Organic & Biomolecular Chemistry

www.rsc.org/obc

Volume 9 | Number 20 | 21 October 2011 | Pages 6853–7256

ISSN 1477-0520

RSC Publishing

FULL PAPER
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International Year of CHEMISTRY 2011

1477-0520(2011)9:20;1-V

Organic & Biomolecular Chemistry

Cite this: *Org. Biomol. Chem.*, 2011, **9**, 6949

www.rsc.org/obc

PAPER

A divalent metal-dependent self-cleaving DNAzyme with a tyrosine side chain†

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Received 8th March 2011, Accepted 15th April 2011
DOI: 10.1039/c1ob05359k

The enzymatic incorporation of a phenol-modified 2′-deoxyuridine triphosphate gave rise to a modified DNA library that was subsequently used in an *in vitro* selection for ribophosphodiester-cleaving DNAzymes in the presence of divalent zinc and magnesium cations. After 11 rounds of selection, cloning and sequencing resulted in 14 distinct sequences, the most active of which was Dz11-17PheO. Dz11-17PheO self-cleaved an embedded ribocytidine with an observed rate constant of 0.20 ± 0.02 min^{-1} in the presence of 10 mM Mg^{2+} and 1 mM Zn^{2+} at room temperature. The activity was inhibited at low concentrations of Hg^{2+} cations and somewhat higher concentrations of Eu^{3+} cations.

Introduction

Over the past 30 years, biological catalysis has been extended from proteins to include nucleic acids. Whereas naturally occurring ribozymes including the hammerhead[1] and RNase P were identified,[2,3] powerful *in vitro* selection techniques (or SELEX) have led to the discovery of a large number of unnatural nucleic acid catalysts including deoxyribozymes (DNAzymes).[4–7] Hence, catalytic nucleic acids have been found to catalyze numerous reactions such as RNA/DNA cleavage,[8–12] Diels–Alder reactions[13–16] and Michael additions, to name just a few.[17]

Compared to the diversity of side-chain functionality in proteins, nucleic acids are functionally deprived and often rely on relatively high concentrations of divalent metal cations (M^{2+}) for folding and catalysis. In order to alleviate the paucity of chemical diversity believed to undermine the catalytic repertoire of DNAzymes, many efforts have been made to enzymatically polymerize modified DNA by using 2′-deoxynucleoside triphosphates (dNTPs) endowed with synthetically-appended functional groups. Indeed, dNTPs have been successfully functionalized with almost every amino acid side chain as well as moieties that cannot be found in proteins.[18–27] While modified dNTPs could potentially enhance the catalytic repertoire of nucleic acid catalysts, as reviewed recently,[28] selections of modified catalysts remain few in number and scope.

Selection of modified DNAzymes have been focused extensively on the cleavage of ribophosphodiester bonds.[29] Indeed, RNA-cleaving DNAzymes have been well-studied due to i) the relative ease of selection and ii) their potential to act as sensors[30,31] and to cleave RNA *in vivo*.[32,33] The first DNAzyme that was obtained from an *in vitro* selection using modified nucleotides was Dz16.2-11.[34] This catalyst utilizes imidazole-modified dU's in the presence of Zn^{2+} cations to catalyze RNA cleavage. It was proposed that the pendant imidazoles chelate a Zn^{2+} cation to promote catalysis. Several other selections have been performed using as many as three different modifications to discover M^{2+}-independent ribophosphodiester-cleaving DNAzymes.[35–39] In particular, $Dz9_{25}$-11, which contains two modifications: imidazoles and cationic amines,[35] was shown to mimic RNaseA where two imidazoles appear to be involved in general acid/base catalysis while a single cationic amine appears to be involved in coulombic stabilization of the negative charge build-up in the transition state.[40]

