ACS Combinatorial Science

Research Article

pubs.acs.org/acscombsci

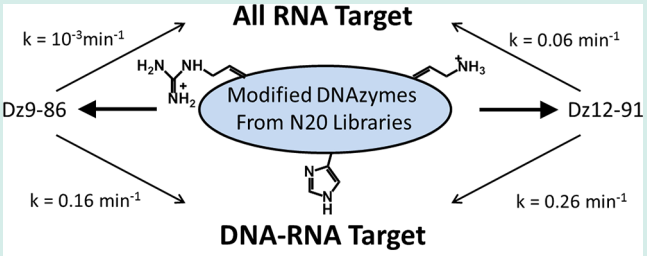
Toward the Combinatorial Selection of Chemically Modified DNAzyme RNase A Mimics Active Against all-RNA Substrates

Marcel Hollenstein, Christopher J. Hipolito, Curtis H. Lam, and David M. Perrin*

Department of Chemistry, University of British Columbia, 2036 Main Mall, Vancouver BC, V6T 1Z1, Canada.

Supporting Information

ABSTRACT: The convenient use of SELEX and related combinatorial methods of in vitro selection provides a formidable gateway for the generation of DNA enzymes, especially in the context of improving their potential as gene therapeutic agents. Here, we report on the selection of DNAzyme 12−91, a modified nucleic acid catalyst adorned with imidazole, ammonium, and guanidinium groups that provide for efficient M^{2+}-independent cleavage of an all-RNA target sequence (k_{obs} = 0.06 min^{-1}). While Dz12−91 was selected for intramolecular cleavage of an all-RNA target, it surprisingly cleaves a target containing a lone ribocytosine unit with even greater efficiency (k_{obs} = 0.27 min^{-1}) than Dz9−86 (k_{obs} = 0.13 min^{-1}). The sequence composition of Dz12−91 bears a marked resemblance to that of Dz9−86 (k_{obs} = 0.0014 min^{-1} with an all-RNA substrate) that was selected from the same library to cleave a target containing a single ribonucleotide. However, small alterations in the sequence composition have a profound impact on the substrate preference and catalytic properties. Indeed, Dz12−91 displays the highest known rate enhancement for the M^{2+}-independent cleavage of all-RNA targets. Hence, Dz12−91 represents a step toward the generation of potentially therapeutically active DNAzymes and further underscores the usefulness of modified triphosphates in selection experiments.



Combinatorial Selection of Chemically Modified DNAzyme RNase A Mimics

KEYWORDS: *deoxyribozyme catalysis, modified nucleic acids, triphosphates, SELEX, RNA cleavage*

INTRODUCTION

DNAzymes (or deoxyribozymes, or catalytic DNA enzymes) represent the newest class of biomolecular catalysts that join the ranks of previously characterized protein enzymes, antibodies, and ribozymes. DNAzymes have been selected to catalyze an increasingly diverse number of reactions as recently reviewed.[1−4] Yet despite the increasing number of reactions that DNAzymes have been selected to catalyze, catalysis has been generally described by single digit rate constants in units of reciprocal minutes while often multiple turnover has not been reported. Of all the potentially useful reactions that DNAzymes have been found to catalyze, RNA cleavage has been the most studied because of its relationship to earlier ribozymes, the relative ease of selection, and its enduring potential import for therapeutic applications to neutralizing viral and oncogenic mRNAs.

Indeed, prior to the discovery of multiple-turnover RNA-cleaving ribozymes, it was suggested that catalysts capable of cleaving RNA at a specific sequence would hold therapeutic promise for gene inactivation against viral infection and cancer. Moreover, the ability to create sequence specific RNases represents a fundamental goal in terms of artificial enzyme design.

Efforts toward this goal have typically followed two different paths. The first involved linking a DNA-oligonucleotide to an otherwise nonspecific RNA-cleaving moiety that positioned imidazoles, amines, or other related functions to mimic the active site of RNaseA.[5−7] The second strategy took a cue from RNA biology that culminated with re-engineering the *Tetrahymena* ribozyme for multiple turnover that could hypothetically target any mRNA sequence.[8] Nevertheless, as ribozymes show very limited serum stability, enthusiasm for RNA-cleaving ribozymes shifted to more stable DNAzymes, which to date have no known natural counterpart and must be combinatorially selected.[9−11] Toward this end, RNA cleaving DNAzymes were selected for therapeutic applications to cleave all-RNA targets with second-order rate constants that approach the limits of catalytic perfection when evaluated at 50 mM Mg^{2+} (>10^9 M^{-1} min^{-1}).[12] The impetus for this elegant work satisfied two important purposes: (1) demonstration of the first example of catalytic perfection in a DNA catalyst placing it on par with protein enzymes (at least in terms of a second order rate constant) and (2) to direct such activity toward viral (HIV) mRNA cleavage for therapeutic applications, which have since caught the attention of many.[11,13−16] While a recent report indeed showed that Dz10−23 catalyzes intracellular mRNA cleavage leading to apparent gene silencing,[17] other reports suggest that the observed gene silencing is due entirely to antisense effects.[18] This latter finding is consistent with previous findings that Dz10−23 adopts a distinct yet

Received: November 16, 2012
Revised: January 25, 2013
Published: March 13, 2013

   dx.doi.org/10.1021/co3001378 | *ACS Comb. Sci.* 2013, 15, 174−182

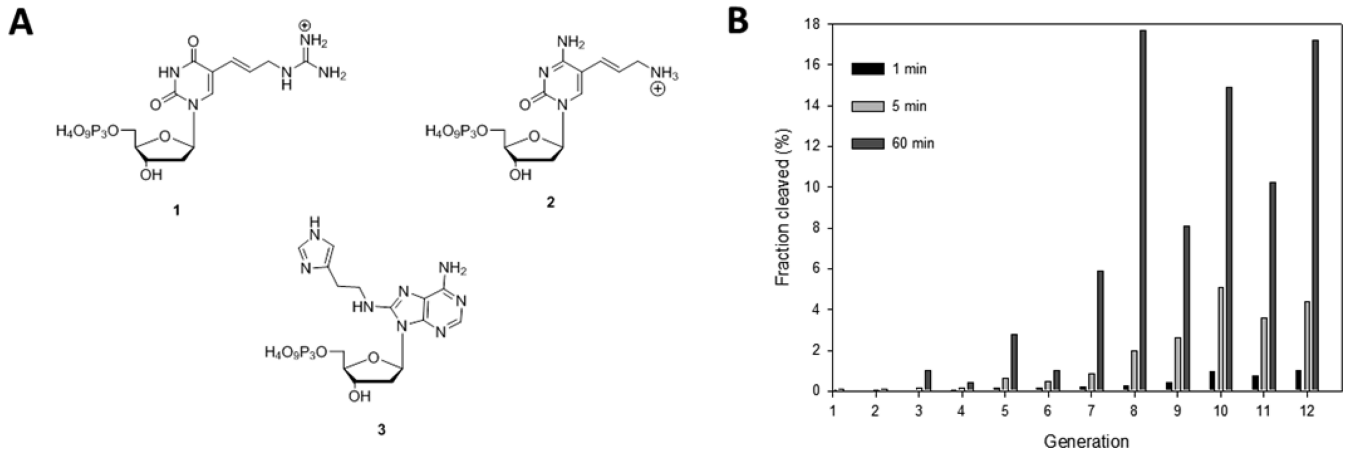


Figure 1. (A) Chemical structures of dC^{aa}TP **1**, dU^{ga}TP **2**, and dA^{im}TP **3**. (B) Progress of the selection: the fraction is shown for each generation (round of selection). For the first five rounds a selection time of 60 min was used. In rounds 6 to 8, the selection time was reduced to 5 min. At round 9, the selection time was again decreased to 1 min and maintained until round 12. Starting in round 4, self-cleavage activity was measured at 1, 5, and 60 min.

catalytically inactive conformation at 0.5 mM Mg^{2+}, which is the intracellular concentration of free Mg^{2+}.[19] Hence, DNAzymes that operate efficiently in the near Mg^{2+}-free environment found inside cells are still unknown yet their identification and application may provide a novel and heretofore untested means of neutralizing gene expression by catalyzed mRNA cleavage.

