Table of Contents

Synthesis of hexapeptides ..... page S1

Synthesis and analysis of DNA-anchored hexapeptides ..... page S2

In vitro selection procedure. ..... page S4

Mass spectrometry data for PAGE-purified deoxyribozyme products. ..... page S5

Diagram of Figure 4 experiment. ..... page S7

References for Supporting Information ..... page S7

Synthesis of hexapeptides

Hexapeptides were prepared by solid-phase synthesis using Fmoc Rink amide MBHA resin, 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 308 mg of Rink amide resin with a loading capacity of 0.65 mmol/g (AAPPTec). All steps were monitored by ninhydrin test. For each coupling, 5 equiv (1.0 mmol) of Fmoc-amino acid, 4.9 equiv (373 mg, 0.98 mmol) of HATU, and 10 equiv (350 μL, 2.0 mmol) of *N*,*N*-diisopropylethylamine (DIPEA) were mixed in 10 mL of anhydrous DMF. The coupling reaction was initiated by adding this mixture to the resin and agitating by bubbling with nitrogen for 45 min, followed by washing with DMF (3 × 10 mL). The N-terminus of the newly installed amino acid was deprotected by agitating the resin in 20% piperidine in DMF under nitrogen for 5 min a total of three times, each time washing with DMF (3 × 10 mL). Hexapeptides with N-terminal cysteine were capped via acetylation of their N-terminus by agitating the resin in 50 equiv (924 μL, 10 mmol) of acetic anhydride and 25 equiv of DIPEA (870 μL, 5 mmol) in 5 mL of DMF under nitrogen for 30 min. The hexapeptide was cleaved from the solid support by stirring the resin in a separate vial with a solution containing 5 mL of trifluoroacetic acid (TFA), 125 μL of water, 125 μL of ethanedithiol, and 50 μL of triisopropylsilane for 90 min. The liquid solution was separated from the resin by filtration. This solution was dried on a rotary evaporator, providing either a solid or an oily liquid depending upon the peptide sequence. To this material 20 mL of cold diethyl ether was added, and the peptide was obtained as a white solid that was filtered and purified by HPLC.

Synthesis and analysis of DNA-anchored hexapeptides

Each hexapeptide was coupled to the DNA anchor oligonucleotide via either the N-terminal α-amino group (Figure S1; linkage created by reductive amination) or the N-terminal cysteine side chain (Figure S2; linkage created by disulfide formation). In the former case, the tether was either short (hexapeptide connected directly to DNA anchor) or long (hexapeptide connected to DNA anchor with an intervening hexa(ethylene glycol), or HEG, tether).

