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Supporting Information

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Highly Emissive Fluorescent Nucleoside Signals the Activity of Toxic Ribosome Inactivating Proteins

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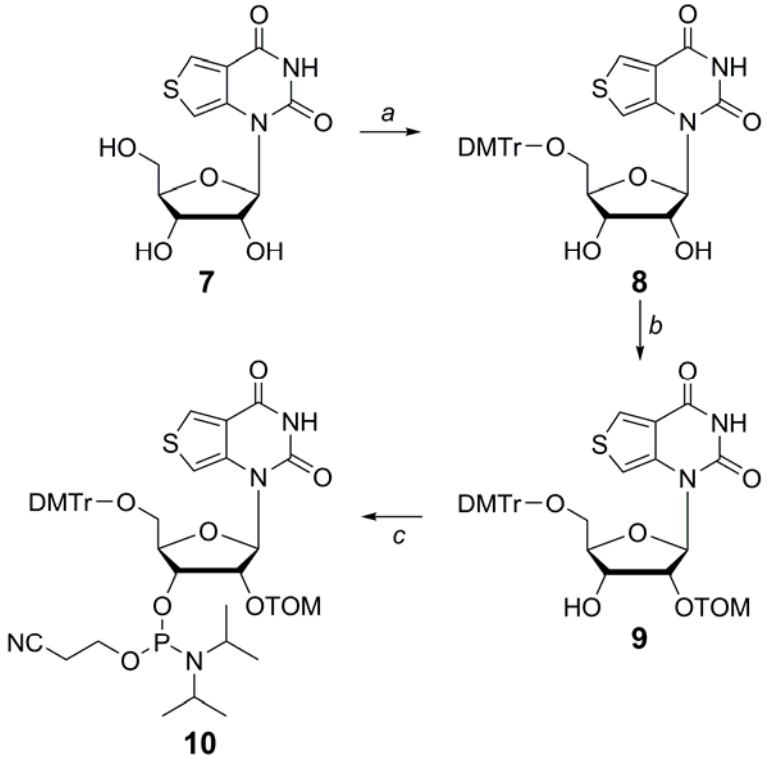
1. Syntheses



Scheme 1. Synthesis of phosphoramidite **10** for solid-phase RNA synthesis. *Reagents and conditions*: (a) DMTr-Cl, pyridine, RT, 62%; (b) (i) nBu$_2$SnCl$_2$, iPr$_2$NEt, DCE, RT; (ii) TOM-Cl, 80°C; (iii) NaHCO$_3$, 39%; (c) iPr$_2$NEt, DCM, iPr$_2$NP(Cl)OEtCN, RT, 67%. TOM = CH$_2$OSi(iPr)$_3$; DMTr = 4,4'-dimethoxytrityl.

1.1 Compound 8: To a solution of nucleoside **7**[S1] (0.470 g, 1.57 mmol, 1.0 eq.) in anhydrous pyridine (6.0 ml) was added dimethoxytrityl chloride (DMTr-Cl, 0.590 g, 1.74 mmol, 1.1 eq.) and the solution was stirred at RT. After ~4 h another 0.4 equivalent of DMTr-Cl was added and the reaction mixture was stirred for 2 h at RT. Pyridine was evaporated and the residue was coevaporated with CH_2Cl_2 twice. The residue was purified by silica gel chromatography to afford the product as off-white foam (0.587 g, 62%). TLC (CH_2Cl_2:MeOH = 95:5 containing 0.5% TEA) R_f = 0.43; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.02 (d, J = 3.6 Hz, 1H), 7.56 (d, J = 3.2 Hz, 1H), 7.41 (d, J = 8.0 Hz, 2H), 7.32−7.25 (m, 8H), 6.83 (d, J = 8.8 Hz, 4H), 6.63 (d, J = 6.4 Hz, 1H), 4.86 (t, J = 6.8 Hz, 1H), 4.65 (t, J = 6.0 Hz, 1H), 4.11 (br, 1H), 3.78 (s, 6H), 3.58−3.51 (m, 2H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 158.8, 158.0, 151.3, 144.4, 135.6, 135.4, 135.2, 130.5, 128.6, 128.2, 127.3, 123.1, 113.5, 108.5, 89.3, 87.2, 83.9, 70.2, 69.9, 62.9, 55.5; ESI-MS (*m/z*) Calculated for $C_{32}H_{30}N_2O_8S$ [M] 602.17, found $[M+Na]^+$ = 625.02.

