DNA-Catalyzed Amide Hydrolysis

Cong Zhou, Joshua L. Avins, Paul C. Klauser, Benjamin M. Brandsen, Yujeong Lee, and Scott K. Silverman*

Department of Chemistry, University of Illinois at Urbana-Champaign, 600 South Mathews Avenue, Urbana, Illinois 61801, United States

Table of Contents

Full version of ref. 6b: McKeague, M.; McConnell, E. M.; Cruz-Toledo, J.; Bernard, E. D.; Pach, A.; Mastronardi, E.; Zhang, X.; Beking, M.; Francis, T.; Giamberardino, A.; Cabecinha, A.; Ruscito, A.; Aranda-Rodriguez, R.; Dumontier, M.; DeRosa, M. C. *J. Mol. Evol.* **2015**, *81*, 150.

Full version of ref. 12b: Gold, L.; Ayers, D.; Bertino, J.; Bock, C.; Bock, A.; Brody, E. N.; Carter, J.; Dalby, A. B.; Eaton, B. E.; Fitzwater, T.; Flather, D.; Forbes, A.; Foreman, T.; Fowler, C.; Gawande, B.; Goss, M.; Gunn, M.; Gupta, S.; Halladay, D.; Heil, J.; Heilig, J.; Hicke, B.; Husar, G.; Janjic, N.; Jarvis, T.; Jennings, S.; Katilius, E.; Keeney, T. R.; Kim, N.; Koch, T. H.; Kraemer, S.; Kroiss, L.; Le, N.; Levine, D.; Lindsey, W.; Lollo, B.; Mayfield, W.; Mehan, M.; Mehler, R.; Nelson, S. K.; Nelson, M.; Nieuwlandt, D.; Nikrad, M.; Ochsner, U.; Ostroff, R. M.; Otis, M.; Parker, T.; Pietrasiewicz, S.; Resnicow, D. I.; Rohloff, J.; Sanders, G.; Sattin, S.; Schneider, D.; Singer, B.; Stanton, M.; Sterkel, A.; Stewart, A.; Stratford, S.; Vaught, J. D.; Vrkljan, M.; Walker, J. J.; Watrobka, M.; Waugh, S.; Weiss, A.; Wilcox, S. K.; Wolfson, A.; Wolk, S. K.; Zhang, C.; Zichi, D. *PLoS ONE* **2010**, *5*, e15004.

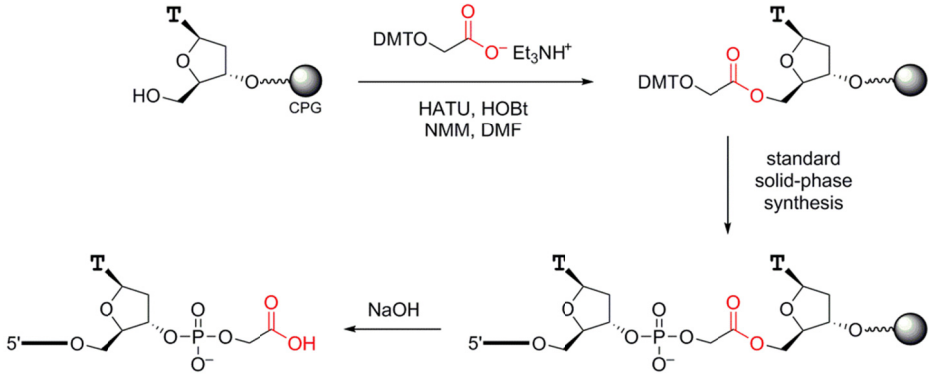
Full version of ref. 12e: Davies, D. R.; Gelinas, A. D.; Zhang, **C**.; Rohloff, J. C.; Carter, J. D.; O'Connell, D.; Waugh, S. M.; Wolk, S. K.; Mayfield, W. S.; Burgin, A. B.; Edwards, T. E.; Stewart, L. J.; Gold, L.; Janjic, N.; Jarvis, T. C. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 19971.

Full version of ref. 12i: Gupta, S.; Hirota, M.; Waugh, S. M.; Murakami, I.; Suzuki, T.; Muraguchi, M.; Shibamori, M.; Ishikawa, Y.; Jarvis, T. C.; Carter, J. D.; Zhang, C.; Gawande, B.; Vrkljan, M.; Janjic, N.; Schneider, D. J. *J. Biol. Chem.* **2014**, *289*, 8706.

Full version of ref. 12j: Gelinas, A. D.; Davies, D. R.; Edwards, T. E.; Rohloff, J. C.; Carter, J. D.; Zhang, C.; Gupta, S.; Ishikawa, Y.; Hirota, M.; Nakaishi, Y.; Jarvis, T. C.; Janjic, N. *J. Biol. Chem.* **2014**, *289*, 8720.

Oligonucleotides and amide substrate

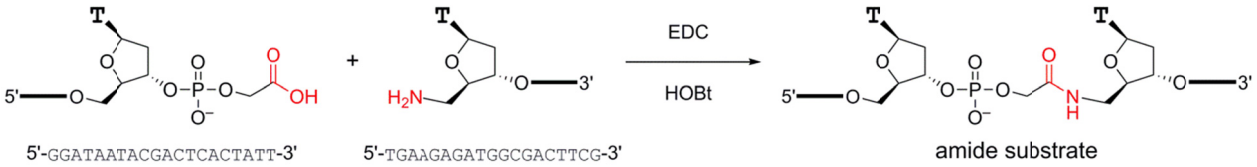
DNA oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA) or prepared by solid-phase synthesis on an ABI 394 instrument using reagents from Glen Research. All oligonucleotides were purified by 7 M urea denaturing PAGE with running buffer 1× TBE (89 mM each Tris and boric acid 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] The amide substrate was synthesized using procedures similar to those used to prepare the ester-linked substrate in our previous report.[2]



Synthesis of 3′-CO_2H oligonucleotide. 5′-DMT-thymidine-derivatized CPG solid support (40 µmol/g; 200 nmol) was deprotected with TCA/CH_2Cl_2 (3 × 1 mL) and washed with CH_2Cl_2 (3 × 5 mL). The support was rinsed with 2% (v/v) NMM/CH_2Cl_2 and CH_2Cl_2 (3 × 5 mL) to improve the efficiency of the next coupling step and dried under a stream of nitrogen. *O*-(4,4′-Dimethoxytrityl)glycolate triethylammonium salt (48 mg, 100 µmol), HATU (38 mg, 100 µmol), and HOBt (14 mg, 100 µmol) were dissolved in 500 µL of dry DMF. NMM (26 µL, 230 µmol) was added to this solution, which was introduced into the DNA synthesis column via two syringes. The coupling step was performed for 16 h with mixing every 5 min for the first 1 h and no mixing for the remaining 15 h. The column was washed with DMF (3 × 5 mL) and CH_2Cl_2 (3 × 5 mL) and dried under a stream of nitrogen. The remainder of the

oligonucleotide was synthesized on the ABI 394 instrument using standard procedures. Cleavage of the oligonucleotide from the CPG and nucleobase deprotection were performed using 200 µL of 400 mM NaOH in 4:1 MeOH:H_2O for 24 h at room temperature. The sample was diluted to 600 µL with water and precipitated with ethanol. The deprotection was completed by treating the sample with concentrated ammonium hydroxide for 16 h at 55 °C. The sample was dried under vacuum and purified by 20% PAGE. MALDI-MS $[M+H]^+$ calcd. 6239.1, found 6237.7.

Synthesis of 5′-amino-5′-deoxythymidine oligonucleotide. Oliogonucleotide synthesis was performed on the ABI 394 instrument using standard procedures and MMT-protected 5′-amino-5′-deoxythymidine phosphoramidite (Glen Research), providing an oligonucleotide terminating in a 5′-amino-5′-deoxythymidine residue.