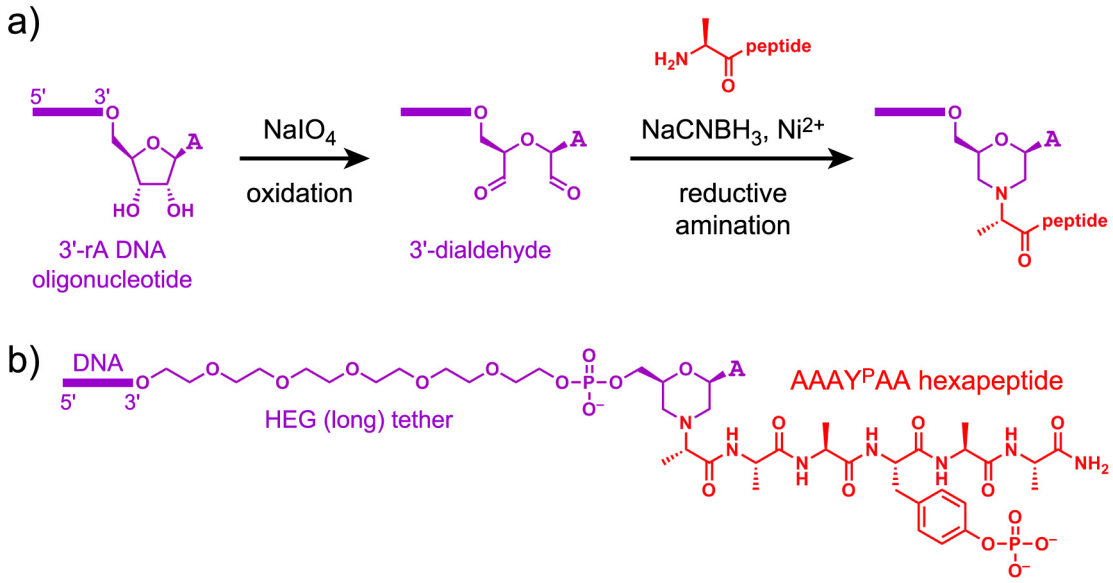

Figure S1. Attachment of hexapeptide to DNA anchor oligonucleotide via the N-terminal α-amino group. a) Conjugation reaction. b) Structure of the DNA-anchored HEG-tethered AAAY^{P}AA hexapeptide substrate. The substrate with the short tether has the 3′-OH of the DNA anchor connected directly to the rA nucleotide that is oxidized by $NaIO_4$, as shown in panel a.

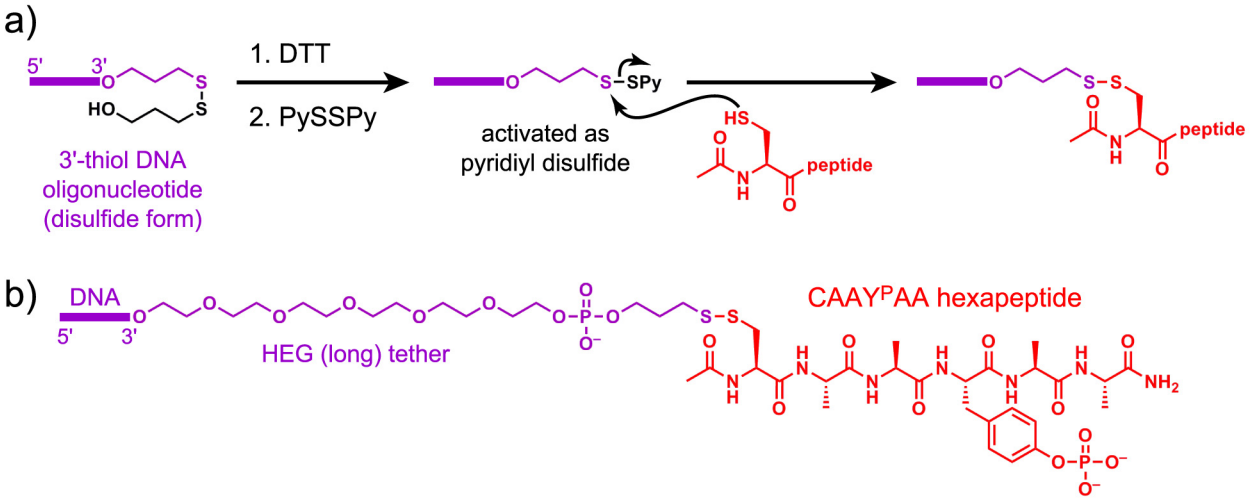

Figure S2. Attachment of hexapeptide to DNA anchor oligonucleotide via the N-terminal cysteine side chain. a) Conjugation reaction. b) Structure of the DNA-anchored HEG-tethered CAAY^{P}AA hexapeptide substrate.