One modification that has yet to be explored in the context of modified DNAzymes is the phenol group, characteristic of the tyrosine side chain, which is found in the active sites of many protein enzymes wherein it may act as a catalytic nucleophile,[41,42] general base[43] or general acid.[44] In order to evaluate the effect of the addition of a phenol modification on the activity of selected DNAzymes, an *in vitro* selection was performed to discover a DNAzyme bearing phenol residues and that catalyzes ribophosphodiester cleavage. We synthesized a phenol-modified dUTP [5-(4-hydroxybenzoylaminomethyl)-2′-deoxyuridine triphosphate] and thereby enzymatically polymerized it to produce a modified DNA library. Selection on the resulting modified single-stranded DNA sequence pool in the presence of Mg^{2+} and Zn^{2+} gave rise to many DNAzymes that catalyze self-cleavage at an embedded RNA linkage, the most active sequence of which was characterized in detail. This work showcases the first example of a phenol-modified DNAzyme that efficiently self-cleaves in the presence of divalent metal cations (M^{2+}).

Results and discussion

Synthesis of phenol-modified DNA

Phenol-modified dUTP (**2**) was readily obtained by either one of two methods (Scheme 1). 5-Aminomethyl-dUTP (**1**)[45] (ESI†)

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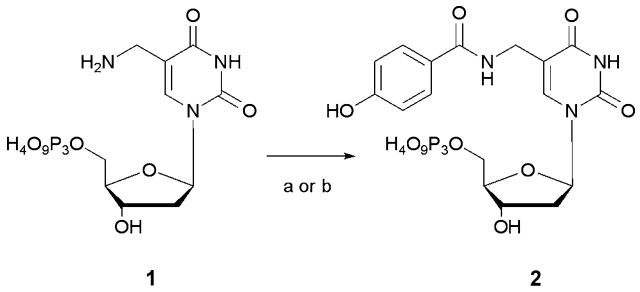
† Electronic supplementary information (ESI) available: Synthesis of **1**, incorporation of **2** by Vent (*exo*-) DNA polymerase, progress of the selection and cloned selection sequences. See DOI: 10.1039/c1ob05359k



Scheme 1 *Reagents and conditions*: a) 4-hydroxybenzoyl-*N*-hydroxysuccinimide ester, $NaHCO_3$, H_2O/DMF; b) i) 4-boronylbenzoyl-*N*-hydroxysuccinimide ester, $NaHCO_3$, H_2O/DMF, ii) H_2O_2, H_2O.

was mixed with the NHS ester of 4-hydroxybenzoic acid in the presence of sodium bicarbonate to give the desired product. Alternatively, triphosphate **1** was reacted with the NHS ester of 4-carboxyphenylboronic acid. The resulting derivative was then oxidized to **2** using hydrogen peroxide. Following the prep-TLC and HPLC purification of **2**, polymerase-mediated reactions revealed that **2** was an excellent substrate for Vent (*exo*-) DNA polymerase. Primer extension reactions requiring five consecutive modified nucleotides to be incorporated were readily accomplished (ESI†). Triphosphate **2** also successfully supports exponential PCR (data not shown). Thus the substrate properties of **2** for DNA polymerases demonstrate that **2** is fully compatible with *in vitro* selection conditions and reinforces the fact that 5-modified dUTP's are generally good substrates for polymerases.[19,23,25,46]

In vitro selection

The selection was performed using the streptavidin-biotin methods used by us and many others.[7,8,35] Briefly, a 5′-biotinylated primer was annealed with a template containing 40 degenerate positions. Triphosphate **2** and the other three unmodified dNTP's were polymerized along the template to give an initial modified oligonucleotide library of more than 10^{13} sequences. Following the removal of the unmodified template strands, the sequence pool was incubated in buffer consisting of 50 mM HEPES pH 7.4, 200 mM NaCl, 0.5 mM $MgCl_2$ and 0.1 mM $ZnSO_4$ at room temperature. At the outset, the selection was intended to generate sugar-sensing DNAzymes because notably tyrosines are relatively abundant in the active sites of lectins.[47] Indeed, the ability of DNA to recognize sugars has recently gained attention.[48–50] Nevertheless, in our hands the use of several sugars in the selection buffer (positive selection) only promoted the release of strands from the streptavidin magnetic particles that had already cleaved in the negative selection.

