Zn^{2+}-Dependent peptide nucleic acid-based artificial ribonucleases with unprecedented efficiency and specificity

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Electronic Supplementary Information

S1. Materials and Methods

All reagents and solvents used were of analytical commercial quality. RNA cleavage experiments were performed in nuclease-free water for molecular biology purchased from Sigma-Aldrich (St. Louis, Missouri, United States). Oligoribonucleotides RNA 1-16 were purchased from Dharmacon (Lafayette, CO, USA). Oligoribonucleotides RNA 2 and 3 were purchased purified. RNAs 1, 4-16 were deprotected according to the manufacturer´s protocol (2'-ACE protecting groups), purified by Ion-Exchange High Performance Liquid Chromatography (IE-HPLC) and desalted by Reversed Phase HPLC (RP-HPLC). IE-HPLC purifications were performed using an analytical Thermo Scientific DNAPac PA-100 BioLC (4 × 250 mm) column (Waltham, MA, USA) with UV detection at 260 nm. A linear gradient of 0–35% buffer B over 15 min was used with a flow rate of 1.5 mL/min at 60 °C, (buffer A) 20 mM NaOAc in 30% MeCN/aq., and (buffer B) 20 mM NaOAc in 0.4 M $LiClO_4$ in 30% MeCN/aq. Purified RNAs were desalted by ion-pairing reversed-phase HPLC on a Jasco HPLC system using the Supelco Discovery BIO Wide Pore C18-5.5 μm (250 × 4.6 mm) column (Sigma-Aldrich, St. Louis, MO, USA). A linear gradient of 0–37 % buffer B over 20 min was used with a flow rate of 1 mL/min at 50 °C, (buffer A) 50 mM triethylammonium acetate (TEAA) in water (pH 6.5) and (buffer B) 50 mM TEAA in water (pH 6.5)-acetonitrile (1:1, v/v). Purified and desalted RNAs were lyophilised three times from water before use and stored as frozen solutions. RNA 17 and 18 were purchased purified from integrated DNA Technologies, Inc (Coralville, Iowa, USA).

RNA 3 and 13 were synthesized on a 32 μmol scale on an ÄKTA Oligopilot 10 system by using commercially available RNA (2' O-TBDMS protection) phosphoramidite buildings blocks and a suitable preloaded PS support (GE Healthcare). Unless otherwise stated all reagents used for RNA synthesis were purchased from Sigma-Aldrich in DNA grade quality and used as supplied. Detritylation was performed by using 3 % dichloroacetic acid in toluene. 5-[3,5-Bis(trifluoromethyl)phenyl]-1H-tetrazole (Activator 42) was used as activating agent. Cyanoethyl deprotection was performed with diethylamine/toluene (20 % v/v). Phosphoramidites were dissolved to a final concentration to 0.1 M in DNA-grade acetonitrile prior to use. Oxidation was carried out with commercially available oxidizer containing pyridine/water/iodine 9/1/12,7 (v/v/w). Recirculation times for phosphoramidites were 10 min for RNA building blocks. After the synthesis oligonucleotides were cleaved from the solid support by treatment with a mixture of aqueous ammonia (26 %) and ethanol (3:1) at 50°C for 5 hours. After cleavage the crude was freeze dried and dissolved in 4 mL of DMSO. 4mL of triethylamine trihydrofluoride were added and the solution was warmed to 50°C for 2 hours. Then, n-butanol (20 mL) was added and the mixture was cooled to -20°C for 30 mins and then centrifuged. The solution was discarded and the pellet washed with butanol, dissolved in water and freeze dried. The products were purified by ion-pairing reverse phase HPLC using a solvent system consisting of A = 100mM diisopropylethylammonium acetate in water (pH 7.0), B=95/5 acetonitrile/water and a gradient from 5% to 25% B on a Xbridge C18 column at room temperature.

Concentrations of RNA sequences were determined by UV absorption at 260 nm on a Varian Cary 300 UV-Vis dual beam spectrophotometer (Varian, Palo Alto, CA, USA) and calculated from extinction coefficients obtained by the nearest neighbour approximation.[2]

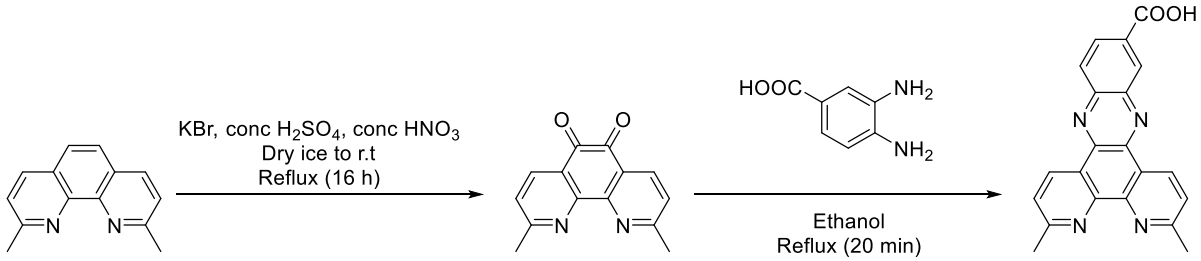
Peptide nucleic acid monomers, Fmoc-PNA-A(Bhoc)-OH, Fmoc-PNA-G(Bhoc)-OH, Fmoc-PNA-C(Bhoc)-OH and Fmoc-PNA-T-OH, were purchased from Link Technologies Ltd. (Glasgow, UK). Fmoc-L-Dap(Mtt)-OH (2,3-diaminopropionic acid), Fmoc-L-Lys(Boc)-OH, N,N'-diisopropylcarbodiimide (DIC) and 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU) were purchased from Iris Biotech GmbH (Marktredwitz, Germany). Ethyl cyano(hydroxyimino) acetate (Oxyma) was purchased from Merck-

Millipore (Burlington, MA, USA). Rink Amide resin (ChemMatrix, 0.47 mmol/g) was purchased from Biotage (Uppsala, Sweden). Acetic anhydride and 2,6-lutidine were purchased from Alfa Aesar (Haverhill, Massachusetts, United States). *N*-methylpyrrolidone (NMP), dichloromethane (DCM), triisopropylsilane (TIS), piperidine and *N*-methylmorpholine (NMM) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Trifluoroacetic acid (TFA) was purchased from Fisher Scientific (Pittsburgh, Pennsylvania, USA). Neocuproine and 3,4-diaminobenzoic acid were purchased from Fluorochem (Hadfield, UK).