Procedure for Figure S1: The DNA anchor oligonucleotide was 5′-GGATAATACGACTCACTAT-rA-3′, where the 3′-terminal ribonucleotide was oxidized by $NaIO_4$. Between the DNA oligonucleotide portion and the rA nucleotide, a HEG spacer was either absent or present for the short or long tether, respectively. A 100 μL sample containing 1.0 nmol of DNA anchor oligonucleotide in 100 mM HEPES, pH 7.5, and 10 mM $NaIO_4$ was incubated at room temperature for 1 h. The oxidized product was precipitated to remove excess $NaIO_4$ by addition of 10 μL of 3 M NaCl and 300 μL of ethanol. The precipitated product was dissolved in 65 μL of water and used directly in the next step. A 100 μL sample containing the $NaIO_4$-oxidized DNA anchor oligonucleotide and 100 nmol (100 equiv) of hexapeptide in 100 mM NaOAc, pH 5.2, 50 mM $NiCl_2$, and 10 mM $NaCNBH_3$ was incubated at 37 °C for 14 h. The DNA-anchored hexapeptide was precipitated by addition of 10 μL of 3 M NaCl and 300 μL of ethanol and purified by 20% PAGE. See Table S1 for MALDI mass spectrometry values.

Procedure for Figure S2: The DNA anchor oligonucleotide was 5′-GGATAATACGACTCACTAT-C_3-SS-C_3-OH-3′, where the 3′-disulfide linker was introduced via standard solid-phase DNA synthesis and unmasked to a 3′-thiol by DTT treatment. A 50 μL sample containing 2 nmol of DNA anchor oligonucleotide in 50 mM HEPES, pH 7.5, and 50 mM DTT was incubated at 37 °C for 2 h. The reduced product was precipitated to remove excess DTT by addition of 50 μL of water, 10 μL of 3 M NaCl, and 300 μL of ethanol. The precipitated product (DNA-C_3-SH) was dissolved in 45 μL of water. Activation as the pyridyl disulfide was achieved by adding 5 μL of 100 mM 2,2′-dipyridyl disulfide in DMF and incubating at 37 °C for 2 h. The product (DNA-C_3-SSPy) was precipitated by addition of 50 μL of water, 10 μL of 3 M NaCl and 300 μL of ethanol and dissolved in 25 μL of water. Conjugation to the hexapeptide was performed by adding 20 μL of 50 mM triethylammonium acetate, pH 7.0, and 5 μL of 20 mM hexapeptide (100 nmol, 100 equiv). The sample was incubated at 37 °C for 2 h, and the DNA-anchored hexapeptide was purified by 20% PAGE. See Table S1 for MALDI mass spectrometry values.

substrate	mass calcd.	mass found	error, % (found – calcd.)
DNA-AAAY^{OH}AA	6625.6	6623.2	–0.04
DNA-AAAS^{OH}AA	6549.5	6545.9	–0.05
DNA-AAAY^{P}AA	6705.6	6704.2	–0.02
DNA-AAAS^{P}AA	6629.5	6627.2	–0.03
DNA-HEG-AAAY^{OH}AA	6969.9	6967.0	–0.04
DNA-HEG-AAAS^{OH}AA	6893.8	6891.8	–0.03
DNA-HEG-AAAY^{P}AA	7049.9	7050.4	+0.01
DNA-HEG-AAAS^{P}AA	6973.8	6973.8	0
DNA-HEG-CAAY^{OH}AA	6901.0	6902.5	+0.02
DNA-HEG-CAAS^{OH}AA	6824.9	6825.5	+0.01
DNA-HEG-CAAY^{P}AA	6980.9	6980.3	–0.01
DNA-HEG-CAAS^{P}AA	6904.8	6903.9	–0.01
DNA-HEG-CAEY^{P}AA	7039.0	7025.7	–0.19
DNA-HEG-CAFY^{P}AA	7057.0	7049.5	–0.11
DNA-HEG-CAKY^{P}AA	7038.0	7028.5	–0.13
DNA-HEG-CAAY^{P}EA	7039.0	7039.7	+0.01
DNA-HEG-CAAY^{P}FA	7057.0	7044.7	–0.17
DNA-HEG-CAAY^{P}KA	7038.0	7028.4	–0.14

Table S1. Mass values from MALDI mass spectrometry assays of DNA-anchored hexapeptide substrates.

