Supporting Information

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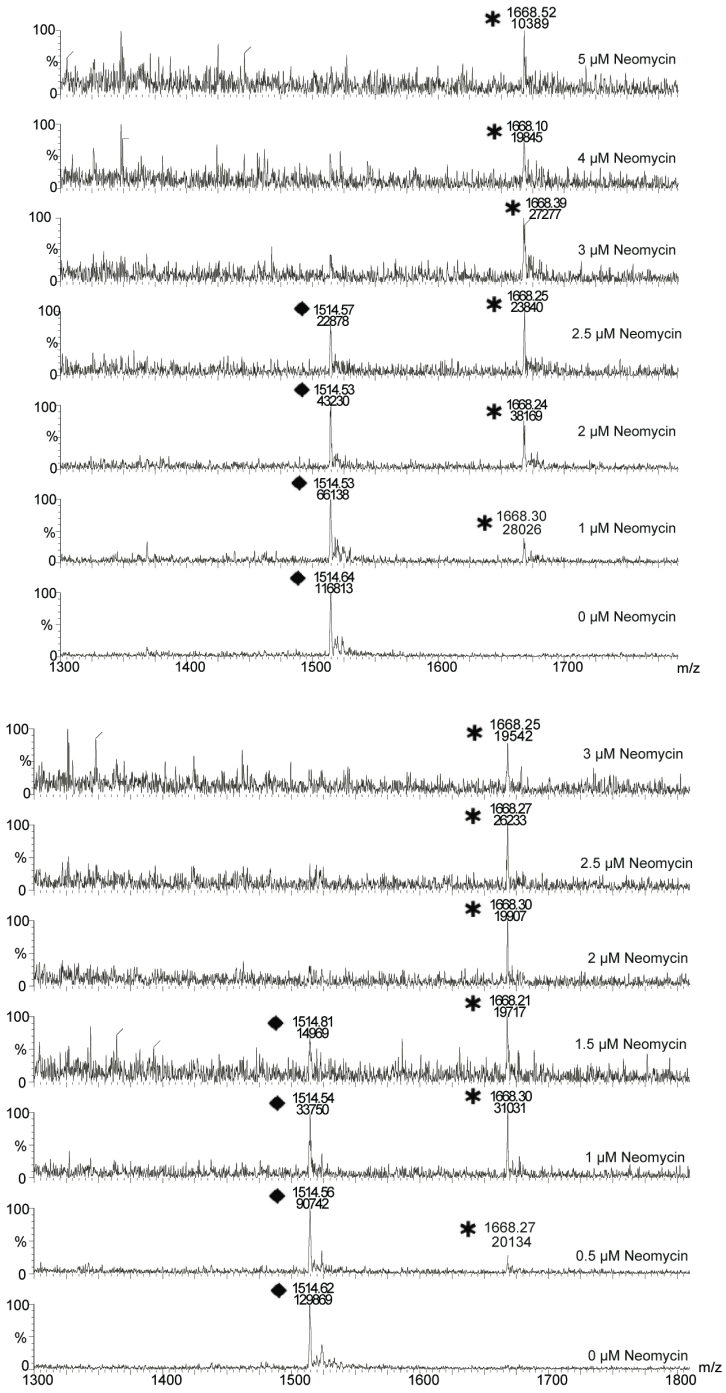

Figure S1. ESI-MS spectra of the binding of neomycin to the 2AP-ΨΨΨ (upper) and ΨΨΨ (lower) H69 RNA are shown. Diamonds (◆) indicate free RNA in the 4– charge states. Stars (✱) represent 1:1 (4– charge state) RNA: drug complex.