1.2. Compound 9: To a solution of 5'-DMTr-protected nucleoside **8** (0.350 g, 0.58 mmol, 1.0 eq.) in anhydrous dichloroethane (4.0 ml) was added diisopropyl ethylamine (0.38 ml, 2.18 mmol, 3.8 eq.). A solution of dibutyltin dichloride (0.180 g, 0.59 mmol, 1.0 eq.) in dichloroethane (0.5 ml) was then added and the reaction mixture was stirred at RT for 1 h. The reaction mixture was heated at 80 °C for 10 min and [(triisopropylsilyl)oxy]methyl chloride (TOM-Cl, 0.175 ml, 0.75 mmol, 1.3 eq.) was added and the heating was continued for another 15−20 min. The reaction flask was cooled to RT, diluted with CH_2Cl_2 (50 ml), and was poured into a stirred solution of saturated $NaHCO_3$ (30 ml). While stirring a white precipitate was formed and the mixture was partitioned in a separatory funnel. The aqueous layer was washed with CH_2Cl_2 (20 ml) and the combined organic extract was evaporated. The residue was purified by silica gel column chromatography to afford the product as white foam (0.180 g, 39%). TLC (hexanes:ethyl acetate = 1:1 containing 0.5% TEA) R_f = 0.67; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.12 (d, J = 3.2 Hz, 1H), 7.58 (d, J = 3.2 Hz, 1H), 7.39−7.25 (m, 10H), 6.80-6.78 (m, 4H), 6.41 (d, J = 6.8 Hz, 1H), 5.13 (d, J = 4.8 Hz, 1H), 4.90 (d, J = 4.4 Hz, 1H), 4.78 (t, J = 6.8 Hz, 1H), 4.67 (dd, J_1 = 6.0 Hz, J_2 = 3.6 Hz, 1H), 4.13−4.12 (m, 1H), 3.76 (s, 6H), 3.54 (dd, J_1 = 10.8 Hz, J_2 = 2.8 Hz, 1H), 3.46 (dd, J_1 = 10.8 Hz, J_2 = 2.0 Hz, 1H), 3.14 (br, 1H), 1.07−1.00 (m, 21H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 158.9, 157.5, 150.4, 144.4, 135.7, 135.3, 135.2, 130.6, 130.5 (d, J = 3.8 Hz), 128.5, 128.2, 127.3, 123.4, 113.5, 108.3, 91.0, 87.2, 86.9, 84.1, 69.6, 63.1, 55.5, 18.0, 12.1; ESI-MS (*m/z*) Calculated for $C_{42}H_{52}N_2O_9SSi$ [M] 788.32, found $[M+Na]^+$ = 811.14.

1.3. Compound 10: To a solution of 2'-TOM protected nucleoside **9** (0.140 g, 0.18 mmol, 1.0 eq.) in anhydrous CH_2Cl_2 (1.0 ml) was added diisopropyl ethylamine and 2-cyanoethyl-*N,N*-diisopropyl chlorophosphoramidite. The resulting solution was stirred at RT for 14 h, and volatile reagents were evaporated. The residue was purified by silica gel column chromatography to afford the product **10** as white foam (0.117 g, 67%). TLC (hexanes:ethyl acetate = 6:4 containing 1% TEA) R_f = 0.63; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.49 (br, 1H), 8.14 (t, J = 3.4 Hz, 1H), 7.53 and 7.51 (2d, J = 3.4 Hz, 1H), 7.42−7.22 (m, 9H), 6.84−6.79 (m, 4H), 6.44 and 6.41 (2d, J = 7.2 Hz, 1H), 5.10 (t, J = 6.8 Hz, 1H),

4.97−4.90 (m, 2H), 4.77−4.67 (m, 1H), 4.26−4.21 (m, 1H), 3.99−3.83 (m, 2H), 3.78 and 3.77 (2S, 6H), 3.63−3.46 (m, 4H), 2.69−2.65 (m, 1H), 2.29−2.25 (m, 1H), 1.20−1.15 (m, 8H), 1.01 (d, J = 6.8 Hz, 4H), 0.92−0.89 (m, 21H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 158.87, 157.63, 157.60, 150.37, 150.28, 144.37, 144.23, 135.75, 135.59, 135.49, 135.28, 130.63, 130.54, 130.47, 130.42, 130.38, 128.77, 128.62, 128.20, 127.33, 123.28, 118.11, 117.53, 113.47, 108.25, 108.13, 89.38, 88.59, 87.47, 87.34, 87.27, 87.15, 84.19, 83.74, 72.79, 71.07, 70.60, 70.44, 69.85, 69.68, 63.03, 62.67, 59.41, 59.26, 57.94, 57.75, 55.48, 55.45, 43.69, 43.57, 43.36, 43.23, 24.87, 24.79, 20.66, 20.61, 20.23, 20.16, 17.87, 17.81, 12.10, 12.04; ESI-MS (*m/z*) Calculated for $C_{51}H_{69}N_4O_{10}PSSi$ [M] 988.42, found $[M+Na]^+$ = 1011.27.

