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Supporting Information

A Mini-Twister Variant and Impact of Residues/Cations on the Phosphodiester Cleavage of this Ribozyme Class

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Solid-phase synthesis of oligoribonucleotides. Standard phosphoramidite chemistry was applied for RNA solid-phase synthesis using 2'-*O*-TOM nucleoside phosphoramidite building blocks (*ChemGenes*) and polystyrene support (*GE Healthcare*, Custom Primer Support^{TM}, 80 µmol g^{-1}; PS 200). All oligonucleotides were synthesized on a ABI 392 Nucleic Acid Synthesizer following standard methods: detritylation (80 s) with dichloroacetic acid/1,2-dichloroethane (4/96); coupling (2.0 min) with phosphoramidites/acetonitrile (0.1 M x 130 µL) and benzylthiotetrazole/acetonitrile (0.3 M x 360 µL); capping (3 x 0.4 min, Cap A/Cap B = 1/1) with Cap A: 4-(dimethylamino)pyridine in acetonitrile (0.5 M) and Cap B: Ac_2O/sym-collidine/acetonitrile (2/3/5); oxidation (1.0 min) with I_2 (20 mM) in tetrahydrofuran (THF)/pyridine/H_2O (35/10/5). The solutions of amidites and tetrazole, and acetonitrile were dried over activated molecular sieves (4 Å) overnight. 5'-*O*-(4,4'-Dimethoxytrityl)-2'-deoxyuridine phosphoramidite was purchased from *ChemGenes*; 5'-*O*-(4,4'-dimethoxytrityl)-2'-*O*-[(triisopropylsilyl)oxy]methyl-2-aminopurine nucleoside phosphoramidite was was purchased from *ChemGenes* and *GlenResearch*; 5'-dimethoxytrityl-N^6-isobutyryl-N^2-methoxyacetyl-2,6-diaminopurine riboside 2'-*O*-triisopropylsilyloxymethyl-3'-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite was purchased from *GlenResearch*; 1,3-Dideazaadenosine phosphoramidite was synthesized based on reference [1]; ^{13}C2-adenine and ^{13}C8-guanosine phosphoramidites were synthesized according to reference [2].

Deprotection of oligonucleotides. The solid support was treated each with $MeNH_2$ in EtOH (33%, 0.5 ml) and $MeNH_2$ in water (40%, 0.5 ml) for 7 h at room temperature. The supernatant was removed and the solid support was washed 3x with ethanol/water (1/1, v/v). The supernatant and the washings were combined and the whole mixture was evaporated to dryness. To remove the 2'-silyl protecting groups, the resulting residue was treated with tetrabutylammonium fluoride trihydrate (TBAF x $3H_2O$) in THF (1 M, 1 mL) at 37 °C overnight. The reaction was quenched by the addition of triethylammonium acetate (1 M, pH 7.4, 1 mL). The volume of the solution was reduced and the solution was desalted with a size exclusion column (GE Healthcare, HiPrep 26/10 Desalting; 2.6 x 10 cm; Sephadex G25) eluting with H_2O, the collected fraction was evaporated to dryness and dissolved in 1 mL H_2O. Analysis of the crude RNA after deprotection was performed by anion-exchange chromatography on a Dionex DNAPac PA-100 column (4 x 250mm) at 80 °C. Flow

rate: 1 mL min^{-1}, eluant A: 25 mM Tris x HCl (pH 8.0), 6M urea; eluant B: 25 mM Tris x HCl (pH 8.0), 0.5 M $NaClO_4$, 6 M urea; gradient: 0–60% B in A within 45 min or 0–40% B in 30 min for short sequences up to 15 nucleotides, ultraviolet detection at 260 nm.

Purification of RNA. Crude RNA products were purified on a semipreparativ Dionex DNAPac PA-100 column (9 x 250 mm) at 80 °C with flow rate 2 mL min^{-1}. Fractions containing RNA were loaded on a C18 SepPak Plus cartridge (Waters/Millipore), washed with 0.1–0.15 M $(Et_3NH)HCO_3^-$, H_2O and eluted with H_2O/CH_3CN (1/1). RNA containing fractions were lyophilized. Analysis of the quality of purified RNA was performed by anion-exchange chromatography with same conditions as for crude RNA; the molecular weight was confirmed by liquid chromatography-electrospray ionization (LC-ESI) mass spectrometry. Yield determination was performed by ultraviolet photometrical analysis of oligonucleotide solutions.

