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A novel strategy of chemical modification for rate enhancement of 10–23 DNAzyme: a combination of A9 position and 8-aza-7-deaza-2'-deoxyadenosine analogs†

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With the help of a divalent-metal ion, 10–23 DNAzyme cleaves RNA. Chemical modification of its catalytic loop to make a more efficient enzyme has been a challenge. Our strategy started from its five 2'-deoxyadenosine residues (A5, A9, A11, A12, and A15) in the loop based on the capability of the N7 atom to form hydrogen bonds in tertiary structures. 8-Aza-7-deaza-2'-deoxyadenosine and its analogs with 7-substituents (3-aminopropyl, 3-hydroxylpropyl, or phenethyl) were each used to replace five dA residues, respectively, and their effect on cleavage rate were evaluated under single-turnover conditions. The results indicated that the N7 atom of five dA residues were necessary for catalytic activity, and the N8 atom and 7-substituents were detrimental to the catalytic behavior of 10–23 DNAzyme, except that all these modifications at A9 were favourable for the activity. Especially, **DZ-3–9** with 7-(3-aminopropyl)-8-aza-7-deaza-2'-deoxyadenosine (3) at A9 position gave a 12- fold increase of k_{obs} , compared to the corresponding parent 10–23 DNAzyme based on their very similar pH-dependent and divalent metal ions-dependent cleavage patterns. Introduction of functional groups at A9 position was demonstrated to be a successful and feasible approach for more efficient 10–23 DNAzyme analogs.

Introduction

The small deoxyribozyme 10–23 DNAzyme is an artificial catalytic DNA molecule selected *in vitro* by Santoro and Joyce. It is comprised of a catalytic loop of 15 2'-deoxyribonucleotides and two flanking substrate-recognition domains.^{1,2} Because of its small size, and chemical and enzymatic stability compared with ribozymes, it has been broadly studied as a general-purpose genetic therapeutic candidate against various disease-related mRNAs.³⁻¹³ However, its fast cleavage on mRNA substrate is seriously dependent on the concentration of divalent metal ion (Mg²⁺), thus, the low Mg²⁺ concentration in cells is an unfavorable factor for its practical applications *in vivo*. Chemical modifications aimed at improving its catalytic properties as well as stability have been carried out since its appearance.¹⁴⁻²⁶ The role of the catalytic motif has been systematically studied by residue deletion or replacement

with other natural or modified nucleosides, as well as backbone modifications. The nucleotide residues at the two edges of the catalytic loop are crucial for its catalytic activity, and generally, any substitution is detrimental to the catalytic efficiency. All of the 2'-deoxyguanosine residues are very important. Two 2'deoxythymidine residues, T4 and T8, behave very differently. The five 2'-deoxyadenosine residues (A5, A9, A11, A12, and A15) contribute to the catalytic reaction positively, albeit differently. Among them, A5 and A15 are the most important residues. However, it is not clear what roles these residues or their functional groups play; a comprehensive understanding regarding the mechanistic details of 10-23 DNAzyme is still absent. Therefore, the basis for a rational modification in the catalytic motif of 10-23 DNAzyme is currently far beyond reach. Nevertheless, in the absence of detailed information of the catalytic active site in 10-23 DNAzyme, chemical modification is still a fundamental tool to have an insight into the catalytic power of this DNA molecule, and for a more efficient version.

In our strategy for more efficient 10–23 DNAzyme analogs with chemical modifications on its catalytic loop, we focused on the five-membered ring part of 2'-deoxyadenosine residues, because their N7 atoms are also active in forming hydrogen bonds, such as Hoogsteen and reversed Hoogsteen hydrogen-bonding involved in various tertiary structures of nucleic acids,²⁷ and nitrogen atom can act as a ligand of metal ions, as testified in ribozyme structures.²⁸ Therefore, five 2'-deoxyadenosine analogs **1–5** were designed, and

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[†] Electronic supplementary information (ESI) available: MALDI-TOF for DNAzyme oligodeoxynucleotides, melting data and example melting curves for DNAzyme-substrate complexes, the cleavage pattern of 10-23 DNAzyme and DZ-3-9 in the presence of different Mg²⁺ concentrations and different divalent metal ions, NMR spectra of compounds **3**, **6**, **10**, and **14**. See DOI: 10.1039/c1ob05065f

each of them were incorporated into the five dA positions in the catalytic loop, respectively. The modified 10–23 DNAzyme analogs were evaluated against a DNA-RNA-DNA chimeric substrate, 5'-d(AGG TGC AGG)-rA-rU-d(GGA GAG CA)-3' (Fig. 1),²⁹ in which the flanking DNA residues were used to avoid ribonuclease cleavage.³⁰ This study would help us to understand the importance of the N7 atoms for the catalytic reaction, and explore the possibility of finding more efficient deoxyribozymes by introducing extra protein-like functional groups into the catalytic loop of 10–23 DNAzyme.



Fig. 1 Secondary structure of 10–23 DNAzyme and the DNA-RNA-DNA substrate complex. Bold letters represent the RNA residues. The arrow denotes the cleavage site on the substrate.

Results and discussion

Chemistry

Nucleosides 1, 2, and 4 and their corresponding phosphoramidites were prepared according to the literature.³¹⁻³⁴ Compound 3 was prepared as described in Scheme 1. The crosscoupling reaction of 7-iodo-8-aza-7-deaza-2'-deoxyadenosine with 3-trifluoroacetamidopropyne offered compound 6. It was hydrogenated with 10% Pd/C to obtain compound 7, which was deprotected in aq. ammonia to offer the nucleoside analog 3. Compound 5 was obtained from the cross-coupling reaction of 7-iodo-8-aza-7-deaza-2'-deoxyadenosine with phenylacetylene³⁵ and subsequent hydrogenation (Scheme 2). The din-butylaminomethylidene group was introduced to protect the 6amino group of compounds 7 and 5 to obtain compounds 8 and 12, respectively. These two compounds were then tritylated with DMT-Cl (to give compounds 9 and 13) and further converted into phosphoramidites 10 and 14, respectively. All new compounds were fully characterized by elemental analysis or HRMS, 1H NMR, ¹³C NMR, and ³¹P NMR. The coupling yields of all of these phosphoramidites (0.15-0.2 M in acetonitrile) in the solidphase synthesis of modified deoxyribozymes were above 95% with coupling time of 2 min. All of the DNA sequences were synthesized in DMT-off mode. After deprotection with conc. aq. ammonia (60 °C, 18 h), the sequences were purified with denaturing PAGE, desalted with a SEP-PAK column. MALDI-TOF MS was used for their characterization (ESI, Table S1[†]).