Whereas 15 rounds of selection were carried out, cloning was performed on the material obtained from generation 11 because no further progress of activity was observed in rounds 12–15 despite increased selection stringency in these later rounds. Each of the 14 sequences identified was different (ESI†). The average number of nucleotides corresponding to the random region was 40, and the average number of modified residues in the random region was 9 (standard deviation of 2), a value that is not substantially lower than the statistically random number of 10. That the PheO-dU is represented with statistical normalcy is not surprising since **2** is a good substrate for Vent (*exo*-) DNA polymerase under both primer extension and PCR conditions. Preliminary kinetic analysis of the identified clones revealed that 9 of the 14 sequences displayed self-cleavage activity (ESI†).

Kinetic experiments on the identified active sequences in various conditions eventually pinpointed two factors that played a significant role in the final outcome of the selection. Firstly, sequences that self-cleaved tended to remain bound to the streptavidin magnetic particles through non-specific interactions instead of eluting readily from the beads, and secondly, the addition of stachyose or other saccharides, *e.g.* sialic acid or D-mannoheptose, promoted the release of these non-specifically bound sequences from the streptavidin magnetic particles that were not removed during the negative selection washes. Consequently, self-cleavage was found to be sugar-independent while the combination of these two factors led to the initially misleading impression that the addition of various sugars promoted sugar-dependent self-cleavage.

Characterization of DNAzyme 11-17PheO

Of the cloned sequences, Dz11-17PheO was the most catalytically active and was characterized further. The hypothetical secondary structure of the DNAzyme as predicted by mfold[51,52] is shown in Fig. 1. Eight modified nucleotides and two stem loops can be found in the random region. A sequence alignment analysis of the most catalytically active sequences obtained from cloning of generation 11 showed that the catalytically active sequences share a common motif with Dz11-17PheO where 19 out of the first 21 residues in the random region are identical (Table S3, ESI†). It should be noted that mfold structure prediction did not take into account any effects that may be caused by the modifications on the dUs, which could dramatically affect the secondary structure.

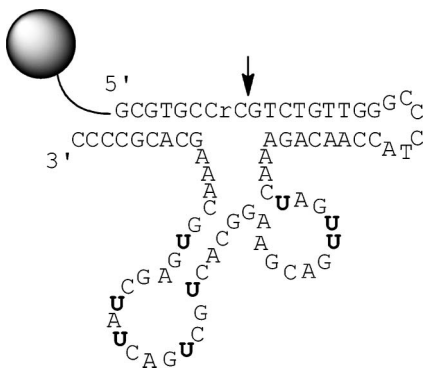


Fig. 1 Predicted 2D structure of Dz11-17PheO. The construct was biotinylated at the 5′ terminus and contained one ribonucleotide which corresponds to the cleavage location (arrow). Modified nucleotides are shown as "U" and the structure was obtained with mfold with hybridization of the rC disallowed.

The self-cleavage kinetic analysis of Dz11-17PheO is shown in Fig. 2. The rate of the reaction was found to be very dependent on the concentrations of the divalent metal cations present. In the presence of 50 mM HEPES pH 7.4, 200 mM NaCl, 1 mM $ZnSO_4$ and 10 mM $MgSO_4$, self-cleavage was more than 90% complete after an hour at room temperature. Under these experimental conditions, an average observed rate constant of 0.20 ± 0.02 min^{-1} was obtained. Based on an estimated background