In vitro selection procedure

The selection experiments designated VM and VP used the DNA-anchored AAAY^{P}AA hexapeptides with the short and long tethers, respectively (Figure S1b). Inclusion of the HEG linker increases the structural freedom of the hexapeptide, which was intended to foster improved reactivity of the corresponding free (unanchored) peptide substrate.

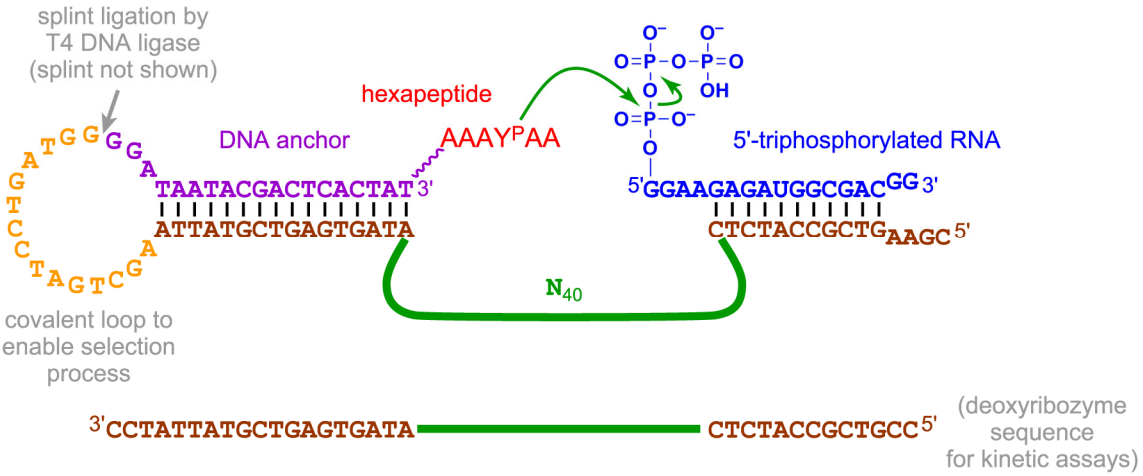

Figure S3. Selection design for identification of deoxyribozymes that covalently tag phosphorylated peptides.

The selection procedure, cloning, and initial analysis of individual clones were performed essentially as described previously,[1] but with a different ligation step. An overview of the selection design is shown in Figure S3. The random deoxyribozyme pool was 5′-CGAAGTCCGCCATCTCTTC-N_{40}-ATAGTGAGTCGTATTA-3′. PCR primers were 5′-CGAAGTCGCCATCTCTTC-3′ (forward primer) and 5′-$(AAC)_4$XCCATCAGATCAGCT-3′, where X is the HEG spacer to stop Taq polymerase (reverse primer). In each round, the ligation step to attach the deoxyribozyme pool at its 3′-end with the 5′-end of the DNA-anchored hexapeptide substrate was performed using a DNA splint and T4 DNA ligase. The splint sequence was 5′-ATAGTGAGTCGTATTATCCTCCATCAGGATCAGCTTAATACGACTCACTAT-3′, where the underlined T is included to account for the untemplated A nucleotide that is added at the 3′-end of each PCR product by Taq polymerase. This T nucleotide was omitted from the splint used for ligation of the initially random N_{40} pool, which was prepared by solid-phase synthesis without the untemplated A.

Procedure for ligation step in round 1. A 34 µL sample containing 500 pmol of DNA pool, 750 pmol of DNA splint, and 1 nmol of 5′-phosphorylated DNA-hexapeptide 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 4 µL of 10× T4 DNA ligase buffer (Fermentas) and 2 µL of 5 U/µL T4 DNA ligase (Fermentas). The sample was incubated at 37 °C for 12 h and purified by 8% PAGE.