An interesting combinatorial approach to discovering M^{2+}-free ribonucleases has been to merge the chemical functionalities found at the active sites of RNaseA with powerful in vitro selection methods to discover DNA-based RNaseA mimics capable of RNA cleavage.[20−22] The resulting catalysts are likely to adopt some of the 2D-folding patterns characteristic of nonmodified DNAzymes, although it is unclear how the added chemical functionalities are integrated into the overall 3D-structure as they must provide entirely new folds that are responsible for greatly increased rate enhancements. Subsequent characterization demonstrated that these functionalities indeed play functional roles analogous to those found in RNaseA.[23−25] This promising combinatorial approach relies on developing modified nucleoside triphosphates along with suitable DNA polymerases that catalyze both modified dNTP incorporation and amplification of the resulting modified DNA.

Over the past decade, we and many others have reported on enzymatic preparation of DNA with additional functionalities, although in most examples only one modified dXTP is incorporated.[26−51] While this approach is broadly hypothesized to lead to enhanced catalytic activities once selected, very few studies have actually shown this. To show this gain of function, we have focused on selections that promote RNA cleavage at neutral pH in the absence of a M^{2+} cofactor,[21,52] which is particularly challenging for unmodified DNAs.[53−55] Hence, given the broad interest in modified dNTPs, we believe the findings herein, which provide a significant gain of function compared to unmodified DNAzymes may be generally important for using modified nucleosides for selecting other activities aside from RNA cleavage.

Previously, we screened for intramolecular cleavage at a single embedded ribophosphodiester linkage within a relatively small library (N20) comprising only 10^{12} sequences that resulted in Dz9−86 (vide infra).[52,56,57] In doing this, a small library was used such that in a 10 pmol primer extension reaction, theoretically all sequences will be represented at 10 copies each and therefore, a priori all of the sequence space would be covered in terms of its potential for being copied into modified DNA. Nevertheless, it is important to note that when using modified nucleosides, and in particular histaminyl-dA whose incorporation is limited to not more than two sequential histaminyl-dAs,[20] sequence space coverage is neither as uniform nor as substantial as governed by theory.

Notwithstanding this obvious limitation on sequence space, the results of several combinatorial selections showed that a loss of sequence space is offset by a gain in "chemical space" as evidenced by the selection of high catalytic activity under M^{2+}-free conditions. Indeed, modified DNAzymes with 3 different functionalities have up to 1000-fold greater M^{2+}-independent rates compared to catalysts selected from unmodified dNTPs that necessarily provide greater sequence space coverage.[52−54] In particular, the selected activity of Dz9−86 was highly specific for a target that contained only a single embedded ribose (k_{obs} = 0.13 min^{-1}), while cleavage at a homologous all-RNA substrate proceeded with only approximately 1% relative efficiency. Consequently, the ensemble of modifications adorning Dz9−86 seem to be highly specific for the overall shape that is presumably largely B-helical in nature compared to an A-helix characteristic of an all-RNA target. Here, we have used the identical N20-library to reselect for a self-cleaving DNAzyme that cleaves an all-RNA target sequence. Its properties and general homology to the antecedent catalyst that cleaved the target with a single embedded ribophosphodiester linkage are discussed.

RESULTS AND DISCUSSION

In vitro Selection and 2D-Structure of Dz12−91. An initial population of modified DNA was generated by polymerizing the three modified nucleoside triphosphates dA^{im}TP, dC^{aa}TP, and dU^{ga}TP (Figure 1A) along with the lone unmodified triphosphate dGTP on a template comprising 20 degenerate positions ($\leq 10^{12}$ sequences) doped with 3.3% of the template of the active Dz9−86 (oligonucleotide **10**, Figure 2A) for the first three rounds of selection by application of a protocol used in other in vitro selection experiments.[12,21,52,56] Surprisingly, doping with a small portion of the active sequence of Dz9−86 in the initial rounds of selection appeared to be

dx.doi.org/10.1021/co3001378 | ACS Comb. Sci. 2013, 15, 174−182

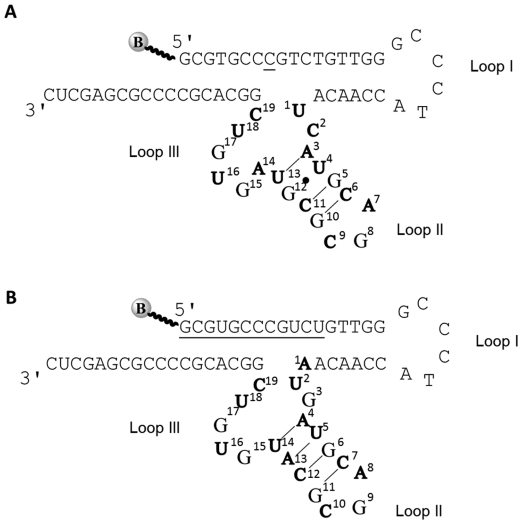


Figure 2. (A) Sequence and hypothetical structure of Dz9−86. (B) Sequence and hypothetical 2D structure of the selected DNAzyme 12−91. Bold-face **A**, **C**, and **U** indicate the position of the modified nucleosides dA^{im}TP, dC^{aa}TP and dU^{ga}TP, respectively, in the catalytic loops, underscored letters indicate the position of the 12 nt all-RNA substrate and the embedded ribocytosine, and all the nucleotides constituting the catalytic cores are numbered (1−19).

crucial for a positive outcome, since a similar selection without the incorporation of the synthetic oligonucleotide **10** failed to give any cleavage activity even after 9 generations (data not shown). A 1:1 mixture of two 5′-biotinylated primers (oligonucleotide **8** containing 12 RNA nucleotides and oligonucleotide **9** containing a 3 nt RNA substrate embedded within a 9-nt long sequence of 2′OMe containing ribonucleotides) was used in order to promote cleavage at a very limited subset of sequences and thereby avoid cleavage at the paired regions of the guiding arms as observed in some DNA-cleaving DNAzymes.[58] From round 6 onward, only primer **8** was used. The biotinylated heteroduplex consisting of functionalized DNA and unmodified cDNA template was captured on streptavidin magnetic particles. Following removal of the template strand by NaOH washing and neutralization, the single stranded modified DNA was incubated in 200 mM NaCl, 50 mM cacodylate (pH 7.4) and 1 mM EDTA at room temperature for 60 min. The stringency was gradually increased over the course of the selection by gradually reducing the reaction time from 60 min (G1-G5), to 5 min (G6-G8), and finally to 1 min (G9-G12) and by increasing the temperature to 37 °C (G9-G12). The selection progress is depicted in Figure 1B where activity was measured at three different time points. Activity was observed at a relatively late stage of the selection (∼2% cleavage after 60 min for round 5) and was sluggish to improve, especially when compared to the selection of Dz9−86.[52,59] While the fraction of sequences that cleaved after 60 min remained in the range of 10−15% even past generation 8, the fraction of those cleaving within 5 min increased in generations 6−10 reflecting that increased stringency eliminated slow-to-cleave species in favor of both fast-to-cleave and inactive species. Following the appearance of an appreciable amount of activity following 1 and 5 min incubations in the 12th (3% cleavage in 5 min and 1.5% in 1 min), cloning and sequencing resulted in 26 distinct sequences (Table S1, Supporting Information).

As we have observed in previous selections using modified dNTPs, the randomized regions of the sequences obtained from the selection varied substantially in length (between 18 and 25 nucleotides) and often contained mutations in the target sequence at the 5′-end. Synthetic oligonucleotides corresponding to the cloned sequences were then used in an initial kinetic survey to assess the activity of the various clones. The individual clone that showed the highest catalytic activity, clone 91, was then further kinetically characterized. The sequence and hypothetical 2D structure of DNAzyme 12−91 is shown in Figure 2B. The degenerate region is slightly shorter than the initial library (19 nucleotides instead of 20) and consists of a single putative hairpin loop. A similar diminution of the randomized region has been observed in the selection of other modified DNAzymes including Dz9−86.[21,52] In addition, the AT and GC contents are roughly equal (47% and 53%, respectively) and only three imidazole-modified residues are present in the catalytic motif.

