Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Characterization of deoxyribozymes with site-specific oxidative cleavage activity against DNA obtained by *in vitro* selection

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1. Method

1.1 General information

T4 polynucleotide kinase(PNK), T4 ligase, and FastAP^{TM} were purchased from MBI Fermentas. [γ-^{32}P]ATP was purchased from Furui Biological Engineering(Beijing, China). All of the DNA oligonucleotides were PAGE-purified by Sangon BiotechCo., Ltd. (Shanghai, China). All reagents and chemicals such as NaN_3, DABCO, DMSO, H_2O_2, catalase , *t*-BuOH, SOD, neocuproine and $Mn(OAc)_3•2H_2O$ were of analytical grade and used without further purifications.

1.2 Labeling reactions

Oligonucleotides were incubated with 10 units of Polynucleotide kinase (PNK) at 37 °C for 0.5 h for DNA phosphorylation in a reaction mixture containing 50 mM Tris-HCl, pH 7.8 at 23 °C, 40 mM NaCl, 10 mM $MgCl_2$, 1 mg/mL BSA, and 10 μCi [γ-^{32}P]ATP. The labeled product was purified by denaturing 10 % PAGE.

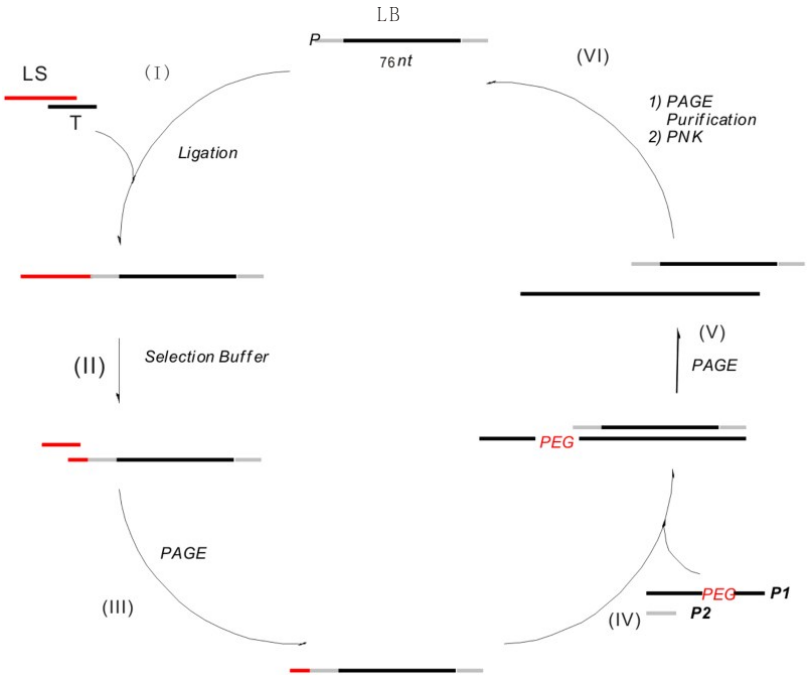
1.3 Standard conditions of deoxyribozyme cleavage assays

Trans-cleaving reaction were carried out with 4.5 μM deoxyribozyme was combined with 15 nM substrate in reaction buffer (50 mM HEPES pH 7.4, 400 mM NaCl, 100 mM KCl, 10mM $MgCl_2$, 7.5 mM $MnCl_2$, 50 μM $CuCl_2$) at 25 °C. Deoxyribozyme-substrate was first heated in H_2O at 90 °C for 30 s, and it was then cooled at room temperature for 5 min. Reaction was initiated by adding the same volume of 2 × Reaction Buffer, and it was stopped after designated period of time by adding EDTA (pH 8.0) to 30 mM. Cleavage products form *trans* reaction were separated by denaturing 10 % or 20 % PAGE and quantitated by phosphorimager.

1.4 Kinetic analyses

Time courses for each deoxyribozyme were conducted at least twice using over 8 time points for each. The experimental data was fit to $Y= Y_{max(}1-e^{(-kobst)})$ exponential equation using non-linear regression analysis in GraphPad Prism 4, from which the observed rate constant (k_{obs}) and maximum cleavage yield (Y_{max}) were determined.

1.5 *In Vitro* selection procedure



Step I: The 5'-phosphorylated 76 nt library LB (5'-A GCT TGT ACT AGT GTT CC N40 TGG ATC GGC ATG ACT AAC) was ligated to 55 nt LS substrate (5'- AAA AAA AAA AAA AAA AAA AA CCT CTG CCG ATC CAT
ACT GCG GAA CAC TTC ATG CG) to yield the LB-LS construct. The reaction mixture containing 1nM of LB, 1nM of LS, and 1 nM of template T (5'-GGA ACA CTA GTA CAA GCT CGC ATG AAG TGT TCC GCA GTA TGG ATC GGC AGA GG) was heated to 94 °C for 1 min and then cooled to room temperature. The 10× ligase buffer 5 ul (Fermentas) and 2 ul of T4 DNA ligase (10 μ/μL) were added to the mixture (50 μM final volume). The reaction mixture was incubated for 12 h at 37 °C. After ethanol precipitation, full library LB-LS was purified by 10% denaturing PAGE.

Step II: The purified LB-LS dissolved into 100 μL H_2O, and a equal volume of 2× Reaction Buffer (composed of 50 mM HEPES, 400 mM NaCl, 100 mM KCl, 10 mM $MgCl_2$, 7.5 mM $MnCl_2$, 50 μM $CuCl_2$) was added. The resultant reaction mixture (with DNA concentration at 7 μM) was allowed to at 37 °C for 12 h. A 2 × volume of stop buffer (45 mM EDTA, pH 8.5) was added to the reaction mixture to quench the reaction.

Step III: The cleavage products were separated by 10% denaturing PAGE. A 5'-P^{32} labeled synthetic oligonucleotide (95nt) with similar length with possible cleaved product (92-98nt) was applied as marker on the gel to help recycle the cleavage product through elution and ethanol precipitation.

Step IV: PCR was used to amplify the recovered cleavage fragments. The PCR in a volume of 100 μL containing 75 mM Tris-HCl (pH 9.0), 2 mM $MgCl_2$, 50 mM KCl, 20 mM $(NH_4)_2SO_4$, 0.5 mM each of four dNTPs, 5 U Tag DNA polymerase, 1μM primer P1 (5'- AAA AAA AAA AAA AAA AAA AA-PEG- GTT AGT CAT GCC GAT CCA) and 1μM primer P2 (5'- AGC TTG TAC TAG TGT TCC). The PCR (Figure S2.1) was carried out for 28 thermo cycles during which the temperature was altered in the following order: 30 s at 94 °C (1 min for the first cycle), 45 s at 59 °C, and 45 s at 72 °C.

Step V: The amplified DNA from PCR reaction above was recovered by ethanol precipitation. The targeted single-stranded DNA was purified by 10% denaturing PAGE.

Step VI: The recovered DNA molecules were incubated with 10 units of PNK in a 10 μL reaction mixture containing 50 mM Tris-HCl, pH 7.6 at 25 °C, 10 mM $MgCl_2$, 5 mM DTT, 1 mM spermidine, and 5μCi [γ-32P]ATP for 30 min, and then added 1ul 100mM ATP for 10 min .The 5'-phosphorylated DNA was used for the next round of selection.

2. Sequences of the deoxyribozymes and substrate.

deoxyribozyme	enzyme region sequence
A-2	5'-AGTGTTCCCACTGTAGCGTCAACCGCTTATTCCTGGTGATAATGGATCGGC-3'
A-3	5'-AGTGTTCCCATCGTAGTGTCAGCCATCGTCTATTCTAGGCGTGGATCGGC-3'
substrate	**sequence**
S1	5'-CCTCTGCCGATCCATACTGCGGAACACT-3'

Fig.S1 Sequence alignments of the deoxyribozymes A-2 and A-3 and substrate S1.

3. Analysis of DNA cleavage sites of A-2 and A-3.

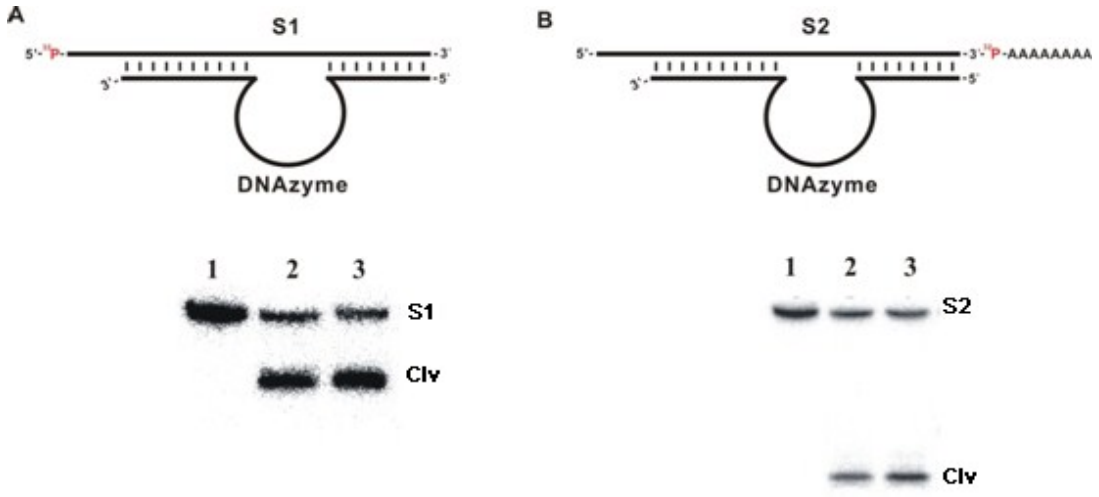


Fig. S2 Cleavage of 5'- (A) and 3'-^{32}P-radiolabeled (B) **S1** substrates by A-2 and A-3. 5'-^{32}P-radiolabeled substrate S1 was prepared using T4 PNK and 3'-^{32}P substrate S2 was prepared by ligating a labeled oligonucleotide (5'-^{32}P-AAAAAAAA-3') using a DNA splint and T4 DNA ligase. All the substrates were purified by denaturing 10 % PAGE. A) Lane 1, DNA substrate (S1) control; lane 2, cleavage by A-2; lane 3, cleavage by A-3; B) Lane 1, DNA substrate (S2) control; lane 2, cleavage by A-2; lane 3, cleavage by A-3. Cleavage assays were under standard single-turnover conditions. Clv identifies 5'-cleavage fragment by deoxyribozyme.