Solution-phase coupling of 3′-CO_2H oligonucleotide and 5′-amino-5′-deoxythymidine oligonucleotide. A 45 µL sample containing 4.0 nmol of 3′-CO_2H oligonuclotide, 6.0 nmol of 5′-amino-5′-deoxythymidine oligonucleotide, and 5.0 nmol of DNA splint was annealed in 110 mM MOPS, pH 7.0, and 1.1 M NaCl by heating at 95 °C for 3 min and cooling on ice for 5 min. The sequence of the DNA splint was 5′-CGAAGT-CGCCATCTCTTCA**T**AATAGTGAGTCGTATTATCCAACAACAACAACAACAAC-3′, where the boldface **T** is unpaired, and the underlined region is included to shift the splint away from the desired product on PAGE. The reaction was initiated by bringing the sample to 50 µL volume containing 100 mM MOPS, pH 7.0, 1 M NaCl, 50 mM EDC, 50 mM HOBt, and 5% (v/v) DMF, which was required to dissolve the HOBt. The sample was incubated at room temperature for 24 h, precipitated with ethanol, and purified by 20% PAGE. MALDI-MS $[M+H]^+$ calcd. 12113.0, found 12113.8.

2′-Deoxynucleotide 5′-triphosphates and 2′-deoxynucleoside 3′-phosphoramidites

The ^{Am}dU 5′-triphosphate (^{Am}dUTP) was from TriLink BioTechnologies (cat. no. N-0249). The ^{HO}dU 5′-triphosphate (^{HO}dUTP) was from TriLink BioTechnologies (cat. no. N-0259). The ^{Am}dU phosphoramidite was from Berry & Associates (cat. no. BA0311). The ^{COOH}dU phosphoramidite was from Glen Research (cat. no. 10-1035). The ^{HO}dU phosphoramidite was from Glen Research (cat. no. 10-1093). The ^{COOH}dU 5′-triphosphate (^{COOH}dUTP) was synthesized from (*E*)-5-(2-carbomethoxyvinyl)-2′-deoxyuridine as described in a subsequent section of this document.

In vitro selection procedure

The selection procedure, cloning, and initial screening of individual clones were performed essentially as described previously,[2] with additional steps to enable incorporation of the modified nucleotides. An overview of the key selection and capture steps of each round is shown in Figure 1C. A depiction of these steps with nucleotide details is shown in Figure S1. The random deoxyribozyme pool was 5′-CGAAGTCGC-CATCTCTTC-N_{40}-ATAGTGAGTCGTATTAAGCTGATCCTGATGG-3′, where the initially random N_{40} region includes modified nucleotides. Each random pool was prepared by solid-phase synthesis, replacing all dT in the N_{40} region with one of ^{Am}dU, ^{COOH}dU, or ^{HO}dU using the corresponding 2′-deoxynucleoside 3′-phosphoramidite. Primers were 5′-CGAAGTCGCCATCTCTTC-3′ (forward primer) and 5′-$(AAC)_{18}$XCCATCA-GGATCAGCT-3′, where X is the HEG spacer to stop Taq polymerase (reverse primer). The $(AAC)_{18}$ tail was especially long to assure PAGE separation of template and ^{Am}dU-modified product strands. For convenience, the same tail length was used with the ^{HO}dU and ^{COOH}dU modifications, although the more standard $(AAC)_4$ tail can be used instead. In each round, the ligation step to attach the deoxyribozyme pool at its 3′-end with the 5′-end of the amide substrate was performed using a DNA splint and T4 DNA ligase. The ligation splint sequence was 5′-ATAGTGAGTCGTATTATCCTCCATCAGGATCAGCTTAATACGACT-CACTAT-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 KOD XL 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. Nucleotide sequences of the amide substrate, the deoxyribozyme binding arms, the 5′-amino capture oligonucleotide, and the capture splint are shown in Figure S1B. T4 DNA ligase was from Thermo Fisher. KOD XL polymerase was from Novagen.

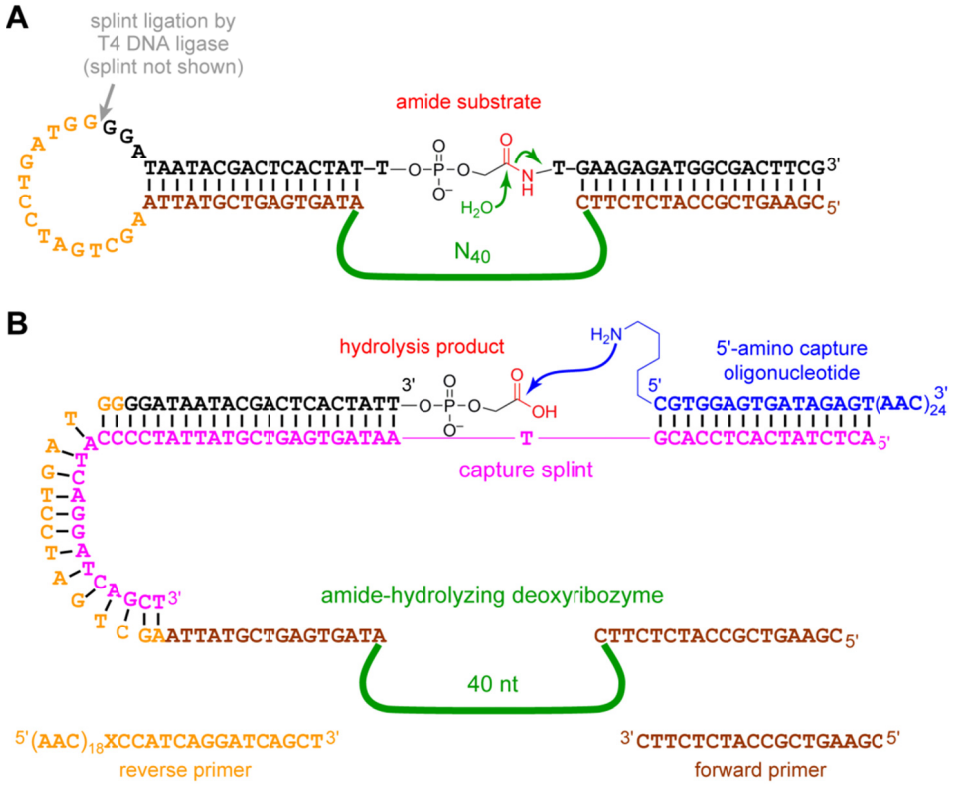


Figure S1. Nucleotide details of the selection and capture steps of in vitro selection. (A) Selection step. (B) Capture step. See text for details of PCR and primer extension using the forward and reverse primers. In the reverse primer, X is the HEG spacer (Glen Spacer 18) that stops extension by DNA polymerase and leads to a size difference between the two PCR product strands. Note that the capture splint enforces proximity of the 5′-amino group of the capture oligonucleotide and the carboxyl group of the hydrolysis product, such that amino or carboxyl groups within the initially random region or 3′-binding arm do not react appreciably during the capture step incubation time.

Procedure for ligation step in round 1. A 25 μL sample containing 600 pmol of modified DNA pool, 700 pmol of DNA splint, and 800 pmol of amide 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 and 2 μL of 5 U/μL T4 DNA ligase. 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 modified DNA pool synthesized by primer extension (~5–10 pmol), 25 pmol of DNA splint, and 50 pmol of amide 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 2 μL of 1 U/μL T4 DNA ligase. The sample was incubated at 37 °C for 12 h and purified by 8% PAGE.

Procedure for selection step in round 1. The selection experiment was initiated with 200 pmol of the ligated N_{40} pool. A 20 μL sample containing 200 pmol of ligated N_{40} 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, 1 mM $ZnCl_2$, 20 mM $MnCl_2$, 40 mM $MgCl_2$, and 150 mM NaCl. 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. A 10 μL sample containing 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, 1 mM $ZnCl_2$, 20 mM $MnCl_2$, 40 mM $MgCl_2$, and 150 mM NaCl. The sample was incubated at 37 °C for 14 h and precipitated with ethanol.

Procedure for alkaline treatment in round 1 of ^{HO}dU selection. For the selection experiment that included ^{HO}dU, a brief alkaline treatment was performed between the selection and capture steps. This treatment accommodated the possibility that a hydroxyl group from a ^{HO}dU nucleotide could serve in place of water as the nucleophile, thereby leading to a macrolactone intermediate that would require subsequent hydrolysis to complete the cleavage process. The precipitated selection sample was dissolved in 45 μL of water, to which was added 5 μL of 500 mM NaOH (50 mM final NaOH concentration). The sample was incubated at room temperature for 30 min and precipitated with ethanol.

Procedure for alkaline treatment in subsequent rounds of ^{HO}dU selection. The precipitated selection sample was dissolved in 9 μL of water, to which was added 1 μL of 500 mM NaOH (50 mM final NaOH concentration). The sample was incubated at room temperature for 30 min and precipitated with ethanol.