Interestingly, both Dz12−91 and Dz9−86 bear certain similarities that might have arisen from the fact that in early rounds of the selection process we had doped with 3% of the 9−86 clone. Indeed, while the catalytic cores of both DNAzymes maintain the same length (i.e., 19 nucleotides), their sequence compositions slightly differ at some positions (see Figure 2) First, an imidazole bearing A^{1} was inserted right after the end of the conserved primer region in Dz12−91 (Figure 2B) instead of U^{1} in Dz9−86 (Figure 2B). Second, the unpaired C^{2} nucleotide at the base of Loop III of Dz9−86 has been mutated into an unmodified G^{3} in Dz12−91. Furthermore, the G^{12}-U^{4} wobble pair in the stem of Loop III in Dz9−86 appears to be replaced with an additional A^{13}-U^{5} Watson−Crick base pair in Dz12−91, a change that occurred through the mutation of the unmodified nucleotide G^{12} (in Dz9−86) to an imidazole bearing A^{13} (in Dz12−91). Finally, the unpaired adenine A^{14} of Dz9−86 is absent in the randomized region of Dz12−91, while the rest of the sequence (G^{15} through C^{19}) is conserved in both DNAzymes. In other words, the most noticeable changes are the appearance of an additional base pair that might rigidify the stem of loop II and the incorporation of an additional imidazole bearing adenosine close to the cleavage site. Thus, this shows that small alterations of the catalytic core of modified DNAzymes can have a significant impact on their substrate preference and cleavage ability (vide infra). Finally, the ability of Dz12−91 to cleave an all-RNA target based on a slight change in sequence composition (as compared to Dz9−86) is reminiscent of the Ca^{2+}-dependent RNA phosphodiester-cleaving DNAzyme Mg5.[54] Indeed, Dz Mg5 is rather proficient at catalyzing scission of a single-embedded ribo-adenosine linkage, but was shown to be inactive in the presence of all-RNA substrates. However, when a few mutations of the canonical bases of the binding arms were introduced in the sequence of Mg5, catalytic activity with all-RNA substrates could be detected (albeit with a ∼20-fold slower rate compared to the cleavage with the single rA-substrate).[60]

Kinetic Analysis of Self-Cleavage. Dz12−91 was synthesized using oligonucleotide **11** by applying the same methodology as described for the selection. Dz12−91 cleaved the 12 nt RNA-target (oligonucleotide **8**) with an average first-order rate constant k_{obs} of 0.058 ± 0.002 min^{-1} (three independent reactions carried out on different days with different modified DNA preparations) in 1 mM EDTA, 200 mM NaCl, 50 mM cacodylate pH 7.4 at 24 °C (Figure 3A and

dx.doi.org/10.1021/co3001378 | ACS Comb. Sci. 2013, 15, 174−182

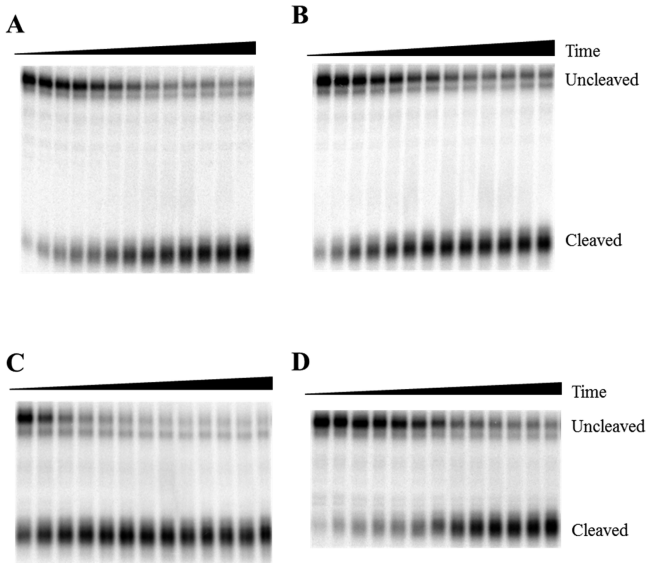


Figure 3. Representative gel images (7% PAGE) of the self-cleavage kinetics for Dz12−91: (A) with the all-RNA substrate oligonucleotide **8**; (B) with the 2′-OMe-RNA chimeric substrate oligonucleotide **9**; (C) with the single rC containing oligonucleotide **1**; (D) with the 17-mer all-RNA substrate oligonucleotide **12**. All reactions were carried out in 200 mM NaCl, 1 mM EDTA, and 50 mM cacodylate (pH 7.4) at room temperature.

Figure S1, Supporting Information). Notably, 9−86 was obligately dependent on the incorporation of all three modifications; even the extension of the imidazole side chain linker by a single methylene resulted in >100 fold drop in activity while replacing the $dU^{ga}TP$ with $dU^{aa}TP$ resulted in a complete loss of activity. Although herein we have not re-examined the effects of deleting any or all of the modifications by enzymatic resynthesis with 1, 2, or 3 more unmodified dXTPs, it would be extraordinary and even more noteworthy, if Dz12−89, which varies only slightly in the extent and kind of modification present compared to Dz9−86, were to entirely lose its dependence on the modifications.

Comparison of the Activity of DNAzyme 12−91 with Different Substrates. The rate of Dz12−91-mediated M^{2+}-independent self-cleavage was investigated as a function of the nature of the target (Table S2, Supporting Information). When a 17 nt all-RNA target (oligonucleotide **12**) was used instead of the 12 nt RNA sequence (Figure 3D and Figure S4, Supporting Information), a slightly lower first-order rate constant was observed (k_{obs} = 0.034 ± 0.001 min^{-1}, n = 3). On the other hand, using the 2′OMe/RNA chimeric substrate **9** (Figure 3B and Figure S2, Supporting Information) led to a slight increase in rate constant (k_{obs} = 0.066 ± 0.007 min^{-1}, n = 3). Interestingly, even though Dz12−91 was selected to cleave an all-RNA substrate, the first-order rate constant observed for the cleavage of a single embedded ribonucleotide (oligonucleotide **1**, Figure 3C and Figure S3, Supporting Information) was nearly 5-fold higher (k_{obs} = 0.272 ± 0.021 min^{-1}, n = 3) and double the rate observed for Dz9−86 (0.134 ± 0.026 min^{-1}).

This preference for a target with a single embedded ribophosphodiester linkage over an all-RNA stretch is somewhat surprising since most RNA-cleaving DNAzymes obtained by in vitro selections follow an opposite trend. Indeed, most DNAzymes that have been selected with all-RNA substrates usually show an equal propensity at cleaving substrates containing single ribonucleotides, as nicely exemplified by Dz8−17.[61] Conversely, DNAzymes that were selected with DNA-RNA chimeric substrates are in general catalytically inept in the presence of substrates containing longer stretches of RNA, a feature that holds for Dz9−86.[52]

Effect of pH and Temperature. As observed for Dz9−86 and other modified M^{2+}-independent DNAzymes, variation of the pH and temperature can have drastic effects on the catalytic efficiency. Consequently, the effect caused by a variation of these two parameters on the first-order rate constant was investigated in the case of Dz12−91. As expected, the pH-rate profile of Dz12−91 adopted a characteristic bell-shaped curve (Figure 4), which was then fit to a theoretical curve