Procedure for ligation step in subsequent rounds. A 16 µL sample containing the PCR-amplified DNA pool (~5–10 pmol), 40 pmol of DNA splint, and 50 pmol of 5′-phosphorylated DNA-hexapeptide 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 (Fermentas) and 2 µL of 1 U/µL T4 DNA ligase (Fermentas). 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 N_{40} pool. A 28 μL sample containing 200 pmol of ligated N_{40} pool and 300 pmol of 5′-triphosphorylated RNA substrate 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 50 mM HEPES, pH 7.5, 40 mM $MgCl_2$, 20 mM $MnCl_2$, 150 mM NaCl, and 2 mM KCl. The sample was incubated at 37 °C for 14 h and separated on 8% PAGE.

Procedure for selection step in subsequent rounds. A 14 μL sample containing ligated pool and 40 pmol of 5′-triphosphorylated RNA substrate 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 50 mM HEPES, pH 7.5, 40 mM $MgCl_2$, 20 mM $MnCl_2$, 150 mM NaCl, and 2 mM KCl. The sample was incubated at 37 °C for 14 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.

Mass spectrometry data for PAGE-purified deoxyribozyme products

As described in the main Experimental Section and repeated here for clarity, deoxyribozyme-catalyzed product identities were verified by MALDI mass spectrometry. Products were prepared using the following procedure. A 15 μL sample containing 300 pmol of DNA-anchored hexapeptide substrate, 330 pmol of deoxyribozyme, and 360 pmol of 5′-triphosphorylated RNA substrate 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 reaction was initiated by bringing the sample to 30 μL total volume containing 50 mM HEPES, pH 7.5, 40 mM $MgCl_2$, 20 mM $MnCl_2$, 150 mM NaCl, and 2 mM KCl and incubating at 37 °C for 14 h. The product was precipitated with ethanol, separated by 20% PAGE, extracted from the polyacrylamide gel in TEN buffer (10 mM Tris, pH 8.0, 300 mM NaCl, and 1 mM EDTA), and precipitated with ethanol. The sample was dissolved in 20 μL of water; 10 μL was desalted by C_{18} ZipTip and used for mass spectrometry. All observed mass values were in accord with expectations. The MALDI mass spectrometry data are shown in Figure S4.

8VM1 product with DNA-AAAY^{P}AA substrate: *m*/*z* calcd. 12372.1, found 12381.9 (Δ = +0.08%).
8VM1 product with DNA-AAAS^{P}AA substrate: *m*/*z* calcd. 12296.0, found 12299.3 (Δ = –0.03%).
8VP1 product with DNA-HEG-AAAY^{P}AA substrate: *m*/*z* calcd. 12719.4, found 12721.0 (Δ = +0.01%).
8VP1 product with DNA-HEG-AAAS^{P}AA substrate: *m*/*z* calcd. 12643.3, found 12645.9 (Δ = +0.02%).