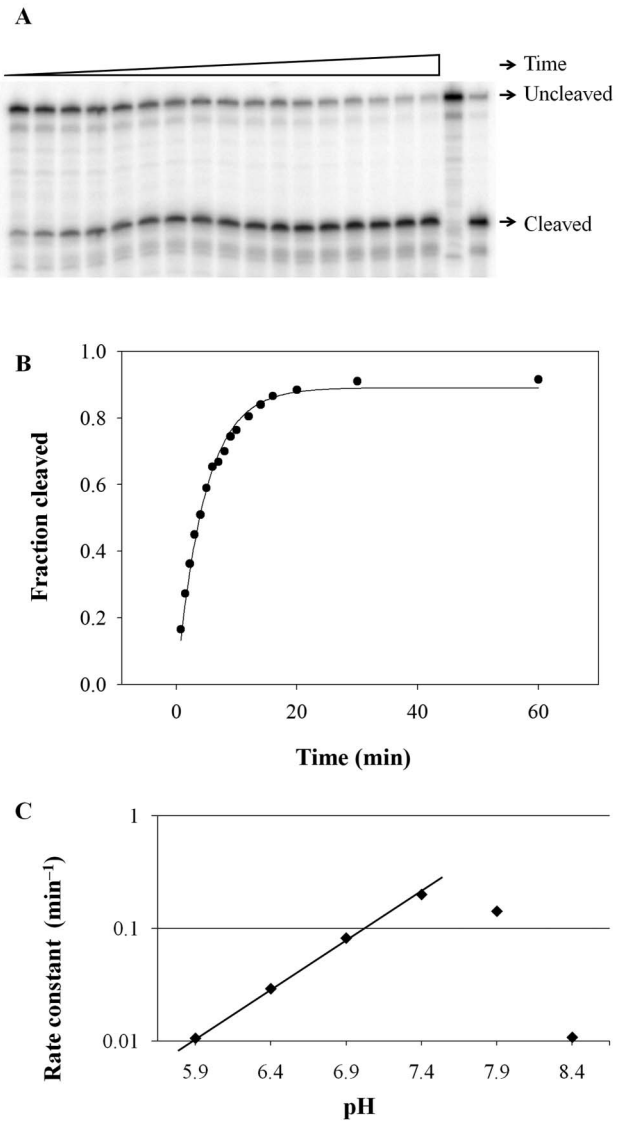


Fig. 2 Self-cleavage kinetics of Dz11-17PheO. A, Denaturing PAGE (7%) of the reaction showing the relative amounts of uncleaved and cleaved material over a period of 60 min. Experiment performed with 50 mM HEPES pH 7.4, 200 mM NaCl, 1 mM $ZnSO_4$, 10 mM $MgSO_4$ at room temperature. Lanes correspond to reaction aliquots quenched at 0.75, 1.5, 2.25, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 20, 30, 60 min, negative and positive controls. B, Graphical analysis of the data. A k_{obs} of 0.20 ± 0.02 min^{-1} was obtained ($R^2 > 0.99$). C, pH rate profile. Reactions were performed in 50 mM buffer, 200 mM NaCl, 1 mM $ZnSO_4$, 10 mM $MgCl_2$ at room temperature. The line was fit to the formula of $\log y = mx + b$, a slope of 0.86 was obtained ($R^2 > 0.99$).

rate of 10^{-5} min^{-1}, the observed rate constant represents a rate acceleration of 2.0×10^4. Dz11-17PheO was active in the presence of either Mg^{2+} or Zn^{2+}, with Zn^{2+} giving superior reaction rates. No activity was observed in the absence of divalent metal cations. Notably, the observed rate constant is comparable to other M^{2+}-dependent ribophosphodiester-cleaving DNAzymes. The very thoroughly studied Dz10-23 cleaves RNA with a $k_{cat} = 0.49$ min^{-1} (10 mM Mg^{2+}, 37 °C),[9] and Dz16.2-11 cleaves RNA with a k_{cat} = 1.5 min^{-1} (1 mM Mg^{2+} and 0.01 mM Zn^{2+}, 37 °C).[34]

When Dz11-17PheO was synthesized with dTTP in place of **2**, the resulting unmodified sequences showed no self-cleavage activity in the presence of both Mg^{2+} and Zn^{2+} suggesting that in this case, as already observed for other modified DNAzymes, the functional groups on dU are essential for ensuring a catalytically competent conformation. Nevertheless, as with all catalytic nucleic acids, the question of whether the appended functionality of a modified nucleotide plays a direct role in catalysis as opposed to an indirect role through folding, or both, is difficult to answer. Indeed, while the preliminary report of a RNA Diels–Alderase containing pyridyl-modified dUs did not establish the role of the pyridine,[14] subsequent reports showed that catalysis was absolutely dependent on a *para*-substituted pyridine[53] and that the pyridine modifications that are involved provide a hydrophobic effect in the mechanism of catalysis.[54] It therefore is likely that the activity herein will similarly depend on the modification's geometry and it is not impossible that the 5-amido group of the dU may also play an important role in catalysis or folding.