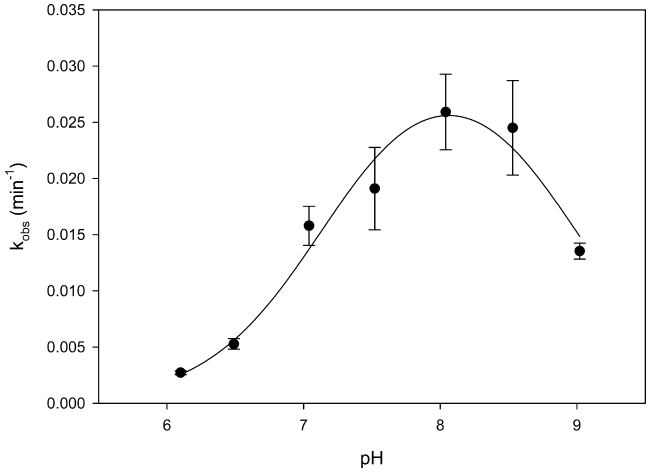


Figure 4. Cleavage rate dependence on pH, measured in (50 mM Tris·HCl, 200 mM NaCl, 1 mM EDTA) at 24 °C, error bars indicate standard error on duplicate runs. Values for pK_a were calculated using eq 2 to be 7.2 ± 0.1 and 9.0 ± 0.2 and k_{max} = 0.033 ± 0.004 min^{-1} (R^2 = 0.96).

corresponding to a two proton transfer process (see Experimental Procedures) yielding pK_a values of 7.2 and 9.0.[62−64] The pK_a of 7.2 of one of the catalytically essential groups is consistent with a pK_a of an imidazole residue (e.g., histidine has a pK_a of 6.4) while the other (pK_a = 9.0) would be more consistent with the pK_a of an amine side chain.[25,52,65] The effect of temperature on the rate of self-cleavage is shown in Figure 5A. As observed in the case of Dz 9−86, the rate constant increases with the temperature until a maximum is reached at 37 °C (k_{obs} = 0.088 ± 0.009 min^{-1}) and the rate then decreases with increasing temperature, a phenomenon that might arise because of the rearrangement of various secondary and/or tertiary folding structures within the catalytic core. In addition, an Arrhenius plot was then constructed (Figure 5B) and fitting the 4−37 °C temperature interval to eq 3 yielded an Arrhenius activation energy of 13.7 kcal·mol^{-1}. However, the Arrhenius plot was not linear over the entire range of temperatures and a significant curvature was observed at T > 37 °C (data not shown), which could reflect some conformational changes.[66,67] By application of transition state theory (using the Eyring eq 4), an enthalpy of activation of $\Delta H^{\ddagger}$ = 13.1 kcal·mol^{-1} and an entropy of activation of $\Delta S^{\ddagger}$ = −21.5 eu were obtained ($\Delta G^{\ddagger}_{298K}$ = 19.5 kcal·mol^{-1}). The enthalpy of activation was comparable to the one observed for Dz 9−86 and to that reported for a high-temperature Zn^{2+}-dependent DNAzyme,[68] but is slightly more favorable than the $\Delta H^{\ddagger}$ of the well-characterized Dz8−17[67] and the hammerhead ribozyme.[66]

dx.doi.org/10.1021/co3001378 | ACS Comb. Sci. 2013, 15, 174−182

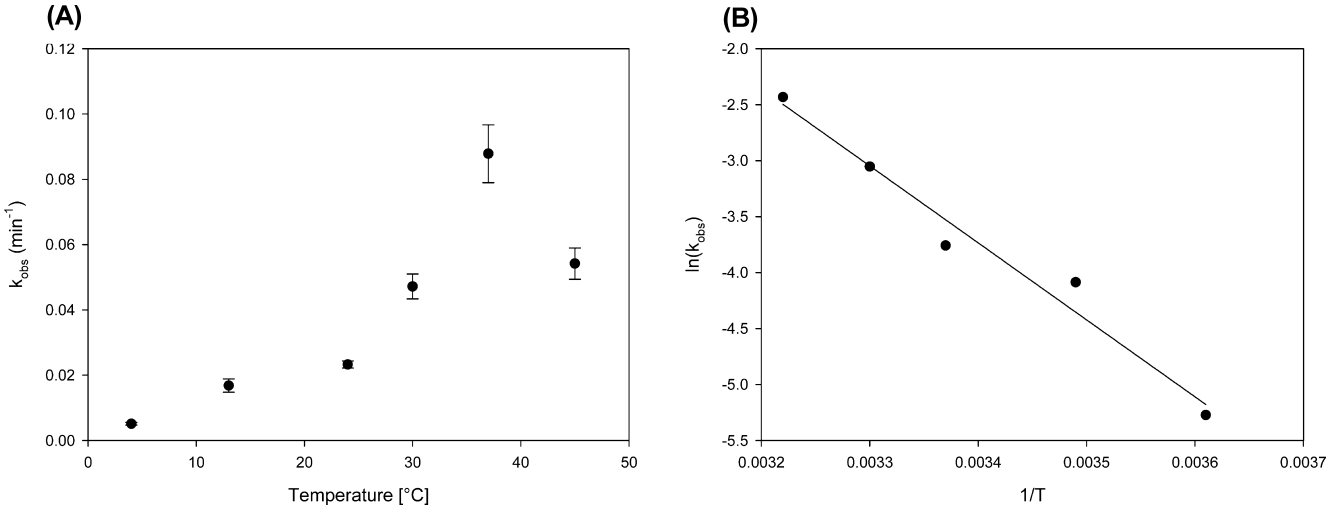


Figure 5. (A) Temperature dependence of the intramolecular RNA cleavage, measured in 200 mM NaCl, 50 mM cacodylate pH 7.4, and 1 mM EDTA over a range of 4−45 °C. Cacodylate was chosen for a relatively constant pH at variable temperature ($\Delta pH/\Delta T = -0.0015$ pH unit/°C), and error bars indicate standard error on triplicate runs. (B) Arrhenius plot of the temperature dependence of DNAzyme 12−91 in the interval 4−37 °C. Fitting the data to a linear regression gave $E_a = 13.7 \pm 1.4$ kcal·mol^{-1} ($R^2 = 0.97$).

On the other hand, the entropy of activation was nearly 2-fold less favorable. Moreover, the apparent entropy penalty observed for the Dz12−91-mediated cleavage reaction is higher than for most RNA-cleaving DNAzymes and ribozymes, which might be due to an unfavorable change of configuration of the catalytic core to encompass the all-RNA substrate.

CONCLUSIONS

The rather limited chemical functionality of unmodified nucleic acids has often been blamed for the strong M^{2+}-dependence of unmodified DNAzymes, which in turn is likely to severely undermine in vivo applications. Here, we used a combinatorial in vitro selection to generate a densely functionalized DNAzyme that is able to cleave an all-RNA target in the absence of any cofactor. Indeed, Dz12−91 is equipped with imidazole, cationic amine, and guanidine residues and cleaves a 12-nt long RNA target with a first-order rate constant of k_{obs} of 0.06 min^{-1}, which compares favorably with the only other all-RNA-cleaving modified DNAzyme selected by Siderov et al.[22]

The sequence composition of the catalytic core of Dz12−91 only differs at few positions from that of Dz9−86, resulting in the formation of an additional base pair that might rigidify the stem of loop II. Whether this was selected directly or resulted from adventitious mutagenesis of the Dz9−86 sequence that was doped into the selection is somewhat unclear, however selection without doping had not afforded the same positive results. In any event, these small mutations have a significant impact on the substrate preference of the DNAzyme since Dz12−91 cleaves an all-RNA substrate with a ∼50-fold higher efficiency than Dz9−86. Furthermore, the effect of the variation of the experimental conditions (i.e., pH and temperature) on the overall kinetic properties of Dz12−91 follows a similar pattern to what has been observed for other modified DNAzymes. Indeed, the pH-rate profile followed a typical bell-shaped curve with calculated pK_a values of 7.2 and 9.0, while a ∼15-fold increase in rate constant was observed when the temperature was raised from 4 to 37 °C (at which point the maximum cleavage efficiency was reached).