Synthesis of 3,6-dimethyl-dipyrido[3,2-a:2',3'-c]phenazine)-11-carboxylic acid

Neocuproine (2.08 g, 10 mmol) and potassium bromide (6.5 g, 55 mmol) were cooled on dry ice. Sulfuric acid (Conc., 98%, 44 ml) was added dropwise, forming a yellow paste. Nitric acid (Conc., 70%, 24 ml) was added dropwise with stirring (on dry ice) and a colour change from yellow to dark orange was observed. The reaction vessel was allowed to react at room temperature for 1.5 hours. After that the reaction mixture was refluxed for 16 h at 86 °C with slow increase in temperature. After the reaction mixture attained room temperature, bromine vapor was removed by blowing a stream of air over the solution and the red solution was poured in ice water mixture (1500 ml) and brought to neutral pH by adding solid potassium carbonate. The resulting yellow solution was extracted with DCM (5x 100 ml). The combined organic layers were washed with brine (2x 100 ml) and dried with sodium sulphate. The solvents were removed in vacuo, resulting in a pale yellow solid, 2,9-dimethyl-1,10-phenanthroline-5,6-dione[1] (2.1 g; 88%).

^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 8.41 (2H, d, $^3J_{H-H}$ = 7.97 Hz), 7.45 (2H, d, $^3J_{H-H}$ = 7:97 Hz), 2.88 (6H, s).



2,9-Dimethyl-1,10-phenanthroline-5,6-dione (1.9 g, 8 mmol) was dissolved in EtOH (160 ml) while the temperature was increased to 50 °C under stirring for 15 min. Then, 3,4-diamino benzoic acid (1.22 g, 8.0 mmol) was added and the mixture heated under reflux for 20 minutes while stirring. As the reaction mixture was allowed to cool to room temperature, the product dimethyl-dppz-COOH precipitated out and was filtered and washed with methanol to give a yellowish orange powder, 3,6-dimethyl-dipyrido[3,2-a:2',3'-c]phenazine)-11-carboxylic acid (2.1 g; 75%). **^{1}H NMR** (300 MHz, $CDCl_3$ with a drop of TFA): δ (ppm) 10.06 -10.04 (2H, d), 9.31 (1H, S), 8.67-8.60 (2H, m), 8.21-8.19 (2H, d), 3.13-3.12 (6H, d). **ESI-TOF ES$^+$** $[M+H]^+$ calculated 355, observed 355

Synthesis of Peptide Nucleic Acid-Based Artificial Ribonucleases

PNA oligomers were prepared on a Biotage Initiator+ Alstra microwave peptide synthesizer. The sequence was assembled automatically on Rink Amide ChemMatrix resin (0.47 mmol/g) on a 10 μmol scale in a 5 mL reactor vial using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry under inert gas (N_2). Before the first synthesis cycle, the resin was allowed to swell in NMP at 70 °C (microwave) for 20 min. Fmoc deprotection was performed at room temperature in two stages by treating the resin with piperidine-NMP (1:4, *v*/*v*) for 3 min followed by piperidine-NMP (1:4, *v*/*v*) for 10 min. The resin was then washed with NMP (×4). PNA couplings were performed using 4 eq. of PNA monomer (or Lys(Boc) or Dap(Mtt) monomers, 0.2 mol/l), 4 eq. Oxyma (0.2 M in NMP), and 4 eq. DIC (0.2 M in NMP). A

coupling time of 6 min at 75 °C (microwave) was employed and the resin was washed with NMP (×4). This was followed by a capping step using NMP-lutidine-acetic anhydride (89:6:5, *v*/*v*/*v*) for 2 min and then washing with NMP (×4). After the synthesis cycles were completed, the resin was washed with NMP (×5) and DCM (×6), and then dried.

Prior to post-conjugations, the methyltrityl (Mtt) protecting group of 2,3-diaminopropionic acid was removed by subjecting the solid-supported PNA (6.4 mg, approx. 0.89 μmol) to 1% trifluoroacetic acid (TFA) in dichloromethane (DCM) for 5 × 1 min, followed by washing with DCM, NMP, 1% NMM in NMP and DCM. Prior to conjugation, DMSO (40 μL) was added to the resin and the mixture subjected to heating at 70 °C in the microwave for 20 minutes to allow for the resin to swell. Dimethyl-dppz-COOH (2.86 mg, 8 μmol) was dissolved in 110 μL of 10% NMM in 1,2-dichlorobenzene/DMSO (1:1 *v*/*v*) by extended sonication while heating at 55-60 °C. The dimethyl-dppz-COOH solution was then pre-activated in the presence of HATU (2.85 mg, 7.5 μmol in 10 μL dry DMSO) over a period of 7 minutes. The preactivated mixture was then added to the resin along with a further portion of NMM (30 μL) and the mixture was subjected to microwave heating (75 °C) for 3 x 6 min. The resin was then washed with 1,2-dichlorobenzene, DMSO, NMP, DCM, and finally dried. The conjugate was cleaved from the resin by treatment with the cleavage cocktail TFA-H_2O-TIS (95:2.5:2.5, *v*/*v*/*v*) for 1.5 h at room temperature, followed by filtration and evaporation to dryness under a flow of N_2. The conjugate was then dissolved in water and evaporated to dryness under reduced pressure. The crude conjugate was redissolved in water and purified by RP HPLC.

RP-HPLC analysis and purification was carried out on a Ascentis Express Supelco Peptide ES-C18 column (2.7 μm, 150 × 4.6 mm) with a linear gradient elution of 0-40% B over 30 min at 60 °C, using a flow rate of 1.0 mL/min and UV detection at 260 nm. The following solvent system was used: solvent A: water containing 0.1% TFA; solvent B: 50% MeCN: water containing 0.1% TFA (1:1; *v*/*v*). Collected products were evaporated to dryness and lyophilised from water (×3). Representative RP HPLC chromatograms are provided. The purified PNA conjugates were identified by mass spectrometry on a Bruker Ultraflex MALDI-TOF mass spectrometer in positive ion mode using a sinapic acid matrix (10 mg/mL sinapic acid in acetonitrile-water (1:1, *v*/*v*) containing 0.1% TFA).