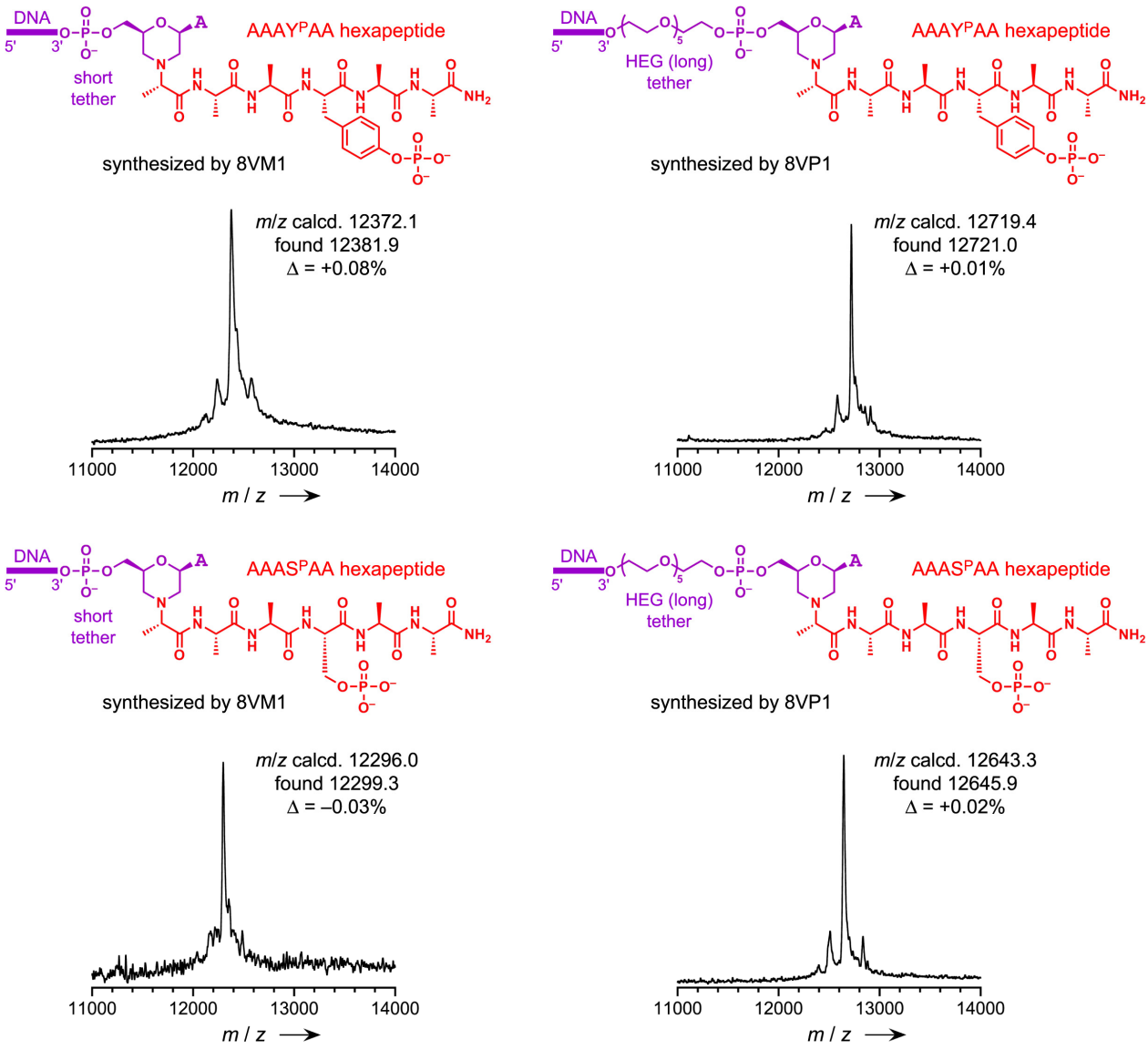

Figure S4. MALDI mass spectrometry data for PAGE-purified deoxyribozyme products.