Procedure for capture step in round 1. A 90 μL sample containing the selection product, 300 pmol of capture splint, and 500 pmol of 5′-amino capture oligonucleotide was annealed in 100 mM MOPS, pH 7.0, and 1 M NaCl by heating at 95 °C for 3 min and cooling on ice for 5 min. The capture reaction was initiated by bringing the sample to 100 μL total volume containing 100 mM MOPS, pH 7.0, 1 M NaCl, 100 mM EDC, and 100 mM HOBt. The sample was incubated at room temperature for 15 h, precipitated with ethanol, and separated by 8% PAGE.

Procedure for capture step in subsequent rounds. A 22.5 μL sample containing the selection product, 30 pmol of capture splint, and 50 pmol of 5′-amino capture oligonucleotide was annealed in 100 mM MOPS, pH 7.0, and 1 M NaCl by heating at 95 °C for 3 min and cooling on ice for 5 min. The capture reaction was initiated by bringing the sample to 25 μL total volume containing 100 mM MOPS, pH 7.0, 1 M NaCl, 50 mM EDC, and 50 mM HOBt. The sample was incubated at room temperature for 15 h and

loaded directly on 8% PAGE. In parallel, during each round a standard capture reaction was performed using the N_{40} pool ligated to the hydrolysis product. The yield is shown as "control" in Figure S2. The PAGE position of this standard capture product was used as a reference to excise the capture product for the selection sample.

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 capture product, 50 pmol of forward primer, 200 pmol of reverse primer, 20 nmol of each dNTP, and 10 μL of 10× KOD XL polymerase buffer, and 2 μL of 2.5 U/μL KOD XL polymerase. This sample was primer-extended and then cycled 10 times according to the following PCR program: 94 °C for 2 min, 47 °C for 2 min, 72 °C for 30 min; then 94 °C for 2 min, 10× (94 °C for 1 min, 47 °C for 1 min, 72 °C for 1 min), 72 °C for 5 min. KOD XL polymerase was removed by phenol/chloroform extraction. Second, a 50 μL sample was prepared containing 1 μL of the 10-cycle PCR product, 25 pmol of forward primer, 100 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 (no modified nucleotides are being incorporated). 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 8% PAGE, and the reverse-complement single strand was isolated for subsequent primer extension. The forward and reverse single-stranded PCR products were separable because formation of the reverse product was initiated using the reverse primer, which contains a nonamplifiable spacer that stops Taq polymerase.

Procedure for primer extension. A 25 μL sample was prepared containing the reverse-complement single strand (which contains a nonamplifiable spacer that stops KOD XL polymerase), 50 pmol of forward primer, 7.5 nmol each of dATP, dGTP, dCTP, and modified ^{X}dUTP (X = Am, COOH, HO), 20 μCi of α-^{32}P-dCTP (800 Ci/mmol), 2.5 μL of 10× KOD XL polymerase buffer, and 0.5 μL of 2.5 U/μL KOD XL polymerase. Primer extension was performed in a PCR thermocycler according to the following program: 94 °C for 2 min, 47 °C for 2 min, 72 °C for 1 h. The sample was separated by 8% PAGE to provide the modified DNA pool for the next selection round.

Procedure for cloning and initial screening. Using 1 μL of a 1:1000 dilution of the 10-cycle PCR product from the relevant selection round, 30-cycle PCR was performed using the same procedure as described, omitting α-^{32}P-dCTP and using primers 5′-CGAAGTCGCCATCTCTTC-3′ (forward primer, 25 pmol) and 5′-TAATTAATTAATTACCCATCAGGATCAGCT-3′ (reverse primer, 25 pmol), where the extensions with TAA stop codons in each frame were included to suppress false negatives in blue-white screening.[3] The PCR product was purified on 2% agarose and cloned using a TOPO TA cloning kit (Invitrogen). Miniprep DNA samples derived from individual *E. coli* colonies were assayed by digestion with EcoRI to ascertain the presence of the expected inserts. Using the miniprep DNA samples, PCR (same conditions as 30-cycle PCR during selection) was performed to synthesize the reverse-complement templates for the subsequent primer extension reactions. For primer extension, 25 μL samples were prepared, each containing a reverse-complement template, 100 pmol of forward primer, 5.0 nmol each of dATP, dGTP, dCTP, and modified ^{X}dUTP (X = Am, COOH, HO), 2 μCi of α-^{32}P-dCTP (800 Ci/mmol), 2.5 μL of 10× KOD XL polymerase buffer, and 0.5 μL of 2.5 U/μL KOD XL polymerase. Primer extension was performed in a PCR thermocycler according to the following program: 94 °C for 2 min, 4× (94 °C for 2 min, 47 °C for 2 min, 72 °C for 1 h), 72 °C for 1 h. The samples were separated by 8% PAGE to provide the modified deoxyribozymes. The concentration of each PAGE-purified deoxyribozyme strand was estimated from the radioactive intensity relative to suitable standards. Each screening assay used ~0.1 pmol of 5′-^{32}P-radiolabeled amide substrate and at least 10 pmol of deoxyribozyme in the single-turnover assay procedure described in a subsequent section of this document.

Selection progressions

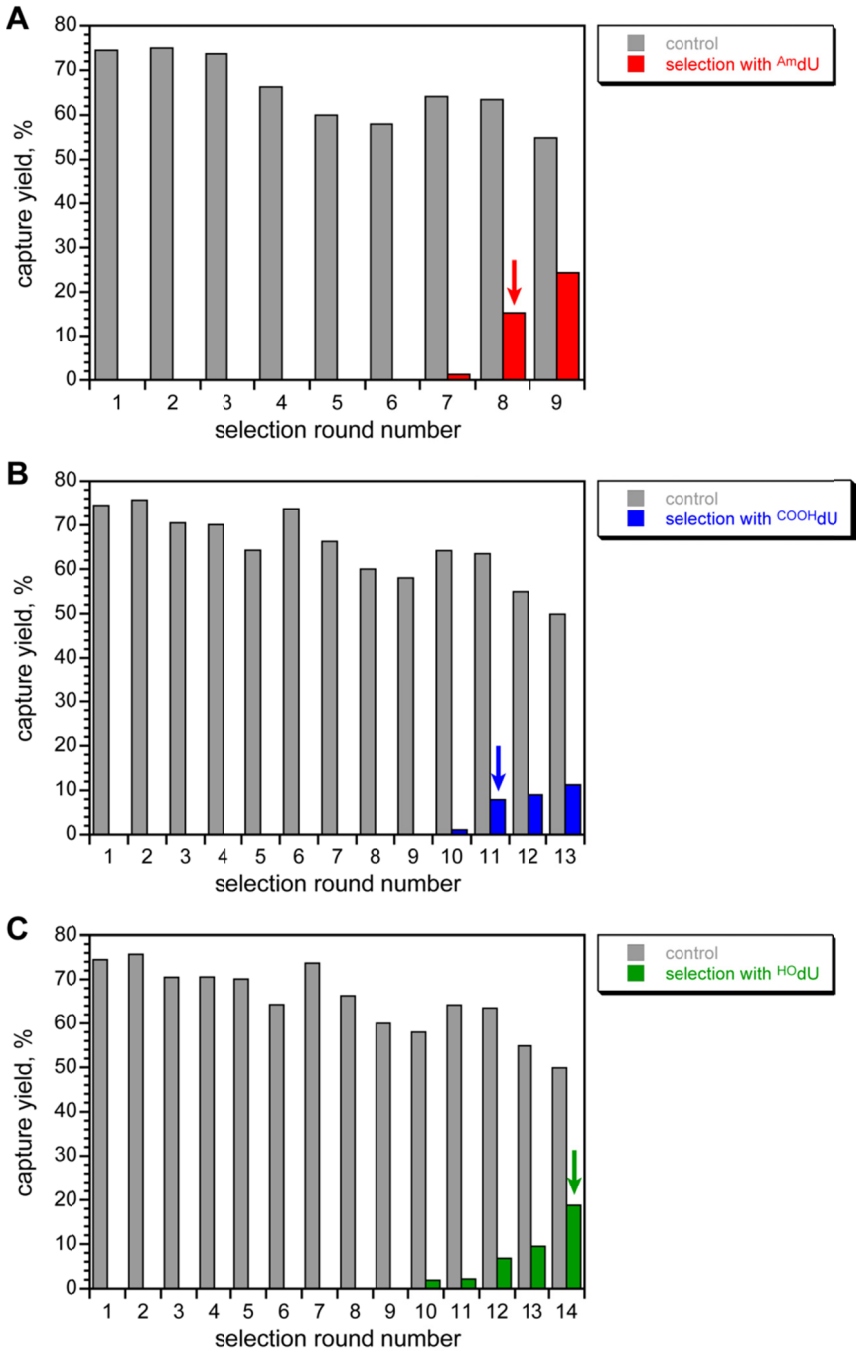


Figure S2. Progressions of the in vitro selection experiments. In each round, "control" refers to the EDC-promoted capture yield for the product that has a 3′-COOH group, and "selection" refers to the EDC-promoted capture yield using the deoxyribozyme pool for that round. Arrows mark the cloned rounds. (A) Selection with ^{Am}dU. (B) Selection with ^{COOH}dU. (C) Selection with ^{HO}dU.