Cleavage reaction of modified 10-23 DNAzyme analogs

In the catalytic loop of 10–23 DNAzyme, there are five 2'-deoxyadenosine residues, assigned as A5, A9, A11, A12, and A15, with some residues contributing more than the others.^{24,25} Single substitutions with **1–5** were conducted at these five positions. All of the modified 10–23 DNAzyme analogs were shown in Table 1 and Table 2.



Scheme 1 Synthesis of nucleoside analog 3 and phosphoramidite 10.

Table 1 Modified deoxyribozymes based on 10–23 DNAzyme with a single substitution in the catalytic loop with 2'-deoxyadenosine analogs 1 and 2 and their k_{obs} measured under single-turnover conditions

Name	Modified 10–23 DNAzyme analogs	$k_{\rm obs}~({\rm min}^{-1})$
10–23 DNAzyme	5'-d(tgc tct cca GGC TAG CTA CAA CGA cct gca cct)-3'	0.0037 ± 0.0007
DZ-1-5	5'-d(tgc tct cca GGC T1G CTA CAA CGA cct gca cct)-3'	ndª
DZ-1-9	5'-d(tgc tct cca GGC TAG CT1 CAA CGA cct gca cct)-3'	0.0018 ± 0.0001
DZ-1-11	5'-d(tgc tct cca GGC TAG CTA C1A CGA cct gca cct)-3'	ndª
DZ-1-12	5'-d(tgc tct cca GGC TAG CTA CA1 CGA cct gca cct)-3'	0.0022 ± 0.0003
DZ-1-15	5'-d(tgc tct cca GGC TAG CTA CAA CG1 cct gca cct)-3'	0.0027 ± 0.0003
DZ-2-5	5'-d(tgc tct cca GGC T2G CTA CAA CGA cct gca cct)-3'	0.0022 ± 0.0002
DZ-2–9	5'-d(tgc tct cca GGC TAG CT2 CAA CGA cct gca cct)-3'	0.0089 ± 0.0008
DZ-2–11	5'-d(tgc tct cca GGC TAG CTA C2A CGA cct gca cct)-3'	0.0020 ± 0.0001
DZ-2–12	5'-d(tgc tct cca GGC TAG CTA CA2 CGA cct gca cct)-3'	0.0032 ± 0.0003
DZ-2–15	5'-d(tgc tet cca GGC TAG CTA CAA CG2 cet gca cet)-3'	0.0008 ± 0.00006

^{*a*} For reactions with no cleavage or an observed rate constant of < 0.0001 min⁻¹, the k_{obs} is denoted as nd.

Table 2 Modified deoxyribozymes based on 10–23 DNAzyme with a single substitution in the catalytic loop with 2'-deoxyadenosine analogs 3, 4, or 5 and their k_{obs} measured under single-turnover conditions

Name	Modified 10–23 DNAzyme analogs	$k_{ m obs}~({ m min}^{-1})$
DZ-3-5	5'-d(tgc tct cca GGC T3G CTA CAA CGA cct gca cct)-3'	0.0003 ± 0.00001
DZ-3–9	5'-d(tgc tct cca GGC TAG CT3 CAA CGA cct gca cct)-3'	0.045 ± 0.004
DZ-3–11	5'-d(tgc tct cca GGC TAG CTA C3A CGA cct gca cct)-3'	0.0015 ± 0.0001
DZ-3–12	5'-d(tgc tct cca GGC TAG CTA CA3 CGA cct gca cct)-3'	0.0027 ± 0.0006
DZ-3–15	5'-d(tgc tct cca GGC TAG CTA CAA CG3 cct gca cct)-3'	0.0022 ± 0.0002
DZ-4-5	5'-d(tgc tct cca GGC T4G CTA CAA CGA cct gca cct)-3'	0.0003 ± 0.00006
DZ-4-9	5'-d(tgc tct cca GGC TAG CT4 CAA CGA cct gca cct)-3'	0.025 ± 0.006
DZ-4–11	5'-d(tgc tct cca GGC TAG CTA C4A CGA cct gca cct)-3'	0.0005 ± 0.00002
DZ-4–12	5'-d(tgc tct cca GGC TAG CTA CA4 CGA cct gca cct)-3'	0.0028 ± 0.0002
DZ-4–15	5'-d(tgc tct cca GGC TAG CTA CAA CG4 cct gca cct)-3'	0.0003 ± 0.00006
DZ-5–9	5'-d(tgc tet cca GGC TAG CT5 CAA CGA cet gca cet)-3'	0.0128 ± 0.0018



Scheme 2 Synthesis of nucleoside analog 5 and phosphoramidite 14.

The $T_{\rm m}$ values of all of the deoxyribozyme-substrate complexes with differently positioned modifications were around 51 °C in a buffer for cleavage reaction (50 mM Tris-HCl, 2 mM Mg²⁺, pH 7.4). Full DNA substrate sequence D19, 5'-d (AGGTGC AGG ATG GAG AGC A)-3' was used to avoid cleavage reaction during the measurement. It was assumed that all of the substrate was associated with the enzyme immediately under the reaction conditions (37 °C), and the modifications in the catalytic loop did not change DNAzyme-substrate complex stability. Singleturnover conditions were used for the measurements of observed rate constant $k_{\rm obs}$, and $k_{\rm obs}$ reflected the rate of the chemical cleavage step.^{36,37}

The effect of N7 atoms of five 2'-deoxyadenosines in the catalytic loop of 10–23 DNAzyme screened with 2'-deoxyadenosine analog 1

Substitution of dA with 7-deaza-2'-deoxyadenosine (1) in the catalytic loop of 10-23 DNAzyme produced five modified deoxyribozymes, each containing 1 instead of dA in a different position in the catalytic loop (Table 1). As shown in Fig. 2A,





Fig. 2 The effect of the N7 atom of dA at five positions in the catalytic loop of 10-23 DNAzyme was demonstrated with compounds 1 (A) and 2 (B) under single-turnover conditions. Solid triangles (A5), solid squares (A9), open diamonds (A11), open squares (A12), open triangles (A15), and solid diamonds (10-23 DNAzyme) were used to indicate the substitutions at different positions.

under single-turnover conditions, almost complete loss of cleavage activity was observed for **DZ-1–5**, indicating the most detrimental effect of compound **1** at A5, compared with 10-23 DNAzyme. The significant negative effect at A11 (**DZ-1–11**) was also observed. This meant that the N7 atom was very important at these two positions. In **DZ-1–9**, **DZ-1–12**, and **DZ-1–15**, the deletion of N7 atom resulted in only moderate loss of activity, as shown by k_{obs} in Table 1. These results suggested that the N7 atom of each 2'-deoxyadenosine residue contributes to the catalytic activity of 10–23 DNAzyme differently.