2. Basic photophysical properties of fluorescent nucleoside 7

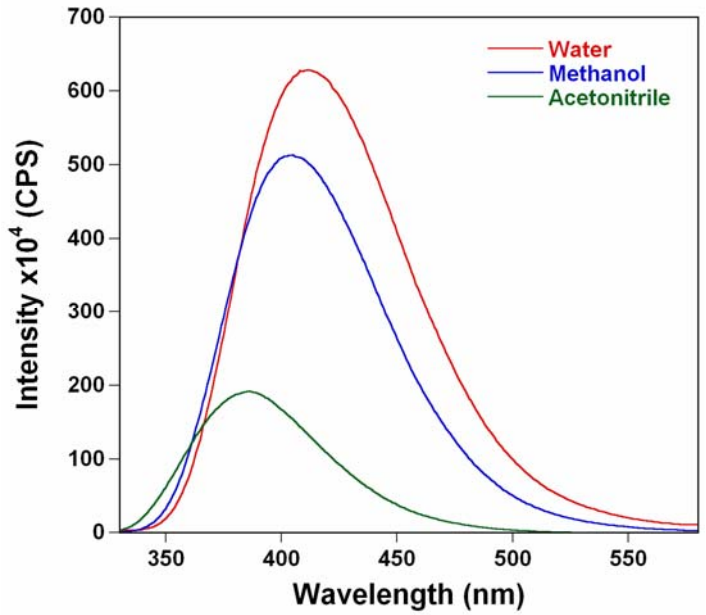


Figure S1. Emission spectra of nucleoside **7** (5.0 μM) in water (red), methanol (blue) and acetonitrile (green). Excitation wavelength was 304 nm, and excitation and emission slit widths were 3 and 5 nm, respectively. All solutions contained 0.5% DMSO. As solvent polarity is decreased from water to methanol, and finally acetonitrile, significant hypsochromic shift (~26 nm) and hypochromic effect (>3-fold) are observed.[S1]

Table S1. Photophysical data for thiophene-modified uridine **7**[a]

Solvent	λ_{max}[b] (nm)	λ_{em} (nm)	I_{rel}[c]
Water	304	412	3.3
Methanol	304	404	2.9
Acetonitrile	304	386	1.0

[a]Conditions for absorption and emission spectra: 100 and 5.0 μM, respectively; [b] the lowest energy maximum is given. [c] relative emission intensity with respect to intensity in acetonitrile.[S1]

3. MALDI-TOF MS measurements: Molecular weight of chemically synthesized fluorescent RNAs were determined via MALDI-TOF MS analysis. 2 μL of a ~100 μM stock solution of the synthesized RNA was combined with 1 μL of 100 mM ammonium citrate buffer (PE Biosystems), 1 μL of a 75 μM DNA standard (17-mer or 25-mer) and 4 μL of saturated 3-hydroxypiccolinic acid. The samples were desalted with an ionexchange resin (PE Biosystems) and spotted onto a gold-coated plate where they were air dried. The resulting spectra were calibrated relative to the +1 and +2 ions of the internal DNA standard, thus the observed oligonucleotides should have a resolution of ±2 mass units (Figure S2−S5).

