would require considerable effort, and the findings would not immediately clarify either tertiary structure or catalytic mechanism.

Single-turnover deoxyribozyme assay procedure

The DNA-anchored peptide substrate was 5′-^{32}P-radiolabeled using γ-^{32}P-ATP and polynucleotide kinase. A 8 μL sample containing 0.1 pmol of 5′-^{32}P-radiolabeled DNA-anchored peptide substrate and 2 pmol of deoxyribozyme was annealed in 5 mM HEPES, 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. The DNA-catalyzed azido-adenylylation reaction was initiated by bringing the sample to 20 μL total volume containing 70 mM HEPES, pH 7.5, 1 mM $ZnCl_2$, 20 mM $MnCl_2$, 40 mM $MgCl_2$, 150 mM NaCl, and 100 μM 2′-Az-dATP or ATP. The sample was incubated at 37 °C. At appropriate time points, 2 μL aliquots were quenched with 6 μL stop solution (80% formamide, 1× TBE [89 mM each Tris and boric acid and 2 mM EDTA, pH 8.3], 50 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol). Samples were separated by 20% PAGE and quantified with a PhosphorImager. Values of k_{obs} were obtained by fitting the yield versus time data directly to first-order kinetics; i.e., yield = Y•$(1 - e^{-kt})$, where $k = k_{obs}$ and Y is the final yield. Each k_{obs} value is reported with error calculated as the standard deviation from the indicated number of independent determinations.

MALDI mass spectrometry data

deoxyribozyme	product	$[M+H]^+$ calcd.	$[M+H]^+$ found	error, % (found – calcd.)
DzAz1	DNA-HEG-CAAY(Az-dAMP)AA [a]	7317.4	7319.6	+0.03
DzAz2	DNA-HEG-CLQTY(Az-dAMP)PRT [a]	7664.6	7667.6	+0.04
DzAz7	DNA-HEG-CQQPY(Az-dAMP)ITN [a]	7639.6	7642.5	+0.04
DzAz8	DNA-HEG-CERSY(Az-dAMP)LMK [a]	7832.7	7835.8	+0.04
DzAz2	peptide A, Prod 1_R [b,c]	862.4	862.5	+0.01
DzAz2	peptide A, Prod 2_R [b,c,d]	1190.0	1190.6	+0.05
DzAz2	peptide A, Prod 2_L [b,c]	1309.6	1309.7	+0.008
DzAz2	peptide A, Prod 1_L [b,c,d]	1637.7	1637.8	+0.006
DzAz2	peptide B, Prod 2_R [b,c]	877.5	877.5	0
DzAz2	peptide B, Prod 1_R [b,c,d]	1205.0	1205.6	+0.05
DzAz2	peptide B, Prod 1_L [b,c]	1294.6	1294.7	+0.008
DzAz2	peptide B, Prod 2_L [b,c,d]	1622.7	1622.8	+0.006
DzAz8	sCT-(Az-dAMP) [b]	3784.8	3787.6	+0.07
DzAz8	ANP-(Az-dAMP) [b]	3433.5	3434.7	+0.03
DzAz8	sCT-AMP [b]	3759.8	3762.9	+0.08
DzAz8	ANP-AMP [b]	3408.5	3410.3	+0.05
–	sCT-Fluorescein [b]	4197.9	4199.4	+0.04
–	ANP-Fluorescein [b]	3846.6	3844.6	–0.05

Table S2. MALDI mass spectrometry data.

[a] "DNA" here refers to the DNA anchor oligonucleotide. The azido-adenylylation product was prepared from an 8 µL sample containing 600 pmol of DNA-anchored HEG-tethered peptide substrate (Table S1) and 660 pmol of deoxyribozyme, which were annealed in 5 mM HEPES, 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. The DNA-catalyzed azido-adenylylation reaction was initiated by bringing the sample to 20 µL total volume containing 70 mM HEPES, pH 7.5, 1 mM $ZnCl_2$, 20 mM $MnCl_2$, 40 mM $MgCl_2$, 150 mM NaCl, and 100 µM 2′-Az-dATP. The sample was incubated at 37 °C for 24 h and separated by 20% PAGE. The PAGE-purified sample was desalted by Millipore C_{18} ZipTip and analyzed by MALDI mass spectrometry (Bruker UltrafleXtreme; matrix 3-hydroxypicolinic acid).