The influence of the shift of N7 to 8-position in 2'-deoxyadenosine on the catalytic reaction of DNAzymes

Based on above N7 atom deletion research, the interactions related to the five N7 atoms of five 2'-deoxyadenosine residues seem to be favorable for the cleavage reaction of 10–23 DNAzyme. In order to find if more favorable interactions could be produced by this nitrogen atom, a micro-perturbation strategy at the N7 atom was realized with compound **2** in which the N7 atom was shifted to the 8-position without significantly altering the whole adenine structure. As shown in Table 1 and Fig. 2B, the N8 atom had a distinct position-dependent effect on the catalytic activity. Firstly, the N8 atom at A5 (**DZ-2–5**) and A11 (**DZ-2–11**) compensated the loss by the absence of N7 atom, to some extent, as evidenced by their k_{obs} . It implied that either N7 or N8 atom was necessary for the catalytic reaction at these two positions, however, N7 atom in 2'-deoxyadenosine was more favorable than N8 atom of compound **1**. But for A15 located next to the cleavage site, it seems that the new interactions related to N8 produced detrimental effect on the catalytic reaction, a significant loss of activity of **DZ-2–15** $(k_{obs} = 0.0008 \text{ min}^{-1})$ (Table 1) was observed. Secondly, the N8 atom in A12 position (**DZ-2–12**) almost equally contributed to the catalytic reaction as the N7 atom. The k_{obs} of **DZ-2–12** was 0.0032 min⁻¹ under present single-turnover conditions, very close to that of 10-23 DNAzyme. Finally, 2.4-fold faster cleavage was observed with **DZ-2–9** ($k_{obs} = 0.0089 \text{ min}^{-1}$), indicating that the N8 atom at A9 position was more appropriately located for its positive role.

These primary screening studies with nucleoside analogs 1 and 2 demonstrated that the N7 atom of each 2'-deoxyadenosine in the loop is necessary for activity. At A9, its contribution could be improved by shifting to the 8-position with 2. More importantly, we learned that the catalytic potential of 10-23 DNAzyme could be further optimized by chemical modification. We hypothesized that A9 could be used as a potential position for further modification and nucleoside analog 2 as the lead compound in the search for more efficient 10-23 DNAzyme analogs.

Further modification of A9 with compounds 3–5 to introduce protein-like functional groups in the catalytic loop of 10–23 DNAzyme

Based on the small improvement on the cleavage rate of DZ-2-9, it was recognized as the lead structure for further chemical modifications with protein-like functional groups. Its residue (compound 2) at A9 was substituted with compound 3 or 4, to produce two novel modified DNAzymes, DZ-3-9 and DZ-4-9, respectively. Thus, an amino or a hydroxyl group was introduced to A9 of the catalytic loop. Amino and hydroxyl groups were selected because they are well-known for hydrogen bonding and proton transferring in catalytic reactions of protein enzymes. On the other hand, the amino group is also known to offer electrostatic stabilization of the transition state in the catalytic reaction of ribozymes 28,38,39 and Ribonuclease A.40 As shown in Table 2 and Fig. 3A, DZ-3-9 exhibited faster cleavage than DZ-2-9. Under single-turnover conditions, k_{obs} for **DZ-3–9** was 0.045 min⁻¹, which was about 5 times faster than DZ-2-9, and 12 times faster than 10-23 DNAzyme. Interestingly, when such substitution was used at the other four dA positions (A5, A11, A12, and A15), slower cleavage than 10-23 DNAzyme was always observed. In other words, the effect of compound 3 on the cleavage rate of the modified deoxyribozymes was position-dependent.

The same tendency was observed with compound **4** (see Table 2 and Fig. 3B), the modified DNAzyme **DZ-4–9** exhibited about 6.7 times faster cleavage than the parent 10–23 DNAzyme and 2.8 times faster than **DZ-2–9**. The replacement of compound **4** at the other four positions in the loop always resulted in a slower cleavage than 10–23 DNAzyme.

The positive effect of compounds **3** and **4** at A9 position in **DZ-3-9** and **DZ-4-9** suggested that A9 and 7-substituted 8-aza-7-deaza-2'-deoxyadenosine could be combined as a novel approach for more efficient 10–23 DNAzyme analogs, **DZ-2-9** could be recognized as the new leading DNAzyme structure.

This approach for more efficient 10–23 DNAzyme analog was further testified with DNAzyme **DZ-5–9**, in which compound **5** was introduced to replace A9 in the catalytic loop (Table 2),



Fig. 3 The effect of 7-substituents of compound 2 at five positions in the catalytic loop of 10-23 DNAzyme was demonstrated with compounds 3 (A) and 4 (B) under single-turnover conditions. Solid triangles (A5), solid squares (A9), open diamonds (A11), open squares (A12), open triangles (A15), and solid diamonds (10-23 DNAzyme) were used to indicate the substitutions at different positions.

The k_{obs} of **DZ-5–9** was 0.0128 min⁻¹, which was slower than **DZ-3–9** and **DZ-4–9**, but still about 3.5 times faster than 10–23 DNAzyme and 1.4 times faster than **DZ-2–9**. It is notable that a phenyl group at A9 was also favorable for the cleavage reaction, although it could not participate in hydrogen-bonding as an amino or hydroxyl group. The new hydrophobic interaction and spacial occupation related to the phenyl group probably resulted in a favorable conformational change. The different contributions of compounds **1–5** at A9 demonstrated the importance of 7-substituents, more efficient version of 10–23 DNAzyme could be expected by optimizing the design of 7-substituents of 8-aza-7-deaza-2'-deoxyadenosine, hydrogen-bonding ability might be one of the important properties to be considered in the design of the 7-substituents.

Preliminary mechanistic studies on the modified DNAzyme DZ-3-9

10-23 DNAzyme has been characterized as a divalent metal iondependent enzyme.^{1,2} The metal ion might act as a Lewis base to strengthen the nucleophilicity of the 2'-hydroxyl group or as a Lewis acid to coordinate the 5'-oxygen atom to facilitate the cleavage of the 5'-end fragment, as well as play a structural role to stabilize and/or pre-organize the active catalytic conformation. The log-linear relationship of catalytic rate *versus* pH indicated that the deprotonation of 2'-OH is embedded in the rate-limiting step.^{1,2,38,41} Therefore, the influence of pH and divalent metal ions on the cleavage rates of the modified DNAzyme **DZ-3–9** was evaluated, and a comparison with the parent 10–23 DNAzyme was made.