Mass spectrometry of RNA. All experiments were performed on a Finnigan LCQ Advantage MAX ion trap instrumentation connected to an Amersham Ettan micro LC system. RNA sequences were analysed in the negative-ion mode with a potential of –4 kV applied to the spray needle. LC: Sample (200 pmol RNA dissolved in 30 µL of 20 mM EDTA solution; average injection volume: 30 µL); column (Waters XTerraMS, C18 2.5 µm; 1.0 x 50 mm) at 21 °C; flow rate: 30 µL min^{-1}; eluant A: 8.6 mM TEA, 100 mM 1,1,1,3,3,3-hexafluoroisopropanol in H_2O (pH 8.0); eluant B: methanol; gradient: 0–100% B in A within 30 min; ultraviolet detection at 254 nm.

Cleavage assays. Aliquots from aqueous millimolar stock solutions of the two RNA strands (R: ribozyme; S: substrate) were mixed and lyophilized. After addition of reaction buffer (30 mM HEPES, pH 7.5, 100 mM KCl, 2 mM $MgCl_2$) to yield a final concentration of c_{RNA} = 55 µM (each strand) in a total volume of 20 µL, the reaction was stopped by the addition of EDTA solution (20 µL; 3 mM) after 10, 45 and 120 min, and analysed by anion exchange HPLC (analytical Dionex DNAPac column) using the conditions as described above.

NMR spectroscopy of RNA. The 19 nt plus 37 nt *env22* twister complex used for the NMR experiments contained two G-C instead of the two U-A base pairs in stem

P1 (U2G, U3G, A54C, A55), and 2'-OCH_3-U5. All NMR spectra were recorded on a Bruker 600 MHz Avance II+ at 298 K. One-dimensional ^{1}H-NMR spectra were acquired with excitation sculpting for solvent suppression (Bruker pulse program zgesgp, gradient duration: 1 ms, recovery delay: 100 µs), recording 256 to 1024 scans. Two-dimensional ^{1}H-^{13}C-HSQC NMR spectra were recorded using a phase-sensitive ge-2D HSQC with water flip-back pulses and PEP with gradients in back-INEPT (Bruker pulse program hsqcetfpgpsi2): number of scans 32, spectral width in proton dimension 24 ppm, spectral width in carbon dimension 10 ppm, 2048x48 complex data points, $^1J(^1H,^{13}C)$ = 175 Hz, increment for delay 165 µs, interscan delay 1.2 s.

NMR sample preparation: The purified RNA (see above) was diluted with an equal volume of 100 mM sodium acetate solution and applied onto a C18 SepPak cartridge (Waters/Millipore). The cartridge was rinsed with water, followed by elution of the RNA with H_2O–acetonitrile 1:1. After lyophilization, the RNA sodium salt was redissolved in stock solutions of sodium cacodylate buffer (250 mM, pH 7.0), of potassium chloride (4 M), and of magnesium chloride (200 mM), followed by addition of water and D_2O to reach a final volume of 0.5 mL and a ratio of 1/9 for D_2O–water. Final concentrations were as indicated in the captions of Figure 4 and Supporting Figure S3.

Samples containing the bimolecular twister complex were heated to 90 °C for two minutes and slowly cooled to room temperature to facilitate annealing of the two RNA strands. For pH dependent experiments, the pH of the RNA containing buffer solution was determined with a *SenTix (R) Mic* pH electrode, and adjusted by the addition of µL-amounts of 200 mM hydrochloric acid and sodium hydroxide solutions, respectively.

Data analysis of pH dependent NMR experiments: Two dimensional ^{1}H-^{13}C-HSQC FIDs were multiplied with appropriate QSINE window functions, Fourier transformed, and the phase adjusted as appropriate. The peak positions in the ^{13}C dimension were plotted as a function of pH and least-squares fitted to a generalized logistic function (equation 1),