To further characterize Dz11-17PheO, kinetic experiments were performed where Mg^{2+} and Zn^{2+} were replaced with other metal cations that included Ca^{2+}, Ba^{2+}, Mn^{2+}, Fe^{3+}, Ni^{2+}, Cu^{2+}, Hg^{2+}, Eu^{3+} and La^{3+} (each at 1 mM). Of these cations, only Ca^{2+} and Mn^{2+} supported appreciable activity and interestingly, in the presence of Mn^{2+} self cleavage was especially rapid ($k_{obs} = 0.35 \pm 0.03$ min^{-1}).

In order to determine whether Dz11-17PheO contained binding sites for metal cations, inhibition experiments were performed. It was hypothesized that if the activity of the DNAzyme were inhibited by one or a few select metal cations, the DNAzyme could potentially function as a sensor for those metal cations.[55] Thus, experiments were carried out by incubating Dz11-17PheO in a buffer containing 10 mM Mg^{2+} and 1 mM Zn^{2+} and 100 μM of another metal cation. The same 9 metal cations that were mentioned in the previous paragraph were used for the inhibition experiments as well. Of all the metal cations used, inhibition of Dz11-17PheO was observed only with Eu^{3+} and Hg^{2+}. At 30 μM and 100 μM Hg^{2+}, no appreciable Dz11-17PheO cleavage was observed after 10 min whereas at 10 μM Hg^{2+} or less, the extent of self-cleavage was more than 50% complete over the same period. Inhibition by Hg^{2+} is consistent with T-Hg^{2+}-T interactions[56,57] while inhibition by the oxophilic lanthanide Eu^{3+} likely reflects phenoxide-Eu^{3+} interactions that distort the active conformation. We also investigated the inhibitory effects of two organophosphate pesticides, fenitrothion and diazinon, which normally phosphorylate a serine nucleophile at the active site of acetylcholinesterase.[58] As the phenolic groups in Dz11-17PheO are at least partially ionized at pH 7.5 (4-hydroxybenzamide has a pK_a of 8.56),[60] we hypothesized that these agents might have phosphorylated a critical phenolate in Dz11-17PheO resulting in inhibition and thereby providing a prototypical nerve agent sensor. Although these pesticides did not appear to inhibit Dz11-17PheO, the presence of a phenolate group might eventually suggest enhanced potential for selecting nerve agent sensors using modified nucleotides, as unmodified DNA normally lacks a good oxygen nucleophile for catalytic sensing.

Lastly, the self-cleavage of Dz11-17PheO was investigated as a function of pH. The reaction was monitored at 6 additional values of pH from 5.9 to 8.9, and the pH rate profile is shown in Fig. 2C. The shape of the curve is similar to that of several other ribophosphodiester-cleaving DNAzymes that are dependent on

metal cations for activity.[10,34,59] Not surprisingly, the rate constant was highest at pH 7.4, the pH at which the selection was performed. Between pH 5.9 and 7.4, the log of the observed rate constant increased linearly with pH. The slope of the line was close to unity (0.86) and suggests that the rate-determining step involves the abstraction of a proton. After pH 7.4, the observed rate constant decreased significantly, possibly due to the deprotonation of a catalytically essential group such as a phenol group.[60]

Conclusions

An *in vitro* selection carried out using a phenol-modified dUTP gave rise to Dz11-17PheO, an efficient self-cleaving DNAzyme that requires Ca^{2+}, Mg^{2+}, Mn^{2+} or Zn^{2+} for catalysis. The DNAzyme was found to be inhibited by the presence of 30 μM Hg^{2+} or 100 μM Eu^{3+} and suggests that this strategy may also be used to expand the sensitivity for detecting either Hg^{2+} or Eu^{3+}. This work demonstrates that the phenol functionality is fully compatible with the selection of an efficient M^{2+}-dependent self-cleaving DNAzyme that can provide a new chemical handle for post-synthetic modification.