Under single-turnover conditions, it was observed that the cleavage rates of **DZ-3–9** and 10–23 DNAzyme were dependent on the concentration of Mg^{2+} in the same way, at least from 0.1 mM to 50 mM used in our experiments, and no cleavage was observed when EDTA was used as a scavenger of divalent metal ions (ESI, Fig. S2†). In addition, the effect of two other divalent metal ions, Mn^{2+} and Ca^{2+} , was also examined for both DNAzymes. As shown in Fig. 4, the effect of these three metal ions are in the same order, $Mn^{2+} > Ca^{2+} \approx Mg^{2+}$ for both **DZ-3–9** and 10–23 DNAzyme, and **DZ-3–9** always reacted faster than 10–23 DNAzyme with each divalent metal ion. The presence of Mn^{2+} led to the fastest cleavage.² These results indicated that the divalent metal ion plays a similar role in the reactions conducted by both modified DNAzyme **DZ-3–9** and the parent DNAzyme.



Fig. 4 The effect of divalent metal ions on the cleavage reactions of 10-23-DNAzyme (A) and **DZ-3-9** (B). Mn²⁺ (solid triangle), Ca²⁺ (open square), and Mg²⁺ (solid diamond) were used to indicate different metal ions.

The effect of pH on cleavage of **DZ-3–9** was measured under multiple-turnover conditions.^{1,2} With pH ranging from 7.5 to 8.5 for 10–23 DNAzyme (Fig. 5, A) and pH from 6.0 to 8.5 for **DZ-3–9** (Fig. 5, B), a roughly linear log k_{obs} fashion against increasing pH was observed for both **DZ-3–9** and 10–23 DNAzyme,^{1,2} faster cleavage was always observed under higher pH values. This pH-dependence indicated that the deprotonation of the 2'-hydroxyl group was also part of the rate-limiting step of **DZ-3–9**, as reported for 10–23 DNAzyme.^{1,2}

The same effect of the two critical factors (divalent metal ion and pH) indicated that 10–23 DNAzyme and **DZ-3–9** probably



Fig. 5 pH-dependence of 10–23-DNAzyme (A) and **DZ-3–9** (B) under multiple-turnover conditions with different pH values ranging from 6 to 8.5.

conducted the cleavage reaction in the same way. We speculated that the functional groups at A9 might be involved in the optimization of catalytic conformation, and their different influence (NH₂ > OH > phenyl) might reflect their ability on driving conformational changes, probably through forming new hydrogen bonds or hydrophobic interactions in the catalytic loop.

In the cleavage reaction of 10-23 DNAzyme, three steps are thought to be mainly responsible for the whole cleavage profile: association of its binding arms with the target sequence, the chemical cleavage step, and the dissociation between the binding arms and the cleaved products.²⁷ The first and the third steps are related to the length of the binding arms and their hybridization affinity to the substrate. Therefore, the length of the binding arms has to be balanced between the rate and specific recognition. It has been reported that the incorporation of LNA (locked nucleic acid) residues in the binding arms significantly increase the rate by improving hybridization affinity to the substrate.²⁴ The present chemical modification on the catalytic loop of 10-23 DNAzyme conferred a faster chemical cleavage step; therefore, the modified DNAzymes are intrinsically faster than 10-23 DNAzyme. LNA modification also might be applied to these modified DNAzymes for a further increase in reaction rates.

Chemical modification was demonstrated again to be a powerful tool for more efficient deoxyribozymes. Currently, much efforts have been being devoted to explore the catalytic potential of nucleic acid structures by chemical modifications with protein-like functional groups,⁴²⁻⁴⁵ although it is not known whether the maximum catalytic power of deoxyribozyme or ribozyme could approach to that of nucleases.

Conclusion

Based on the chemical modifications with 2'-deoxyadenosine analogs 1–5 on the catalytic loop of 10–23 DNAzyme, the importance and potential of the five-membered ring part of 2'deoxyadenosine residues for catalytic activity were explored for the first time. A9 is the only position for a positive effect of these funtional groups: N8 atom, amino, hydroxyl, and phenyl groups which were located in the five-membered ring part of 2'deoxyadenosine. A9 position and 7-substituted 8-aza-7-deaza-2'deoxyadenosine analogs were combined to offer a prosperous approach for more efficient 10–23 DNAzyme analogs. More efficient deoxyribozymes will undoubtedly offer a better choice in deoxyribozyme applications, such as for genetic therapeutics.

Experimental section

General

Most of the commercially available chemicals were used without further purification. Pyridine was dried by refluxing with CaH₂. Thin layer chromatography (TLC) was run on HS GF₂₅₄ (Yantai Institute of Chemical Industry, China). Silica gel (200–300 mesh, Qingdaohaiyang Chemicals Co., China) was used for flash column chromatography. ¹H, ¹³C and ³¹P NMR were recorded on a JNM-ECA-400 spectrometer (JEOL, Japan), internal trimethylsilane (TMS) (¹H, ¹³C) or external 85% H₃PO₄ (³¹P) was used as the standard. The *J* values are given in Hz. Elemental analysis on a Fisons-1108 (Fisons, Italy), and HR-MS on Q-FT-MS (Apex Qe, Brucker) were performed by the National Center of Biomedical Analysis (Beijing, China).

Oligodeoxynucleotides

Normally protected phosphoramidites of canonical residues were purchased from Proligo (Sigma–Aldrich). Oligodeoxynucleotide synthesis was run on an ABI 392 DNA/RNA synthesizer (Applied Biosystems, USA) on a 1 µmol scale with the DMT-off mode according to the User Protocol. The DMT-off oligodeoxynucleotides were deprotected in conc. aq. ammonia for 18 h at 60 °C. All were then purified by a 20% polyacrylamide/7 M urea denaturing gel and extracted with 0.3 M sodium acetate.²⁰ Desalting was conducted using SEP-PAK cartridges (Oasis MAX, C18, Waters, USA) with repeated washing with sterilized doublydistilled water. The product was lyophilized and stored at –18 °C. The chimera substrate was purchased from Takara (Dalian, China). A Cary-100 Bio UV-Visible spectrophotometer equipped with a Cary temperature controller (Varian, USA) was used for T_m measurements.

The homogeneous oligodeoxynucleotides were characterized by MALDI-TOF with 2',4',6'-trihydroxyacetophenone (THAP) as the matrix. MALDI-TOF MS were acquired on an AXIMA-CFP plus mass spectrometer (KRATOS Analytical, Shimadzu Group Company, Japan) equipped with a nitrogen laser (337.1 nm) in positive ion and linear mode, with an acceleration voltage of 20 kV and averaged over 100 laser shots. Each spectrum was externally calibrated with Cytochrome C (m/z 12361) providing mass measurement accuracies of approximately 500 ppm across the 4 kDa ~20 kDa mass range.