[b] See procedure elsewhere in Supporting Information.

[c] These peptide fragments are listed in the order depicted in Figure 5.

[d] The azido group was reduced to an amino group by the DTT used to cleave the peptide from the DNA anchor oligonucleotide.[5] The calculated mass takes this reaction into account.

Metal ion dependence assays for DzAz1 deoxyribozyme and variants

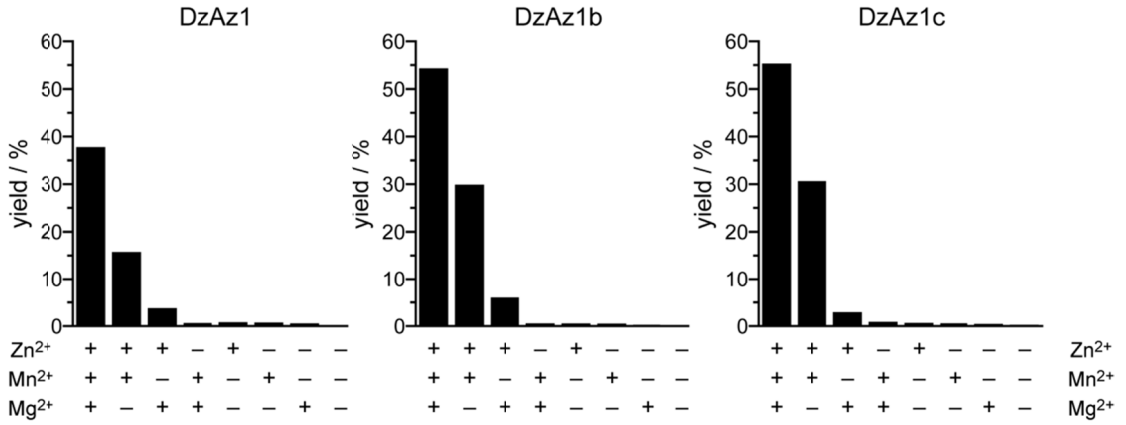

Figure S4. Assays of metal ion dependence of the DzAz1 deoxyribozyme and its DzAz1b and DzAz1c sequence variants. Each assay used the DNA-anchored CAAYAA peptide substrate. Incubation conditions: 70 mM HEPES, pH 7.5, combinations of 1 mM $ZnCl_2$, 20 mM $MnCl_2$, and 40 mM $MgCl_2$ as appropriate, and 150 mM NaCl at 37 °C with 100 μM 2′-Az-dATP. The yield at 24 h for each metal ion combination is shown.