Experimental

General

Unless indicated otherwise, all reagents and solvents were obtained from either Sigma Aldrich or Fisher Scientific. Anhydrous solvents were prepared by either distillation or treatment with molecular sieves (4 Å). Flash chromatography was performed using silica gel (230–400 mesh) from Silicycle. Thin layer chromatography was performed using precoated glass-backed plates of silica gel 60 F_{254} from EMD Chemicals. NMR spectra were obtained using either a Bruker AV-300 or inverse AV-400 instrument. Spectra were referenced to the signal of the solvent. ESI-MS were collected with a Waters LC/MS instrument, and MALDI-TOF mass spectra were collected using a Bruker Biflex instrument. HPLC purification was performed with an Agilent 1100 system and Phenomenex Jupiter 10 μ C4 300A column. Metal salts used: $Fe(NO_3)_3{\cdot}9H_2O$, $BaCl_3$, $EuCl_3{\cdot}xH_2O$, $CuSO_4{\cdot}5H_2O$ $ZnSO_4{\cdot}7H_2O$, $MnCl_2$, $NiCl_2{\cdot}6H_2O$ CaCl2, $La(OAc)_3$, $MgCl_2{\cdot}6H_2O$, $MgSO_4{\cdot}7H_2O$, $Hg(OAc)_2$.

Vent (*exo*-) DNA polymerase, *Taq* DNA polymerase and λ-exonuclease were obtained from New England Biolabs. Oligonucleotides were purchased from Integrated DNA Technologies. dNTP's were purchased from Fermentas. α-^{32}P-dGTP was obtained from Perkin Elmer. Acrylamide/bis-acrylamide solution (40%) was purchased from Bio-Rad Laboratories. Streptavidin magnetic particles were purchased from Roche and the pGEM-T-Easy Vector Systems kit was purchased from Promega. Radioactivity was detected with a Typhoon 9200 phosphorimaging scanner from GE Healthcare. PCR was performed using a Techne TC-312 machine.

Oligonucleotides

Biotin-T_{20}GCGTGCC**r**CGTCTGTTGGGCCCTACCAACA, **O1**; GAGCTCGCGGGGCGTGCN$_{40}$CTGTTGGTAGGGCCCAACAGACG, **O2**; phosphate-CGTCTGTTGGGCCCTACCA, **O3**; GAGCTCGCGGGGCGTGC, **O4**; phosphate-ACGACACA GAGCGTGCCCGTCTGTTGGGCCCTACCA, **O5**; GGGGCGTGCTTTGCACTCGATAGTCAGCAGTGCCTTCGTCAACTAGTTTCTGTTGGTAGGGCCCAACAGACGGGCACGCTCTGTGTC, **O6**.

Buffers/solutions

TEN (Tris, EDTA, NaCl): 40 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl. *Loading solution*: formamide (27 mL), $EDTA_{(aq)}$ (3 mL, 0.5 M), xylene $cyanol_{(aq)}$ (300 μL, 0.05%) and bromophenol $blue_{(aq)}$ (300 μL, 0.05%). *Elution buffer*: 1% $LiClO_4$, 10 mM Tris pH 7.0 in water. *Selection buffer 1*: 50 mM HEPES pH 7.4, 200 mM NaCl, 0.1 mM $ZnSO_4$, 0.5 mM $MgCl_2$. *Selection buffer 2*: 50 mM HEPES pH 7.4, 200 mM NaCl, 0.1 mM $ZnSO_4$, 1 mM $MgCl_2$. *Selection buffer 3*: 50 mM HEPES pH 7.4, 200 mM NaCl, 1 mM $ZnSO_4$, 10 mM $MgCl_2$. *First amplification cocktail* (5X): **O3** (7.5 nmol), **O4** (9.0 nmol), dNTP's (345 nmol each), $MgSO_4$ (1.15 μmol), thermopol (115 μL, 10X) and water for a final volume of 230 μL. *Second amplification cocktail* (5X): **O4** (20 nmol), **O5** (15 nmol), dNTP's (690 nmol each), $MgSO_4$ (3.45 μmol), thermopol (230 μL, 10X) and water for a final volume of 460 μL. *Kinetics quenching buffer*: loading solution/100 mM biotin in DMF (99 : 1).