MALDI-MS *m*/*z*: **PNAzyme I** $C_{151}H_{181}N_{73}O_{38}$ $[M+H]^{+1}$ calculated 3625.34, observed 3625.36. **PNAzyme II** $C_{151}H_{182}N_{70}O_{40}$ $[M+H]^{+1}$ calculated 3616.44, observed 3616.47. **PNAzyme III** $C_{153}H_{181}N_{77}O_{36}$ $[M+H]^{+1}$ calculated 3673.48, observed 3673.08.

Concentrations of PNA conjugates were determined by UV absorption at 260 nm on a Varian Cary 300 UV-Vis dual beam spectrophotometer (Varian, Palo Alto, CA, USA) and calculated from extinction coefficients obtained by the nearest neighbour approximation,[2] and the reported extinction coefficient for dipyridophenazine.[3]

RNA Cleavage Experiments

RNA cleavage experiments were performed in nuclease-free water for molecular biology. RNA cleavage reactions were carried out in sealed tubes immersed in a thermostated water bath (37 °C). Experiments were performed at pH 7.0 in HEPES buffer (10 mM HEPES, 0.1 M NaCl), except in the pH profile study where buffers ranging from pH 6.8 to pH 8.0 were used. Experiments were performed with a 100 μM effective final concentration of Zn^{2+} ions, except in the Zn^{2+} concentration dependence study where final Zn^{2+} concentrations from 2 to 200 μM were used. The total volume of each RNA cleavage experiment was 40 μL, which was analysed as a single timepoint. Low retention pipette tips were used to transfer PNA.

RNA targets (0.16 nmol, 4 μM final concentration, except in turnover experiments where 100 μM RNA was used) were equilibrated in appropriate amounts of water, HEPES buffer (10 mM HEPES, 0.1 M

NaCl final concentration, except in the experiments where buffer concentration was varied and NaCl concentration also varied to keep constant ionic strength) and EDTA (10 μM final concentration) over a 15-minute period at 37 °C. This was followed by the addition of $Zn(NO_3)_2$ (*aq*) solution (110 μM Zn^{2+} which in the presence of the previously added 10 μM EDTA results in 100 μM accessible zinc ions), the PNAzyme (1.4 equiv relative to RNA, 5.6 μM, except in turnover experiments where 3 μM PNAzyme was used). The reaction mixtures were then allowed to incubate at 37 °C. The reactions were quenched at specified time points with EDTA (70 μL of 2 mM EDTA in 30% MeCN/milliQ). The samples were analysed by anion exchange HPLC (IE-HPLC) using a Dionex NucleoPac PA-100 (4 × 250 mm) column with a linear gradient elution of 0–45% buffer B over 30 min at 60 °C. A flow rate of 1.5 mL/min was used and UV detection was carried out at 260 nm. The following solvent system was used: (A) 20 mM NaOAc in 30% aq. MeCN and (B) 20 mM NaOAc, 0.4 M $LiClO_4$ in 30% aq. MeCN. The extent of cleavage of RNA substrates was based on the quantification of the remaining intact RNA target and the sum of the formed RNA fragments detected in the IE-HPLC analysis. Representative chromatograms are provided. The % RNA cleavage values presented are average values of at least two experiments with a standard deviation of less than ± 5% unless otherwise specified.

Determination of RNA Cleavage Sites

RNA cleavage experiments were performed as detailed above, but with a 2-hour incubation time in order to isolate and identify the cleaved fragments. The quenched reaction aliquots were analysed by ion-pairing reversed phase HPLC using a Supelco Discovery BIO Wide Pore C18 column (250 x 4.6 mm, 5 μm particle size, 300 Å pore size) and a linear gradient of 0-20% buffer B in 20 min with a flow rate of 1 mL/min at 50 °C with the following solvent system: buffer A: 50 mM triethylammonium acetate (TEAA) in water, pH 6.5 and buffer B: 50% 50 mM TEAA in water (pH 6.5)-acetonitrile (1:1, v/v).

The cleaved RNA fragments were collected, lyophilised, dissolved in water, and analysed as follows. HRMS spectra were collected by elution of the oligonucleotide on a XBridge C18 column using a 0.3 ml/min linear gradient from 20 to 70% acetonitrile in 10 mM tributylammonium acetate over 8 min at 60 °C on Waters BioAccord system with a TOF mass analyser. Ionization mode: ESI negative. Source capillary voltage: 800 V. Source desolvation temperature: 550 °C. Full scan with fragmentation, mass range: 400–5000 and 2 Hz scan rate.

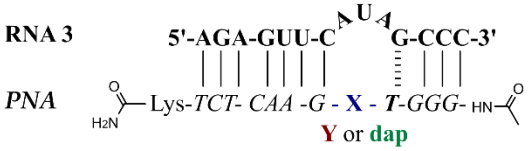
HRMS *m*/*z*: **RNA 3** Fragment 1 (5'-AGCCC-3') $C_{47}H_{61}N_{19}O_{32}P_4$ $[M-2H]^{-2}$ calc. 762.627, obs. 762.626. Fragment 2 (2',3'-cyclic phosphate, 5'-AGAGUUCAU-3') $C_{86}H_{105}N_{34}O_{63}P_9$ $[M-2H]^{-2}$ calc. 1449.1775, obs. 1449.1688. **RNA 13** Fragment 1 (5'-AGCCC-3') $C_{47}H_{61}N_{19}O_{32}P_4$ $[M-2H]^{-2}$ calc. 762.627, obs. 762.625. Fragment 2 (2',3'-cyclic phosphate, 5'-AGAGUUCUU-3') $C_{85}H_{104}N_{31}O_{65}P_9$ $[M-2H]^{-2}$ calc. 1437.664, obs. 1437.663. **RNA 17** Fragment 1 (5'-AGCCG-3') $C_{48}H_{61}N_{21}O_{32}P_4$ $[M-2H]^{-2}$ calc. 782.630, obs. 782.630. Fragment 2 (2',3'-cyclic phosphate, 5'-AGAAUCCUU-3') $C_{85}H_{105}N_{32}O_{63}P_9$ $[M-2H]^{-2}$ calc. 1429.174, obs. 1429.169. **RNA 18** Fragment 1 (5'-AGCCU-3') $C_{47}H_{60}N_{18}O_{33}P_4$ $[M-2H]^{-2}$ calc. 763.119, obs. 763.117. Fragment 2 (2',3'-cyclic phosphate, 5'-ACUUAUCUU-3') $C_{83}H_{103}N_{26}O_{66}P_9$ $[M-2H]^{-2}$ calc. 1398.150, obs. 1398.147.