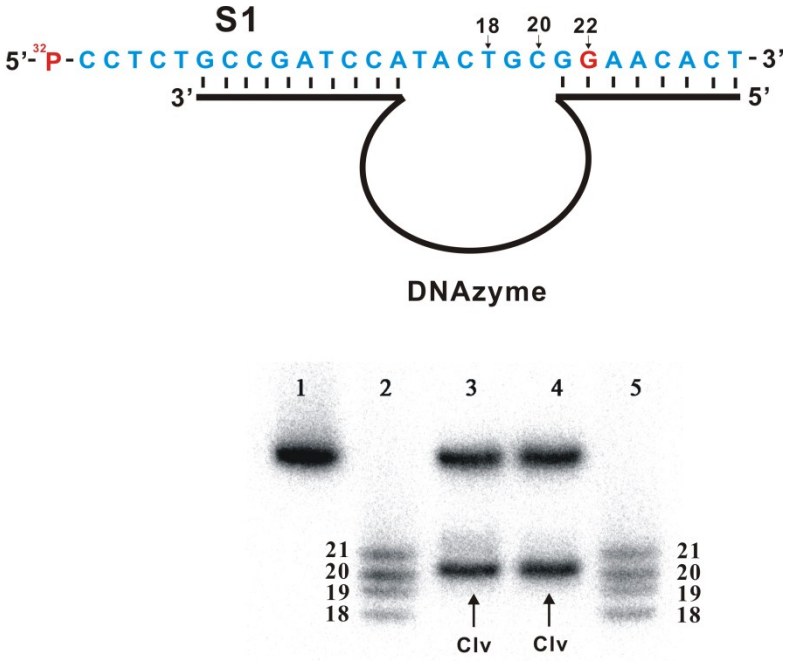


Fig. S3 PAGE gel (20 %) analysis of cleaving reaction catalyzed by A-2 and A-3. Lane 1: 5'-^{32}P labeled DNA substrate S1; lanes 2 and 5: 5'-^{32}P labeled DNA markers with different lengths; lane 3: 5'-^{32}P labeled DNA substrate was cleaved by A-2 under standard conditions (50 mM HEPES pH 7.4, 400 mM NaCl, 100 mM KCl, 10 mM $MgCl_2$, 7.5 mM $MnCl_2$, 50 µM $CuCl_2$) at 37 °C for 12 h. lane 4: 5'-^{32}P labeled DNA substrate was cleaved by A-3 under standard conditions. Numbers identify the length of 5'-^{32}P labeled DNA markers according to S1. Cleaved 5'-cleavage fragment has slightly faster mobility than corresponding 21nt marker maybe

because of the presence of phosphate group at the 3’ end. Clv identifies 5’-cleavage fragment by deoxyribozyme.

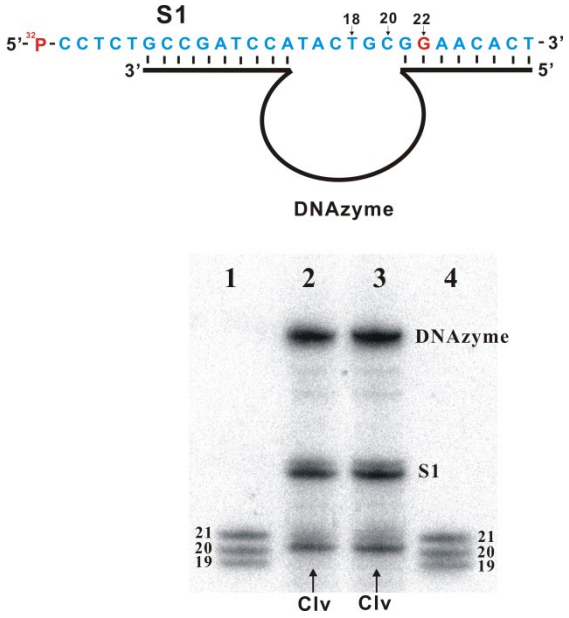


Fig. S4 Treatment of cleaved product with FastAP™ alkaline phosphatase. Lanes 1 and 4 were 5’-^{32}P-radiolabeled synthetic oligonucleotides with different lengths; lane 2: 5’-cleavage product by A-2 was firstly treated with FastAP™ enzyme (37 °C for 30 min) and labeled again with PNK and [γ-^{32}P]ATP; lane 3: 5’-cleavage product by A-3 was firstly treated with FastAP™ enzyme (37 °C for 30 min) and labeled again with PNK and [γ-^{32}P]ATP. Numbers identify the length of 5’-^{32}P labeled DNA markers according to S1. Clv identifies 5’-cleavage fragment by deoxyribozyme.

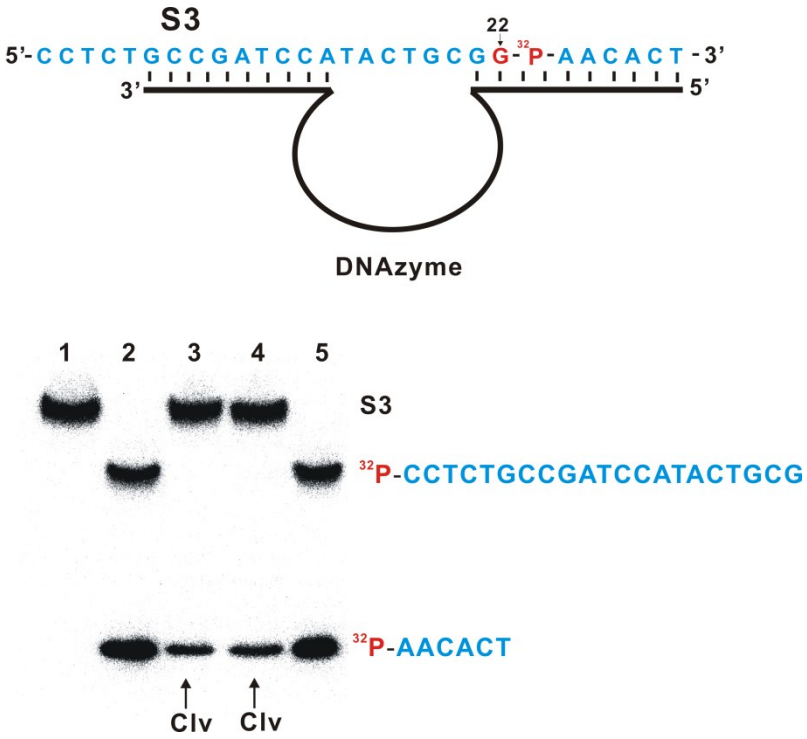


Fig. S5 The PAGE gel analysis of cleavage site of deoxyribozymes A-2 and A-3 by using internally labeled substrates. The internally labeled substrate S3 were prepared using T4 DNA ligase and were purified by denaturing 10% PAGE prior to use. Lane 1, DNA control; lanes 2 and 5, DNA markers (loaded with 5’-^{32}P-radiolabeled synthetic oligonucleotides with different lengths), lanes 3,

cleavage of S3 by A-2; lanes 4, cleavage of S3 by A-3. Clv identifies 5'-cleavage fragment by deoxyribozyme.