Sequences of individual deoxyribozymes

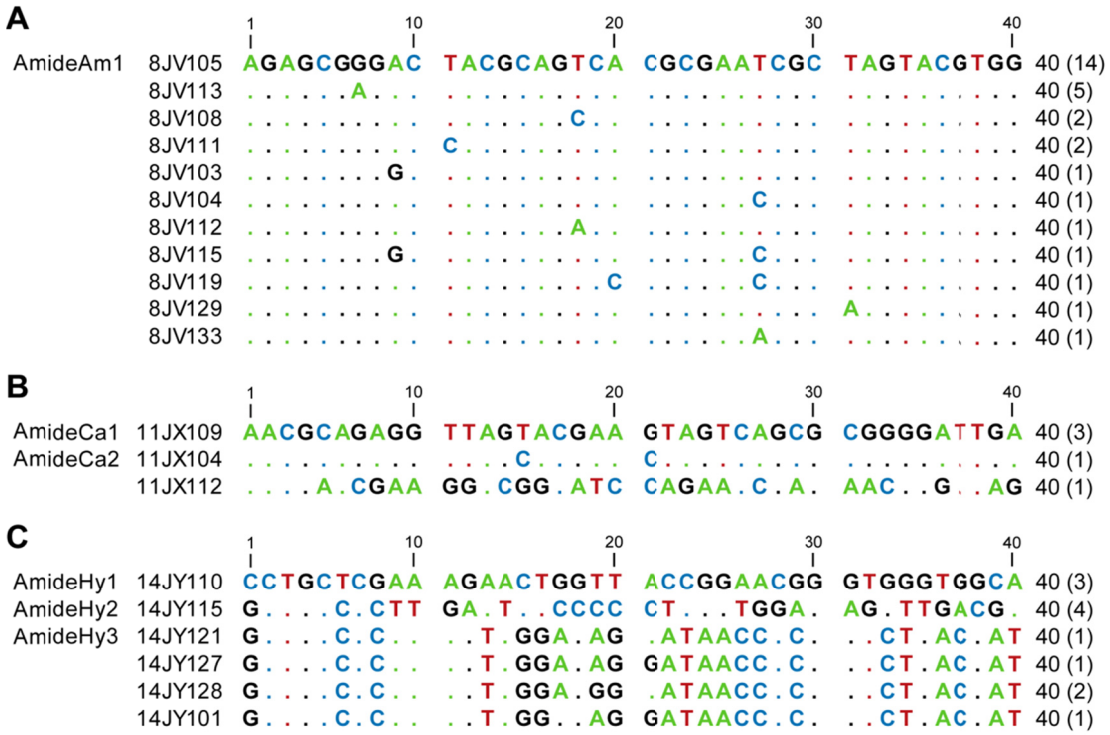


Figure S3. Sequences of the initially random regions of the new deoxyribozymes. (A) ^{Am}dU deoxyribozymes. (B) ^{COOH}dU deoxyribozymes. (C) ^{HO}dU deoxyribozymes. All deoxyribozymes were used as 5′-CGAAGTCGCCATCT-CTTC-N_{40}-ATAGTGAGTCGTATTA-3′, where N_{40} represents the specific 40 nucleotides of the initially random region as determined through the selection process. All alignments show only the initially random region. A dot denotes conservation, i.e., the same nucleotide as in the uppermost sequence. Next to each sequence length in nucleotides on the far right is shown (in parentheses) the number of times that sequence was found during cloning. Each deoxyribozyme is listed with its internal laboratory designation such as 8JV105, where 8 is the round number, JV1 is the systematic alphabetical designation for the particular selection, and 05 is the clone number.

Procedure for preparing modified deoxyribozymes by primer extension

A 50 µL sample was prepared containing 400 pmol of the reverse-complement template 5′-TAATACG-ACTCACTAT-**N**$_{40}$-GAAGAGATGGCGACTTCGGAAAGAGCAATAGTAA-3′ (the boldface **N**$_{40}$ is complementary to the 40 nt initially random region in the deoxyribozyme; the underlined nucleotides are arbitrary sequence included to allow PAGE separation of the primer extension product from the template), 800 pmol of forward primer, 15 nmol each of dATP, dGTP, dCTP, and modified ^{X}dUTP (X = Am, COOH, HO), 5.0 µL of 10× KOD XL polymerase buffer, and 1.0 µL of 2.5 U/µL KOD XL polymerase. Primer extension was performed in a PCR thermocycler according to the following program: 94 °C for 2 min, 4× (94 °C for 2 min, 47 °C for 2 min, 72 °C for 1 h), 72 °C for 1 h. The sample was separated by 12% PAGE to provide the modified deoxyribozyme.

Single-turnover deoxyribozyme assay procedure

The amide substrate was 5′-^{32}P-radiolabeled using γ-^{32}P-ATP and polynucleotide kinase. A 10 µL sample containing 0.2 pmol of 5′-^{32}P-radiolabeled amide substrate and 20 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 hydrolysis 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 $MgCl_2$, and 150 mM NaCl. The sample was incubated at 37 °C. At appropriate time points, 2 µL aliquots were

quenched with 5 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 = $Y \bullet (1 - e^{-kt})$, 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.

Mass spectrometry procedure

To prepare the cleavage products using a deoxyribozyme, a 25 µL sample containing 100 pmol of amide substrate and 120 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 hydrolysis reaction was initiated by bringing the sample to 50 µL total volume containing 70 mM HEPES, pH 7.5, 1.0 mM $ZnCl_2$, 20 mM $MnCl_2$, 40 mM $MgCl_2$, and 150 mM NaCl. The sample was incubated at 37 °C for 48 h, precipitated with ethanol, desalted by Millipore C_{18} ZipTip, and analyzed by MALDI mass spectrometry. 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 (instrument purchased with support from NIH S10RR027109A).

Assaying 3′-binding arm of AmideAm1 for ^{Am}dU requirements

To assess functional contributions of ^{Am}dU nucleotides within the 3′-binding arm of AmideAm1, we used splint ligation to prepare a version of this deoxyribozyme without 3′-binding arm modifications. The 5′-segment (i.e., 5′-binding arm + initially random 40 nucleotides) was prepared by primer extension using the forward primer from Figure S1 and reverse complement template 5′-**CCACGTACTAGCGATTCGCGTGAC-TGCGTAGTCCCGCTCT**GAAGAGATGGCGACTTCGGAAAGAGCAATAGTAA-3′, where the boldface nucleotides are complementary to the initially random 40 nucleotides, the standard nucleotides are complementary to the 5′-binding arm (binding site for forward primer), and the underlined nucleotides are arbitrary sequence included to allow PAGE separation of the primer extension product from the template. The 3′-segment 5′-AGAGTGAGTCGTATTA-3′ was prepared by solid-phase synthesis. The splint sequence was 5′-TAATACG-ACTCACTAT**CCACGTACTAGCGATTCGCG**-3′. To prepare the deoxyribozyme without 3′-binding arm modifications, a 26 µL sample containing 200 pmol of the 5′-segment, 300 pmol of DNA splint, and 400 pmol of 3′-segment 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 and 1 µL of 5 U/µL T4 DNA ligase. The sample was incubated at 37 °C for 12 h and separated by 12% PAGE.