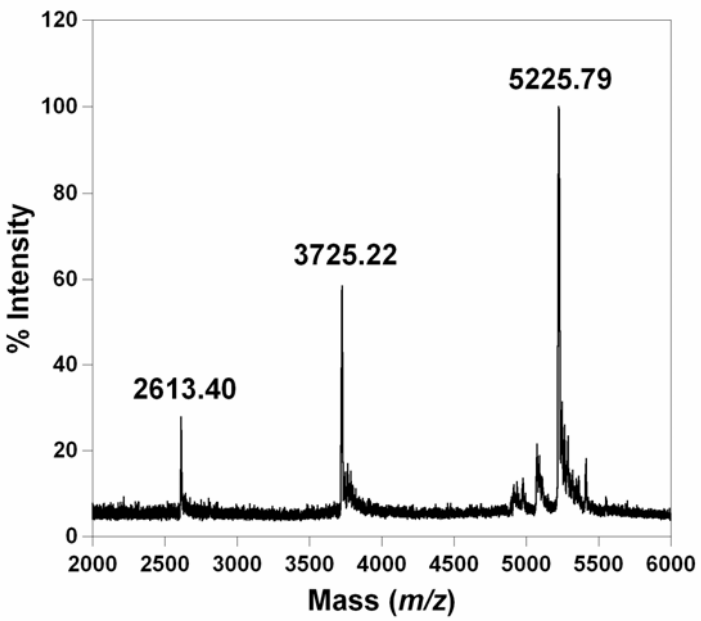


Figure S2. MALDI-TOF MS spectrum of the modified RNA **3** calibrated relative to the +1 and +2 ions of an internal 17-mer DNA standard (m/z: 5225.79 and 2613.40). Calculated mass [M] = 3723.45; observed mass = 3725.22

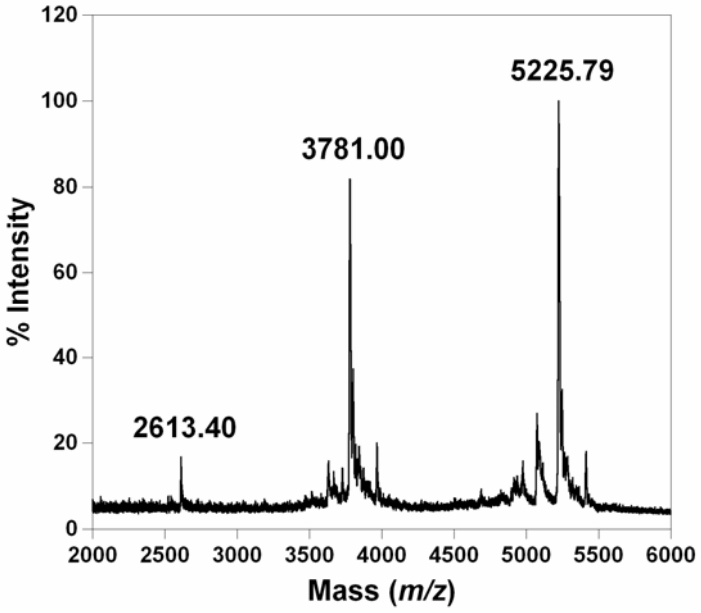


Figure S3. MALDI-TOF MS spectrum of the modified RNA **4** calibrated relative to the +1 and +2 ions of an internal 17-mer DNA standard (m/z: 5225.79 and 2613.40). Calculated mass [M] = 3779.45; observed mass = 3781.00

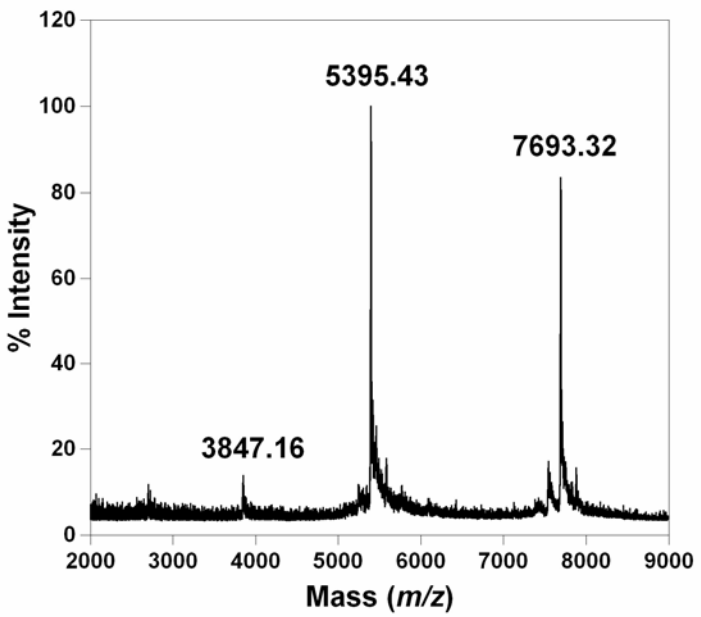


Figure S4. MALDI-TOF MS spectrum of the modified RNA **5** calibrated relative to the +1 and +2 ions of an internal 25-mer DNA standard (m/z: 7693.32 and 3847.16). Calculated mass [M] = 5393.67; observed mass = 5395.43