In addition, even though Dz12−91 was selected to cleave an all-RNA substrate, it cleaves a DNA-RNA chimeric substrate containing a single embedded ribocytosine linkage with a ∼5-fold higher efficiency. While both targets can be cleaved by both Dz9−86 and Dz12−91, selection against what appears to be a more difficult all-RNA target gave catalytic activity in Dz12−91 that was exalted when investigated against what appears to be an easier DNA-RNA chimeric target. Therefore in terms of selection, one might consider starting with a more difficult target, in this case the all-RNA substrate, in order to promote catalysis and enhance the rate constants for both challenging and more modest substrates. In addition, since Dz12−91 catalyzes the cleavage of all-RNA substrates under physiological conditions, this DNAzyme should be considered for potential in vivo applications, provided it can be engineered to a trans-cleaving species;[23,59] a goal that is currently under way.

More generally, this work further shows that when combinatorial selections are undertaken for cleavage of substrates that vary only slightly in their ribonucleotide contents, the outcome of a selection from the same library can be subtle in terms of the catalytic loop composition yet quite significant in terms of the selected catalytic activity.

EXPERIMENTAL PROCEDURES

Materials and Methods. $dA^{im}TP$ and $dU^{aa}TP$, were prepared according to literature procedures[20,59] and $dC^{aa}TP$ was obtained from TriLink. All oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA) and were purified by 10−15% 7 M urea denaturing PAGE. Ultrapure dNTPs were obtained from Fermentas, Sequenase Version 2.0 was purchased from GE Healthcare and Single Stranded DNA binding protein (SSB) was acquired from Epicenter Biotechnologies. Lambda exonuclease, *Taq* DNA polymerase, T4 polynucleotide kinase and Vent exo(-) DNA polymerase were obtained from New England Biolabs. Streptavidin magnetic particles were purchased from Roche. The nucleoside triphosphates dGTP α-[^{32}P] and ATP γ-[^{32}P] were purchased from Perkin-Elmer. pGEM-T-Easy Vector Systems kit was obtained from Promega. $LiClO_4$ was purchased from J.T. Baker.

dx.doi.org/10.1021/co3001378 | *ACS Comb. Sci.* 2013, 15, 174−182

Oligonucleotides. The following oligonucleotides (shown 5′−3′) were used: Biotin-T_{20}GCGTGCC**rC**GTCTGTTGG-GCCCTACCAACA **1**, GAGCTCGCGGGGCGTGCN$_{20}$-CTGTTGGTAGGGCCCAACAGACG **2**, phosphate-CGT-CTGTTGGGCCCTACCA **3**, GAGCTCGCGGGGCGTGC **4**, phosphate-ACGACACAGAGCGTGCCCGTCTGTTGG-GCCCTACCA 5, TTTTTTTTTTTTTTTTTTTTGAGC-TCGCGGGGCGTGC **6**, phosphate-TAATACGACTCACTA-TAGGGAGCTCGCGGGGCGTGC **7**, biotin-T_{40}r(GCG-UGCC**C**GUCU)GTTGGGCCCTACCAACA **8**, biotin-T_{40}m(GCGUGC)r(C**C**G)m(UCU)GTTGGGCCCTACCAA-CA **9**, GGGGCGTGCGACACTACGCGCTGCATGATG-TTGGTAGGGCCCAACAGACGGGCACGCTCGTGTCGT **10**, GGGGCGTGCGACACATGCGCTGCATCATTGTTG-GTAGGGCCCAACAGACGGGCACGCTCGTGTCGT **11**, and biotin-T_{20}r(GCGUGCC**C**GUCUGUUGG)GCCCTA-CCAACA **12**. Bold-faced letters indicate the position of the embedded **rC** in oligonucleotide **1** and the corresponding site in oligonucleotides **8**, **9**, and **12**, whereas r designates a stretch of RNA bases and m a stretch of 2′OMe bases.

Buffers. Buffer 1 (cleavage buffer): 50 mM sodium cacodylate (pH 7.4), 200 mM NaCl, 1 mM EDTA. Buffer 2 (elution buffer): 1% $LiClO_4$/Tris-HCl 10 mM (pH 8) in water. Buffer 3 (pH variance): 50 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA. The pH of all buffers was adjusted to 6.10, 6.49, 7.04, 7.52, 8.04, 8.53, or 9.02.

In Vitro Selection. Sixteen picomoles of oligonucleotide **8** (5′ biotin-T_{40}r(GCGUGCC**C**GUCU)GTTGGGCCCTAC-CAACA) and 16 pmol of oligonucleotide **9** (biotin-T_{40}m-(GCGUGC)r(C**C**G)m(UCU)GTTGGGCCCTACCAACA) were annealed to 29 pmol of template DNA (T_{20}GAGCTC-GCGGGGCGTGCN$_{20}$CTGTTGGTAGGGCCCAACAGACG prepared by nested PCR using primers **5** and **6**) and 1 pmol of synthetic template of Dz9−86 (oligonucleotide **10**, for rounds 1 to 3 only) and then enzymatically polymerized at 37 °C for 3 h using 9.1 units of Sequenase in a mixture containing Single Stranded Binding protein (SSB, 5 units), 5 mM DTT, 50 μM dA^{im}TP, 10 μM of each dU^{ga}TP, dC^{aa}TP, dGTP, and 5−15 μCi of dGTP α-[^{32}P] to give a final volume of 40 μL. The reaction was quenched by adding EDTA (25 mM final). The extension product was immobilized on 50 μL of prewashed magnetic streptavidin particles by incubating at room temperature for 30 min. After 2 short washes with 100 μL TEN buffer (10 mM Tris HCl, 1 mM EDTA, 100 mM NaCl), the template strand was removed by five washes of 100 μL 0.1 M NaOH and 1 mM EDTA, followed by a neutralization wash of 200 μL 25 mM cacodylate (pH 6) and one 100 μL water wash. The modified DNA was then incubated in reaction buffer **1** for varying time spans. The reaction time was decreased from 60 min down to 1 min over the selection (rounds 1−5 = 60 min; rounds 6−8 = 5 min; rounds 9−12 = 1 min). However, from round 4 onward, self-cleavage activity was measured at 1, 5, and 60 min. Moreover, from round 6 onward, only primer **9** was used for the selection. Finally, the modified ssDNA was incubated at room temperature for the initial rounds (G1−G8) and at 37 °C for later rounds (G9−G12). Following magnetization, the supernatant was precipitated (1% $LiClO_4$ in acetone), washed (EtOH), resuspended and resolved by 7% 7 M urea denaturing PAGE. The species corresponding to the cleaved product was eluted using buffer **2**, precipitated and desalted. The PCR amplification of the resulting modified DNA followed the nested double PCR amplification method outlined in the selection of DNAzyme 9_{2S}-11.[21] In the first amplification step, the modified DNA was PCR amplified using primers **3** and **4** and an internal label (10 μCi dGTP α-[^{32}P]) for 30 cycles (15 s at 54 °C, 40 s at 75 °C and 15 s at 95 °C). The reaction buffer included 0.07 units/μL Vent(exo-) DNA polymerase, 20 mM Tris-HCl (pH 8.8 at 25 °C), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 3 mM $MgSO_4$, 0.1% gelatin, 7 μM oligonucleotides and 0.3 mM of each natural dNTP. Prior to purification by 10% 7 M urea denaturing PAGE, the amplicon was treated with lambda exonuclease. An aliquot was then further amplified using primers **5** and **6** with 0.1 unit/μL Vent(exo-) DNA polymerase over 30 PCR cycles (using the same program as for the first amplification). The resulting product was precipitated (phenol-chloroform followed by an EtOH wash) and the phosphorylated strand was digested using lambda exonuclease. The single stranded DNA product was then purified by 10% 7 M urea denaturing mini-PAGE and identified by UV-shadowing. The resulting DNA was used in the ensuing round of selection. A total of 12 rounds of selection were performed.