4-Amino-1-(2-deoxy-β-D-erythro-pentofuranosyl)-3-(3-trifluoroacetamido-propyn-1-yl)-1H-pyrazolo[3,4-d]pyrimidine (6). 7-Iodo-8-aza-7-deaza-deoxyadenosine (0.8 g, 2.1 mmol) was dissolved in dried DMF (30 ml). To the solution was added Pd(Ph₃)₂Cl₂ (0.14 g, 2.0 mmol), CuI (0.1 g, 0.6 mmol), the mixture was stirred at r.t. under nitrogen atmosphere. Triethylamine (2 ml, 10.5 mmol) was added to the reaction mixtrure, followed by N-propynyltrifluoroacetamide (0.4 g, 2.5 mmol). The reaction mixture was then stirred at r.t. overnight. DMF was vacuum-evaporated off and the residue was subjected to flash chromatography. The product was obtained as colorless solid (0.74 g, 87.5%). R_f (CH₂Cl₂/CH₃OH 9:1) 0.4. ¹H NMR (DMSO d_6): $\delta 2.25$ (m, 1 H, C2'-H_{α}), 2.76 (m, 1 H, C2'-H_{β}), 3.36, 3.51 (2 m, 2 H, C5'-H), 3.82 (m, 1 H, C4'-H), 4.42 (m, 3 H, C3'-H, CH₂), 4.75 (t, J = 5.6, 1 H, C5' - OH), 5.28 (d, J = 4.5, 1 H, C3' - OH), 6.55 (t, J =6.3, 1 H, C1'-H), 8.26 (s, 1 H, C2-H), 6.74, 8.17 (2 br, 2 H, NH₂), 10.16 (s, 1 H, NH). ¹³C NMR (DMSO-*d*₆): δ 30.0, 38.9, 62.3, 70.9, 74.3, 84.0, 87.7, 90.2, 101.0, 126.1, 153.7, 156.7, 157.6. HRMS Calcd for C₁₅H₁₆F₃N₆O₄ (MH⁺): 401.1180, Found: 401.1181.

4-Amino-1-(2-deoxy-β-D-erythro-pentofuranosyl)-3-(3-trifluoroacetamido-prop-1-vl)-1*H*-pvrazolo[3,4-d]pvrimidine (7). The suspension of compound 6 (2 g, 5 mmol) and Pd/C (10%, 1.0 g) in methanol (200 ml) was sealed in an oven under hydrogen (5 atm). The mixture was stirred at 30 °C for 5 h. The catalyst was filtered off, and the filtrate was concentrated to obtain a colorless solid (1.9 g, 95%). R_f (CH₂Cl₂/CH₃OH 10:1) 0.45. ¹H NMR (DMSO d_6): δ 1.87 (m, 2 H, 5-CH₂CH₂CH₂), 2.20 (m, 1 H, C2'-H_a), 2.79 $(m, 1 H, C2'-H_{\beta}), 2.97 (m, 2 H, 5-CH_2CH_2CH_2), 3.30 (m, 2 H, 2)$ 5-CH₂CH₂CH₂), 3.37, 3.52 (2 m, C5'-H), 3.79 (m, 1 H, C4'-H), 4.42 (m, 1 H, C3'-H), 4.78 (m, 1 H, C5'-OH), 5.22 (d, J = 4.8, 1 H, C3'-OH), 6.50 (t, J = 6.6, 1 H, C1'-H), 7.35 (br, 2 H, NH₂), 8.16 (s, 1 H, C2–H), 9.41 (m, 1 H, NHCO). ¹³C NMR (DMSO-d₆): δ 25.2, 27.3, 37.9, 62.5, 71.2, 83.7, 87.5, 98.8, 111.6, 114.5, 117.4, 120.2, 145.0, 154.8, 155.9, 158.1. Anal. Calcd for C₁₅H₁₉F₃N₆O₄ 0.25 H₂O (M 408.85): C, 44.07; H, 4.81; N, 20.56. Found: C, 44.13; H, 4.82; N, 20.06.

4-Amino-3-(3-amino-prop-1-yl)-1-(2-deoxy-*β*-D-*erythro*-pentofuranosyl)-1*H*-pyrazolo[3,4-d]pyrimidine (3). The suspension of compound 7 (100 mg, 0.25 mmol) in aq. ammonia (10 ml) was sealed in a bottle. After stirring at r.t. for 4 h, the solution was concentrated and the product was crystallized from the residue as a colorless solid (70 mg, 93.3%). R_r (CH₂Cl₂/CH₃OH/NH₃·H₂O, 1:1:0.02) 0.26. ¹H NMR (DMSO*d*₆): δ 1.76 (m, 2 H, 5-CH₂CH₂CH₂), 2.21 (m, 1 H, C2'-H_α), 2.67 (m, 2 H, 5-CH₂CH₂CH₂), 2.78 (m, 1 H, C2'-H_β), 2.99 (m, 2 H, 5-CH₂CH₂CH₂), 3.38, 3.54 (2 m, 1 H, C5'-OH), 5.22 (d, *J* = 4.8, 1 H, C3'-OH), 6.50 (t, *J* = 6.6, 1 H, C1'-H), 7.35 (br, 2 H, NH₂), 8.15 (s, 1 H, C2-H). ¹³C NMR (DMSO-*d*₆): δ 24.8, 30.6, 37.9, 62.5, 71.2, 83.6, 87.5, 98.8, 145.5, 154.7, 155.8, 158.2. HRMS Calcd for C₁₃H₂₁N₆O₃ (MH⁺): 309.1670. Found: 309.1671.