5-(4-Hydroxybenzoylaminomethyl)-2′-deoxyuridine triphosphate (2)

A solution of 4-hydroxybenzoyl-*N*-hydroxysuccinimide ester[61] (0.24 mg, 1.0 μmol) in DMF (5 μL) was added to 5-aminomethyl-2′-deoxyuridine triphosphate (0.25 μmol) in aqueous sodium bicarbonate (10 μL, 0.4 M). After 3 h of periodic agitation, the reaction was concentrated, resuspended with water and purified by preparative-TLC with dioxane/H_2O/NH_4OH (6 : 4 : 1); R_f 0.13. Finally, **2** was purified by HPLC (retention time of 8.7 min) using a 50 mM triethylammonium acetate (pH 7.0) acetonitrile/H_2O system (0–10 min, 0–1%; 10–18 min, 1–25% acetonitrile/H_2O). Product (571 nmol, 57% yield) was isolated as the tetrakis(triethylammonium) salt. ε_{258} = 17 900 $cm^{-1}M^{-1}$. ^{1}H NMR (400 MHz, D_2O, 25 °C, spectrum acquired with solvent suppression pulse program): δ = 7.92 (s, 1H), 7.81–7.75 (m, 2H), 7.06–6.99 (m, 2H), 6.35–6.29 (m, 1H), 4.75–4.60 (m, 1H), 4.42–4.12 (m, 4H), 2.47–2.39 (m, 2H) MS (MALDI$^-$): m/z = 615.9 (M–1)$^-$. λ_{max} = 258 nm. Alternately, a solution of 4-boronylbenzoyl-*N*-hydroxysuccinimide ester[62] (0.24 mg, 1.0 μmol) in DMF (5 μL) was added in 3 portions over 1 h to 5-aminomethyl-2′-deoxyuridine triphosphate (0.25 μmol) in aqueous sodium bicarbonate (10 μL, 0.4 M). After a total reaction time of 2 h with periodic agitation, the reaction was concentrated, resuspended with water and purified by preparative-TLC with 8 : 4 : 1 MeCN/H_2O/NH_4OH. R_f 0.4 (6 : 4 : 1 MeCN/H_2O/NH_4OH). Half of the isolated material was dissolved in H_2O and mixed with H_2O_2 (0.75 μL, 1%). Triphosphate **2** was successfully obtained from this oxidation.

In vitro selection

An initial sequence pool was obtained by the primer extension of **O1** (30 pmol) on **O2** (30 pmol) in the presence of dATP (2.0 nmol), **2** (2.0 nmol), dCTP (1.0 nmol), dGTP (1.0 nmol), α-^{32}P-dGTP (2.0 μL), DTT (40 nmol), thermopol buffer (10X) and Vent (*exo*-) DNA polymerase (3U). The reaction (final volume of 40 μL) was incubated at 72 °C and then quenched by the addition of $EDTA_{(aq)}$