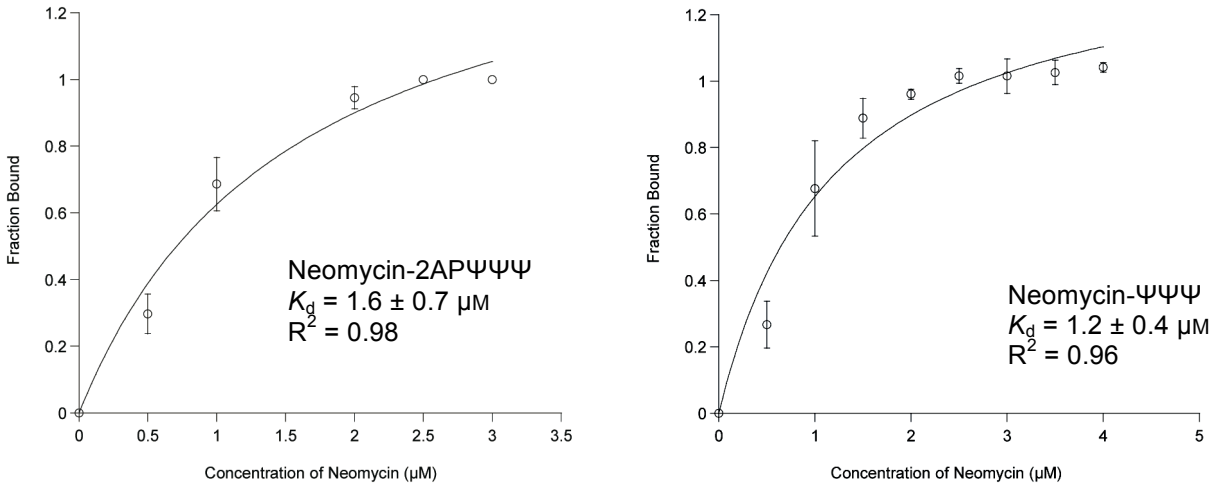

Figure S2. Curve fittings of the fraction of 2AP-ΨΨΨ (left) and ΨΨΨ (right) RNAs bound (calculated from the 4– charge state in ESI-MS) vs. neomycin concentration (μM).

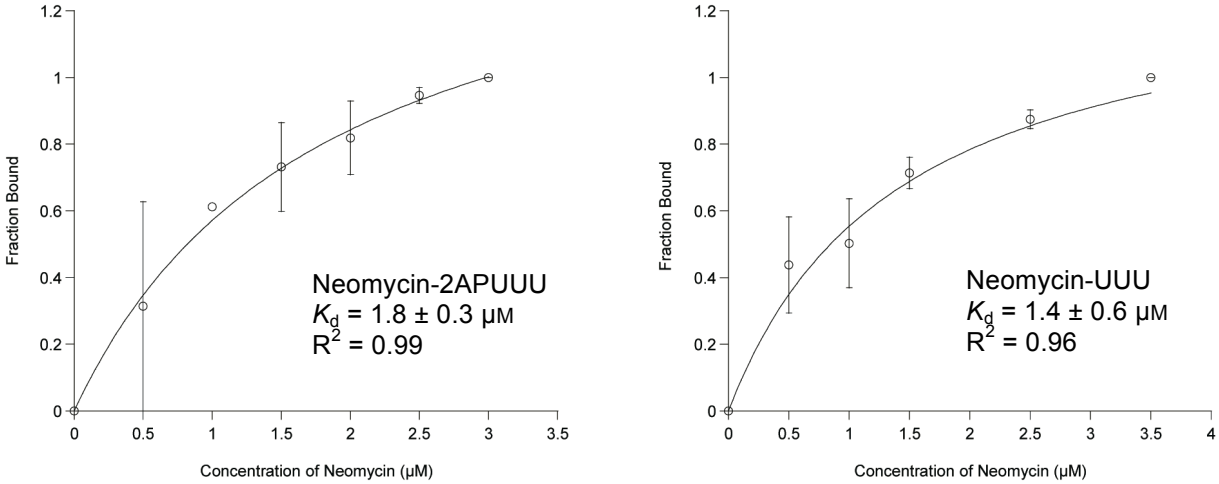

Figure S3. Curve fittings of the fraction of 2AP-UUU (left) and UUU (right) RNAs bound (calculated from the 4– charge state in ESI-MS) vs. neomycin concentration (μM).