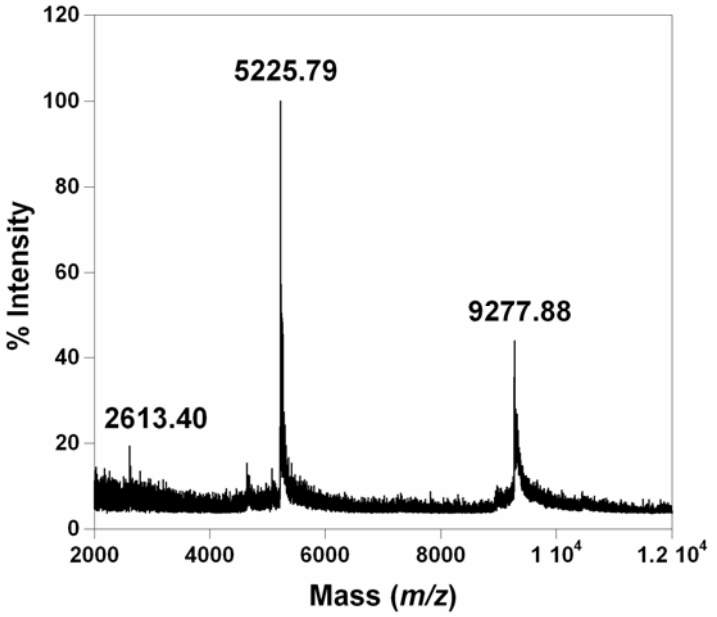


Figure S5. MALDI-TOF MS spectrum of the modified RNA **6** calibrated relative to the +1 and +2 ions of an internal 17-mer DNA standard (m/z: 5225.79 and 2613.40). Calculated mass [M + K] = 9278.28; observed mass = 9277.88

4. Fluorescence of Duplexes derived from RNA probes 3−6: Custom RNAs **1** and **2b** were annealed to fluorescent probes (**3**−**6**) by heating a 1:1 mixture (12.5 μM) of the oligonucleotides in 20 mM cacodylate buffer (pH 7.0, 100 mM NaCl, 0.5 mM EDTA) at 90 °C for 3 min and cooling the samples slowly to room temperature, followed by incubating the solutions in crushed ice (~15 min). Samples were diluted to give a final concentration of 1 μM in cacodylate buffer. Annealed RNA constructs (1 μM) were excited at 304 nm with an excitation slit width of 10 nm and emission slit width of 12 nm, and the fluorescence was monitored at the emission maximum, 408 nm (25 °C). Fluorescence experiments were carried out in a micro fluorescence cell with a path length of 1.0 cm (Hellma GmH & Co KG, Mullenheim, Germany) on a Horiba Jobin Yvon (FluoroMax-3) spectrometer.

5. Thermal denaturation experiments: RNA constructs were hybridized by heating a 1:1 mixture of the oligonucleotides in 20 mM cacodylate buffer (pH 7, 100 mM NaCl, 0.5 mM EDTA) at 90°C for 3 min and cooling the solutions slowly to room temperature. Hybridized samples were diluted with cacodylate buffer to give a final concentration of 1 μM. Thermal denaturation was monitored at 260 nm.

Table S2. Thermal melting of **1**, **2b** and hybridized constructs derived from **1** and **2b**

RNA construct	Type	Tm (°C)
1	Stem loop	62.0 ± 0.1
2b	Stem loop	63.8 ± 0.9
1•3	Duplex	62.5 ± 1.0
2b•3	Abasic	64.7 ± 0.2
1•4	Duplex	63.2 ± 1.0
2b•4	Abasic	63.7 ± 0.6
1•5	Duplex	69.8 ± 0.3
2b•5	Abasic	61.6 ± 0.5
1•6	Duplex	88.7 ± 0.1
2b•6	Abasic	83.9 ± 0.2

Higher T_m values for duplexes **1•6** and **2b•6** (88.7 ± 0.1 and 83.9 ± 0.2 °C, respectively) as compared to **1** and **2b** (62.0 ± 0.1 and 63.8 ± 0.9 °C, respectively) strongly indicates that the RIPs substrate **1** and the depurinated model **2b** form stable duplexes with probe **6**.

6. Gel mobility shift experiments to assess the hybridization efficiency of model duplexes: Non-radiolabeled RNA **1** was doped with 5'-^{32}P-labeled RNA **1**, and was annealed to fluorescent RNA oligonucleotides (**3**−**6**) by heating a 1:1 mixture (12 μM) in 20 mM cacodylate buffer (pH 7.0, 100 mM NaCl, 0.5 mM EDTA) at 90 °C for 3 min and cooling the samples slowly to room temperature, followed by incubating the solutions in crushed ice (total volume 10 μL). Same hybridization procedure was also followed for RNA **2b**. To each sample was added 4 μL of the loading buffer (10 mM Tris-HCl, pH 8.0, 30% sucrose, 10% glycerol, 0.05% bromophenol blue) and samples (4 μL) were loaded onto an analytical 15% non-denaturing polyacrylamide gel containing 100 mM NaCl. Electrophoresis was performed in TBE buffer (90 mM Tris, 90 mM boric acid and 2 mM EDTA) containing 100 mM NaCl at 175 V. The buffer was recirculated using peristaltic pump. Products on the gel were analyzed using a phosphorimager (Figure S6).