Peptide sequence discrimination assays for DzAz1–DzAz9 deoxyribozymes

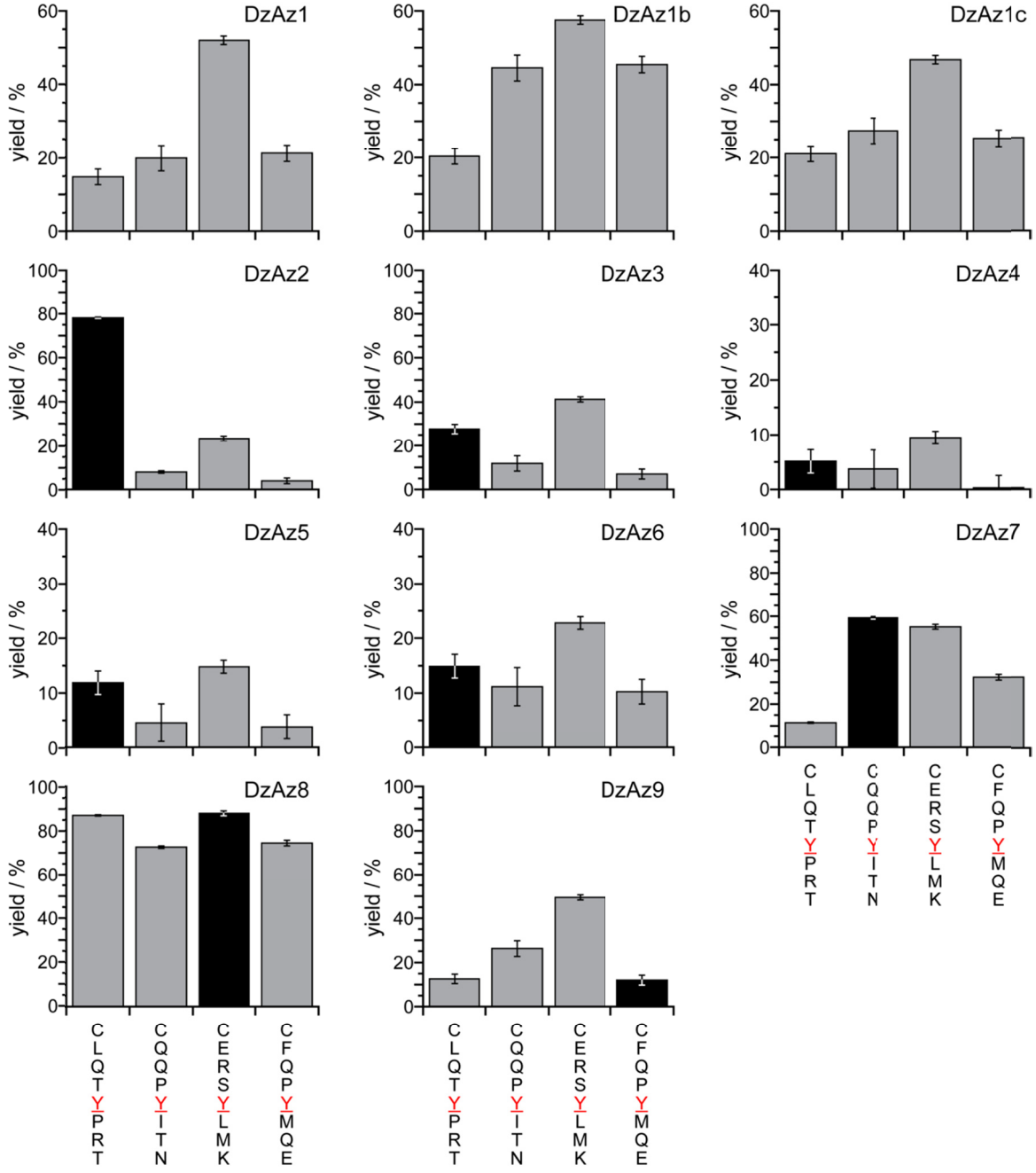

Figure S5. Activities of DzAz1–DzAz9 with the four DNA-anchored peptide substrates (yield at 24 h; $n = 3$, ± sd). Black bars denote the particular peptide substrate used during identification of that deoxyribozyme; grey bars are for the other peptide substrates (DzAz1 and variants were identified using CAAYAA). To facilitate comparisons, data are shown for all deoxyribozymes; the same data for DzAz2, DzAz7, and DzAz8 are also in Figure 3b.