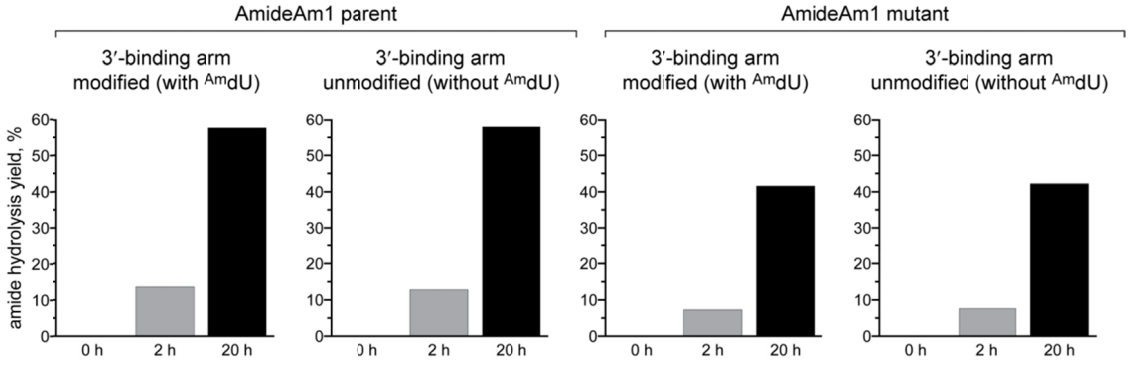


Figure S4. Assaying 3′-binding arm of the AmideAm1 deoxyribozyme for ^{Am}dU requirement. Both the parent version of AmideAm1 (with all six ^{Am}dU nucleotides marked in Figure 2A) and the mutant version of AmideAm1 (with only positions 34 and 38 of the initially random N_{40} region as ^{Am}dU) were assayed when ^{Am}dU was either included or not included within the 3′-binding arm.

Assaying T34 and T38 of AmideAm1 for ^{Am}dU requirements

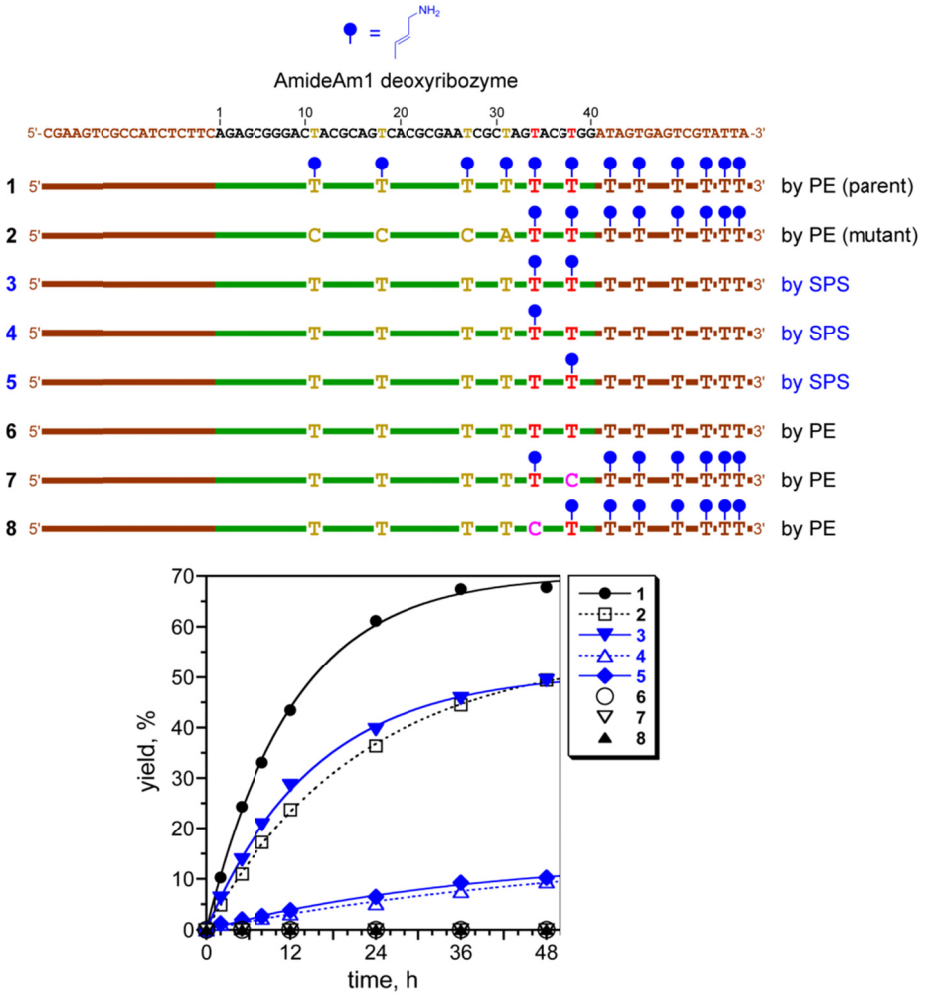


Figure S5. Assaying T34 and T38 of the AmideAm1 deoxyribozyme for ^{Am}dU requirements. Shown are assays for eight variants of AmideAm1. Variants **1** and **2** are the parent and mutant deoxyribozymes from Figure 2, respectively, each prepared by primer extension ("PE"). Therefore, all dT nucleotides in the initially random N_{40} region and 3′-binding arm include the primary amino modification, and removing the four N_{40} primary amino groups at positions T11, T18, T27, and T31 required mutation to dC/dA (with choice of nucleotide dictated by the phylogeny in Figure S3A), because primer extension does not allow site-specific replacements. Variants **3**, **4**, and **5** were each prepared by solid-phase synthesis ("SPS"), which allows individual replacement of amino-modified dT with unmodified dT. Variant **3** includes amino-modified dT at only positions 34 and 38. Variants **4** and **5** include amino-modified dT at only positions 34 or 38, respectively (k_{obs} ~0.02 h^{-1}). Variants **6**, **7**, and **8** were each prepared by primer extension (although variant **6** could equally well be prepared by solid-phase synthesis), and each had no detectable activity. Comparison of the results with variants **4** and **5** to those with variants **7** and **8** indicate that the T nucleobases at positions 34 and 38 are important for catalysis.