Cloning of cDNAs. The 12th generation was amplified using *Taq* DNA polymerase with primers **3** and **7** to produce PCR products with 3′-A overhangs. These amplicons were then TA-cloned using the pGEM-T-Easy Vector Systems kit and were used to transform *E.coli* DH10B via electroporation. The transformation was plated on LB Agar containing 100 mg/L ampicillin. White colonies were picked and used to inoculate 1 mL of TB containing Plasmid Miniprep Kit and were subjected to restriction digest using *EcoR* I. Plasmids containing a single insert of the correct size (as controlled by 2% agarose gels), were submitted for sequencing. The Nucleic Acid Protein Service Unit of UBC carried out the sequencing of the most active clones using an SP6 sequencing primer. From the 100 random clones, 47 contained single inserts. Synthetic oligonucleotides corresponding to the various clones were used as templates to synthesize modified DNA as described previously.[52,56,59] Consequently, 5 pmol of each individual synthetic oligonucleotide were immobilized on streptavidin magnetic particles. Following 2 short washes with 100 μL TEN buffer, the template strands were removed by three washes with 100 μL NaOH 0.1 M and EDTA 1 mM, followed by a neutralization wash of 200 μL cacodylate 25 mM (pH 6) and one 100 μL water wash. The modified DNAs were then incubated at room temperature in 40 μL of buffer **1**. Eight time points were taken (2, 5, 10, 15, 30, 60, 120, and 1000 min) and resolved by 7% 7 M urea denaturing PAGE. Clone 91 showed the highest activity and was thus fully characterized.

Kinetic Analysis of Intramolecular Cleavage. Thirty-two picomoles of primer containing the substrate of interest (oligonucleotides **1**, **8**, **9**, or **12**) were annealed to 30 pmol of synthetic template DNAzyme 12−91 (oligonucleotide **11**), then enzymatically polymerized at 37 °C for 3 h using Sequenase 2.0 in a mixture containing SSB protein (5 U), 50 μM dA^{im}TP, 10 μM of each dU^{ga}TP, dC^{aa}TP, dGTP, and 5−15 μCi of dGTP α-[^{32}P] giving a total volume of 40 μL. The reaction was quenched by adding EDTA (25 mM final). Three microliters of the extension product was immobilized on 5 μL of prewashed magnetic streptavidin particles by incubating at room temperature for 30 min. After 2 short washes with 100 μL TEN buffer, the template strands were removed by three washes with 100 μL 0.1 M NaOH and 1 mM EDTA, followed by a neutralization wash of 200 μL 25 mM cacodylate (pH 6), 4 °C (to reduce self-cleavage) and one 100 μL water wash (at 4 °C as well). The avidin-bound modified DNA was incubated in 100 μL of cleavage buffer **1**, which formed a slurry of bead-

dx.doi.org/10.1021/co3001378 | *ACS Comb. Sci.* 2013, 15, 174−182

bound and cleaved material. Five microliters of the slurries were removed and quenched in 15 μL formamide containing 1 mM biotin, 25 mM EDTA, 0.01% bromophenol blue and 0.01% xylene cyanole). Samples were then heated (95 °C, 5 min.), cooled (0 °C), magnetized, and resolved by 7% 7 M urea denaturing PAGE. Visualization was carried out by means of a phosphorimager (Amersham Typhoon 9200) and polygons were drawn around the bands corresponding to the cleaved and uncleaved species. The autoradiographic data of the cleavage reactions (represented by pixel volumes on the Imagequant software program) were then fitted to first-order reactions with Sigmaplot 2001 (version 7.101) using eq 1

$$P_t = P_\infty \cdot (1 - e^{-kt}) \quad (1)$$

where P_t and P_∞ are the fractions cleaved at time t and the end point of the reaction, respectively, and k is the first-order rate constant. At least three independent sets of data were collected.

pH Rate Profile. The dependence of the rate of self-cleavage of DNAzyme 12−91 on pH was measured by incubating single-stranded modified DNAs obtained as described above in 100 μL buffer **3**. The pH range was 6.10−9.02. The data were fitted to eq 2[63]

$$k_{obs} = \frac{k_{max}}{[1 + 10^{(pK_a - pH)} + 10^{(pH - pK_{a'})} + 10^{(pK_a - pK_{a'})}]} \quad (2)$$

where k_{obs} is the observed rate constant, k_{max} is the maximum rate constant and K_a and $K_{a'}$ are the ionization constants of the two catalytically essential groups.

Temperature Dependence. The single stranded modified DNAs were incubated in 90 μL of buffer **1** (which was preincubated at the appropriate temperature for 30 min prior to the experiment) at various temperatures. Buffer **1** was prepared at 24 degrees and cacodylate was chosen on account of the fact that the pH of a cacodylate solution remains relatively constant with temperature ($\Delta pH/\Delta T = -0.0015$ pH units/°C).[69] All experiments were carried out under mineral oil to prevent evaporation. Temperature varied <0.5 °C in a VWR temperature controlled water bath. First order rate constants (k_{obs}) were then obtained by fitting the fraction cleaved to eq 1. The results shown are the average of two independent reactions. Data were then fit to the Arrhenius eq 3

$$\ln(k_{obs}) = \ln A - \frac{E_a}{R \times T} \quad (3)$$

where E_a is the activation energy and A is the pre-exponential factor. Activation parameters were obtained by application of transition state theory using the Eyring equation (eq 4)[68]

$$\ln\left(\frac{k_{obs}}{T}\right) = -\frac{\Delta H^{\neq}}{R \times T} + \ln\left(\frac{k_B}{h}\right) + \frac{\Delta S^{\neq}}{R} \quad (4)$$

■ ASSOCIATED CONTENT

Supporting Information

Sequences of the clones and rate constants of Dz12−91 with various substrates. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +1 604 822 0567. Fax: +1 604 822 2847. E-mail: dperrin@chem.ubc.ca.

Funding

M.H. was supported by fellowships from the Swiss National Science Foundation (grant PBBE2-108568). This work was funded by grants from the CIHR Operating Funds and NSERC.

Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Schlosser, K.; Lam, J. C. F.; Li, Y. F. A genotype-to-phenotype map of in vitro selected RNA-cleaving DNAzymes: implications for accessing the target phenotype. *Nucleic Acids Res.* **2009**, *37* (11), 3545−3557.

(2) Hobartner, C.; Silverman, S. K. Modulation of RNA tertiary folding by incorporation of caged nucleotides. *Angew. Chem., Int. Ed.* **2005**, *44* (44), 7305−9.

(3) Baum, D. A.; Silverman, S. K. Deoxyribozymes: Useful DNA catalysts in vitro and in vivo. *Cell. Mol. Life Sci.* **2008**, *65*, 2156−2174.

(4) Silverman, S. K. DNA as a versatile chemical component for catalysis, encoding, and stereocontrol. *Angew. Chem., Int. Ed.* **2010**, *49* (40), 7180−7201.

(5) Gnaccarini, C.; Peter, S.; Scheffer, U.; Vonhoff, S.; Klussmann, S.; Gobel, M. W. Site-specific cleavage of RNA by a metal-free artificial nuclease attached to antisense oligonucleotides. *J. Am. Chem. Soc.* **2006**, *128*, 8063−8067.

(6) Niittymäki, T.; Lönnberg, H. Artificial ribonucleases. *Org. Biomol. Chem.* **2006**, *4*, 15−25.

(7) Lonnberg, H. Cleavage of RNA phosphodiester bonds by small molecular entities: A mechanistic insight. *Org. Biomol. Chem.* **2011**, *9* (6), 1687−1703.

(8) Zaug, A. J.; Been, M. D.; Cech, T. R. The *Tetrahymena* ribozyme acts like an RNA restriction endonuclease. *Nature* **1986**, *324*, 429−433.

(9) Joyce, G. F. Directed evolution of nucleic acid enzymes. *Annu. Rev. Biochem.* **2004**, *73*, 791−836.

(10) Peracchi, A. DNA catalysis: Potential, limitations, open questions. *ChemBioChem* **2005**, *6*, 1316−1322.