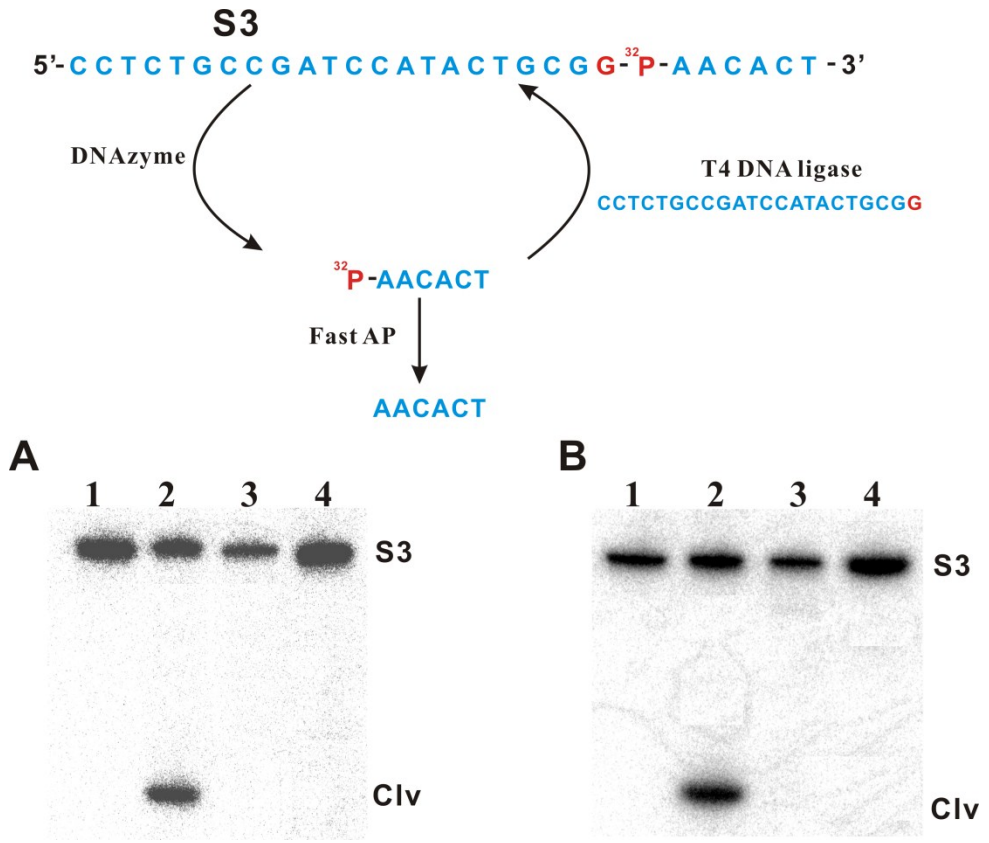


Fig. S6 Dephosphorylation with FastAP™ enzyme and ligation assays with T4 DNA ligase to assign 5'-terminal of 3'-cleavage product by deoxyribozymes A-2 and A-3. Cleavage reactions were carried out by using internally labeled substrate S3. Then, the 3'-cleavage product was treated with FastAP™ or ligated with 5'-CCT CTG CCG ATC CAT ACT GCGG-3' to get substrate S3 again**.** A) Lane 1: internally-labeled S3; lane 2: cleavage of S3 by A-2; lane 3: treatment of cleavage product in lane 3 with FastAP™ enzyme; lane 4, the cleaved product in lane 2 was ligated with a synthetic 22-nucleotide oligonucleotide. B) Lane 1: internally-labeled S3; lane 2: cleavage of S3 by A-3; lane 3: treatment of cleavage product in lane 3 with FastAP™ enzyme; lane 4, the cleaved product in lane 2 was ligated with a synthetic 22-nucleotide oligonucleotide. These results demonstrated that 3'-cleavage product carries an unmodified phosphate group at its 5' terminus. Cleavage assays were under standard single-turnover conditions. Clv identifies 5'-cleavage fragment by deoxyribozyme.

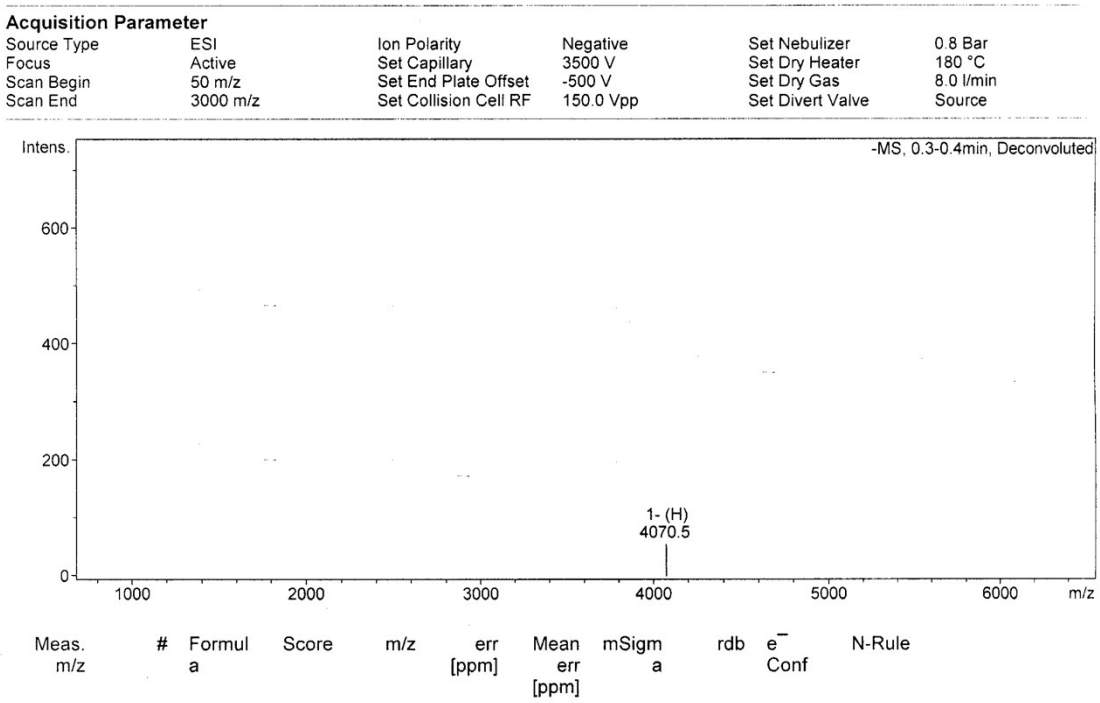


Fig. S7 Mass spectrometry analysis of the 5'-cleavage product (5'-GATCCATACTGCG-modified phosphoester, in Fig S11) of the substrate S4 by **A-2** which separated from HPLC (100 mM TEAA, pH 7.0, HPLC gradient 5-40% acetonitrile/20 min at 0.8 mL/min, 50 °C.). ESI^-, Calcd. 4071.5, found. 4070.5.

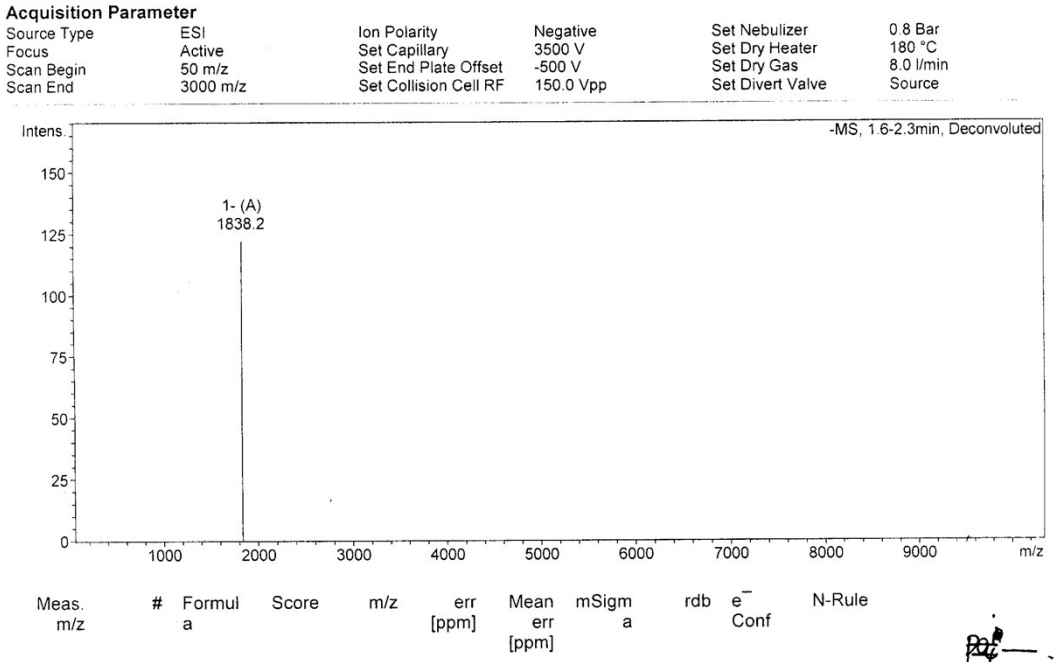


Fig. S8 Mass spectrometry analysis of the 3'-cleavage product (pAACACT-3', Fig S11) of the substrate S4 by **A-2** which separated from HPLC.(100 mM TEAA, pH 7.0, HPLC gradient 5-40% acetonitrile/20 min at 0.8 mL/min, 50 °C.) ESI^-, Calcd. 1838.3, found. 1838.2.

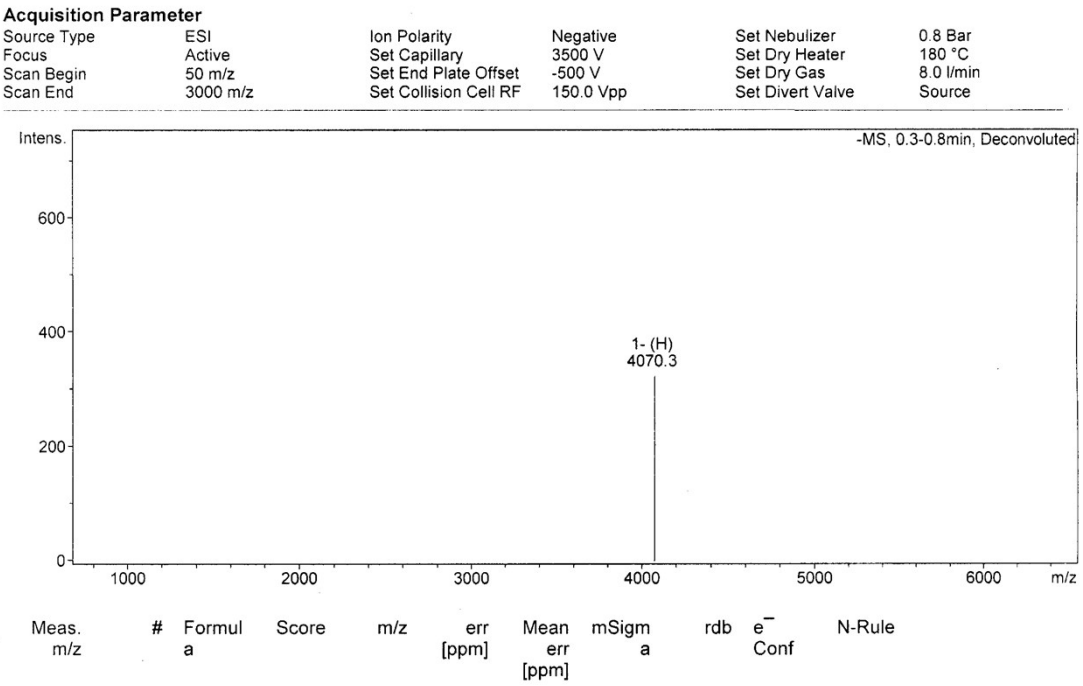


Fig. S9 Mass spectrometry analysis of the 5'-cleavage product (5'-GATCCATACTGCG-modified phosphoester, Fig S11) of the substrate S4 by **A-3** which separated from HPLC (100 mM TEAA, pH 7.0, HPLC gradient 5-40% acetonitrile/20 min at 0.8 mL/min, 50 °C.). ESI^-, Calcd. 4071.5, found. 4070.3.