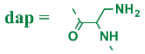
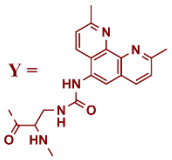
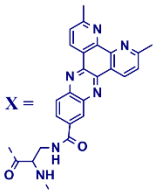
Thermal Melting Analysis

UV melting experiments were performed on a Varian Cary 300 UV/VIS dual beam spectrophotometer (Varian) at 260 nm. Samples of RNA 3 and PNA (either unconjugated PNA, PNA-neocuproine conjugate[4] or PNA-dimethyl-dipyridophenazine conjugate), each at a concentration of 4 μM, were analysed in 10 mM phosphate buffer containing 100 mM NaCl and 0.1 mM EDTA at pH 7.0. Initially, the temperature was rapidly increased (5 °C/min) from 20 to 90 °C and the samples heated at 90 °C for 5 min. The temperature was then decreased (3 °C/min) back to 20 °C and the samples equilibrated for 10 min. Melting points were then determined by slowly increasing (0.2 °C/min) the temperature from

20 to 90 °C and the midtransition point was calculated to yield the melting temperature (Tm) quoted in Table 1 below. The Tm for the neocuproine conjugate is consistent with the previously reported values.[4]

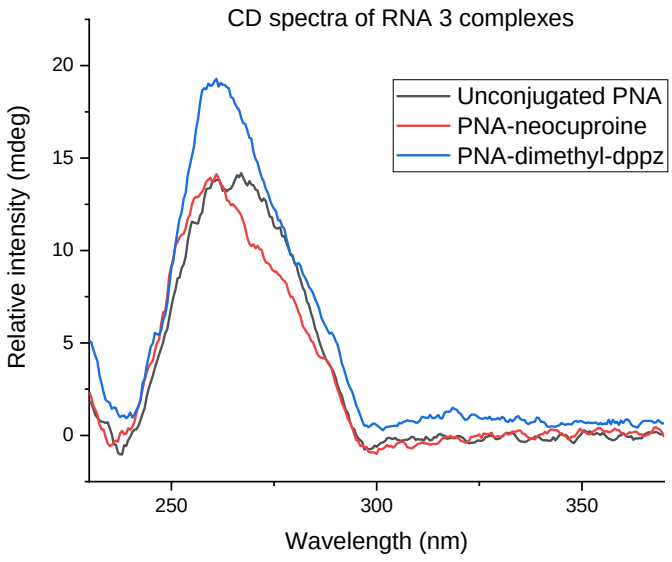
Table 1. Structures and melting temperatures of RNA/PNA complexes where the PNA is either unconjugated or conjugated to neocuproine or dimethyl-dppz



PNA	Structure	Tm (°C)
Unconjugated		52
Neocuproine conjugate		54
Dimethyl-dppz conjugate		56

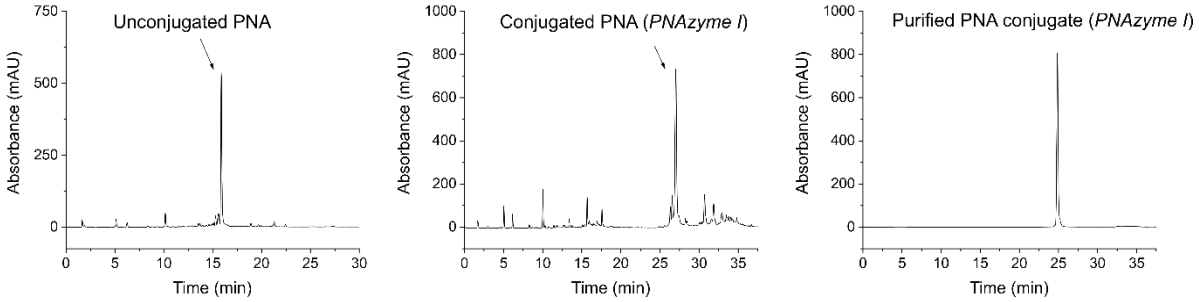
Circular Dichroism (CD) spectroscopy

CD spectra of RNA 3 complexes with unconjugated PNA, PNA-neocuproine conjugate[4] and PNA-dimethyl-dipyridophenazine conjugate (*PNAzyme I*) were measured between 230 and 370 nm on a Jasco J-1500 CD Spectrometer using 10 mm path length cuvettes. The spectra were recorded as an average of five scans at 25°C and normalised by subtracting the background buffer scans. The samples of RNA 3/PNA complexes (4 μM) were analysed in 10 mM phosphate buffer containing 100 mM NaCl and 0.1 mM EDTA at pH 7.0. The spectra were smoothed over 5 points.



S2. Analysis of PNA conjugation reactions

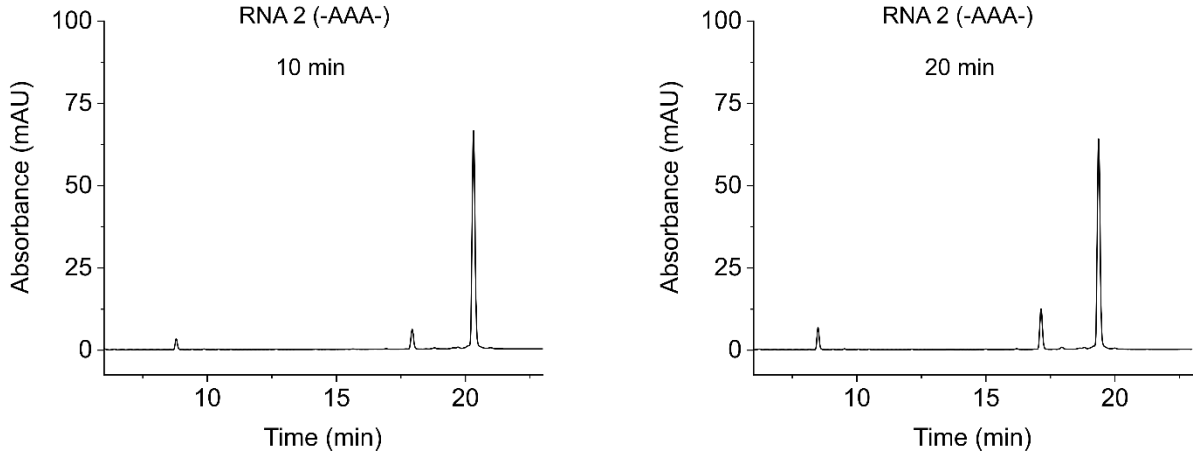
Representative RP HPLC chromatograms from the analysis of unconjugated PNA and conjugated PNA (*PNAzyme I*), as well as the final purified dimethyl-dppz-PNA conjugate (*PNAzyme I*):