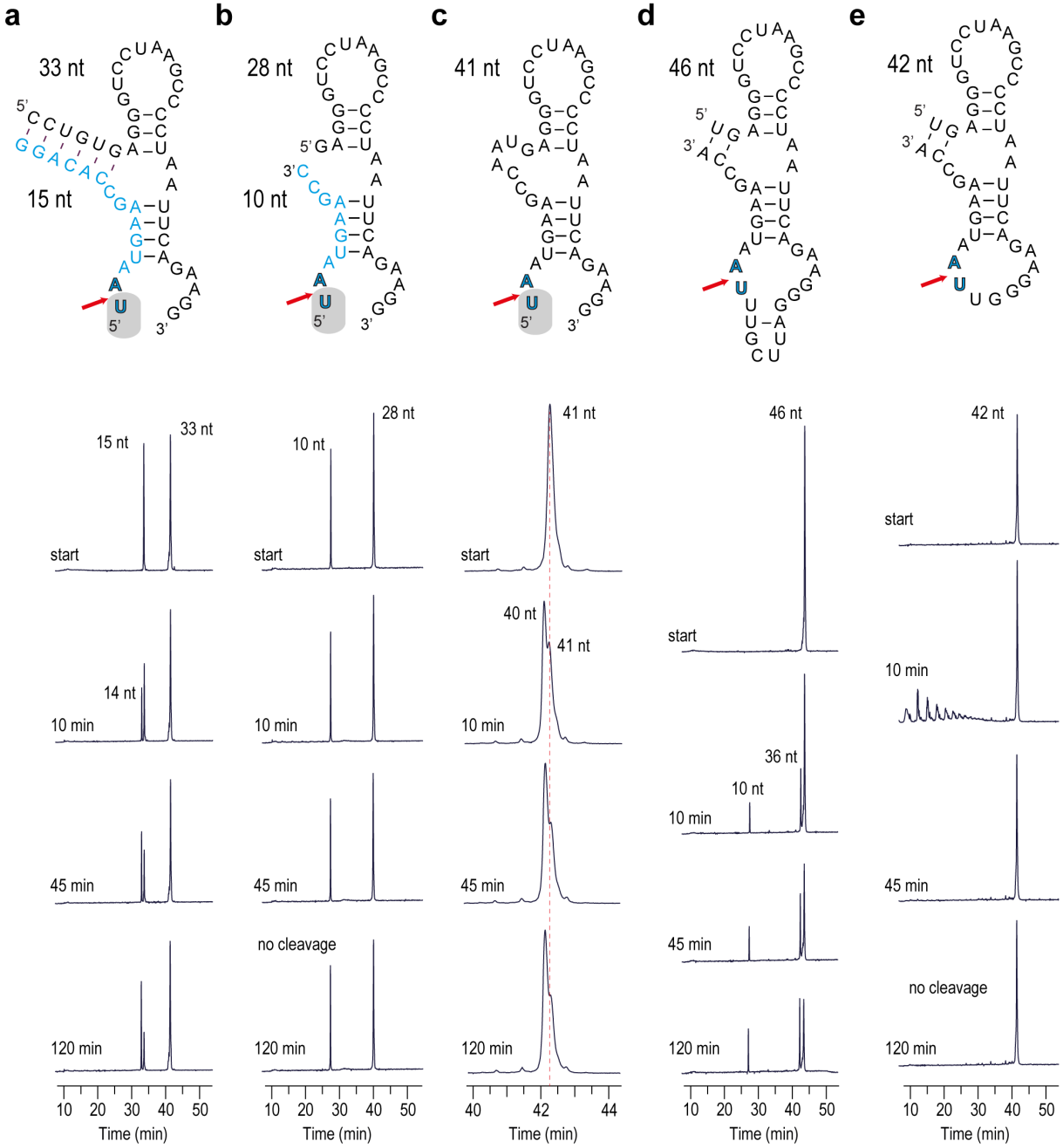
$$\delta^{13}\mathrm{C}(\mathrm{pH}) = D + \frac{A-D}{1+\left(\frac{\mathrm{pH}}{C}\right)^{\mathrm{B}}} \qquad \text{(equation 1)}$$

where A and D are parameters related to the upper and lower asymptotes, parameters B and C are linked to the slope and the positioning of the inflection point, respectively. The pKa value was then determined by numerical analysis of the fit to identify the inflection point .