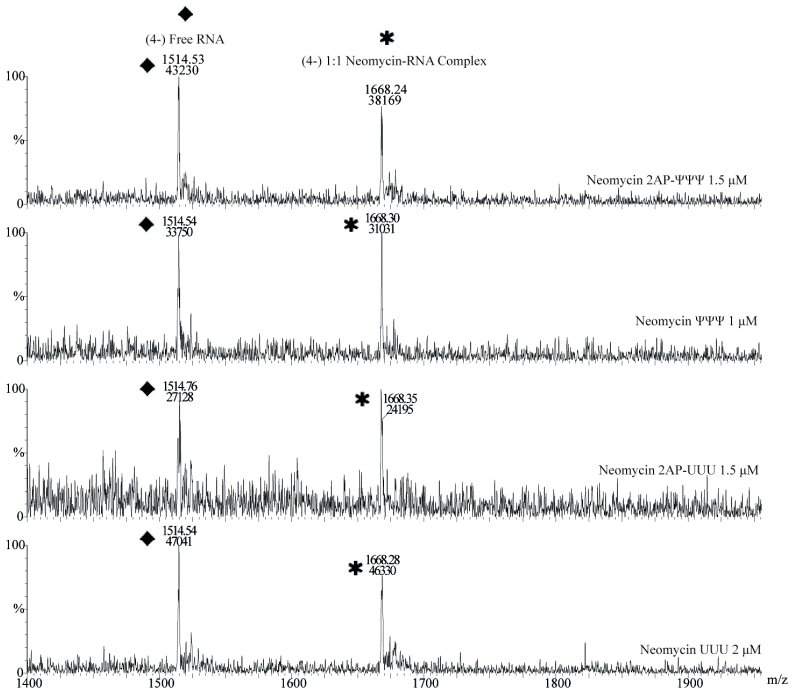

Figure S4. ESI-MS spectra of the binding of neomycin to the 2AP-ΨΨΨ, ΨΨΨ, 2AP-UUU, and UUU H69 RNA (from top to bottom) at a single concentration near the K_d value are shown. Diamonds (◆) indicate free RNA in the 4– charge states. Stars (✱) represent 1:1 (4– charge state) RNA: drug complex.

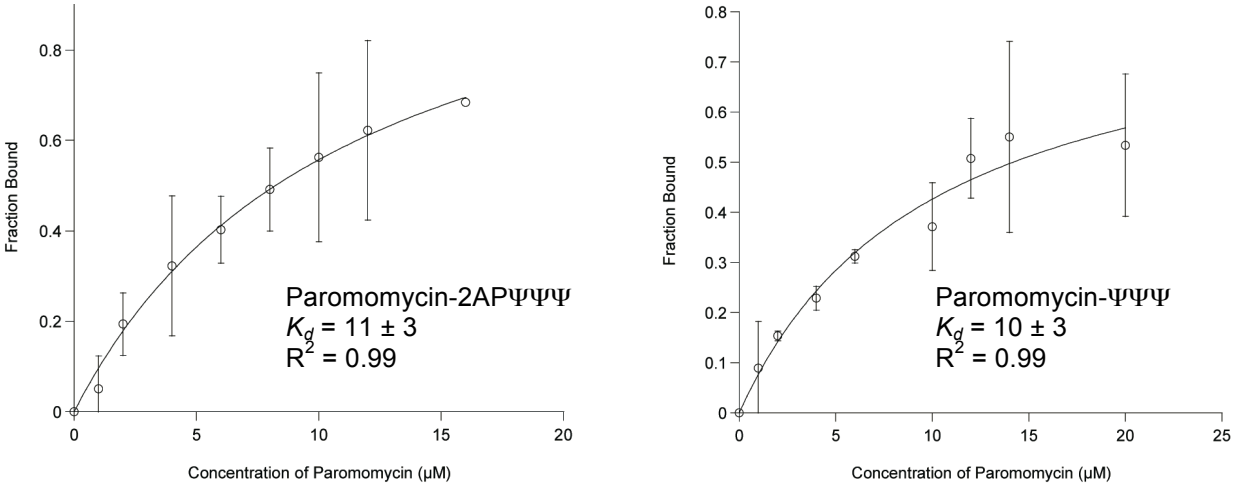

Figure S5. Curve fittings of the fraction of 2AP-ΨΨΨ (left) and ΨΨΨ (right) RNAs bound (calculated from the 4– charge state in ESI-MS) vs. paromomycin concentration (μM).