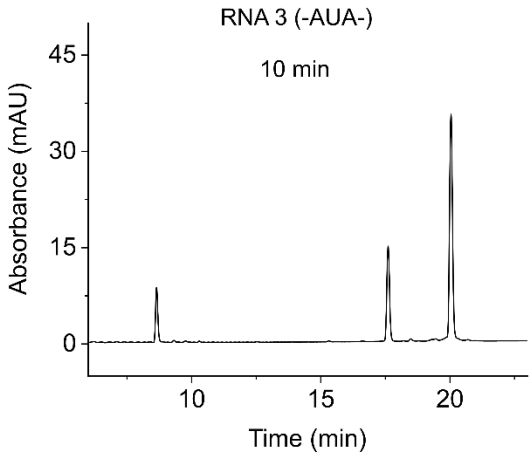


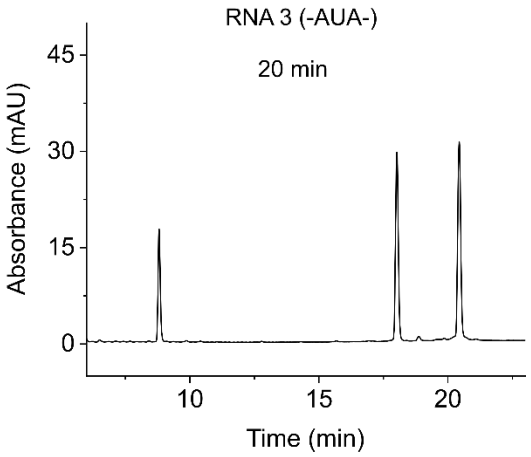
S3. Anion-Exchange HPLC Analysis of the Extent of RNA Cleavage by PNAzymes

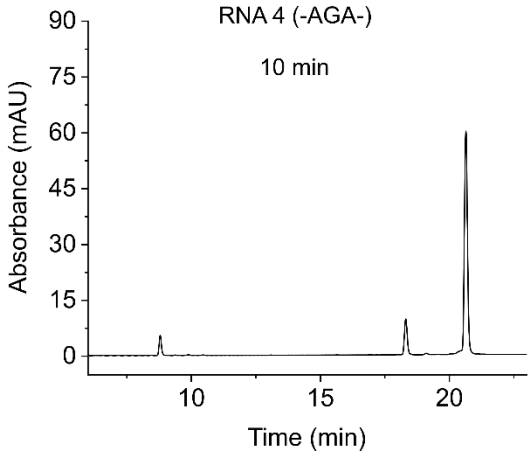
The Extent of Cleavage of RNAs 2-16 by PNAzyme I

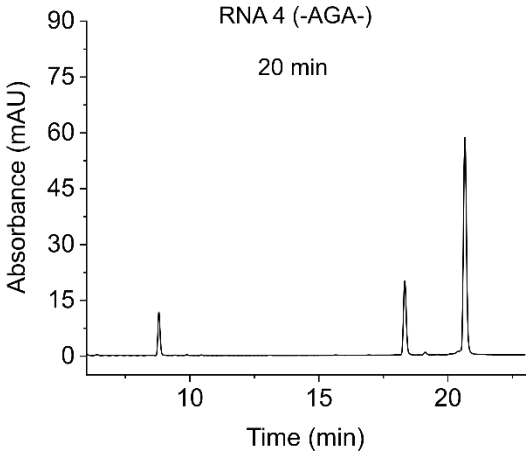
Representative chromatograms corresponding to the data presented in Fig 2a.
Timepoints at which the experiments were quenched are specified for each chromatogram.

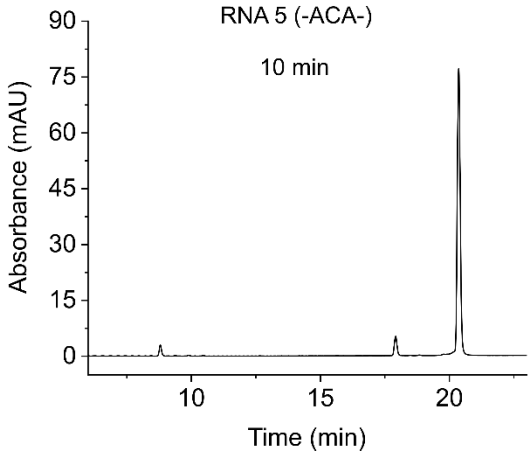


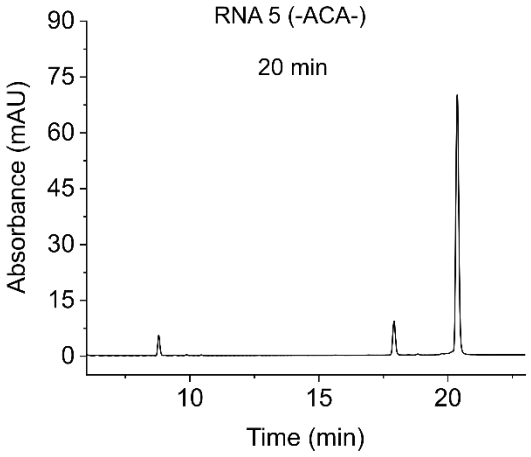

RNA 3 (-AUA-)
10 min
Absorbance (mAU)
Time (min)

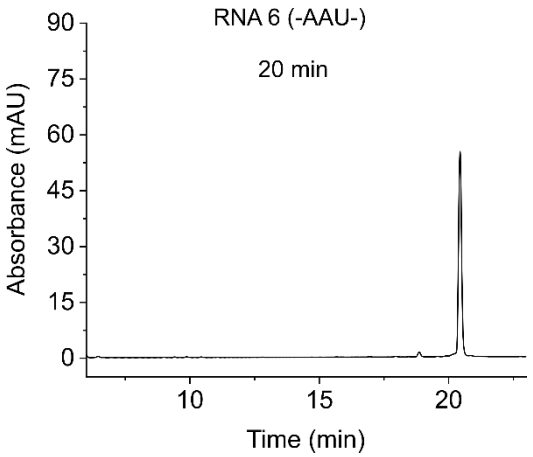

RNA 3 (-AUA-)
20 min
Absorbance (mAU)
Time (min)