Additional kinetic data for DzAz2–DzAz9 deoxyribozymes

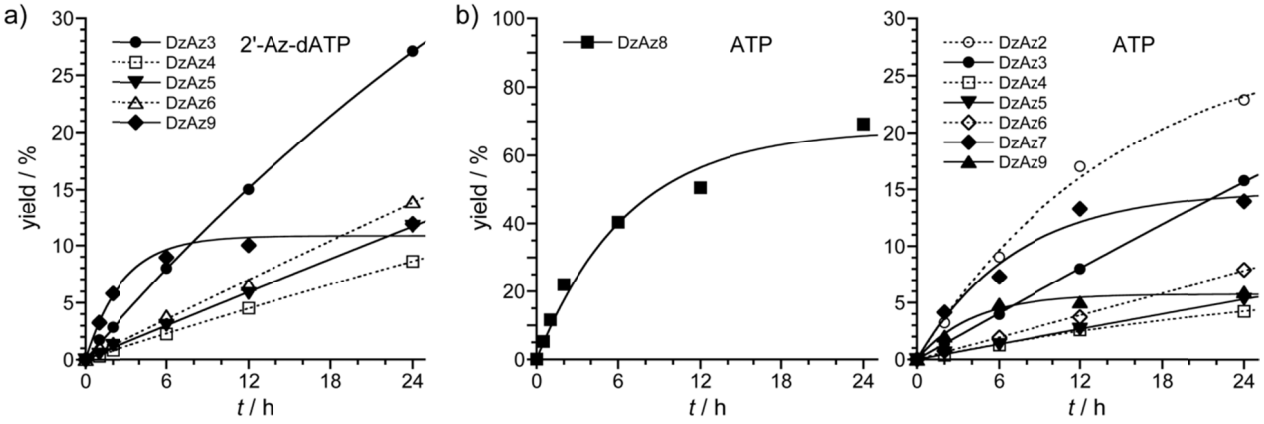

Figure S6. Additional kinetic data for DzAz2–DzAz9 deoxyribozymes. a) Kinetic data for deoxyribozymes that are not included in Figure 3a. Incubation conditions: 70 mM HEPES, pH 7.5, 1 mM $ZnCl_2$, 20 mM $MnCl_2$, 40 mM $MgCl_2$, and 150 mM NaCl at 37 °C with 100 μM 2′-Az-dATP. b) Kinetic data for deoxyribozymes with ATP rather than 2′-Az-dATP (see Figure 2 for DzAz1 data with ATP).

Validating DzAz2 requirement for Tyr at modification site

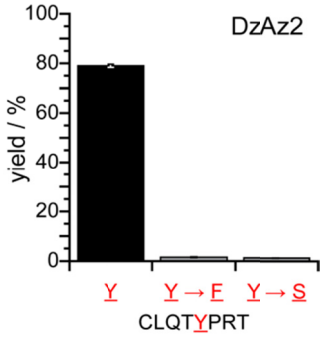

Figure S7. Activity of DzAz2 upon mutation of Tyr to Phe or Ser in the DNA-anchored peptide substrate. Either mutation abolished activity (yield at 24 h; $n = 3$, ± sd).

Establishing YPR motif as necessary and sufficient for DzAz2 reactivity

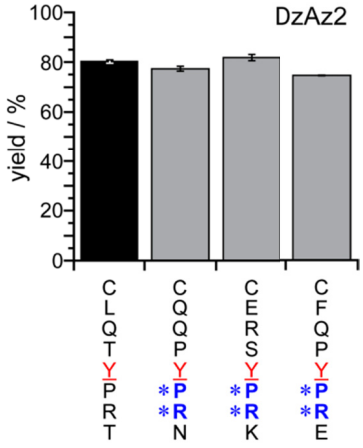

Figure S8. DzAz2 activity requires only the YPR motif in the peptide substrate. Each of the three alternative DNA-anchored peptide sequences was mutated to introduce the YPR motif while retaining all other existing amino acids. In all three cases, full activity was observed (yield at 24 h; $n = 3$, ± sd). Compare Figure S5 for DzAz2 activity with the unmutated peptide sequences.