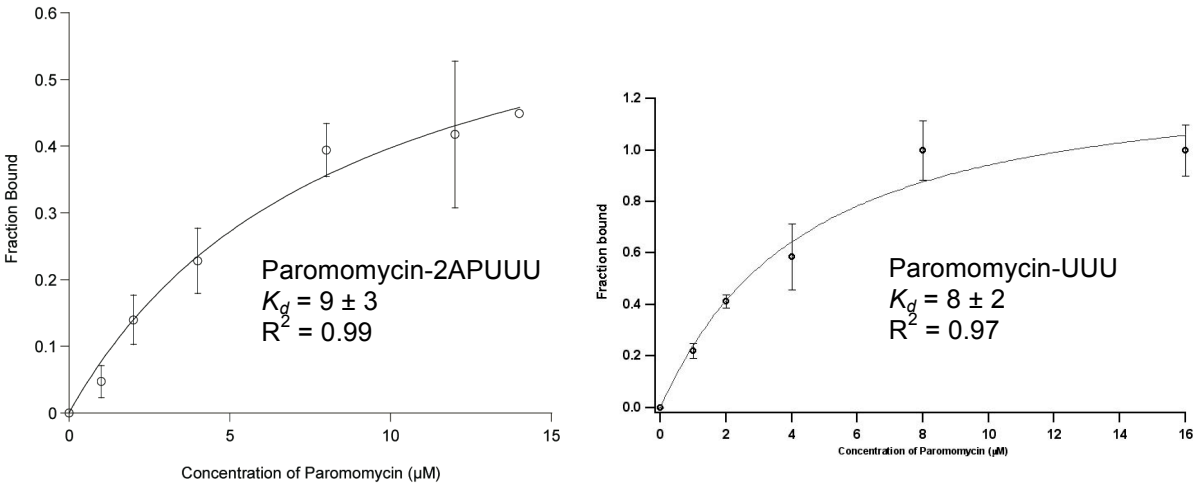

Figure S6. Curve fittings of the fraction of 2AP-UUU (left) and UUU (right) RNA bound (calculated from the 4– charge state in ESI-MS) vs. paromomycin concentration (μM).

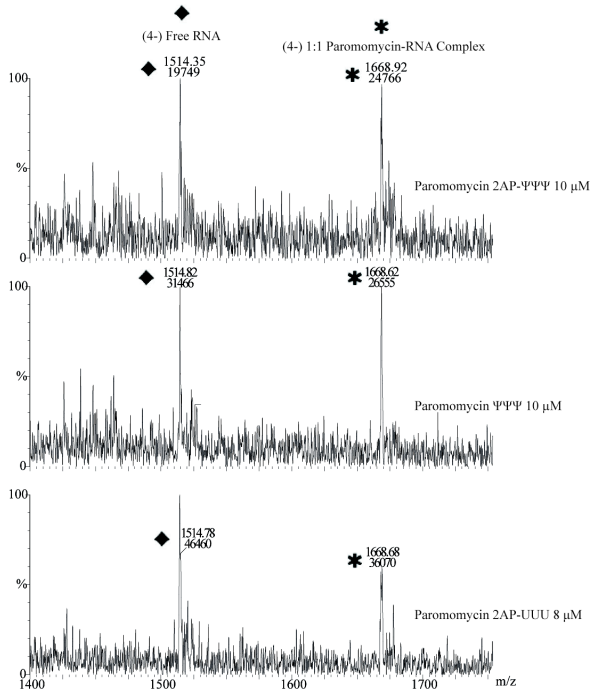

Figure S7. ESI-MS spectra of the binding of paromomycin to the 2AP-UUU, 2AP-ΨΨΨ, and ΨΨΨ H69 RNA (from top to bottom) at a single concentration near the K_d value are shown. Diamonds (◆) indicate free RNA in the 4– charge states. Stars (✱) represent 1:1 (4– charge state) RNA: drug complex.