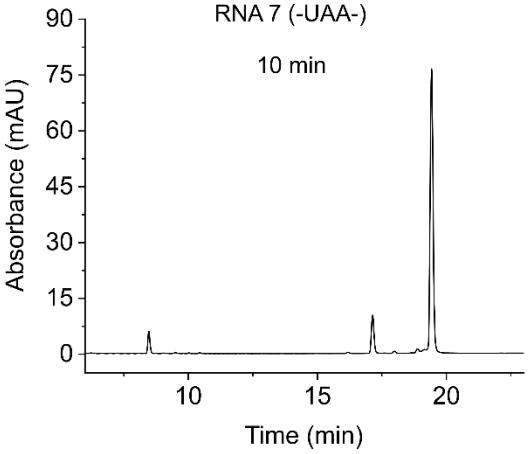

RNA 4 (-AGA-)
10 min
Absorbance (mAU)
Time (min)

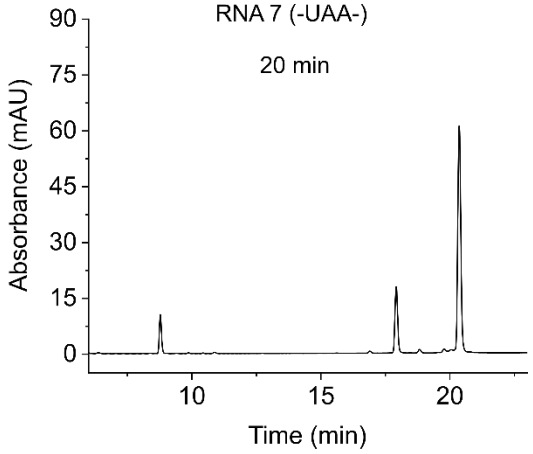

RNA 4 (-AGA-)
20 min
Absorbance (mAU)
Time (min)

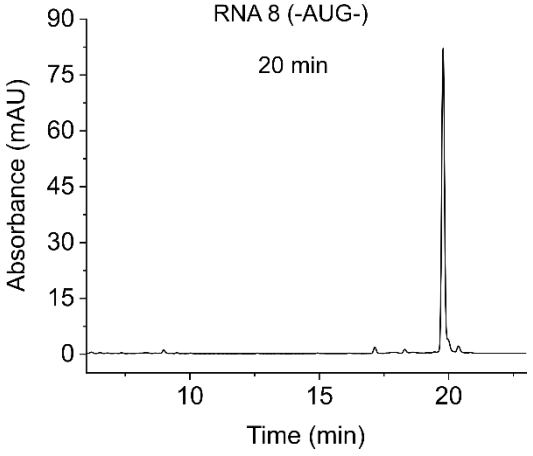

RNA 5 (-ACA-)
10 min
Absorbance (mAU)
Time (min)

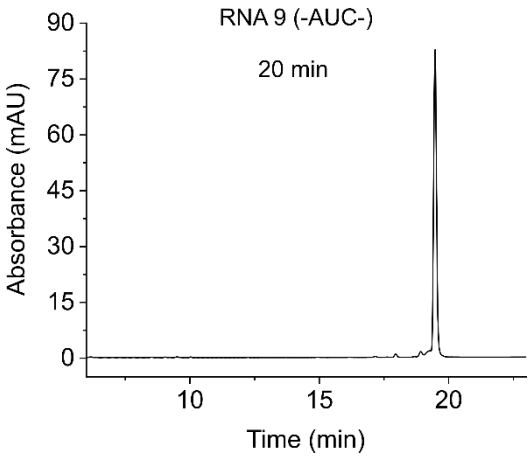

RNA 5 (-ACA-)
20 min
Absorbance (mAU)
Time (min)

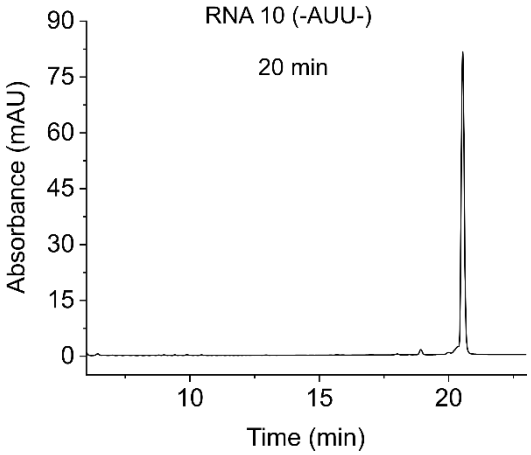

RNA 6 (-AAU-)
20 min
Absorbance (mAU)
Time (min)

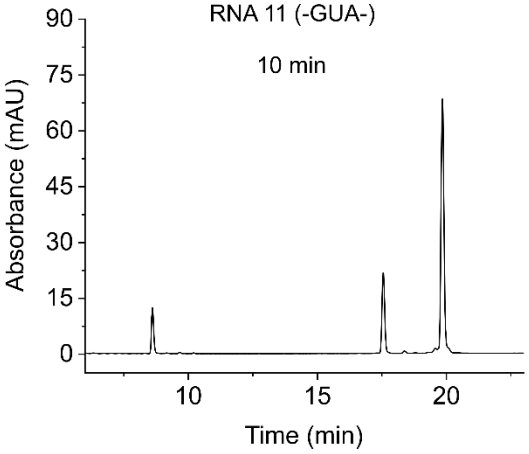

RNA 7 (-UAA-)
10 min
Absorbance (mAU)
Time (min)

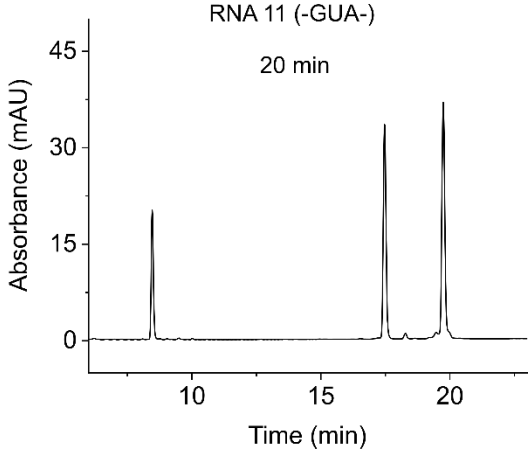

RNA 7 (-UAA-)
20 min
Absorbance (mAU)
Time (min)