Assaying catalysis by ^{COOH}dU deoxyribozymes AmideCa1 and AmideCa2

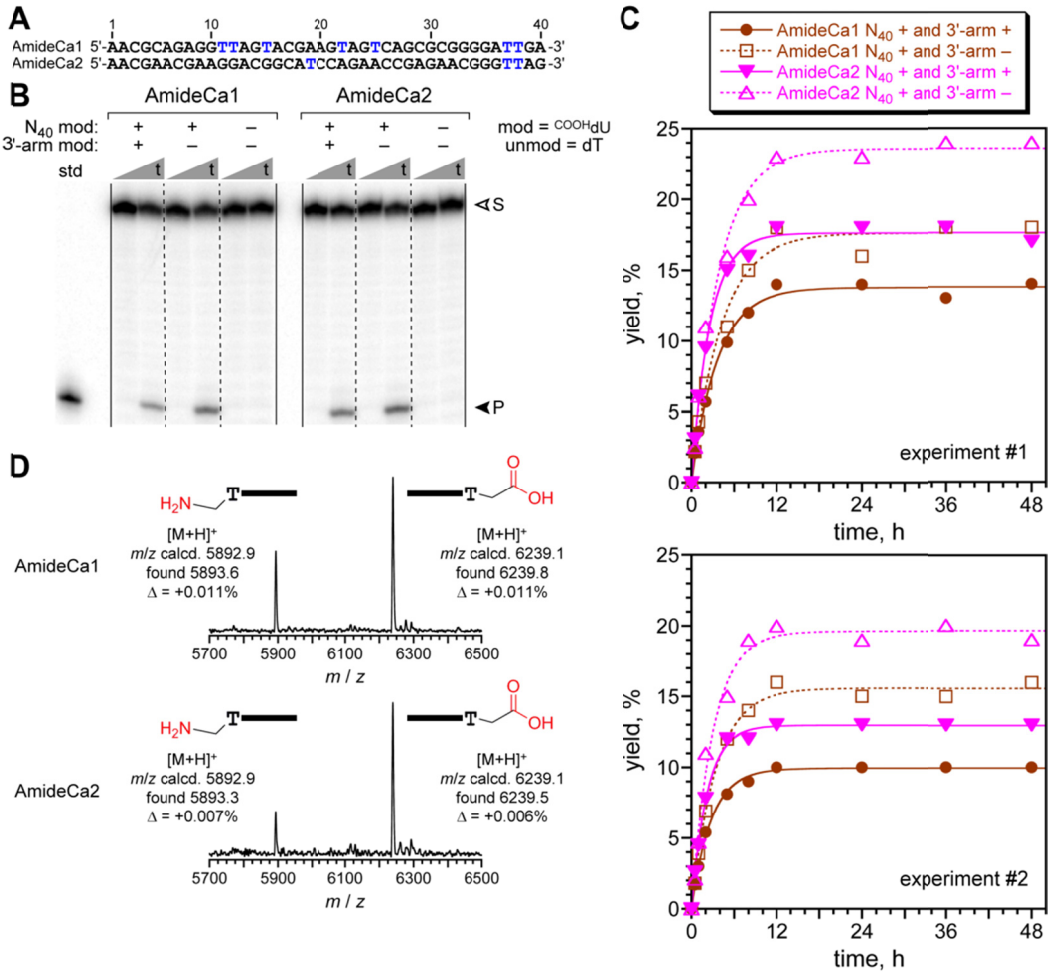


Figure S6. ^{COOH}dU-modified deoxyribozymes for amide hydrolysis. (A) Sequence of the initially random (N_{40}) regions of AmideCa1 and AmideCa2. Each blue T is ^{COOH}dU in the deoxyribozyme. (B) PAGE assay (t = 0, 48 h). The ^{COOH}dU modifications were either present (+) or absent (–) in the initially random region and 3′-binding arm as marked. (C) Kinetic plots. Shown are results from duplicate experiments performed on different days. k_{obs}, h^{-1}: AmideCa1 N_{40} + and 3′-arm + 0.27, 0.36; AmideCa1 N_{40} + and 3′-arm – 0.24, 0.29; AmideCa2 N_{40} + and 3′-arm + 0.39, 0.45; AmideCa2 N_{40} + and 3′-arm – 0.26, 0.34. (D) Mass spectrometry analysis of products.

Assaying 3′-binding arm of ^{HO}dU deoxyribozymes for ^{HO}dU requirement

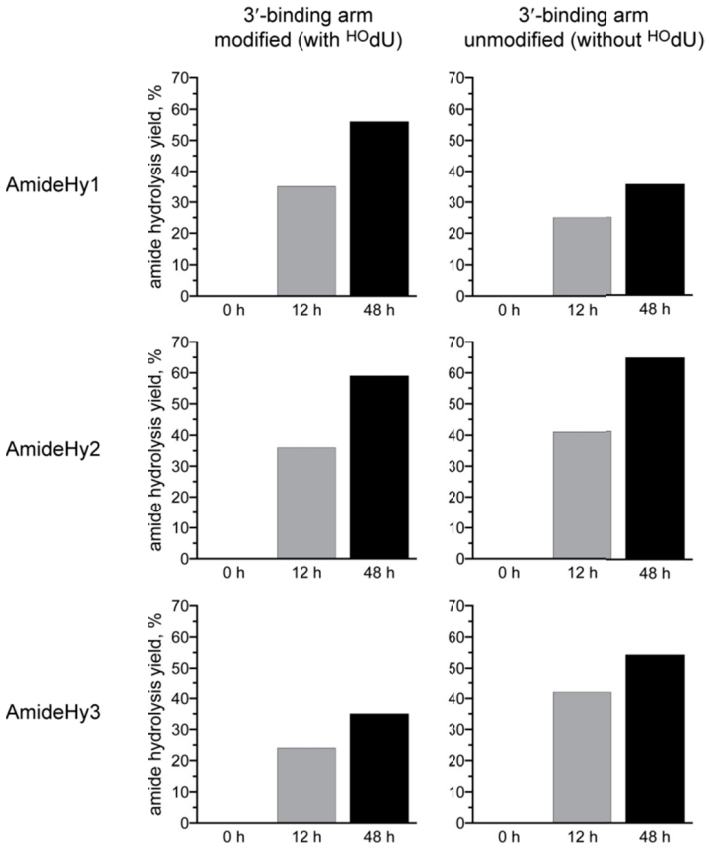


Figure S7. Assays of AmideHy1, AmideHy2, and AmideHy3, each prepared with or without ^{HO}dU modifications in the 3′-binding arm. The results reveal that ^{HO}dU modifications are not required in the 3′-binding arm for any of these three deoxyribozymes. Indeed, for AmideHy3 the yield was higher without 3′-binding arm modifications. Each deoxyribozyme with ^{HO}dU modifications in the 3′-binding arm was synthesized by primer extension from a reverse complement template. Each deoxyribozyme without ^{HO}dU modifications in the 3′-binding arm was synthesized by splint ligation as described in Figure S4.