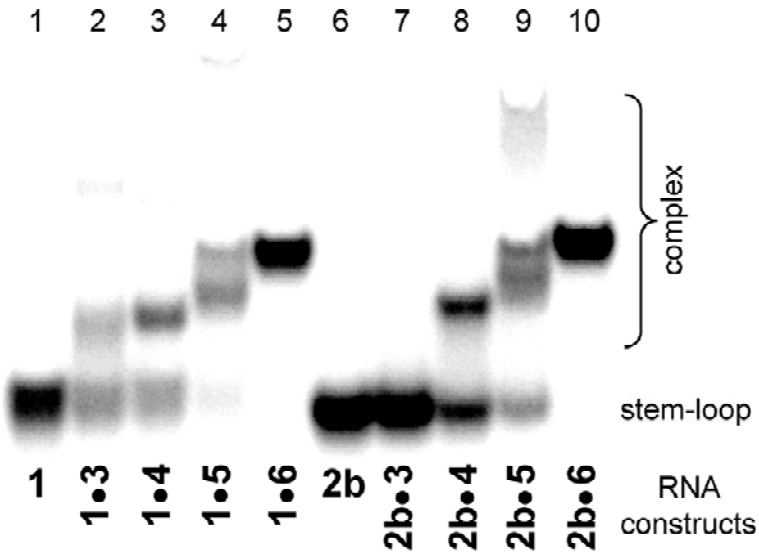


Figure S6. Gel mobility shift experiments to analyze the hybridization efficiency of duplexes derived by annealing ^{32}P-labeled RNA **1** or **2b** to fluorescent probes **3**−**6**. Oligos **3**, **4** and **5** show multiple structures and ineffective hybridization, while duplex **1•6** and **2b•6** formed using fluorescent probe **6** that encompasses the entire stem-loop structure displays complete hybridization.

7. Saporin catalyzed depurination reaction

7.1. Gel electrophoresis method: A stock solution (5 μM) containing non-radiolabeled substrate RNA **1** and trace amount of 5'-^{32}P-labeled RNA **1** was prepared in water. From the above stock a solution of doped RNA **1** (1 μM) in 30 mM Tris buffer (pH 6.0, 25 mM NaCl, 2 mM $MgCl_2$) was hybridized into stem-loop structure by heating the sample at 75 °C for 3 min and slowly cooling the solution to RT. The sample was placed in crushed ice for 15 min and then at RT for 10 min. The sample was incubated at 37 °C for 5 min and the depurination reaction was initiated by adding saporin (2 μM). The final volume of the reaction solution was 50 μL. Aliquots (5 μL) of the reaction mixture were mixed with 10 μL of aniline-acetate solution (1M, pH 4.5) at regular time intervals and were incubated at 37 °C for 10 min in dark. After this period the samples were evaporated to dryness in a Speed Vac and 15 μL of loading buffer (7 M urea in 10 mM Tris-HCl, 100 mM EDTA, pH 8 and 0.05% bromophenol blue) was added. Samples (4 μL) were loaded onto an analytical 20% denaturing polyacrylamide gel and were analyzed using a

phosphorimager. Strand scission with aniline gave rise to two products for each of the abasic site, probably due to the formation aniline adducts of β–eliminated keto-enol intermediates (P. A. Küpfer, C. J. Leumann, *Nucleic Acid Res*., **2007**, *35*, 58−68). RNase T1 (G-specific) and alkaline hydrolysis ladders were used to identify the site of depurination. See below for procedures.

RNase T1 ladder: A solution (2.5 μM) of non-radiolabeled substrate RNA **1** and radiolabeled RNA **1**, and yeast RNA (5 μg/μL) in water was evaporated to dryness in a Speed Vac. 4 μL of CEU buffer (25 mM citric acid, 25 mM potassium citrate, 1 mM EDTA, 7 M urea, pH 5.0) was added. The sample was incubated at 50 °C for 5 min and RNase T1 was added to give a final concentration of 0.8 U/μL. The sample was incubated at 50 °C for 15 min and denaturing loading buffer (5 μL) was added. 5 μL of the sample was loaded onto the 20% denaturing polyacrylamide gel.