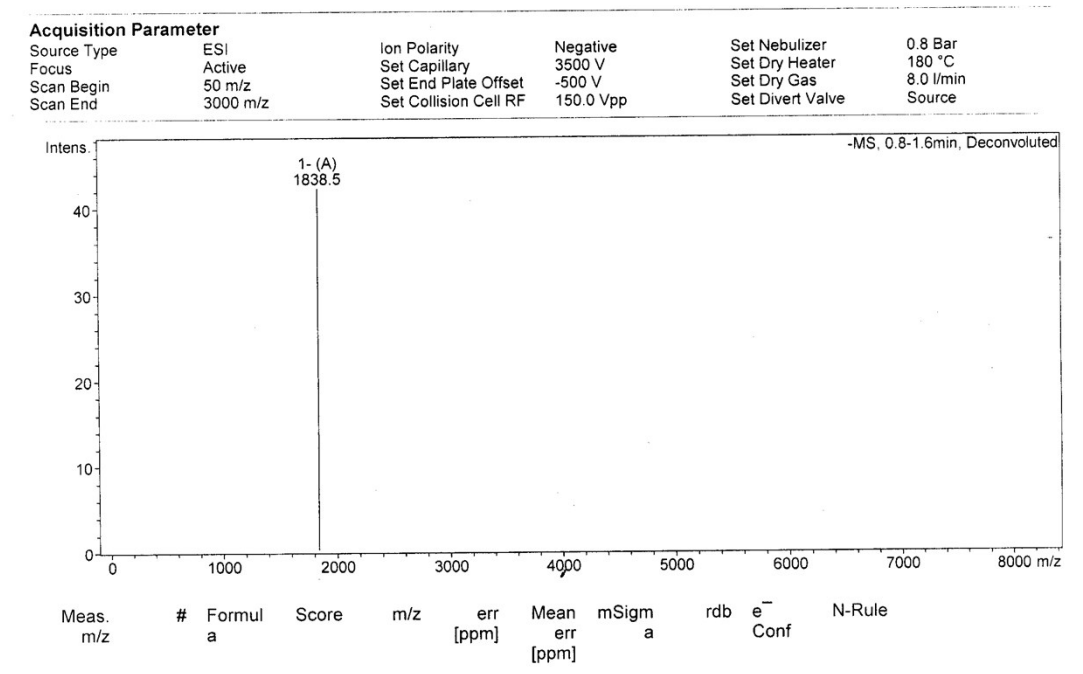


Fig. S10 Mass spectrometry analysis of the 3'-cleavage product (pAACACT-3', Fig S11) of the substrate S4 by **A-3** which separated from HPLC.(100 mM TEAA, pH 7.0, HPLC gradient 5-40% acetonitrile/20 min at 0.8 mL/min, 50 °C.) ESI^-, Calcd. 1838.3, found.

1838.5.

DNAzyme	substrate	mass (L,calcd.)	mass (L found)	mass (R,calcd.)	mass (R found)
A-2	S4	4071.5	4070.5	1838.3	1838.2
A-3	S4	4071.5	4070.3	1838.3	1838.5

Table S1. Mass spectrometry analysis of the cleavage products formed by deoxyribozymes A-2 and A-3 using substrate S4 (5'-GATCCATACTGCGGAACACT-3'). L and R in the table respectively represent 5'- and 3'-cleavage fragments. The date are consistent with denaturing PAGE indicating the cleavage leads to 3'-modified phosphate and 5'- phosphate termini.

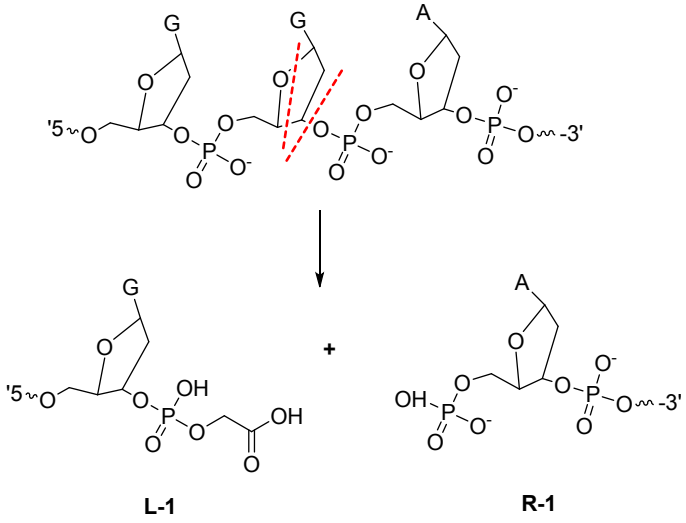


Fig. S11 Chemical structure of cleavage product formed by deoxyribozymes A-2 and A-3.

4. Metal ions requirements for deoxyribozymes.

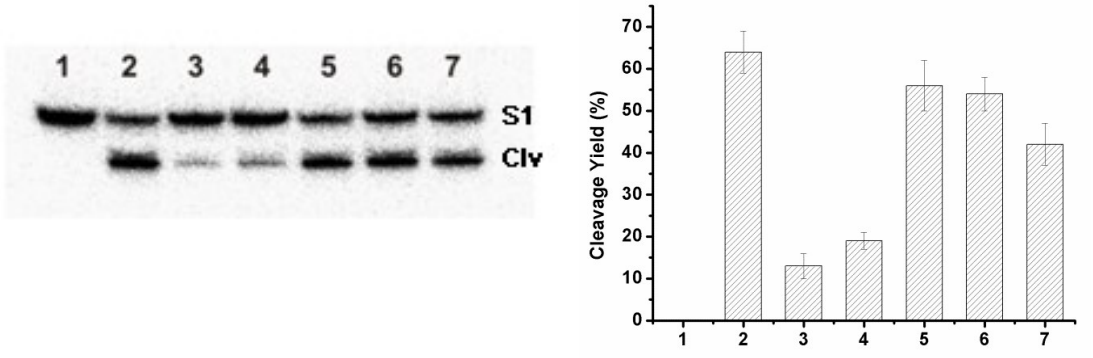


Fig. S12. Metal ions requirements for the deoxyribozyme A-2. (A) Denaturing PAGE image. Lane 1: 5'-^{32}P-radiolabeled substrate; lane 2: Cleaving reaction by A-2 was carried out in reaction buffer; lane 3: minus Cu^{2+};lane 4: minus Mn^{2+}; lane 5: minus Na^{+} and K^{+}; lane 6: minus Mg^{2+}; lane 7: minus Na^{+}, K^{+} and Mg^{2+}; (B): Quantization of cleavage yield to corresponding reactions of A. Clv identifies 5'-cleavage fragment by deoxyribozyme.

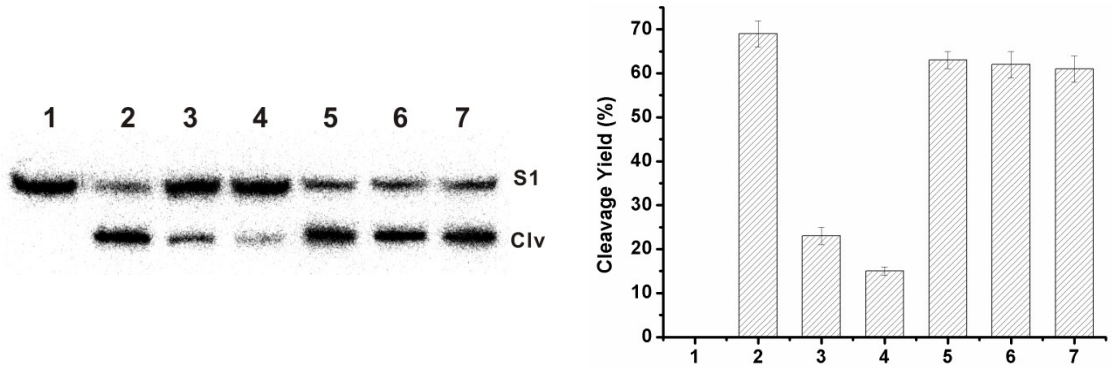


Fig. S13 Metal ions requirements for the deoxyribozyme A-3. (A) Denaturing PAGE image. Lane 1: 5'-^{32}P-radiolabeled substrate; lne 2: Cleaving reaction by A-3 was carried out in buffer; lne 3: minus Cu^{2+}; Lane 4: minus Mn^{2+}; lne 5: minus Na^{+} and K^{+}; lne 6: minus Mg^{2+}; lne 7: minus Na^{+}, K^{+} and Mg^{2+}; (B): Quantization of cleavage yield to corresponding reactions of A. Clv identifies 5'-cleavage fragment by deoxyribozyme.