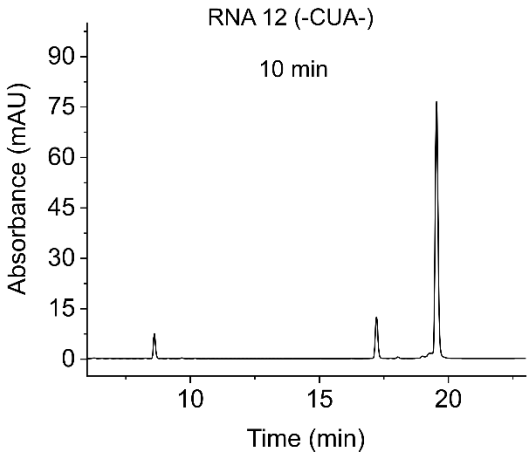

RNA 8 (-AUG-)
20 min
Absorbance (mAU)
Time (min)

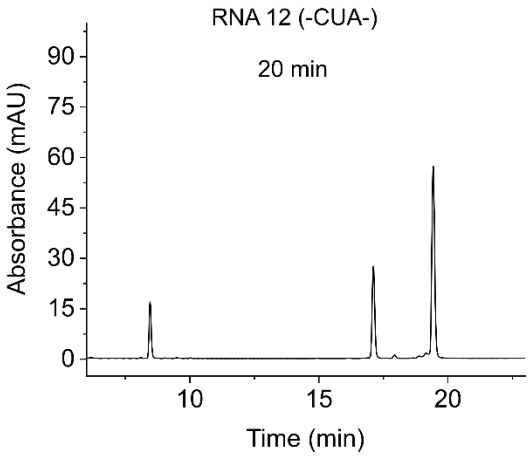

RNA 9 (-AUC-)
20 min
Absorbance (mAU)
Time (min)

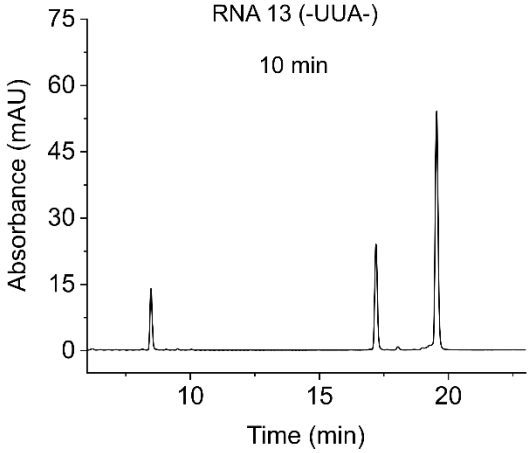

RNA 10 (-AUU-)
20 min
Absorbance (mAU)
Time (min)

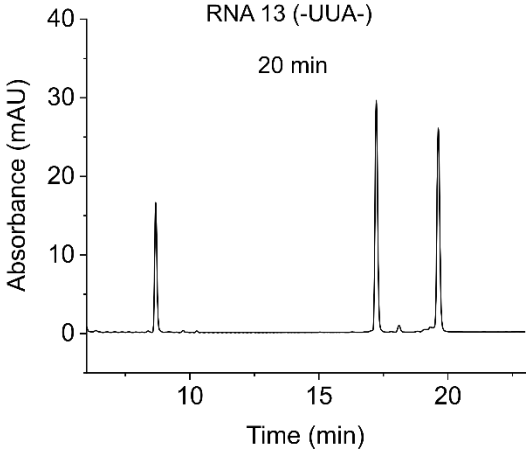

RNA 11 (-GUA-)
10 min
Absorbance (mAU)
Time (min)

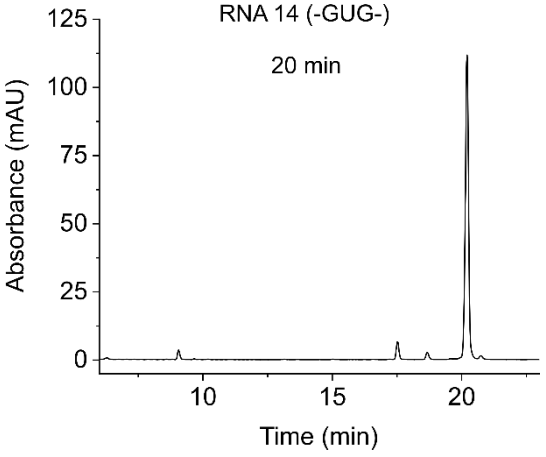

RNA 11 (-GUA-)
20 min
Absorbance (mAU)
Time (min)

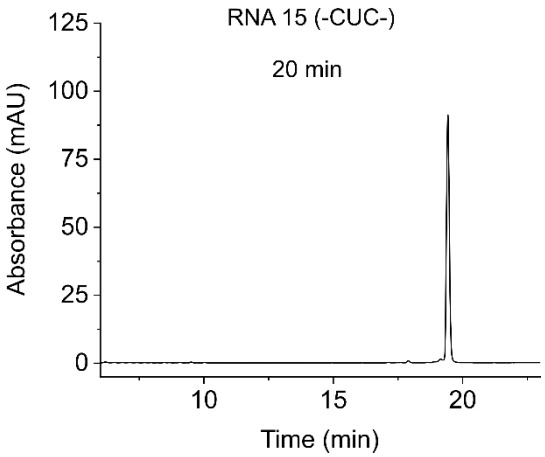

RNA 12 (-CUA-)
10 min
Absorbance (mAU)
Time (min)

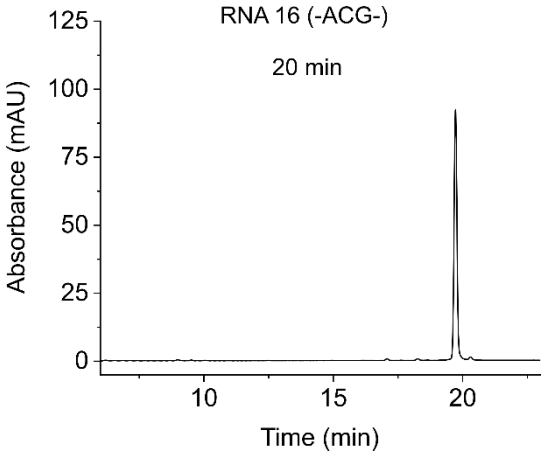

RNA 12 (-CUA-)
20 min
Absorbance (mAU)
Time (min)