Assay procedure for Figure 5, and calculation of discrimination factors

The azido-adenylylation product of peptide A or peptide B was prepared from an 8 μL sample containing 600 pmol of DNA-anchored HEG-tethered peptide substrate and 660 pmol of deoxyribozyme, which were annealed in 5 mM HEPES, 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. The DNA-catalyzed azido-adenylylation reaction was initiated by bringing the sample to 20 μL total volume containing 70 mM HEPES, pH 7.5, 1 mM $ZnCl_2$, 20 mM $MnCl_2$, 40 mM $MgCl_2$, 150 mM NaCl, and 100 μM 2′-Az-dATP. The sample was incubated at 37 °C for 24 h and separated by 20% PAGE. The product was quantified by absorbance (A_{260}); in each case, ~400 pmol was obtained. From each sample, 50 pmol was dissolved in 5 μL of water and brought to a total volume of 10 μL containing 100 mM Tris, pH 8.0, 40 mM DTT, and 0.6 ng/μL Lys-C (Roche cat. no. 11420429001). The sample was incubated at 37 °C for 12 h, desalted by Millipore C_{18} ZipTip, and analyzed by MALDI mass spectrometry (Bruker UltrafleXtreme, matrix 2,5-dihydroxybenzoic acid).

For each product, treatment with Lys-C and recording of the mass spectrum was done twice. From each mass spectrum, peak intensity ratios were used to calculate two discrimination factors, one as $Prod1_L/Prod2_L$ and the other as $Prod1_R/Prod2_R$, assuming that the response factor for each peptide fragment does not change upon azido-adenylylation. For peptide A, $Prod1_L/Prod2_L$ was 3.8 and 3.4, and $Prod1_R/Prod2_R$ was 139 and 120; the smaller value is reported in Figure 5. For peptide B, $Prod1_L/Prod2_L$ was 605 and 270, and $Prod1_R/Prod2_R$ was 99 and 119.

Evaluating DzAz8 with peptide mutants

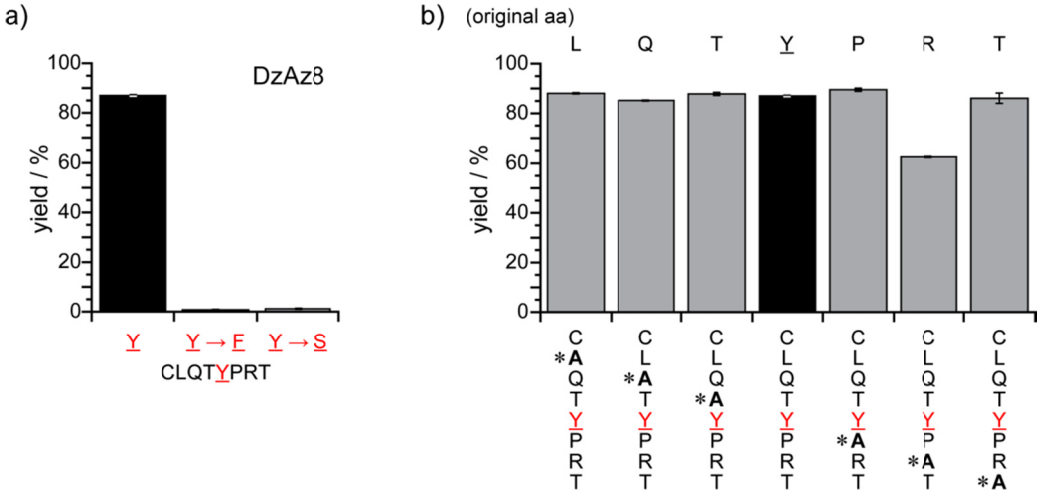

Figure S9. a) Activity of DzAz8 upon mutation of Tyr to Phe or Ser in the DNA-anchored peptide substrate. Either mutation abolished activity (yield at 24 h; $n = 3$, ± sd). b) Activity of DzAz8 with a series of DNA-anchored peptide substrates in which a single amino acid was mutated to Ala (yield at 24 h; $n = 3$, ± sd).