5. Sequence tolerance and secondary structures of deoxyribozymes .

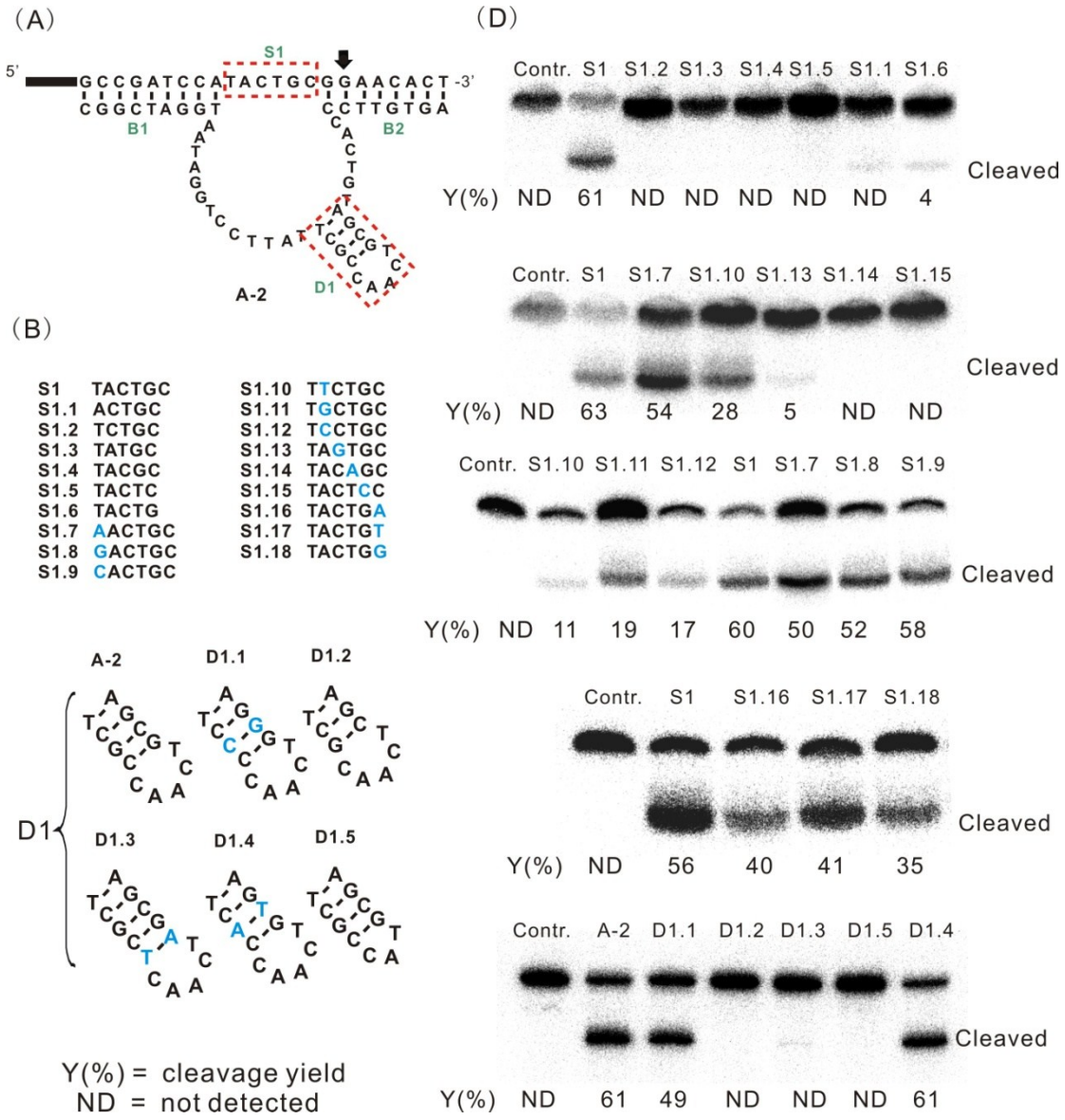


Fig. S14 Secondary structure characterization of deoxyribozyme A-2. (A) A proposed structural mode. (B) Investigating the substrate sequence tolerance and hairpin structure to the function. (C) Denaturing PAGE images corresponding to (B). Cleavage assays were under standard single-turnover conditions. Cleaved identifies 5'-cleavage fragment by deoxyribozyme.

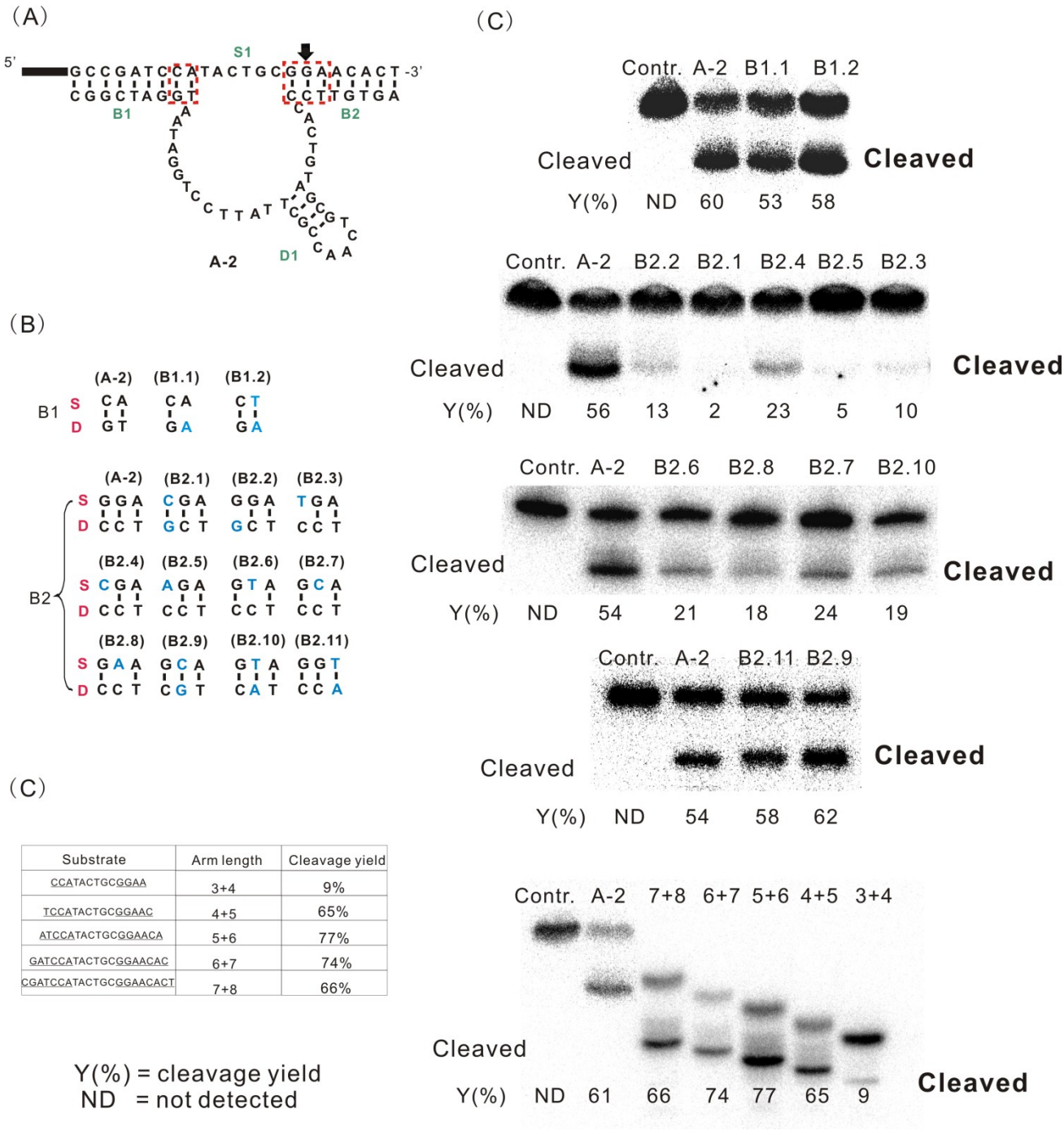


Fig. S15 Secondary structure characterization of deoxyribozyme A-2. (A) A proposed structural mode (B) Investigating the importance of binding arm to the function. (C) Analysis of the effect of varying substrate-recognition domain length. (D) Denaturing PAGE images corresponding to B and C. Cleavage assays were under standard single-turnover conditions. Cleaved identifies 5'-cleavage fragment by deoxyribozyme.