RNA 13 (-UUA-)
10 min
Absorbance (mAU)
Time (min)


RNA 13 (-UUA-)
20 min
Absorbance (mAU)
Time (min)

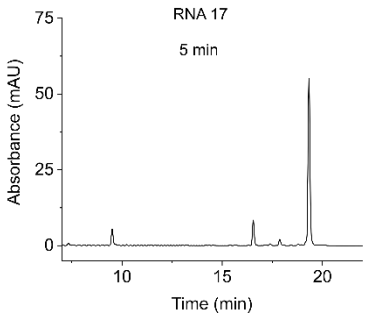

RNA 14 (-GUG-)
20 min
Absorbance (mAU)
Time (min)

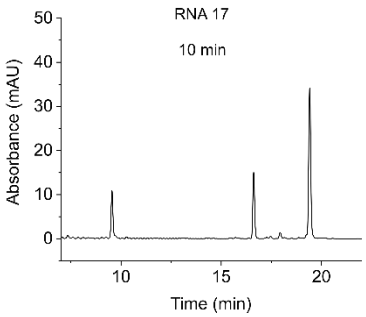


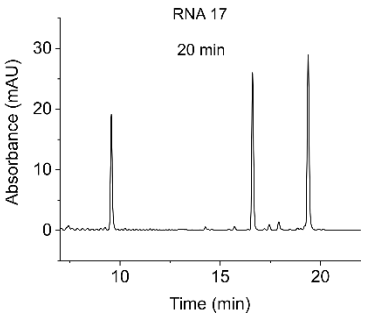


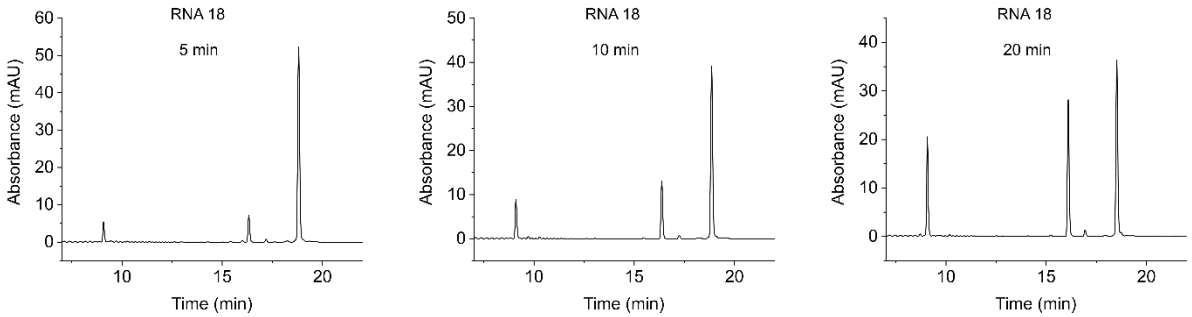
The Extent of Cleavage of RNAs 17 by PNAzyme II and RNA 18 by PNAzyme III

Representative chromatograms corresponding to the data presented in Fig 4.
Timepoints at which the experiments were quenched are specified for each chromatogram.



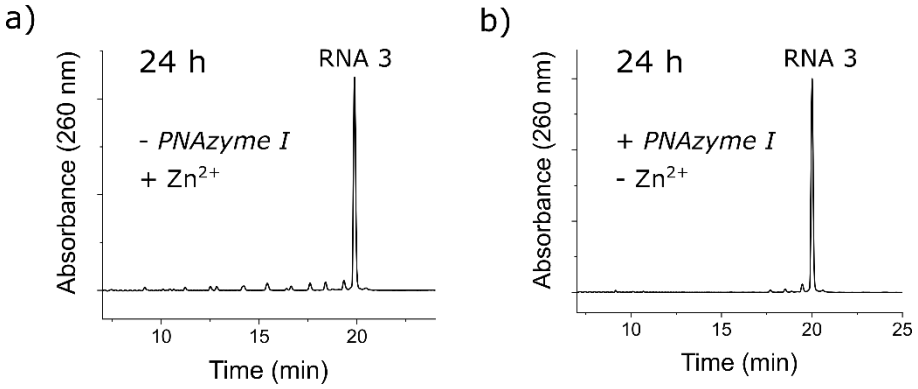






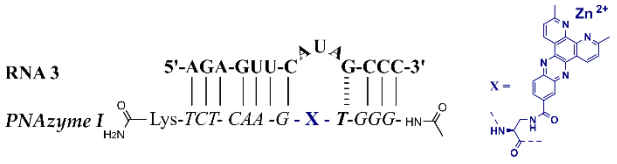
S4. Control Experiments

Control experiments where RNA 3 was incubated over a 24-hour period a) in the presence of Zn^{2+} (100 µM), but in the absence of *PNAzyme I* and b) in the presence of *PNAzyme I* (1.4 equiv), but in the absence of Zn^{2+}.



S5. The effect of varying the HEPES buffer concentration

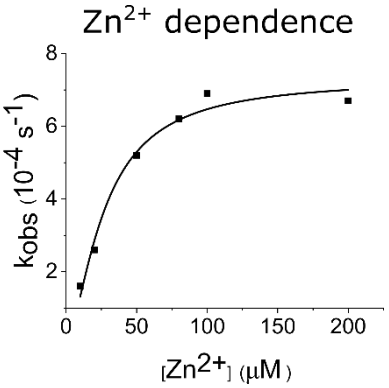
The extent of cleavage of RNA 3 by *PNAzyme I* in 10, 20 and 40 mM HEPES buffers in the presence of 100 µM Zn^{2+} at pH 7, at 37 °C.



HEPES concentration	% RNA cleaved	
	10 min	20 min
10 mM	35	58
20 mM	36	59
40 mM	36	57

S6. Zn^{2+} concentration dependence

Estimated rate constants for the cleavage of RNA 3 by *PNAzyme I* at pH 7, at 37 °C at 2-200 µM Zn^{2+} concentration. The rate constants were calculated based on the cleavage data from 4 different timepoints at each zinc ion concentration, and each cleavage experiment was performed at least twice.



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