Procedures for Figure 6 and Figure S10

The azido-adenylylation or adenylylation product from each untethered peptide was prepared from a 10 μL sample containing 3.3 nmol of free unmodified DNA anchor oligonucleotide (lacking 3′-phosphate, HEG, and thiol) and 3 nmol of deoxyribozyme, which were annealed in 5 mM HEPES, 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. The DNA-catalyzed azido-adenylylation reaction was initiated by bringing the sample to 30 μL total volume containing 70 mM HEPES, pH 7.5, 5 mM $ZnCl_2$ (optimized), 20 mM $MnCl_2$, 40 mM $MgCl_2$, 150 mM NaCl, 100 μM untethered peptide and 1 mM 2′-Az-dATP or ATP. The sample was incubated at 37 °C for 24 h and separated by HPLC (Shimadzu Prominence instrument; Phenomenex Jupiter Proteo C_{12} column, 4 μm, 10 × 250 mm). The reaction for azido-adenylylation or adenylylation of sCT was analyzed with a gradient of 28% solvent A (acetonitrile) and 72% solvent B (0.1% TFA in water) at 0 min to 40% solvent A and 60% solvent B at 60 min with flow rate of 2 mL/min. The reaction for azido-adenylylation or

adenylylation of ANP was analyzed with a gradient of 17% solvent A and 83% solvent B at 0 min to 29% solvent A and 71% solvent B at 60 min with flow rate of 2 mL/min. The HPLC-purified sample was analyzed by MALDI mass spectrometry (Bruker UltrafleXtreme, matrix 2,5-dihydroxybenzoic acid). The HPLC response factor of azido-adenylylated or adenylylated peptide over unmodified peptide was assigned as 1.2 on the basis of UV absorbance data (A_{205}) for sCT, ANP, and AMP (data not shown). The yield of each DzAz8-catalyzed reaction was calculated from the peak integrals, accounting for the response factor.

For subsequent modification of each azido-adenylylated peptide product, 0.4 nmol of HPLC-purified sample was brought to 5 μL total volume containing 100 mM HEPES, pH 7.5, 10 mM PEG_{5k}-alkyne or fluorescein-alkyne, 40 mM tris(3-hydroxypropyltriazolylmethyl)amine (THPTA), 10 mM sodium ascorbate, and 5 mM $CuSO_4$. The final four reagents were added from 100 mM (10×; PEG_{5k}-alkyne in water or fluorescein-alkyne in DMSO), 1 M (25×), freshly prepared 100 mM (10×), and 100 mM (20×) stock solutions, respectively; the four reagents were mixed together and then added at once to the other components. The sample was incubated at 4 °C for 1 h. For SDS-PAGE analysis, the sample was separated by 16.5% Tris-Tricine SDS-PAGE and imaged by Coomassie staining or, for fluorescein labeling, with a Storm 840 PhosphorImager (excitation at 450 nm). For mass spectrometry, the sample was analyzed by MALDI mass spectrometry (Bruker UltrafleXtreme, matrix 2,5-dihydroxybenzoic acid).

Adenylylation of sCT and ANP using the DzAz8 deoxyribozyme with ATP

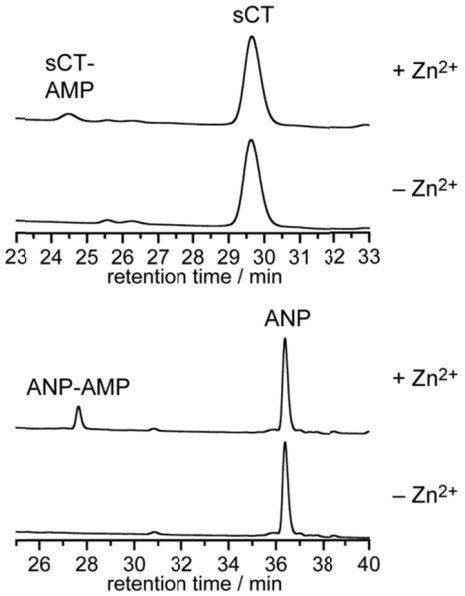

Figure S10. Adenylylation of untethered (free) peptide substrates by DzAz8, assayed by HPLC (t = 24 h).

References for Supporting Information

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