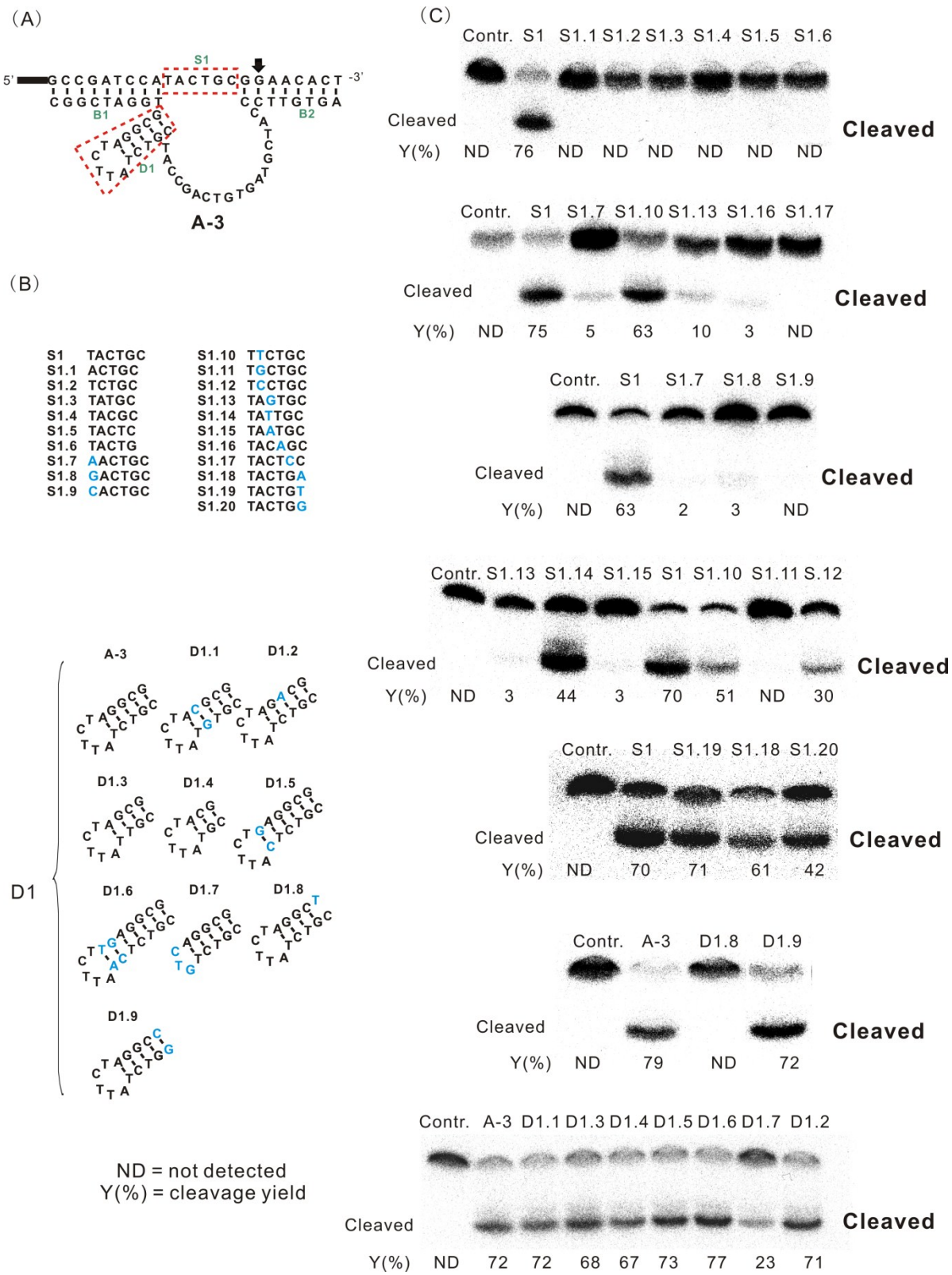


Fig. S16 Secondary structure characterization of deoxyribozyme A-3. (A) A proposed structural mode. (B) Investigating the substrate sequence tolerance and hairpin structure to the function. (C) Denaturing PAGE images corresponding to (B). Cleavage assays were under standard single-turnover conditions. Cleaved identifies 5'-cleavage fragment by deoxyribozyme.

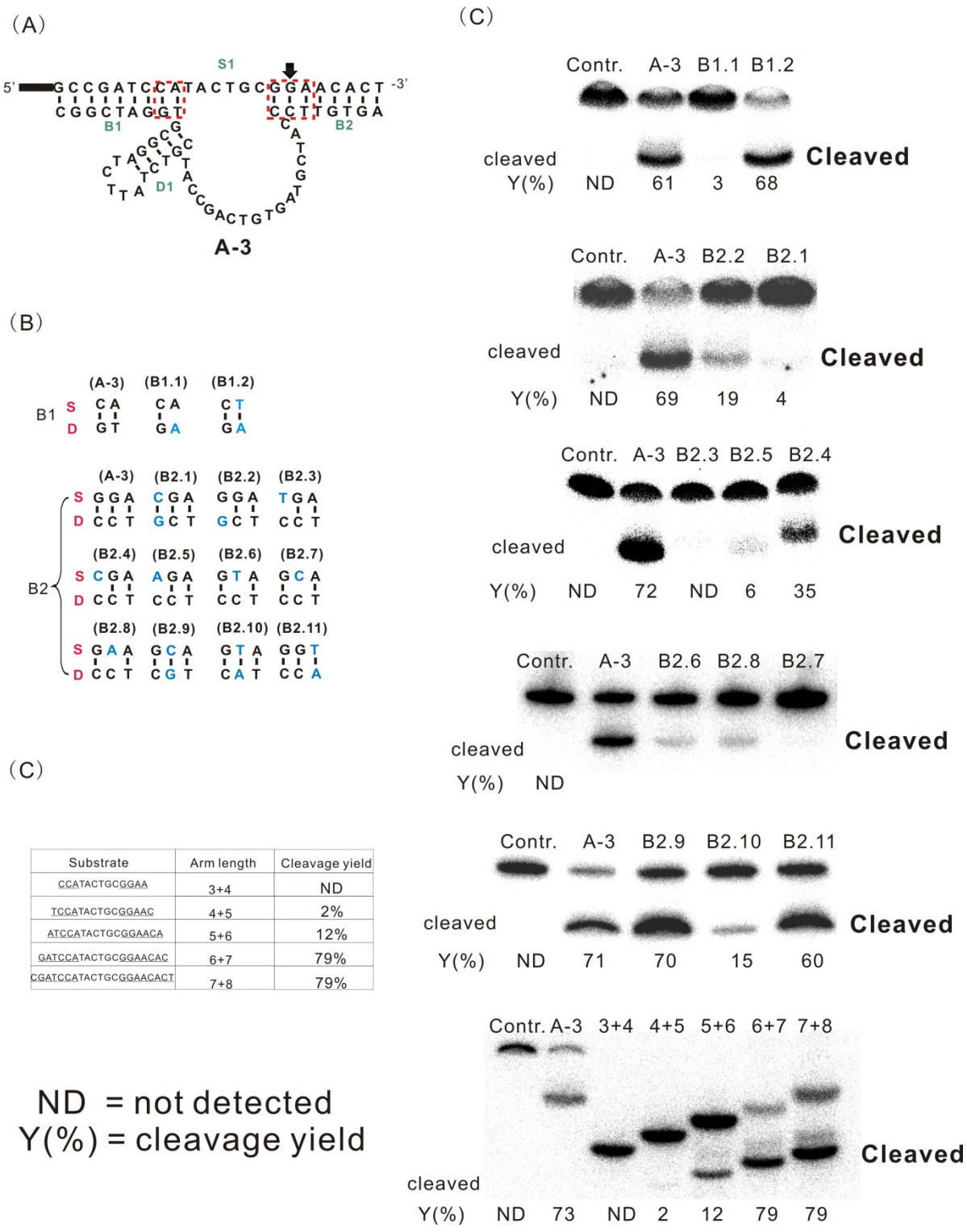


Fig. S17 Secondary structure characterization of deoxyribozyme A-3. (A) A proposed structural mode (B) Investigating the importance of binding arm to the function. (C) Analysis of the effect of varying substrate-recognition domain length. (D) Denaturing PAGE images corresponding to B and C. Cleavage assays were under standard single-turnover conditions. Cleaved identifies 5'-cleavage fragment by deoxyribozyme.

6. Chemical mechanism of the deoxyribozymes.

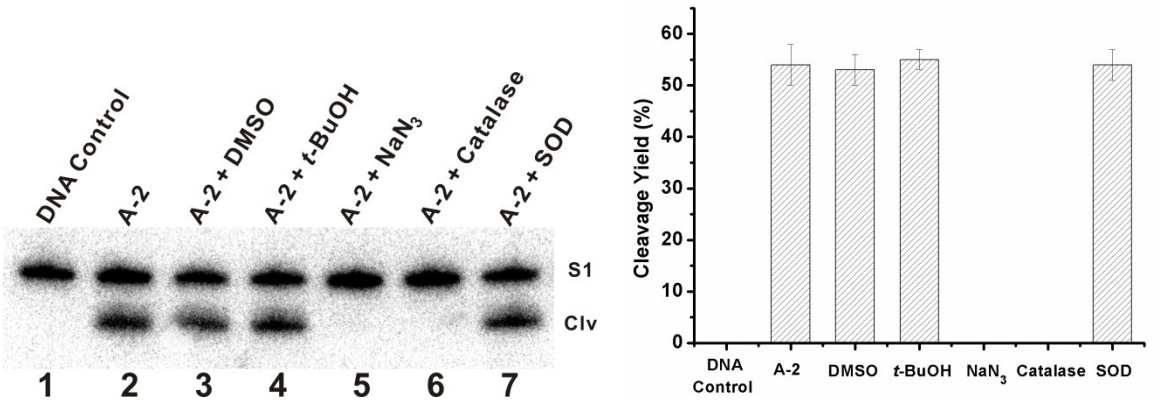


Fig. S18. Effect of ROS scavengers on the cleavage reaction of deoxyribozyme A-2 under the standard reaction conditions. A) lane 1: 5'-^{32}P labeled DNA substrate; lanes 2: cleavage of A-2 in the absence of additional scavengers; lanes 3-7: cleavage of **A-2** in the presence of additional scavengers 0.1 M DMSO, 0.1 M *t*-BuOH, 0.1 M NaN_3, 10 U catalase, 10 U SOD respectively; B) Quantitation of % cleaved DNA of corresponding reactions in A. Clv identifies 5'-cleavage fragment by deoxyribozyme.