(11) Schubert, S.; Kurreck, J. Ribozyme- and deoxyribozyme-strategies for medical applications. *Curr. Drug Targets* **2004**, *5* (8), 667−681.

(12) Santoro, S. W.; Joyce, G. F. A general purpose RNA-cleaving DNA enzyme. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94* (9), 4262−4266.

(13) Achenbach, J. C.; Chiuman, W.; Cruz, R. P. G.; Li, Y. DNAzymes: From creation in vitro to application in vivo. *Curr. Pharm. Biotechnol.* **2004**, *5*, 321−336.

(14) Asif-Ullah, M.; Levesque, M.; Robichaud, G.; Perreault, J. P. Development of ribozyme-based gene-inactivations; The example of the hepatitis delta virus ribozyme. *Curr. Gene Ther.* **2007**, *7* (3), 205−216.

(15) Pan, W. H.; Clawson, G. A. Catalytic DNAzymes: derivations and functions. *Expert Opin. Biol. Ther.* **2008**, *8* (8), 1071−1085.

(16) Baum, D. A.; Silverman, S. K. Deoxyribozymes: useful DNA catalysts in vitro and in vivo. *Cell. Mol. Life Sci.* **2008**, *65* (14), 2156−2174.

(17) Fokina, A. A.; Meschaninova, M. I.; Durfort, T.; Venyaminova, A. G.; Francois, J. C. Targeting insulin-like growth factor I with 10−23 DNAzymes: 2′-*O*-Methyl modifications in the catalytic core enhance mRNA cleavage. *Biochemistry* **2012**, *51* (11), 2181−2191.

(18) Young, D. D.; Lively, M. O.; Deiters, A. Activation and deactivation of DNAzyme and antisense function with light for the photochemical regulation of gene expression in mammalian cells. *J. Am. Chem. Soc.* **2010**, *132* (17), 6183−6193.

(19) Cieslak, M.; Szymanski, J.; Adamiak, R. W.; Cierniewski, C. S. Structural rearrangements of the 10−23 DNAzyme to beta-3 integrin subunit mRNA induced by cations and their relations to the catalytic activity. *J. Biol. Chem.* **2003**, *278*, 47987−47996.

(20) Perrin, D. M.; Garestier, T.; Helene, C. Expanding the catalytic repertoire of nucleic acid catalysts: simultaneous incorporation of two

modified deoxyribonucleoside triphosphates bearing ammonium and imidazolyl functionalities. *Nucleosides Nucleotides* **1999**, *18* (3), 377–91.
(21) Perrin, D. M.; Garestier, T.; Helene, C. Bridging the gap between proteins and nucleic acids: a metal-independent RNAseA mimic with two protein-like functionalities. *J. Am. Chem. Soc.* **2001**, *123* (8), 1556–63.
(22) Sidorov, A. V.; Grasby, J. A.; Williams, D. M. Sequence-specific cleavage of RNA in the absence of divalent metal ions by a DNAzyme incorporating imidazolyl and amino functionalities. *Nucleic Acids Res.* **2004**, *32* (4), 1591–601.
(23) Lermer, L.; Roupioz, Y.; Ting, R.; Perrin, D. M. Toward an RNaseA mimic: A DNAzyme with imidazoles and cationic amines. *J. Am. Chem. Soc.* **2002**, *124* (34), 9960–1.
(24) Ting, R.; Thomas, J. M.; Lermer, L.; Perrin, D. M. Substrate specificity and kinetic framework of a DNAzyme with an expanded chemical repertoire: a putative RNaseA mimic that catalyzes RNA hydrolysis independent of a divalent metal cation. *Nucleic Acids Res.* **2004**, *32* (22), 6660–72.
(25) Thomas, J. M.; Yoon, J. K.; Perrin, D. M. Investigation of the catalytic mechanism of a synthetic DNAzyme with protein-like functionality: An RNaseA mimic? *J. Am. Chem. Soc.* **2009**, *131* (15), 5648–5658.
(26) Hocek, M.; Fojta, M. Cross-coupling reactions of nucleoside triphosphates followed by polymerase incorporation. Construction and applications of base-functionalized nucleic acids. *Org. Biomol. Chem.* **2008**, *6*, 2233–2241.
(27) Lam, C.; Hipolito, C.; Perrin, D. M. Synthesis and enzymatic incorporation of modified deoxyadenosine triphosphates. *Eur. J. Org. Chem.* **2008**, 4915–4923.
(28) Lam, C. H.; Hipolito, C. J.; Hollenstein, M.; Perrin, D. M. A divalent metal-dependent self-cleaving DNAzyme with a tyrosine side chain. *Org. Biomol. Chem.* **2011**, *9* (20), 6949–6954.
(29) Hollenstein, M. Nucleoside triphosphates—Building blocks for the modification of nucleic acids. *Molecules* **2012**, *17* (11), 13569–13591.
(30) Hollenstein, M. Synthesis of deoxynucleoside triphosphates that include proline, urea, or sulfonamide groups and their polymerase incorporation into DNA. *Chem.—Eur. J.* **2012**, *18* (42), 13320–13330.
(31) Bergen, K.; Steck, A. L.; Strutt, S.; Baccaro, A.; Welte, W.; Diederichs, K.; Marx, A. Structures of KlenTaq DNA polymerase caught while incorporating C5-modified pyrimidine and C7-modified 7-deazapurine nucleoside triphosphates. *J. Am. Chem. Soc.* **2012**, *134* (29), 11840–11843.
(32) Baccaro, A.; Weisbrod, S. H.; Marx, A. DNA conjugation by the Staudinger ligation: New thymidine analogues. *Synthesis.* **2007**, *13*, 1949–1954.
(33) Jäger, S.; Famulok, M. Generation and enzymatic amplification of high-density functionalized DNA double strands. *Angew. Chem., Int. Ed.* **2004**, *43*, 3337–3340.
(34) Jäger, S.; Rasched, G.; Kornreich-Leshem, H.; Engeser, M.; Thum, O.; Famulok, M. A versatile toolbox for variable DNA functionalization at high density. *J. Am. Chem. Soc.* **2005**, *127* (43), 15071–15082.
(35) Raindlova, V.; Pohl, R.; Sanda, M.; Hocek, M. Direct polymerase synthesis of reactive aldehyde-functionalized DNA and its conjugation and staining with hydrazines. *Angew. Chem., Int. Ed.* **2010**, *49* (6), 1064–1066.
(36) Raindlova, V.; Pohl, R.; Klepetarova, B.; Havran, L.; Simkova, E.; Horakova, P.; Pivonkova, H.; Fojta, M.; Hocek, M. Synthesis of hydrazone-modified nucleotides and their polymerase incorporation onto DNA for redox labeling. *ChemPlusChem* **2012**, *77* (8), 652–662.
(37) Vaught, J. D.; Bock, C.; Carter, J.; Fitzwater, T.; Otis, M.; Schneider, D.; Rolando, J.; Waugh, S.; Wilcox, S. K.; Eaton, B. E. Expanding the chemistry of DNA for in vitro selection. *J. Am. Chem. Soc.* **2010**, *132* (12), 4141–4151.
(38) Sawai, H.; Nagashima, J.; Kuwahara, M.; Kitagata, R.; Tamura, T.; Matsui, I. Differences in substrate specificity of C(5)-substituted or C(5)unsubstituted pyrimidine nucleotides by DNA polymerases from thermophilic bacteria, archaea, and phages. *Chem. Biodiversity* **2007**, *4*, 1979–1995.
(39) Kuwahara, M.; Takahata, Y.; Shoji, A.; Ozaki, A. N.; Ozaki, H.; Sawai, H. Substrate properties of C5-substituted pyrimidine 2′-deoxynucleoside 5′-triphosphates for thermostable DNA polymerases during PCR. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 3735–3738.
(40) Kuwahara, M.; Tamura, T.; Kitagata, R.; Sawai, H.; Ozaki, H. Comparison study on PCR amplification of modified DNA by using various kinds of polymerase and modified nucleoside triphosphates. *Nucleic Acids Symp. Ser.* **2005**, No. 49, 275–6.
(41) Masud, M. M.; Kuwahara, M.; Ozaki, H.; Sawai, H. Sialyllactose-binding modified DNA aptamer bearing additional functionality by SELEX. *Bioorg. Med. Chem.* **2004**, *12* (5), 1111–1120.
(42) Mehedi Masud, M.; Ozaki-Nakamura, A.; Kuwahara, M.; Ozaki, H.; Sawai, H. Modified DNA bearing 5(methoxycarbonylmethyl)-2′-deoxyuridine: preparation by PCR with thermophilic DNA polymerase and postsynthetic derivatization. *ChemBioChem* **2003**, *4* (7), 584–8.
(43) Obayashi, T.; Masud, M. M.; Ozaki, A. N.; Ozaki, H.; Kuwahara, M.; Sawai, H. Enzymatic synthesis of labeled DNA by PCR using new fluorescent thymidine nucleotide analogue and superthermophilic KOD Dash DNA polymerase. *Bioorg. Med. Chem. Lett.* **2002**, *12* (8), 1167–1170.
(44) Ohbayashi, T.; Kuwahara, M.; Hasegawa, M.; Kasamatsu, T.; Tamura, T.; Sawai, H. Expansion of repertoire of modified DNAs prepared by PCR using KOD dash DNA polymerase. *Org. Biomol. Chem.* **2005**, *3* (13), 2463–2468.
(45) Ohmichi, T.; Kuwahara, M.; Sasaki, N.; Hasegawa, M.; Nishikata, T.; Sawai, H.; Sugimoto, N. Nucleic acid with guanidinium modification exhibits efficient cellular uptake. *Angew. Chem., Int. Ed.* **2005**, *44*, 6682–6685.
(46) Ohsawa, K.; Kasamatsu, T.; Nagashima, J. I.; Hanawa, K.; Kuwahara, M.; Ozaki, H.; Sawai, H. Arginine-modified DNA aptamers that show enantioselective recognition of the dicarboxylic acid moiety of glutamic acid. *Anal. Sci.* **2008**, *24* (1), 167–172.
(47) Saitoh, H.; Nakamura, A.; Kuwahara, M.; Ozaki, H.; Sawai, H. Modified DNA aptamers against sweet agent aspartame. *Nucleic Acids Res. Suppl.* **2002**, No. 2, 215–6.
(48) Sawai, H.; Ozaki, A. N.; Satoh, F.; Ohbayashi, T.; Masud, M. M.; Ozaki, H. Expansion of structural and functional diversities of DNA using new 5-substituted deoxyuridine derivatives by PCR with superthermophilic KOD Dash DNA polymerase. *Chem. Commun.* **2001**, *24*, 2604–2605.
(49) Shoji, A.; Kuwahara, M.; Ozaki, H.; Sawai, H. Modified DNA aptamer that binds the (*R*)-isomer of a thalidomide derivative with high enantioselectivity. *J. Am. Chem. Soc.* **2007**, *129* (5), 1456–1464.
(50) Held, H. A.; Benner, S. A. Challenging artificial genetic systems: thymidine analogs with 5-position sulfur functionality. *Nucleic Acids Res.* **2002**, *30*, 3857–3869.
(51) Held, H. A.; Roychowdhury, A.; Benner, S. A. C-5 modified nucleosides: Direct insertion of alkynyl-thio functionality in pyrimidines. *Nucleosides Nucleotides* **2003**, *22*, 391–404.
(52) Hollenstein, M.; Hipolito, C.; Lam, C.; Perrin, D. M. A self-cleaving DNA enzyme modified with amines, guanidines and imidazoles operates independently of divalent metal cations (M2+). *Nucleic Acids Res.* **2009**, *37*, 1638–1649.
(53) Geyer, C. R.; Sen, D. Evidence for the metal-cofactor independence of an RNA phosphodiester-cleaving DNA enzyme. *Chem Biol* **1997**, *4* (8), 579–93.
(54) Faulhammer, D.; Famulok, M. Characterization and divalent metal-ion dependence of in vitro selected deoxyribozymes which cleave DNA/RNA chimeric oligonucleotides. *J. Mol. Biol.* **1997**, *269* (2), 188–202.
(55) Carrigan, M. A.; Ricardo, A.; Ang, D. N.; Benner, S. A. Quantitative analysis of a RNA-cleaving DNA catalyst obtained via in vitro selection. *Biochemistry* **2004**, *43* (36), 11446–59.
(56) Hollenstein, M.; Hipolito, C.; Lam, C.; Dietrich, D.; Perrin, D. M. A highly selective DNAzyme sensor for mercuric ions. *Angew. Chem., Int. Ed.* **2008**, *47* (23), 4346–4350.