1-(2-Deoxy-β-D-*erythro***-pentofuranosyl)-4-{[(di-n-butylamino)**methylidene]amino}-**3-(3-trifluoroacetamido-prop-1-yl)-1***H*-pyrazolo[**3,4-d]pyrimidine (8).** To the solution of compound **7** (1.9 g, 4.7 mmol) in methanol (40 ml) was added *N*,*N*-di-nbutylformamide dimethyl acetal (1.75 ml, 7 mmol). The solution was stirred at 40 °C for 4 h. Then, it was concentrated for flash chromatography. The product was obtained as a colorless solid (2.1 g, 82%). R_f (CH₂Cl₂/CH₃OH 20:1) 0.35. ¹H NMR (DMSO- d_6): δ 0.92 [2 t, J = 7.4, 6 H, CHN(CH₂CH₂CH₂CH₃)₂], 1.32 (m, 4 H, CHN($CH_2CH_2CH_2CH_3$)₂], 1.61 (m, 4 H, CHN(CH₂CH₂CH₂CH₃)₂], 2.00 (m, 2 H, 5-CH₂CH₂CH₂), 2.24 $(m, 1 H, C2'-H_{\alpha}), 2.82 (m, 1 H, C2'-H_{\beta}), 3.03 (m, 2 H, 5-$ CH₂CH₂CH₂), 3.23–3.60 [m, 8 H, CHN(CH₂CH₂CH₂CH₃)₂, C5'-H, 5-CH₂CH₂CH₂], 3.82 (m, 1 H, C4'–H), 4.45 (m, 1 H, C3'– H), 4.77 (t, J = 5.7, 1 H, C5'–OH), 5.24 (d, J = 4.5, 1 H, C3'– OH), 6.55 (t, J = 6.4, 1 H, C1'-H), 8.42 (s, 1 H, C2-H), 8.98 [s, 1 H, CHN(CH₂CH₂CH₂CH₃)₂], 9.41 (m, 1 H, NHCO). ¹³C NMR (DMSO-*d*₆): δ 13.5, 13.6, 19.1, 19.8, 25.7, 27.3, 28.7, 30.4, 37.9, 45.4, 51.4, 62.5, 71.2, 83.8, 87.5, 106.0, 111.6, 114.5, 117.4, 120.2, 146.5, 155.4, 157.2, 162.5. Anal. Calcd for C₂₄H₃₆F₃N₇O₄ (M 543.58): C, 53.03; H, 6.68; N, 18.04. Found: C, 52.65; H, 6.49; N, 17.87.

1-[2-Deoxy-5-O-(4,4'-dimethoxytriphenylmethyl)-β-D-erythropentofuranosyl]-4-{[(di-n-butylamino)methylidene]amino}-3-(3trifluoroacetamido-prop-1-yl)-1*H*-pyrazolo[3,4-*d*]pyrimidine (9). Compound 8 (1.0 g, 1.85 mmol) was co-evaporated with dried pyridine three times before it was dissolved in dried pyridine (3 ml). 4,4'-Dimethoxytrityl chloride (DMT-Cl) (0.75 g, 2.2 mmol) was added to the solution in portions. After stirring at r.t. for 1 h, methanol (3 ml) was added to the reaction mixture. The solution was concentrated for flash chromatography. The product was obtained as a colorless solid (0.95 g, 62.5%). R_f (CH₂Cl₂/CH₃OH 20:1) 0.7. ¹H NMR (DMSO- d_6): δ 0.93 [t, 6 H, CHN(CH₂CH₂CH₂CH₂CH₃)₂], 1.33 (m, 4 H, $CHN(CH_2CH_2CH_2CH_3)_2$), 1.62 (m, 4 H, $CHN(CH_2CH_2CH_2CH_3)_2$, 1.77 (m, 2 H, 5- $CH_2CH_2CH_2$), 2.30 (m, 1 H, C2'-H_α), 2.78 (m, 1 H, C2'-H_β), 2.89 (m, 2 H, 5-CH₂CH₂CH₂), 3.03–3.13 [m, 4 H, CHN(CH₂CH₂CH₂CH₂CH₃)₂], 3.50-3.60 (m, C5'-H, 5-CH₂CH₂CH₂], 3.67, 3.69 (2 s, 2 OCH₃), 3.95 (m, 1 H, C4'-H), 4.52 (m, 1 H, C3'-H), 5.27 (m, 1 H, C3'-OH), 6.60 (m, 1 H, C1'-H), 6.73-7.30 (m, arom. H), 8.44 (s, 1 H, C2-H), 9.00 [s, 1 H, CHN(CH₂CH₂CH₂CH₃)₂], 9.37 (m, 1 H, NHCO). ¹³C NMR (DMSO- d_6): δ 13.5, 13.6, 19.1, 19.8, 25.7, 27.2, 28.6, 30.4, 38.2, 45.4, 51.4, 54.8, 54.8, 64.9, 71.3, 83.4, 85.1, 85.4, 106.0, 112.8, 112.9, 114.5, 117.4, 126.3, 127.5, 127.6, 129.5, 129.6, 135.6, 145.0, 146.5, 155.4, 157.1, 157.8, 157.9, 162.4. Anal. Calcd for C₄₅H₅₄F₃N₇O₆ 0.25 H₂O (M 850.45): C, 63.55; H, 6.46; N, 11.53. Found: C, 63.32; H, 6.20; N, 11.40.

1-[2-Deoxy-5-O-(4,4'-dimethoxytriphenylmethyl)-β-D-erythropentofuranosyl]-4-{[(di-n-butylamino)methylidene]amino}-3-(3-trifluoroacetamido-prop-1-yl)-1H-pyrazolo[3,4-d]pyrimidine 3'-[(2-Cyanoethyl) N,N-diisopropylphosphoramidite] (10). To the solution of compound 9 (0.45 g, 0.55 mmol) in dichloromethane (5 ml), diisopropylamine tetrazolide (0.17 g, 1.1 mmol) and 2-cyanoethyl N, N, N', N'-tetraisopropylphosphorodiamidite (0.55 g, 1.1 mmol) were added. The reaction mixture was stirred at rt for 30 min. It was washed with 5% aq. NaHCO₃ and subsequently brine, and dried with anhydrous Na₂SO₄. The solution was concentrated for flash chromatography to afford the product as colorless foam (0.45 g, 80.5%). R_f $(CH_2Cl_2/CH_3OH 40:1)$ 0.48, 0.50. ¹H NMR (DMSO- d_6): δ 0.90-1.81 [m, 28 H, CHN(CH₂CH₂CH₂CH₃)₂, 5-CH₂CH₂CH₂, 2 CH(CH₃)₂], 2.47-3.18 [m, 10 H, C2'-H, 5-CH₂CH₂CH₂), CHN(CH₂CH₂CH₂CH₃)₂, CH₂CH₂CN], 3.35–3.77 [m, 14 H,

C5'-H, 5-CH₂CH₂CH₂, 2 OCH₃, 2 CH(CH₃)₂, CH₂CH₂CN], 4.10 (m, 1 H, C4'-H), 4.78 (m, 1 H, C3'-H), 6.61–6.75, 7.14, 7.28 (3 m, 14 H, C1'-H, arom. H), 8.47 (2 s, 1 H, C2-H), 9.01 [s, 1 H, CHN(CH₂CH₂CH₂CH₃)₂], 9.40 (m, 1 H, NHCO). ³¹P NMR (CDCl₃): 147.16, 147.83. ¹³C NMR (CDCl₃): δ 13.6, 13.8, 19.0, 19.7, 20.0, 20.3, 22.8, 22.9, 24.4, 24.5, 25.6, 26.9, 29.1, 31.0, 37.5, 39.2, 43.0, 43.1, 45.2, 46.0, 46.5, 52.4, 55.1, 58.0, 58.2, 64.4, 84.1, 85.0, 85.2, 85.3, 85.8, 107.0, 112.8, 116.9, 117.5, 126.4, 127.5, 128.1, 128.2, 130.0, 136.0, 144.9, 146.9, 155.8, 156.0, 156.7, 157.0, 158.2, 162.9. HRMS Calcd for C₅₄H₇₂F₃N₉O₇P (MH⁺): 1046.5239. Found: 1046.5253.