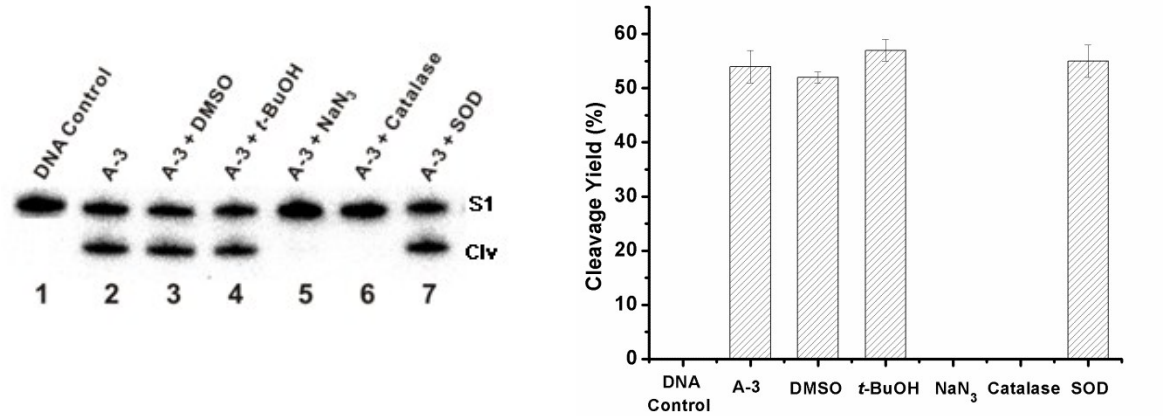


Fig. S19. Effect of ROS scavengers on the cleavage reaction of deoxyribozyme A-3 under the standard reaction conditions. A) lane 1: 5'-^{32}P labeled DNA substrate; lanes 2: cleavage of A-3 in the absence of additional scavengers. Lanes 3-7: cleavage of A-3 in the presence of additional scavengers 0.1 M DMSO, 0.1 M *t*-BuOH, 0.1 M NaN_3, 10 U catalase, 10 U SOD respectively; B) Quantitation of % cleaved DNA of corresponding reactions in A. Clv identifies 5'-cleavage fragment by deoxyribozyme.

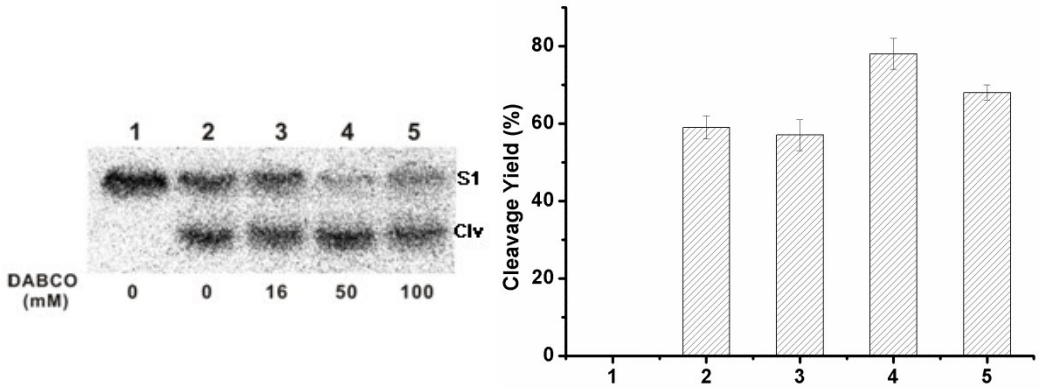


Fig. S20 Effect of DABCO on the cleavage reaction of deoxyribozyme A-2 under the standard reaction conditions. A) Lane 1, 5'-^{32}P labeled DNA substrate S1; lanes 2: cleavage of A-2 in the absence of additional scavengers. lanes 3-5: cleavage of A-2 in the presence of additional 0.016-0.1 M DABCO respectively; (B) Quantitation of % cleaved DNA of corresponding reactions in A. Clv identifies 5'-cleavage fragment by deoxyribozyme.

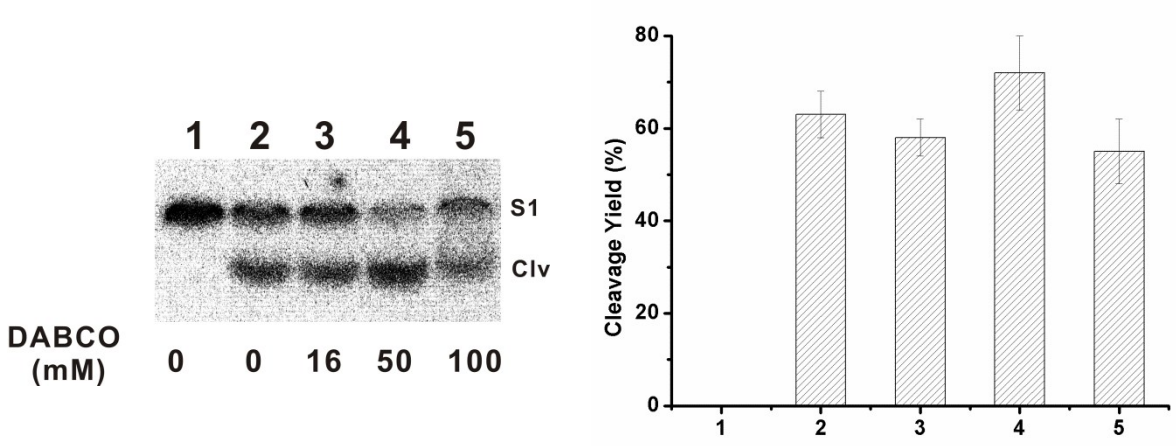


Fig. S21 Effect of DABCO on the cleavage reaction of deoxyribozyme A-3 under the standard reaction conditions. A) Lane 1, 5'-^{32}P labeled DNA substrate; lanes 2: cleavage of A-3 in the absence of additional scavengers; lanes 3-5: cleavage of **A-3** in the presence of additional 0.016-0.1 M DABCO respectively; (B) Quantitation of % cleaved DNA of corresponding reactions in A. Clv identifies 5'-cleavage fragment by deoxyribozyme.

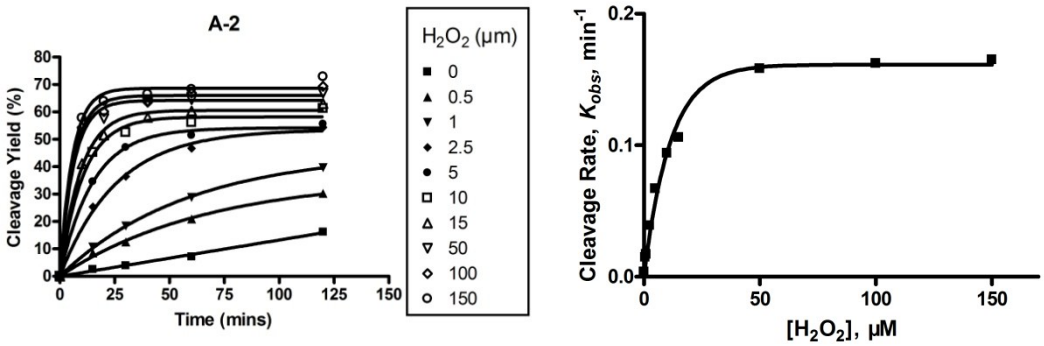


Fig. S22 Single-turnover experiments to validate the participation of H_2O_2 in the DNA cleavage reactions catalyzed by A-2. Assays were performed under the standard reaction conditions except for the variation of the H_2O_2 concentration (ranging from 0 to 150 μM). Kinetic characterizations were shown respectively. In the presence of 50 μM H_2O_2, the cleavage rate for A-2 was increased rapidly with maximal $k_{obs} \sim 0.17$ min^{-1} for A-2.

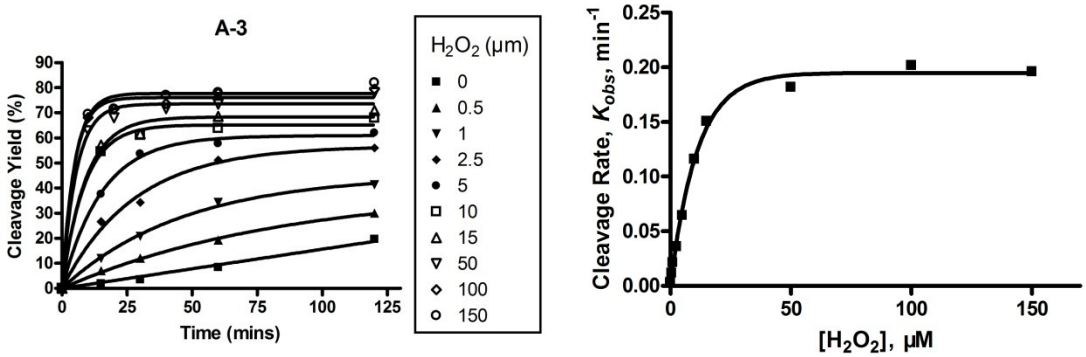


Fig. S23 Single-turnover experiments to validate the participation of H_2O_2 in the DNA cleavage reactions catalyzed by A-3. Assays were performed under the standard reaction conditions, except for the variation of the H_2O_2 concentration (ranging from 0 to 150 μM). Kinetic characterizations were shown respectively. In the presence of 50 μM H_2O_2, the cleavage rate for A-3 was increased rapidly with maximal $k_{obs} \sim 0.20$ min^{-1} for A-3.