Alkaline hydrolysis ladder: To a solution (1 μM) of non-radiolabeled substrate RNA **1** and radiolabeled RNA **1** in water was added Na_2CO_3-EDTA (25 mM, pH 9.2) to give a final volume (10 μL). The sample was heated at 90 °C for 25 min and was flash cooled on crushed ice. To the above solution was added CEU buffer (5 μL) and denaturing loading buffer (10 μL). 5 μL of the sample was loaded onto the 20% denaturing polyacrylamide gel.

7.2. Hybridization efficiency by gel mobility shift experiments: Abasic sites are known to be susceptible to strand cleavage. An untreated reaction sample after 75 min showed trace amounts of spliced products as a result of auto strand scission (Figure 3, lane 10, main text). This loss in the concentration of the depurinated product would lead to ineffective hybridization with the fluorescent probe **6**, and hence, excess of more emissive probe. To account for the self cleavage, saporin catalyzed depurination reaction mix was hybridized with probe **6** in different molar ratios. Native gel retardation experiments (discussed below) revealed an optimal concentration ratio of 1:0.9 for substrate **1** to probe **6**.

Radiolabeled RIPs substrate **1** (1 μM) was incubated with saporin (2 μM) under above conditions for 60 min. The depurinated product **2a** was mixed with fluorescent probe **6** in different molar ratios such that the final solutions contained 100 mM NaCl in 30 mM Tris buffer. Samples were hybridized by heating at 90 °C for 3 min and cooling the samples slowly to RT. To each of the sample was added 10 μL of the native gel loading buffer (10 mM Tris-HCl, pH 8.0, 30% sucrose, 10% glycerol, 0.05% bromophenol blue) and samples (4 μL) were loaded onto an analytical 20% non-denaturing polyacrylamide gel containing 100 mM NaCl. Electrophoresis was performed in TBE buffer (90 mM Tris, 90 mM boric acid and 2 mM EDTA) containing 100 mM NaCl at 200 V. The buffer was recirculated using peristaltic pump. Products on the gel were analyzed using a phosphorimager (Figure S7).

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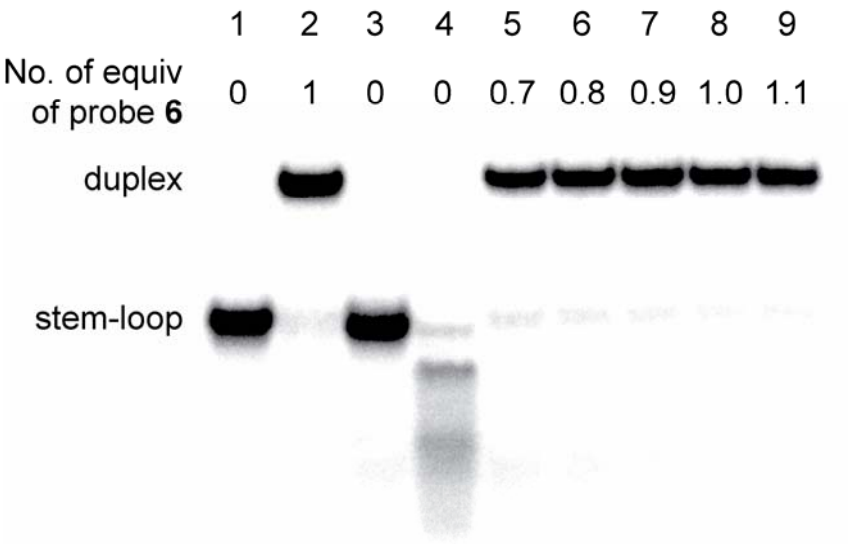

Figure S7. Native gel mobility shift experiments to determine the ratio of RNA **1** to probe **6** for fluorescence-hybridization assay. Lane 1, control stem-loop RNA **1**; lane 2, control duplex **1•6**; lane 3, saporin catalyzed depurination of RNA **1** (reaction sample 60 min); lane 4, reaction sample treated with aniline; lanes 5−9; reaction sample hybridized to probe **6** at different molar ratios.