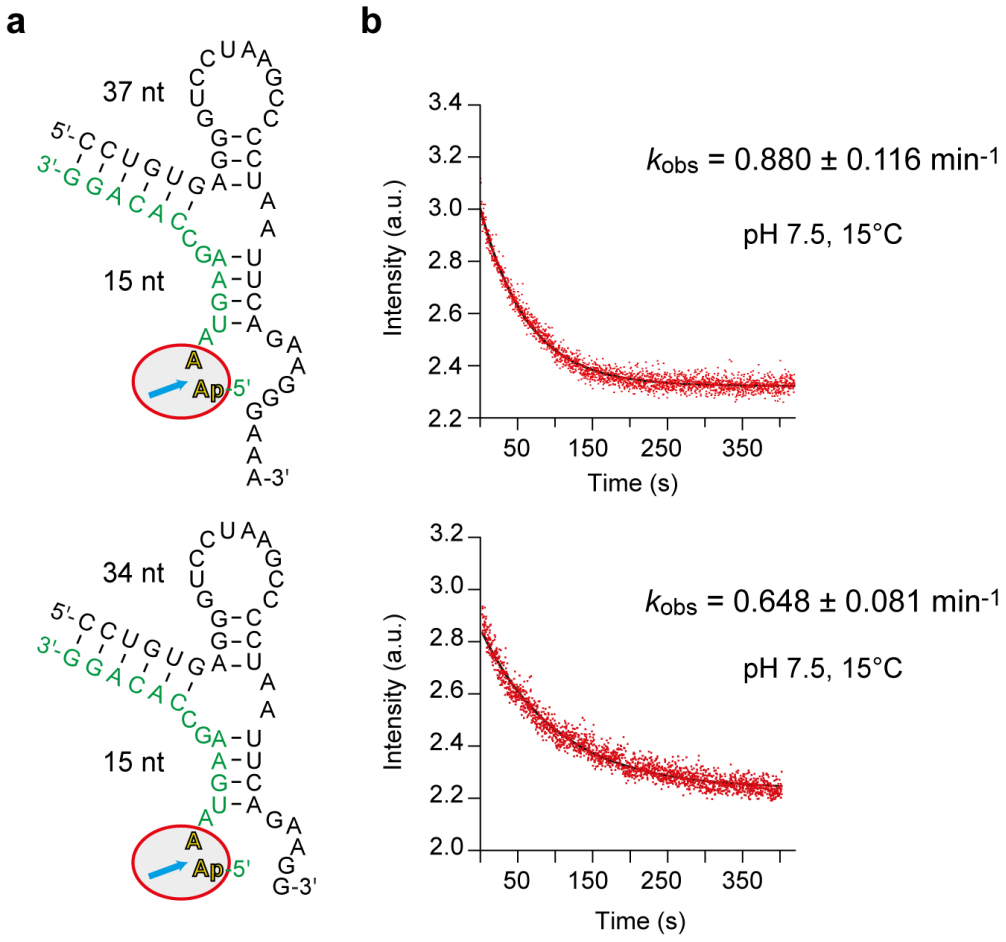
Crystallization and structure determination. Crystals of the *env22* twister ribozyme (lacking C20 and A56) and containing a U5(2'-OCH_3) group were grown under conditions similar to those reported by us previously for the *env22* ribozyme [3]. The structure was solved by molecular replacement using the previously reported structure of the twister ribozyme [3], with the model built using COOT and refined employing PHENIX using a 2.64 Å data set. Metal ions and their coordinated waters were identified based on *2Fo-Fc* and *Fo-Fc* maps guided by coordination geometries. The x-ray statistics are listed in Supporting Table S1. The coordinates of the structure have been deposited in the Protein Data Bank (PDB code: 5DUN).