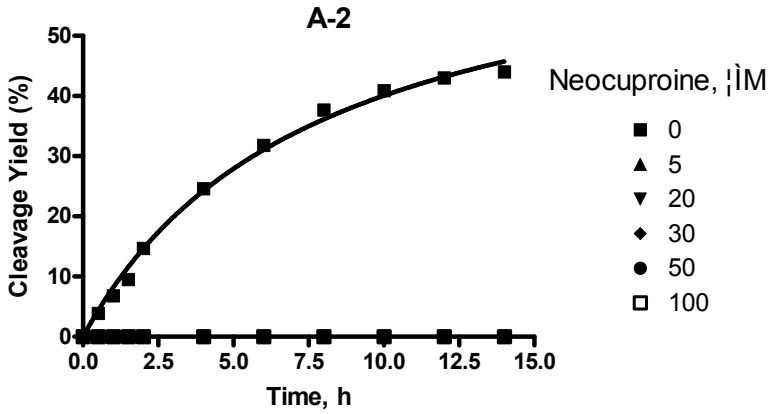


Fig. S24 Single-turnover experiments to validate the presence of Cu(I) chelator neocuproine in DNA cleavage by A-2. A 1 mM stock solution of neocuproine in ethanol was prepared. Assays were performed under the standard incubation conditions supplemented with neocuproine (5-100 μM final ethanol concentration 10 % v/v). The assay without any added neocuproine but with 10 % ethanol revealed a modest decrease in rate and yield due solely to the ethanol, which is required for neocuproine solubility. Inclusion of neocuproine was detrimental to catalytic function. We could not find any cleavage products.

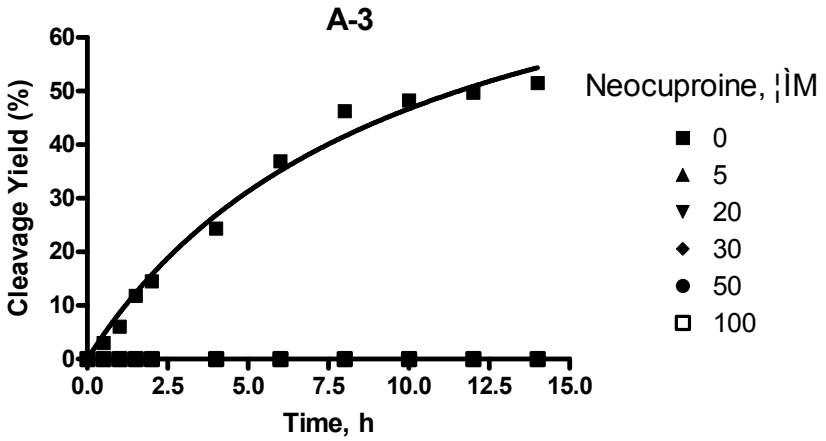


Fig. S25 Single-turnover experiments to validate the presence of Cu(I) chelator neocuproine in DNA cleavage by A-3. Assays were performed under the standard incubation conditions supplemented with neocuproine (5-100 μM final ethanol concentration 10 % v/v). We also could not find any cleavage products.

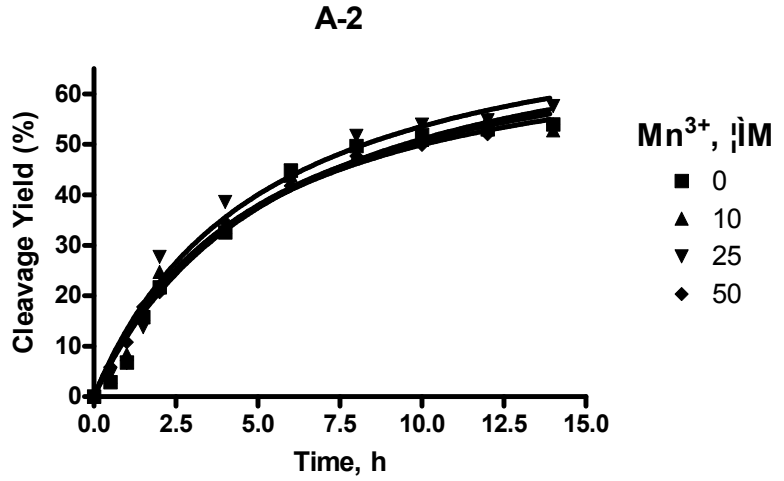


Fig. S26 Single-turnover experiments to validate the absence of Mn(III) in DNA cleavage by **A-2**. A 1 mM stock solution of $Mn(OAc)_3 \bullet 2H_2O$ in ethanol was prepared. Assays were performed under the standard incubation conditions supplemented with Mn(III) (5-50 μM final ethanol concentration 5% v/v). The assay without any added Mn(III) but with 5 % ethanol revealed a modest decrease in rate and yield.

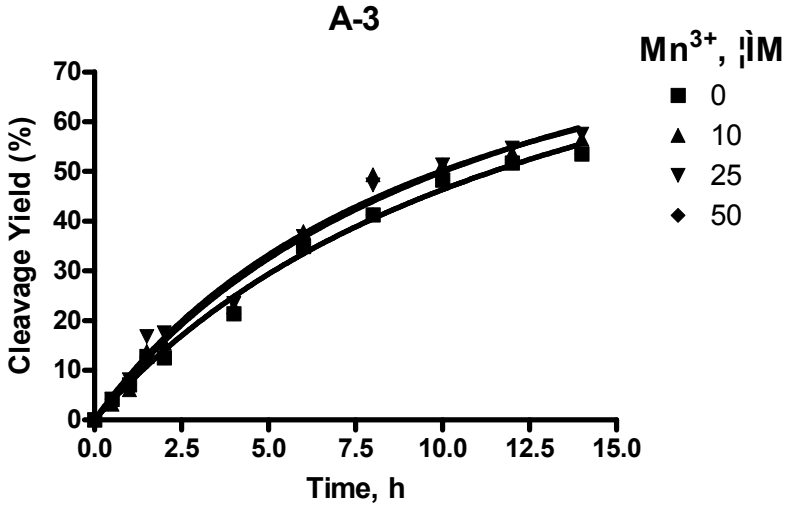


Fig. S27 Single-turnover experiments to validate the absence of Mn(III) in DNA cleavage by **A-3**. A 1 mM stock solution of $Mn(OAc)_3 \bullet 2H_2O$ in ethanol was prepared. Assays were performed under the standard incubation conditions supplemented with Mn(III) (5-50 μM final ethanol concentration 5% v/v). The assay without any added Mn(III) but with 5 % ethanol revealed a modest decrease in rate and yield.

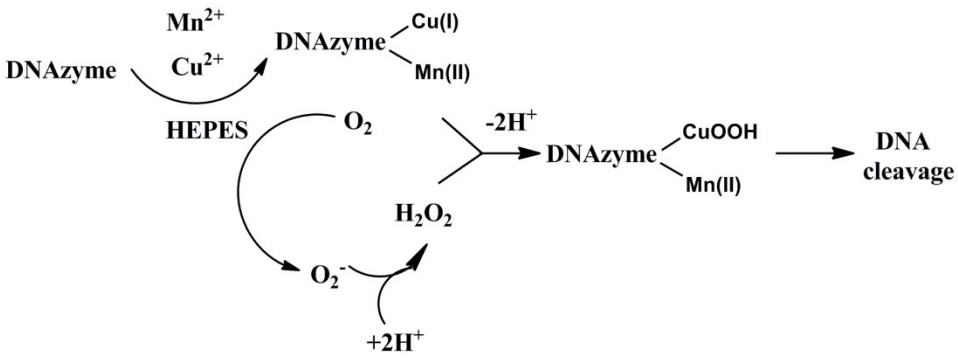


Fig. S28 Proposed plausible mechanism for DNA cleavage promoted by deoxyribozymes.

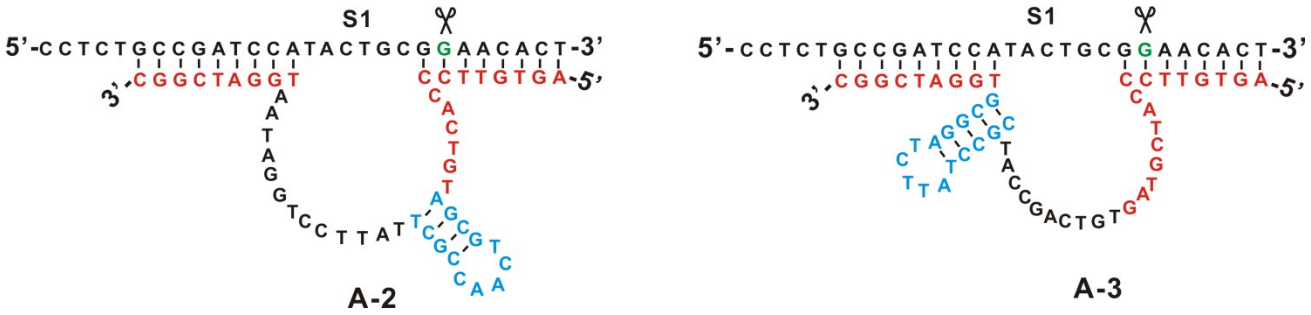


Fig. S29 Sequence analysis and structures of individual deoxyribozymes.