(57) Hipolito, C. J.; Hollenstein, M.; Lam, C. H.; Perrin, D. M. Protein-inspired modified DNAzymes: Dramatic effects of shortening side-chain length of 8-imidazolyl modified deoxyadenosines in selecting RNaseA mimicking DNAzymes. *Org. Biomol. Chem.* **2011**, *9* (7), 2266−2273.
(58) Chandra, M.; Sachdeva, A.; Silverman, S. K. DNA-catalyzed sequence-specific hydrolysis of DNA. *Nature Chem. Biol.* **2009**, *5* (10), 718−720.
(59) Hollenstein, M.; Hipolito, C. J.; Lam, C. H.; Perrin, D. M. A DNAzyme with three protein-like functional groups: Enhancing catalytic efficiency of M2+-independent RNA cleavage. *ChemBioChem* **2009**, *10* (12), 1988−1992.
(60) Peracchi, A. Preferential activation of the 8−17 deoxyribozyme by Ca^{2+} Ions: Evidence for the identity of 8−17 with the catalytic domain of the Mg5 deoxyribozyme. *J. Biol. Chem.* **2000**, *275*, 2875−2882.
(61) Schlosser, K.; Li, Y. F. A versatile endoribonuclease mimic made of DNA: Characteristics and applications of the 8−17 RNA-cleaving DNAzyme. *ChemBioChem* **2010**, *11* (7), 866−879.
(62) Bevilacqua, P. C. Mechanistic considerations for general acid-base catalysis by RNA: Revisiting the mechanism of the hairpin ribozyme. *Biochemistry* **2003**, *42*, 2259−2265.
(63) Roychowdhury-Saha, M.; Burke, D. H. Extraordinary rates of transition metal ion-mediated ribozyme catalysis. *RNA* **2006**, *12* (10), 1846−1852.
(64) Breaker, R. R.; Joyce, G. F. A DNA enzyme with Mg^{2+}-dependent RNA phosphoesterase activity. *Chem. Biol.* **1995**, *2* (10), 655−660.
(65) Battersby, T. R.; Ang, D. N.; Burgstaller, P.; Jurczyk, S. C.; Bowser, M. T.; Buchanan, D. D.; Kennedy, R. T.; Benner, S. A. Quantitative analysis of receptors for adenosine nucleotides obtained via in vitro selection from a library incorporating a cationic nucleotide analog. *J. Am. Chem. Soc.* **1999**, *121* (42), 9781−9789.
(66) Peracchi, A. Origins of the temperature dependence of hammerhead ribozyme catalysis. *Nucleic Acids Res.* **1999**, *27*, 2875−2882.
(67) Bonaccio, M.; Credali, A.; Peracchi, A. Kinetic and thermodynamic characterization of the RNA-cleaving 8−17 deoxyribozyme. *Nucleic Acids Res.* **2004**, *32* (3), 916−925.
(68) Nelson, K. E.; Bruesehoff, P. J.; Lu, Y. In vitro selection of high temperature Zn^{2+}-dependent DNAzymes. *J. Mol. Evol.* **2005**, *61*, 216−225.
(69) Gore, M. G. Spectrophotometry and spectrofluorimetry. In *Spectrophotometry and Spectrofluorimetry*; Oxford University Press: Oxford, U.K., 2000; p 339.