References

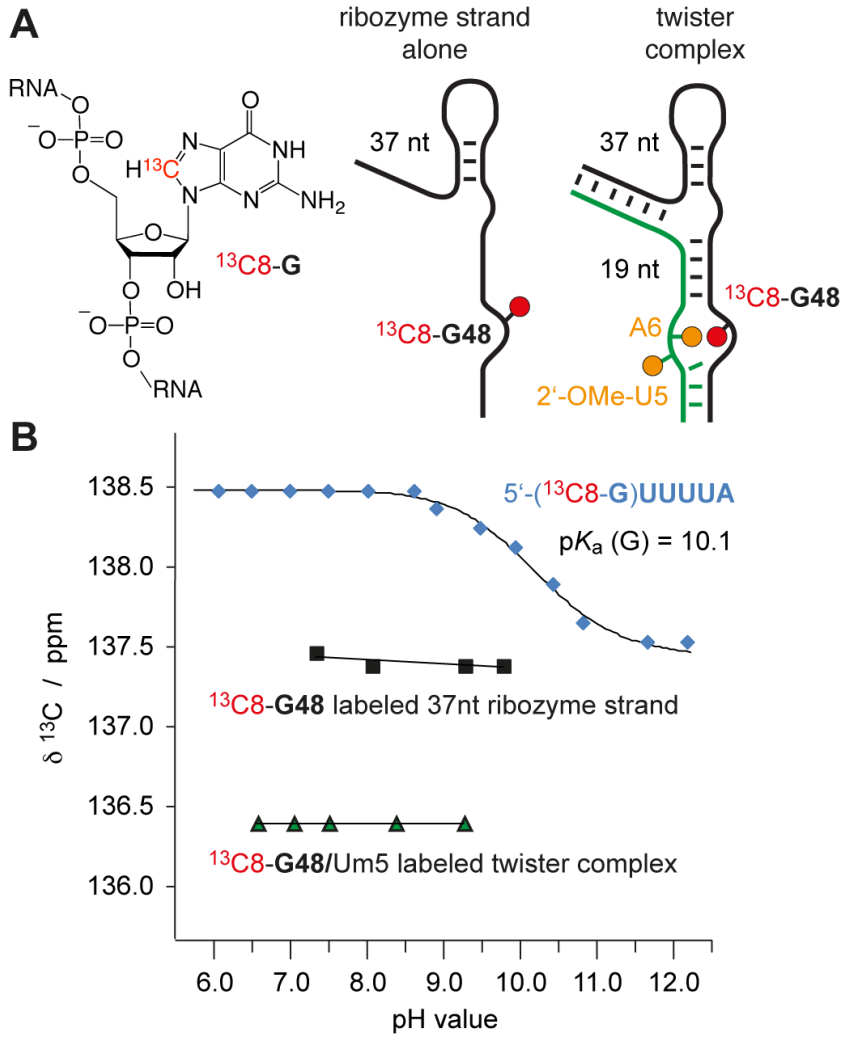
1. Devlin, T. A., & Jebaratnam, D. J. (2006). An Improved Procedure for the Synthesis of 1,3-Dideazaadenosine. *Synthetic Communications*, *25*(5), 711–718.
2. Wunderlich, C. (2015) Advanced stable isotope labeling for NMR of RNA. *PhD thesis*, Leopold-Franzens University, Innsbruck, Austria.
3. Ren, A., Košutić, M., Rajashankar, K. R., Frener, M., Santner, T., Westhof, E., Micura, R. & Patel D. J. (2014). In-line alignment and Mg^{2+} coordination at the cleavage site of the *env22* twister ribozyme. *Nature Communications*, *5*, 5534.



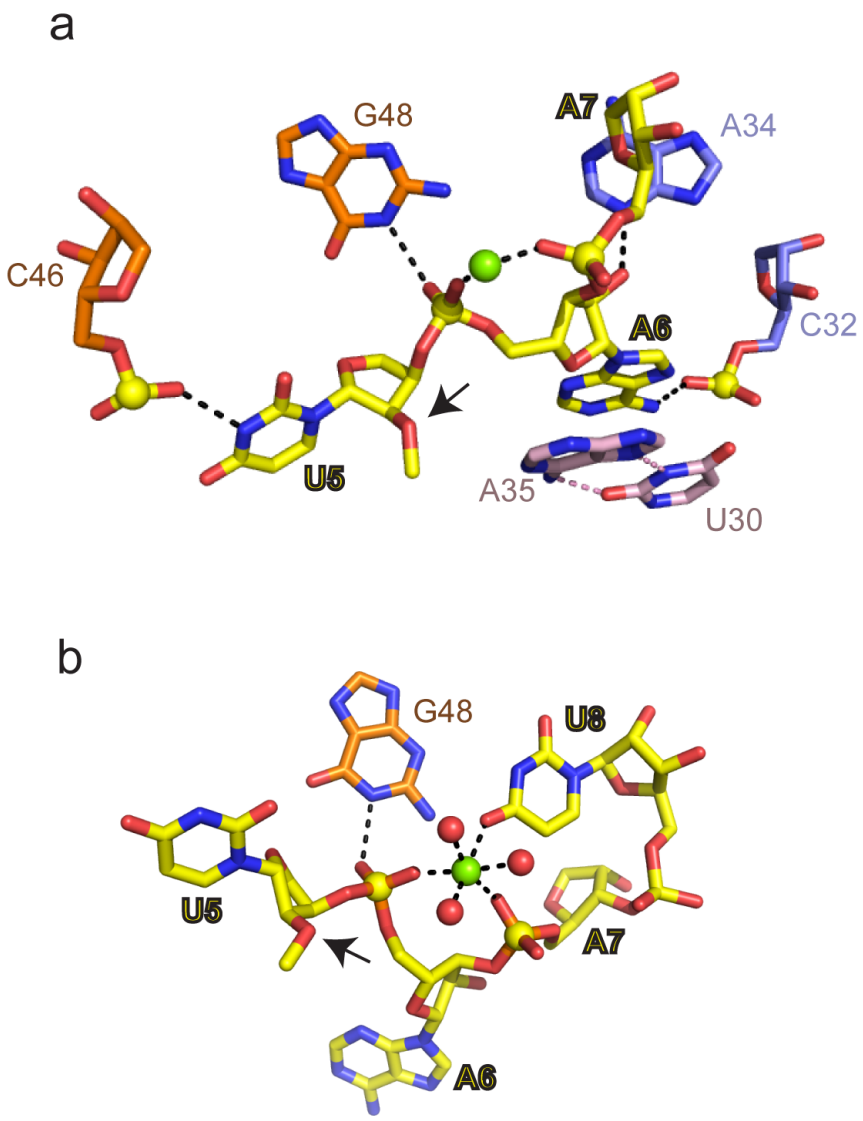
Supporting Figure S1. HPLC cleavage assays of bimolecular (**a**,**b**) and monomolecular (**c**-**e**) RNA assemblies with the twister motive.