Assaying deoxyribozymes for divalent metal ion requirements

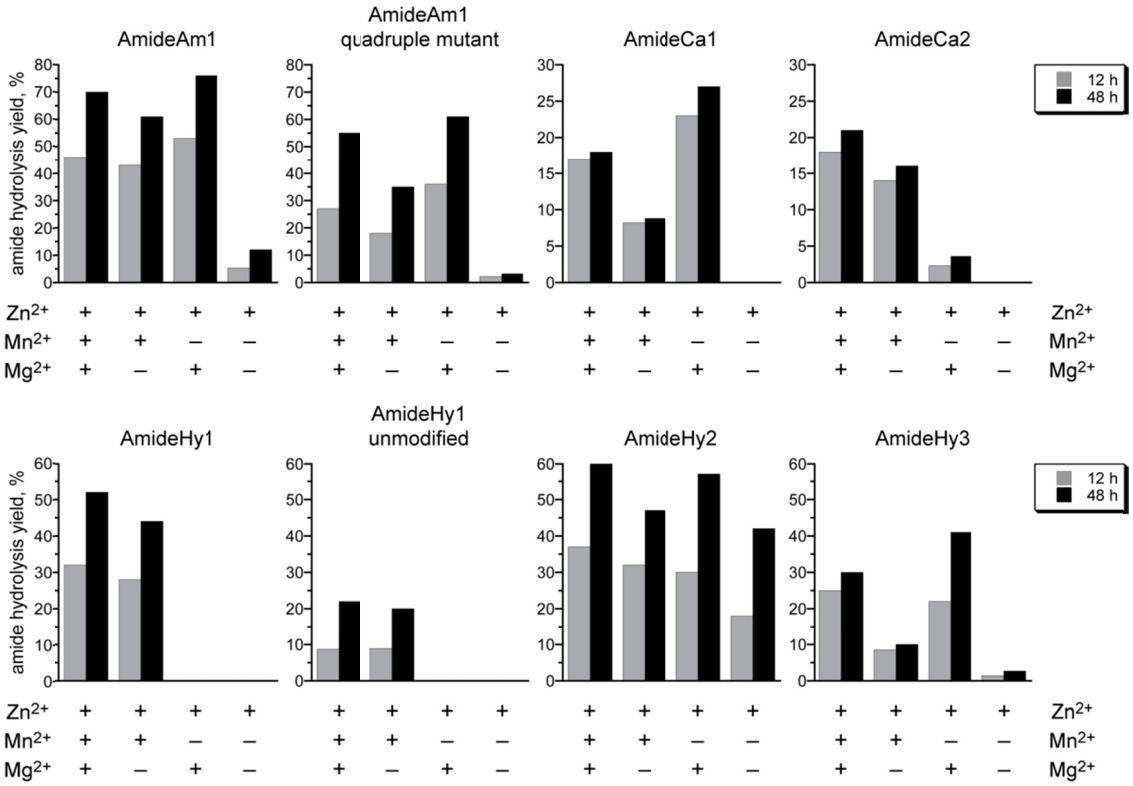


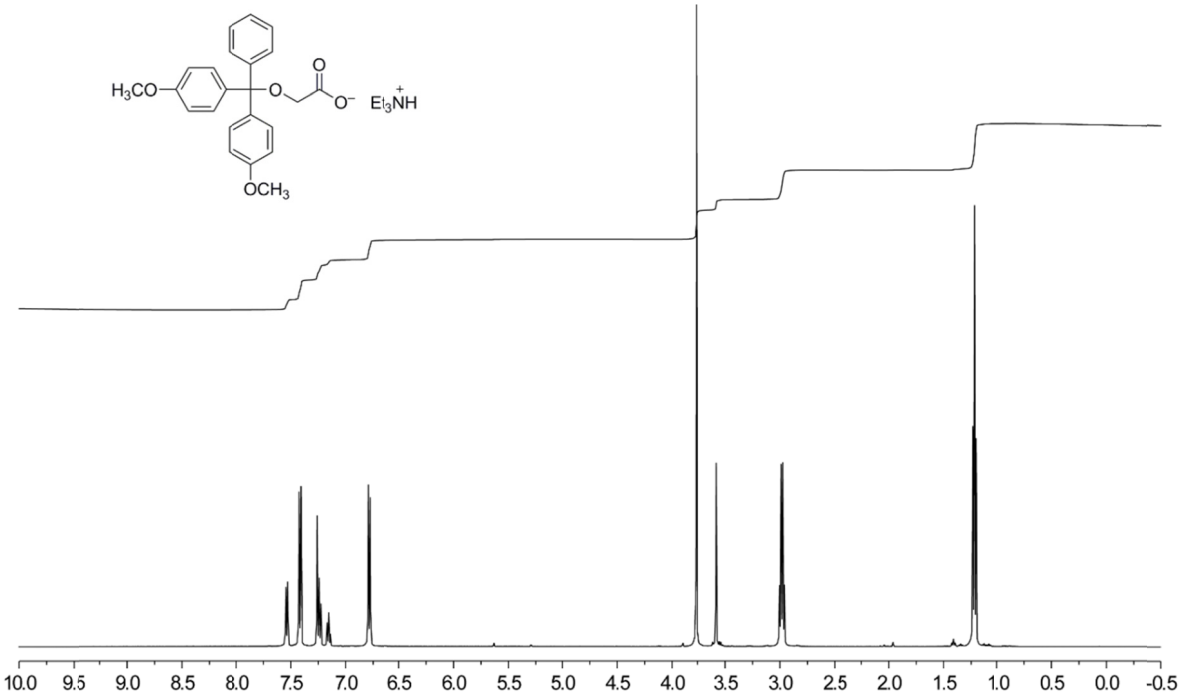
Figure S8. Divalent metal ion requirements of the amide-hydrolyzing deoxyribozymes. Incubation conditions were 70 mM HEPES, pH 7.5, combinations of 1 mM $ZnCl_2$, 20 mM $MnCl_2$, and 40 mM $MgCl_2$ as indicated, and 150 mM NaCl at 37 °C. All deoxyribozymes were prepared by primer extension with modifications throughout the sequence, including the 3′-binding arm. For each of the eight deoxyribozymes, omitting all three metal ions led to no observed amide hydrolysis (<0.5%).

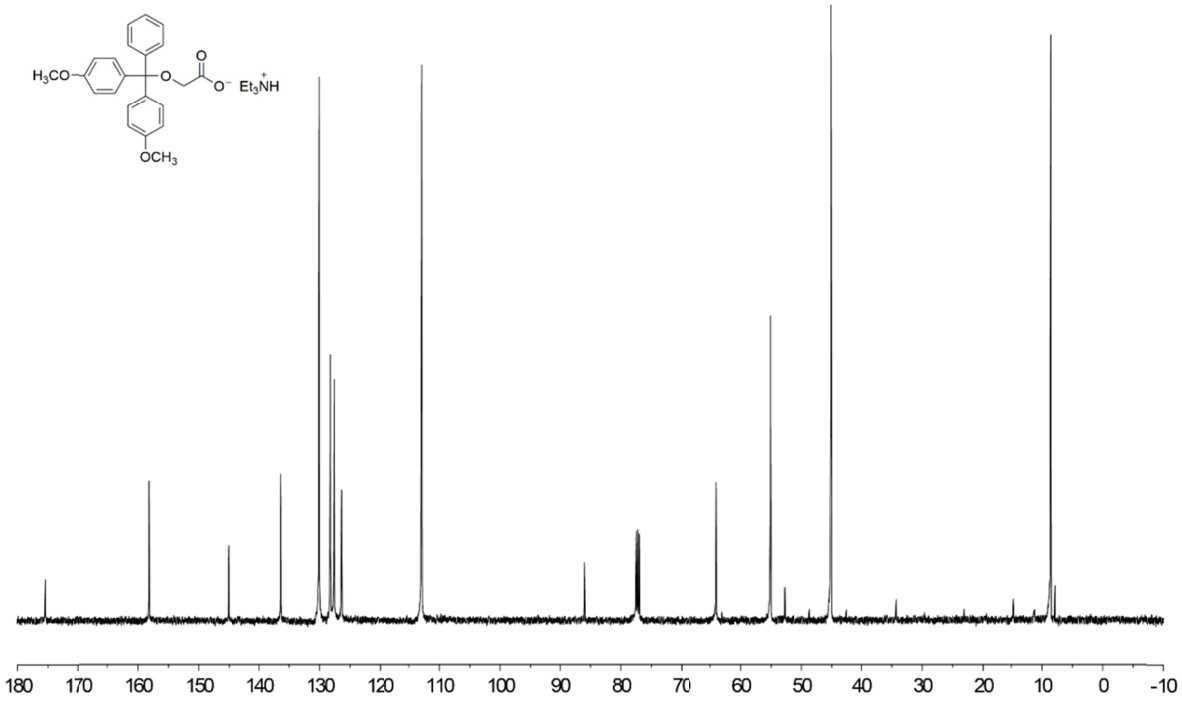
Organic synthesis procedures

Reagents were commercial grade and used without purification unless otherwise indicated. Dry pyridine was obtained from Acros Acroseal bottles. Thin-layer chromatography (TLC) was performed on silica gel plates pre-coated with fluorescent indicator, with visualization by UV light (254 nm). Flash column chromatography was performed with silica gel (230-400 mesh). NMR spectra were recorded on a Varian Unity instrument. The chemical shifts in parts per million (δ) are reported downfield from TMS or DSS (0 ppm) and referenced to the residual proton signal for $CDCl_3$ (7.26 ppm) or to DSS (0 ppm). Apparent multiplicities of ^{1}H NMR peaks are reported as s (singlet), d (doublet), t (triplet), q (quartet), or m (multiplet and overlapping spin systems), along with values for apparent coupling constants (*J*, Hz). Mass spectrometry data were obtained at the UIUC School of Chemical Sciences Mass Spectrometry Laboratory using a Waters Quattro II instrument (LR-ESI).

Synthesis of O-*(4,4′-dimethoxytrityl)glycolate triethylammonium salt.* Glycolic acid (228 mg, 3.0 mmol; Alfa Aesar) was dissolved in dry pyridine (25 mL) under argon. 4,4′-Dimethoxytrityl chloride (1.21 g, 3.6 mmol; Sigma) and a catalytic amount of DMAP (35 mg, 0.3 mmol; Sigma) were added to the solution, which was stirred overnight at room temperature. A portion of CH_3OH (1.5 mL, 36 mmol) was added to quench any unreacted 4,4′-dimethoxytrityl chloride. The solution was diluted with ethyl acetate (100 mL) and washed with 80% (w/v) aqueous $NaHCO_3$ (30 mL) and saturated NaCl (30 mL). The organic layer was dried over anhydrous $MgSO_4$ and concentrated on a rotary evaporator. The oily residue was purified by silica gel column chromatography, eluting with 0–3% CH_3OH in CH_2Cl_2 containing 2% (v/v) Et_3N to yield the desired compound as a colorless oil (906 mg, 63%).

TLC: R_f = 0.19 [1% CH_3OH in CH_2Cl_2 with 2% (v/v) Et_3N].
^{1}H NMR: (500 MHz, $CDCl_3$) δ 7.54 (dd, *J* = 8.3, 1.3 Hz, 2H), 7.42 (d, *J* = 8.8 Hz, 4H), 7.24 (t, *J* = 7.9 Hz, 2H), 7.15 (tt, *J* = 7.3, <2 Hz, 1H), 6.78 (d, *J* = 8.8 Hz, 4H), 3.76 (s, 6H), 3.58 (s, 2H), 2.98 (q, *J* = 7.3 Hz, 6H), 1.22 (t, *J* = 7.3 Hz, 9H) ppm.
^{13}C NMR: (125 MHz, $CDCl_3$) δ 175.4, 158.2, 145.1, 136.4, 130.1, 128.2, 127.6, 126.4, 112.9, 86.0, 64.0, 55.1, 45.1, 8.6 ppm
ESI-MS: *m/z* for carboxylic acid calcd. for $C_{23}H_{21}O_5$ $[M–H]^-$ 377.4; found 377.4.