Diagram of Figure 4 experiment

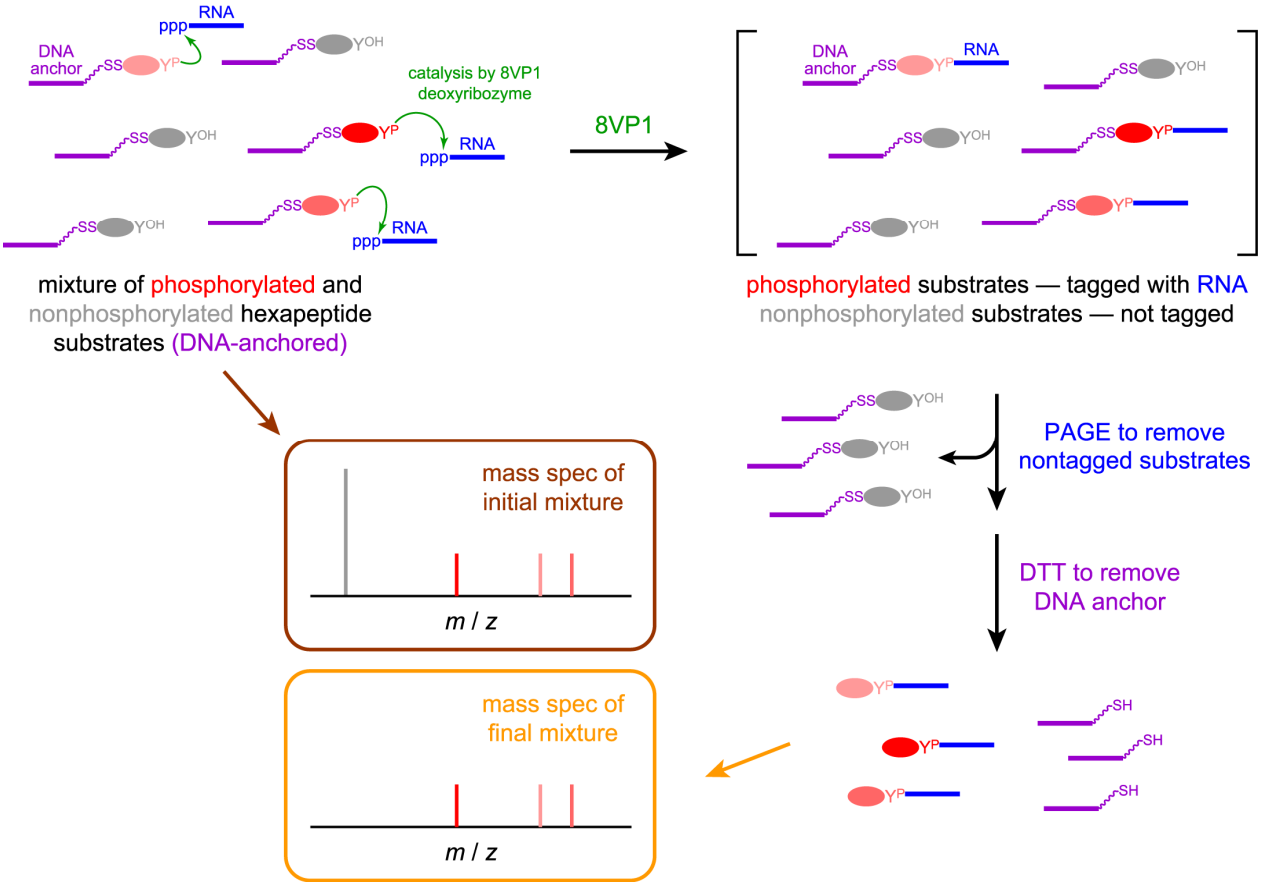

Figure S5. Diagram of Figure 4 experiment. The mass spectrometry data in Figure 4 was obtained using the illustrated procedure (details in Experimental Section), in which PAGE separation of the RNA-tagged DNA-anchored phosphopeptides was performed after the DNA-catalyzed RNA tagging reaction. A similar spectrum to that shown on the bottom of Figure 4, albeit with weaker signal, was observed when the PAGE separation step was omitted (data not shown).

References for Supporting Information

[1] A. Flynn-Charlebois, Y. Wang, T. K. Prior, I. Rashid, K. A. Hoadley, R. L. Coppins, A. C. Wolf, S. K. Silverman, *J. Am. Chem. Soc.* **2003**, *125*, 2444-2454; D. M. Kost, J. P. Gerdt, P. I. Pradeepkumar, S. K. Silverman, *Org. Biomol. Chem.* **2008**, *6*, 4391-4398.