Supporting Figure S2. Cleavage kinetics of mini-twister ribozymes analysed by 2-aminopurine fluorescence spectroscopy. (**a**) RNA sequences used in the assays (blue arrows indicate the cleavage position. (**b**) Fluorescence response of 2-aminopurine nucleoside cleavage upon $MgCl_2$ addition; conditions: c_{RNA} = 0.3 μM, 50 mM potassium 3-(*N*-morpholino)propanesulfonate (KMOPS), 100 mM KCl, 15 °C, pH 7.5; mixing was performed manually in less than 2 s resulting in 10 mM Mg^{2+} concentration. Experiments were performed and analyzed as described in Ren, A., Košutić, M., Rajashankar, K. R., Frener, M., Santner, T., Westhof, E., Micura, R., Patel, D. J. (2014). In-line alignment and Mg^{2+} coordination at the cleavage site of the *env22* twister ribozyme. *Nature Communications*, 5, 5534.



Supporting Figure S3. NMR spectroscopic investigation to evaluate the pK_a of guanine-48 in twister. (**a**) Chemical structures of ^{13}C8-labeled guanosine (left) and cartoon of the strands used for the experiments. (**b**) Chemical-shift changes of ^{13}C8-G in a short reference RNA, the 37 nt ribozyme strand, and the twister complex, with changes in the pH value derived from $^{1}J(^{1}H,^{13}C)$ HSQC spectra. Conditions: $c_{(RNA)}$ = 0.5 mM; 100 mM KCl, 2 mM $MgCl_2$, 10 mM Na cacodylate, H_2O/D_2O 9/1, 298 K.



Supporting Figure S4. Residue alignments centered about the U5(2'-OCH_3)-A6 step in the 2.64 Å structure of the *env22* twister ribozyme (lacking C26 and A56) and containing a U5(2'-OCH_3) group (PDB 5DUN). (**a**)**,** Positioning of ribozyme residues and Mg^{2+} (green ball) relative to the U5(2'-OCH_3)-A6-A7 step, with hydrogen bonds labeled by dashed lines. (**b**) An alternate view focused on positioning of G48 and hydrated Mg^{2+} relative to the scissile phosphate.

Supporting Table S1. Crystallographic statistics for the *env22* twister ribozyme (lacking C20 and A56) and containing a U5(2'-OCH_3) group.

Crystal	
Data collection	24-ID-E
Space group	$P4_32_12$
Cell dimensions	
a, *b*, *c* (Å)	46.8, 46.8, 143.7
α, β, γ (º)	90, 90, 90
Wavelength	0.9792
Resolution (Å)	143.7-2.64 (2.73-2.64)*
R_{pim}	0.035(0.397)
$I/\sigma I$	14.5 (1.7)
Completeness (%)	99.9 (100)
Redundancy	2.0 (2.0)
Unique reflections	5164 (492)
Refinement	
Resolution (Å)	44.5-2.64
No. reflections	5156
R_{work}/R_{free}	0.19/0.25
No. atoms	
RNA	1151
Cations	9
Water	21
B-factors	
RNA	39.4
Cations	41.6
Water	32.8
R.m.s deviations	
Bond lengths (Å)	0.010
Bond angles (º)	1.650

*Values for the highest-resolution shell are in parentheses.