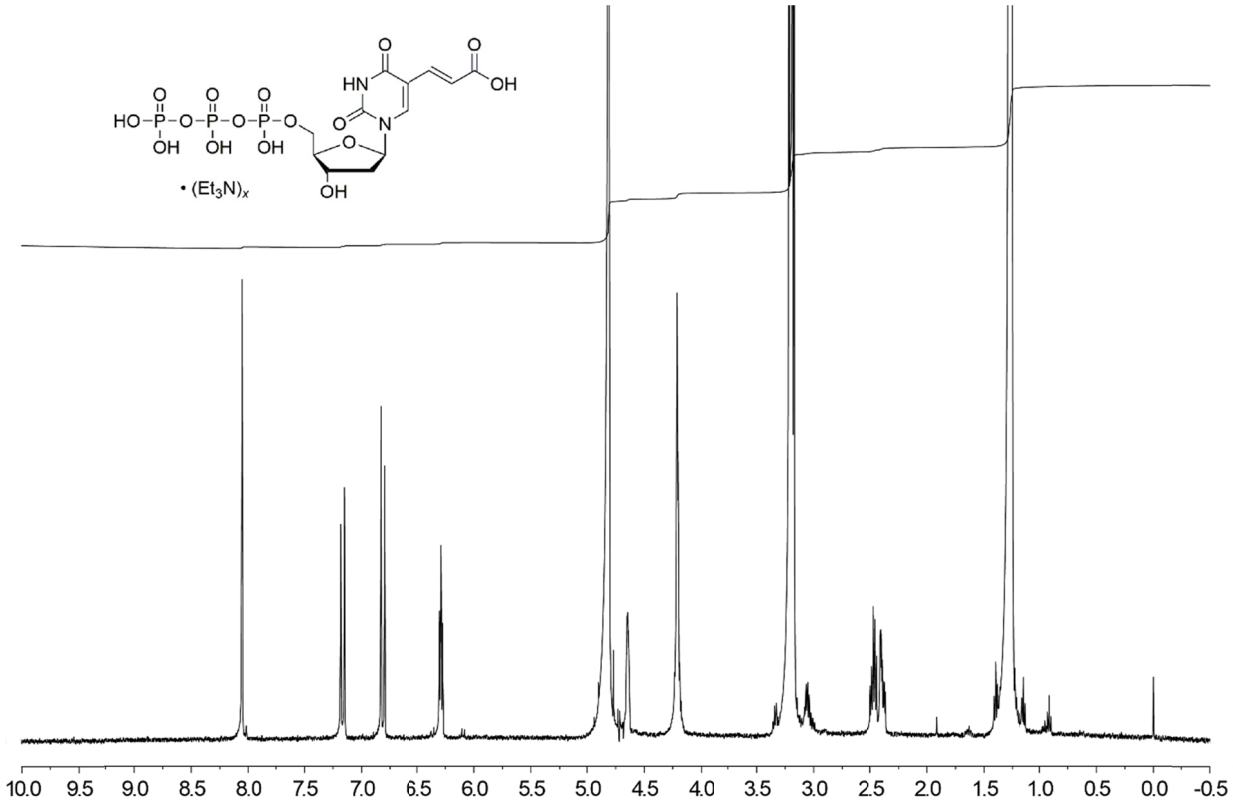




Synthesis of ^{COOH}dU 5′-triphosphate (^{COOH}dUTP). The ^{COOH}dUTP was synthesized from (*E*)-5-(2-carbomethoxyvinyl)-2′-deoxyuridine (Berry & Associates, cat. no. PY7170). The triphosphorylation procedure was modified from the procedure reported by Huang and co-workers.[4] 2-Chloro-1,3,2-benzodioxaphosphorin-4-one (TCI, cat. no. C1210) was portioned into a dried vial in a glove box. 2′-Deoxyuridine and tributylammonium pyrophosphate (Sigma, cat. no. P8533) were dried under vacuum. Tributylamine was dried over 4 Å molecular sieves. Tributylamine (0.65 mL, 2.74 mmol) was added to a solution of tributylammonium pyrophosphate (188 mg, 0.34 mmol) dissolved in 1 mL of anhydrous DMF under argon. This solution was added into a stirred solution of 2-chloro-1,3,2-benzodioxaphosphorin-4-one (50 mg, 0.25 mmol) in 1 mL of anhydrous DMF under argon. The resulting solution was stirred at room temperature for 1 h and then added dropwise over 5 min to a solution of 2′-deoxyuridine (50 mg, 0.16 mmol) in 1 mL of anhydrous DMF under argon, cooled in an ice-salt bath at 0 to –10 °C. The solution was stirred for 5 h and raised to room temperature for 30 min. A solution of 0.02 M iodine in THF/pyridine/water (Glen Research, 8 mL) was added until a permanent brown color was maintained. The brown solution was stirred at room temperature for 30 min. Two sample volumes of water were added. The two-layer mixture was stirred vigorously at room temperature for 1.5 h, followed by addition of 0.1 volumes of 3 M NaCl and 3 volumes of ethanol. The thoroughly mixed sample was precipitated at –80 °C overnight and centrifuged for 30 min at $10{,}000 \times g$ and 4 °C. The precipitated sample of ^{COOMe}dUTP was dried, dissolved in water, and purified by HPLC [Shimdazu Prominence instrument; Phenomenex Gemini-NX C_{18} column, 5 μm, 10×250 mm; gradient of 0.5% solvent A (20 mM triethylammonium acetate in 50% acetonitrile/50% water, pH 7.0) and 99.5% solvent B (20 mM triethylammonium acetate in water, pH 7.0) at 0 min to 25% solvent A and 75% solvent B at 45 min with flow rate of 3.5 mL/min]. To a solution of the ^{COOMe}dUTP in 50 μL of water was added 50 μL of 0.2 M NaOH, forming ^{COOH}dUTP. The sample was incubated at room temperature for 2 h, neutralized with 10 μL of 2.5 M NaOAc buffer, pH 5.2, and purified by HPLC (same gradient as before). The ^{COOH}dUTP was isolated as its triethylammonium salt [12 mg, 8% from (*E*)-5-(2-carbomethoxyvinyl)-2′-deoxyuridine].

^{1}H NMR: (500 MHz, D_2O with 0.01 mg/mL DSS) δ 8.05 (s, 1H), 7.17 (d, J = 16.0 Hz, 1H), 6.81 (d, J = 16.0 Hz, 1H), 6.29 (dd, J = 7.6, 6.3 Hz, 1H), 4.81 (D_2O), 4.64 (m, 1H), 4.21 (m, 3H), 3.19 (q, J = 7.3 Hz, 24H), 2.46 (m, 1H), 2.39 (ddd, J = 14.2, 6.4, 3.4 Hz, 1H), 1.27 (t, J = 7.3 Hz, 36H) ppm. From the integrations, $x \approx 4$ triethylamine molecules were present for each ^{COOH}dUTP molecule.

ESI-MS: *m/z* calcd. for $C_{12}H_{17}N_2O_{16}P_3$ $[M–H]^–$ 537.0; found 537.1.



References for Supporting Information

(1) (a) Flynn-Charlebois, A.; Wang, Y.; Prior, T. K.; Rashid, I.; Hoadley, K. A.; Coppins, R. L.; Wolf, A. C.; Silverman, S. K. *J. Am. Chem. Soc.* **2003**, *125*, 2444. (b) Wang, Y.; Silverman, S. K. *Biochemistry* **2003**, *42*, 15252.

(2) Brandsen, B. M.; Hesser, A. R.; Castner, M. A.; Chandra, M.; Silverman, S. K. *J. Am. Chem. Soc.* **2013**, *135*, 16014.

(3) Langner, J.; Klussmann, S. *BioTechniques* **2003**, *34*, 950.

(4) Caton-Williams, J.; Hoxhaj, R.; Fiaz, B.; Huang, Z. *Curr. Protoc. Nucleic Acid Chem.* **2013**, *52*, 1.30.1.