7.3. Fluorescence-hybridization method: A solution of substrate RNA **1** (2.2 μM) in 30 mM Tris buffer (pH 6.0, 25 mM NaCl, 2 mM $MgCl_2$) was hybridized into stem-loop structure by heating the sample at 75 °C for 3 min and slowly cooling the solution to RT. The sample was placed in crushed ice for 15 min and then at RT for 10 min. The sample was incubated at 37 °C for 5 min and the depurination reaction was initiated by adding saporin (4.4 μM) so that the ratio of **1**:saporin was 1:2. At regular time intervals aliquots of reaction solution (75 μL) were mixed with fluorescent probe **6** (2 μM, 75 μL) to give a molar ratio of 1:0.9 (**1**:**6**) in 30 mM Tris buffer (pH 6.0, 100 mM NaCl, 2 mM $MgCl_2$) and was immediately hybridized by heating at 90 °C for 4 min and flash cooling on crushed ice over 15 min. Hybridized samples were brought to RT over 10 min, centrifuged and the fluorescence spectrum was measured. Samples were excited at 304 nm with an excitation slit width of 10 nm and emission slit width of 12 nm, and changes in fluorescence was monitored at the emission maximum, 410 nm (25 °C). Fluorescence experiments were carried out in a micro fluorescence cell with a path length of 1.0 cm (Hellma GmH & Co KG, Mullenheim, Germany) on a Horiba Jobin Yvon (FluoroMax-3) spectrometer.

7.4. Curve fitting

Fluorescence-hybridization method: Fluorescence intensity at each time point of the saporin catalyzed depurination of substrate **1** correspond to the sum of fluorescence from a less emissive perfect duplex (**1•6**) and more emissive duplex of the depurinated product and the probe (**2a•6**, equation 1). To determine the amount of depurination at a given time, the residual fluorescence associated with the duplex **1•6** has to be subtracted from the observed fluorescent enhancement at each time point. The rate of consumption of substrate is proportional to the rate of increase of fluorescence. Hence, the amount of fluorescence contribution from the duplex **1•6** at given time can be calculated from equation 2. The depurinated fraction obtained from equation 1 and 2 was plotted against time.

$$I_t = I_s + I_p \qquad (1)$$

$$I_s = I_0 - (I_t - I_0/I_F)I_0 \qquad (2)$$

I_t = fluorescence intensity at time = t

I_s = fluorescence intensity of duplex **1•6** at time = t

I_p = Fluorescence of **2a•6** at time = t

I_0 = fluorescence of duplex **1•6** at t = 0 min

I_F = fluorescence of duplex (**2a•6**) at t = 60 min, where complete depurination of substrate **1** to product **2a** took place.

Radioactive gel electrophoresis method: Radioactive band corresponding to the consumption of the substrate **1** was used to determine the formation of the product from the control substrate band without saporin as a function of time. The depurinated fraction thus obtained was plotted against time.

Apparent rate constant (k_{ap}) was determined by fitting the fluorescence and gel electrophoresis data using an exponential rate equation (3).

$$y = a + be^{-k_{ap}t} \qquad (3)$$

y is fractional depurination, k_{ap} is apparent rate constant, t is time, a and b are coefficients, respectively. Igor Pro 6.0 software was used for curve fitting.

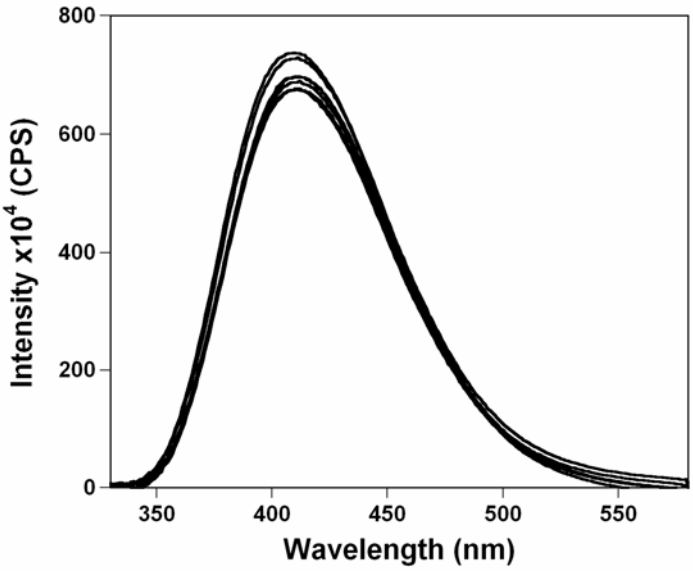

Figure S8. Fluorescence spectra of probe **6** (1 μM) in the presence of saporin (2 μM) as a function of time. Reaction was performed under conditions described above. At regular time intervals, aliquots of reaction solution were excited at 304 nm and fluorescence was recorded. The emission profile clearly indicates that there is no apparent change in the fluorescence intensity upon addition of toxin to the probe **6**.

8. Reference

[S1] S. G. Srivatsan, Y. Tor, *Org. Biomol. Chem*. **2008**, *6*, 1334–1338.