1-(2-Deoxy-β-D-*erythro***-pentofuranosyl)-3-(2-phenylethyl)-1***H***-pyrazolo[3,4-***d***]pyrimidin-4-amine (5).** Compound **11** (1.8 g, 5.12 mmol) and 10% Pd/C (0.66 g) in methanol (50 ml) was sealed under hydrogen (5 kg) in an oven. The mixture was stirred at 30 °C for 6 h. The catalyst was filtered off, and the filtrate was concentrated to obtain a colorless solid (1.7 g, 93.4%). R_f (CH₂Cl₂/CH₃OH 9 : 1) 0.38. ¹H NMR (DMSO-*d*₆): δ 2.19 (m, 1 H, C2'–H_α), 2.74 (m, 1 H, C2'–H_β), 3.01 (m, 2 H, 5-CH₂CH₂), 3.31 (2 m, 4 H, C5'–H, 5-CH₂CH₂), 3.80 (m, 1 H, C4'–H), 4.43 (d, *J* = 4.2, 1 H, C3'–H), 4.79 (t, *J* = 5.7, 1 H, C5'–OH), 5.22 (d, *J* = 4.2, 1 H, C3'–OH), 6.50 (t, *J* = 6.5, 1 H, C1'–H), 7.27 (m, 5 H, arom. H), 8.2 (s, 1 H, C2–H). ¹³C NMR (DMSO-*d*₆): δ 29.4, 33.5, 38.0, 62.6, 71.3, 83.6, 87.6, 98.9, 125.9, 128.2, 128.6, 141.1, 145.1, 154.8, 155.8, 158.2. Anal. Calcd for C₄₈H₅₂N₆O₅ (M 355.39): C 60.83, H 5.96, N 19.71. Found C 60.56, H 5.99, N 19.22.

1-(2-Deoxy-β-D-erythro-pentofuranosyl)-4-{[(di-n-butylamino)methylidene|amino}-3-(2-phenylethyl)-1H-pyrazolo[3,4-d]pyrimidine (12). Using the same procedure as for 8, compound 5 (1.0 g, 2.85 mmol) was treated with N-di-n-butylformamide dimethyl acetal (0.58 g, 2.85 mmol) in methanol (4 ml) at 40 °C. After purification by flash chromatography, an oily product was obtained (1.13 g, 94.0%). R_f (CH₂Cl₂/CH₃OH 15:1) 0.57. ¹H NMR (DMSO- d_6): δ 0.67, 0.91 [2 t, 6 H, CHN(CH₂CH₂CH₂CH₂CH₃)₂], 1.16, 1.29 [2 m, 4 H, CHN(CH₂CH₂CH₂CH₃)₂], 1.56 [m, 4 H, CHN(CH₂CH₂CH₂CH₃)₂], 2.22 (m, 1 H, C2'-H_a), 2.78 (m, 1 H, C2'-H₈), 3.09 (m, 2 H, 5-CH₂CH₂), 3.33, 3.48 [2 m, 8 H, C5'-H, CHN(CH₂CH₂CH₂CH₃)₂, 5-CH₂CH₂], 3.80 (m, 1 H, C4'-H), 4.43 (m, 1 H, C3'–H), 4.81 (t, J = 5.5, 1 H, C5'–OH), 5.25 (d, J = 4.5, 1 H, C3'-OH), 6.54 (t, J = 6.4, 1 H, C1'-H), 7.24 (m, 5 H, arom. H), 8.42 (s, 1 H, C2-H), 8.98 [s, 1 H, CHN(CH₂CH₂CH₂CH₃)₂]. ¹³C NMR (DMSO-*d*₆): δ 30.1, 34.7, 34.8, 37.9, 40.8, 62.5, 71.2, 83.7, 87.5, 106.6, 125.9, 128.2, 128.3, 141.4, 146.8, 155.4, 157.6, 162.5. Anal. Calcd for $C_{27}H_{38}N_6O_3 \cdot 0.5H_2O$ (M 499.13): C 64.91, H 7.71, N 16.83. Found C 65.08, H 7.97, N 16.33.

1-[2-Deoxy-5-*O*-(4,4'-dimethoxytriphenylmethyl)-β-D-*erythro*pentofuranosyl)-4- {**[(di-n-butylamino)methylidene]amino**}-3-(2-phenylethyl)-1*H*-pyrazolo[3,4-*d*]pyrimidine (13). Using the same procedure as for compound 9, compound 12 (0.87 g, 2.07 mmol) was tritylated with DMT-Cl (0.78 g, 2.28 mmol) in dried pyridine (2 ml). The colorless product was obtained after purification by flash chromatography (1.1 g, 66.7%). R_f (CH₂Cl₂/CH₃OH 20:1) 0.45. ¹H NMR (DMSO-*d*₆): δ 0.74, 0.91 [2 t, 6 H, CHN(CH₂CH₂CH₂CH₃)₂], 1.10, 1.30 [2 m, 4 H, CHN(CH₂CH₂CH₂CH₃)₂], 1.56 (m, 4 H, CHN(CH₂CH₂CH₂CH₂)₂], 2.33 (m, 1 H, C2'-H_α), 2.75 (m, 3 H, C2'-H_β, 5-CH₂CH₂), 3.13 (m, 4 H, C5'-H, 5-CH₂CH₂), 3.48 [m,

4 H, CHN(CH₂CH₂CH₂CH₃)₂], 3.63 (2 s, 6 H, 2 OCH₃), 3.98 (m, 1 H, C4'–H), 4.54 (m, 1 H, C3'–H), 4.81 (t, J = 5.5, 1 H, C5'–OH), 5.31 (d, J = 4.8, 1 H, C3'–OH), 6.63 ~7.18 (m, 19 H, arom. H, C1'–H), 8.47 (s, 1 H, C2–H), 9.01 [s, 1 H, CHN(CH₂CH₂CH₂CH₃)₂]. ¹³C NMR (DMSO- d_6): δ 13.6, 19.2, 19.6, 28.6, 29.8, 30.4, 34.0, 38.4, 45.3, 51.4, 54.8, 54.9, 65.1, 71.4, 83.4, 85.2, 85.6, 106.0, 112.9, 125.8, 126.4, 127.6, 127.7, 127.9, 128.2, 129.5, 129.7, 135.7, 141.5, 145.1, 146.9, 155.4, 157.2, 157.8, 157.9. Anal. Calcd for C₄₈H₅₆N₆O₅ (M 797.00): C 72.34, H 7.08, N 10.54. Found C 72.04, H 7.08, N 10.27.