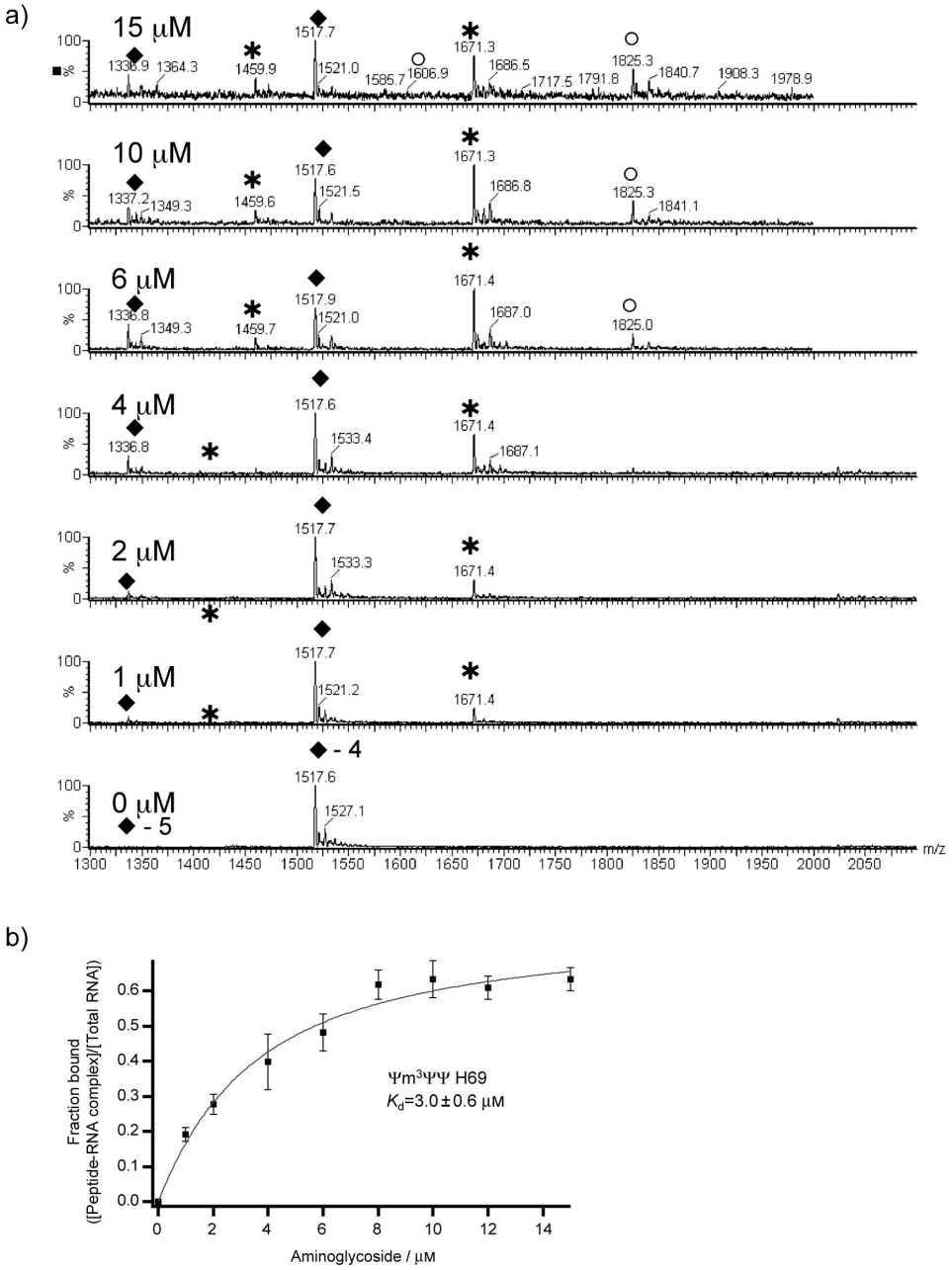

Figure S8. (a) ESI-MS spectra of the binding of neomycin to the $\Psi m^3\Psi\Psi$ H69 RNA are shown. Diamonds (◆) indicate free RNA in the 4– and 3– charge states. Stars (✱) represent 1:1 (4– and 3– charge states, as well as Na^+/K^+ ion adducts) RNA:drug complex. Open circles (○) designate 1:2 RNA/drug complex (at 3– and 4– charge states). (b) Curve fitting of the fraction of $\Psi m^3\Psi\Psi$ RNA bound *vs.* neomycin concentration, K_d = 3.0 μM, average of three independent experiments, is shown.