(2.0 μL, 0.5 M). A suspension of streptavidin magnetic particles was magnetized and then washed with 3 samples of TEN buffer (100 μL each). The extension reaction was bound to streptavidin magnetic particles, and the resulting suspension was left at room temperature with periodic agitation over 30 min. The material was magnetized and then washed with a series of solutions: 2 solutions of TEN buffer (100 μL each), 5 × 100 μL solutions containing NaOH (0.1 M) and EDTA (1.0 mM), a solution of HEPES buffer (200 μL, 50 mM, pH 7.4) and water (100 μL). The particles were then incubated in selection buffers 1, 2 or 3 in the presence or absence of sugars. Eluted sequences were precipitated with 1% $LiClO_4$, washed with EtOH and purified by denaturing PAGE (7%). The properly-sized material was eluted with elution buffer, precipitated and desalted (G25 Sephadex). The material was amplified with 2 PCR reactions. Each reaction was performed for 30 cycles: 15 s at 54 °C, 40 s at 75 °C and 15 s at 95 °C. First PCR, material was added 1st amplification cocktail (8 μL), α-^{32}P-dGTP (0.75 μL) and Vent (*exo*-) DNA polymerase (1 μL, 2U). After PCR was performed, the reactions were treated with a phenol/chloroform extraction, washed with EtOH and digested with λ-exonuclease prior to purification on denaturing PAGE (10%). An aliquot of the gel-purified first PCR product was added 2nd amplification cocktail (40 μL), water (150 μL) and Vent (*exo*-) DNA polymerase (9 μL, 18 U). The 2nd PCR product was obtained in the same way as the 1st PCR product except that the product was identified by UV-shadowing. The product was used as a template for the next round of selection.

Selection conditions

The selection was performed at room temperature. The negative selection was performed with selection buffer 1 (rounds 1–3) for 1 h, selection buffer 2 for 1 h (rounds 4–11), selection buffer 2 for 2 h (rounds 12–13) and selection buffer 3 for 2 h (rounds 14–15). The positive selection was carried out with negative selection buffer + sugar (rounds 1–9, 1 mM each of stachyose, sialic acid, D-mannoheptose and ATP; rounds 10–11, 1 mM stachyose; rounds 12–15, 0.2 mM stachyose). Positive selection was carried out with 1 buffer solution sample for 1 h (rounds 1–7) or 3 buffer solutions for 3 consecutive time intervals (0–1, 1–5, 5–60 min, rounds 8–15).

Cloning

The product stemming from the second PCR of generation 11 was used as template for PCR with *Taq* DNA polymerase to produce double stranded material with overhanging 3′-dA's. TA-cloning was then performed on this product using the pGEM-T-Easy Vector Systems kit. Plasmids were transformed into *E. coli* DH10B cells using electroporation, and the cells were spread onto LB agar containing ampicillin (100 mg L^{-1}). Individual white colonies were picked and used to inoculate 1 mL samples of TB. Plasmids were harvested using the Plasmid Miniprep Kit (Invitrogen) and subjected to restriction enzyme digestion using EcoRI to screen for appropriately sized inserts. Single insert-containing plasmids were sequenced by the UBC Nucleic Acid Protein Service Unit.

Kinetic analysis of self-cleavage reaction

Primer **O1** (30 pmol) and 11-17PheO template **O6** (30 pmol) were annealed and primer extended in the same manner as described for the synthesis of the sequence pool of the selection. The reaction mixture was then bound to streptavidin magnetic particles and washed in the same manner as during the selection. The suspension of streptavidin magnetic particles in the final water wash was divided into 3 portions for 3 individual reactions, and the water was decanted after magnetization. Each sample was incubated with selection buffer 3 (80 μL). Aliquots (4 μL each) were removed and added directly to quenching buffer (12 μL each). The quenched aliquots were analyzed by denaturing PAGE (10%). The relative amounts of uncleaved catalyst and cleaved product were obtained by autoradiographic densitometry. The data were fit to a single exponential rate equation. Each observed rate constant value was the average of at least 3 identical experiments. For the inhibition experiments, either 100 μM of metal cation or saturated organophosphate pesticide was used.

pH rate profile

DNAzyme 11-17PheO bound to streptavidin magnetic particles was incubated in the presence of 50 mM buffer, 200 mM NaCl, 1 mM $ZnSO_4$, 10 mM $MgCl_2$. Experiments were performed at 6 additional values of pH: 5.9, 6.4, 6.9, cacodylic acid; 7.9, HEPES; 8.4, 8.9, TrisHCl. The reaction rate at pH 8.9 was so slow that an observed rate constant could not be accurately obtained from the data.

Acknowledgements

Funding for this research was provided by CIHR Operating Funds and NSERC.

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