1-[2-Deoxy-5-O-(4,4'-dimethoxytriphenylmethyl)-β-D-erythropentofuranosyl)-4-{[(di-n-butylamino)methylidene]amino}-3-(2phenylethyl) - 1H - pyrazolo[3,4 - d]pyrimidine3'-[(2 - cyanoethyl) N, N-diisopropylphosphoramidite] (14). Using the same procedure as for compound 10, compound 13 (0.25 g, 0.31 mmol) was treated with N,N-di-isopropylchlorophosphoramidite 2-cyanoethyl (0.1 g, 0.42 mmol) in dried dichloromethane (2 ml) in the presence of N,N-diisopropylethylamine (DIEA) (1 ml, 5.75 mmol). The product was obtained after flash chromatography as a colorless solid (120 mg, 38.3%). R_f (CH₂Cl₂/CH₃OH 30:1): 0.61, 0.67. ¹H NMR (CDCl₃): δ 0.80, 0.95 [2 t, 6 H, CHN(CH₂CH₂CH₂CH₃)₂], 1.07-1.72 [m, 20 H, 2 CH(CH₃)₂, CHN(CH₂CH₂CH₂CH₃)₂], 2.40 ~3.10 (m, 6 H, C2'-H, CH2CH2CN, 5-CH2CH2), 3.24-3.82 [m, 16 H, 2 OCH₃, C5'-H, CH₂CH₂CN, 5-CH₂CH₂, CHN(CH₂CH₂CH₂CH₃)₂], 4.23 (m, 1 H, C4'–H), 4.83 (m, 1 H, C3'-H), 6.66-7.28 (m, 19 H, aromatic H, C1'-H), 8.49 (s, 1 H, C2-H), 8.88 [s, 1 H, CHN(CH₂CH₂CH₂CH₃)₂]. ³¹P NMR (CDCl₃): 148.75, 148.57. ¹³C NMR (CDCl₃): δ 13.7, 13.6, 19.8, 20.1, 20.3, 24.4, 24.5, 24.6, 37.6, 43.0, 43.1, 43.2, 74.0, 74.2, 74.5, 74.7, 84.1, 85.0, 85.2, 85.9, 125.6, 126.4, 126.5, 127.5, 128.1, 128.2, 130.0, 136.2, 142.0, 144.9, 147.7, 155.5, 156.0, 156.6, 158.2, 162.9, HRMS Calcd for C₅₇H₇₄N₈O₆P (MH⁺): 997.5464. Found: 997.5478.

$T_{\rm m}$ measurement

An equimolar mixture of deoxyribozyme (1.3 μ M) and full DNA substrate sequence D19, 5'-d(AGG TGC AGG ATG GAG AGC A)-3' (1.3 μ M) in a buffer (1 ml) was used for T_m measurements. The buffer consisted of 50 mM Tris-HCl (pH 7.5) and 2 mM MgCl₂. The solution was heated to 85 °C. After halting for 10 min, it was cooled to 10 °C at a rate of 0.5 °C/min. The UV absorbance was recorded at 260 nm during the cooling process. The T_m values were obtained from the melting curves, and each melting curve was fit to a non-self-complementary two-state model.

Radio-labeling of the chimeric substrate

The substrate 5'-d(AGG TGC AGG)-rA-rU-d(GGA GAG CA)-3' (11 nmol) was 5'-³²P-labeled with 50 μ Ci of [γ -³²P] ATP and 10 U of T4 polynucleotide kinase (Takara, Dalian, China) at 37 °C for 60 min. After deactivation at 70 °C for 5 min, the radioactive substrate was extracted using a SEP-PAK column. After washing with sterilized water several times, it was eluted with methanol/water (70/30, v/v), lyophilized, and stored at -30 °C.

The cleavage reaction under single-turnover conditions

The reaction between deoxyribozyme (2 μ M) and the substrate (20 nM) was conducted in a buffer of 50 mM Tris-HCl (pH 7.4)

containing 2 mM MgCl₂.¹ The mixture of deoxyribozyme and the substrate was incubated in 50 mM Tris-HCl (pH 7.4) at 90 °C for 3 min. After cooling to 37 °C, an equal volume of 50 mM Tris-HCl (pH 7.4) containing Mg²⁺ (4 mM) was added to initiate the reaction. The reaction was monitored by taking aliquots from the reaction mixture at different time points that were quenched by an equal volume of stopping solution (100 mM EDTA, 8 M Urea). The cleavage process was analyzed by electrophoresis on a 20% polyacrylamide gel containing 7 M urea, and quantified by the densitometry of the gel images with a Molecular Dynamics Storm 840 Phosphoimager.¹⁴ The equation $P\% = P_{\infty}\% - C \cdot exp$ $[-k_{obs}t]$ was used to calculate the observed rate constant, where P% is the cleavage percentage of product at time t, $P_{w}\%$ is the final percentage of the product at $t = \infty$, C is the difference in P% between $t = \infty$ and t = 0, and k is the observed rate constant. The data was given as the averaged results of three independent experiments. Less than 20% variation was observed for identical experiments performed on different days.

Analysis of pH dependence under multiple-turnover conditions

The reactions were conducted in different buffers according to the required pH ranging from 6.0 to 8.5. Multiple-turnover conditions were employed.² A mixture of the substrate (200 nM) and deoxyribozyme (20 nM) was incubated in 50 mM Tris-HCl buffer containing 2 mM Mg²⁺. Aliquots were withdrawn after various time intervals for analysis as described above.

Analysis of divalent metal ion dependence under single-turnover conditions

The cleavage rate of deoxyribozymes in the presence of either Mg^{2+} , Mn^{2+} , or Ca^{2+} (2 mM) were measured under single-turnover conditions in a buffer of 50 mM Tris-HCl (pH 7.5).² A mixture of the substrate (20 nM) and deoxyribozyme (2 μ M) in a buffer containing different divalent metal ions was incubated at 37 °C. Aliquots were withdrawn after various time intervals for analysis as described above.

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