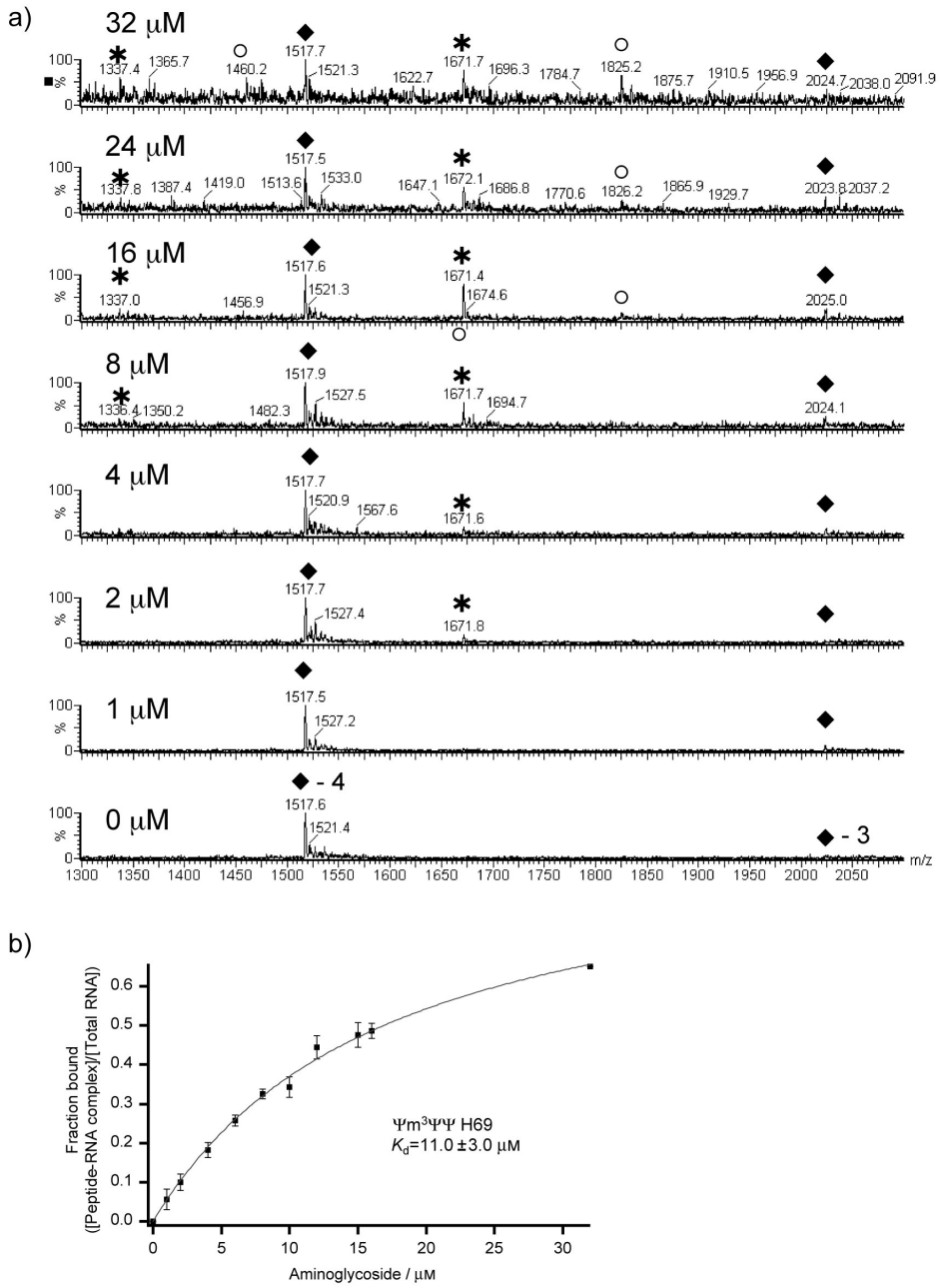

Figure S9. (a) ESI-MS spectra of the binding of paromomycin to the $\Psi m^3\Psi\Psi$ H69 RNA are shown. Diamonds (◆) indicate free RNA in the 4– and 3– charge states. Stars (✱) represent 1:1 (4– and 3– charge states, as well as Na^+/K^+ ion adducts) RNA:drug complex. Open circles (○) designate 1:2 RNA/drug complex (at 4– and 3– charge states). (b) Curve fitting of the fraction of $\Psi m^3\Psi\Psi$ RNA bound *vs.* paromomycin concentration, K_d = 11 μM, average of four independent experiments, is shown.

Experimental Section

2AP-incorporated RNA preparation. All 19-nt RNA oligonucleotides were synthesized by Dharmacon Research, Inc. (Lafayette, CO). RNAs were purified by 20% denaturing polyacrylamide gel electrophoresis and elution in 20 mM Tris-HCl, 250 mM NaCl, 1 mM EDTA, pH 7.5, at 4 °C overnight. Eluted RNAs were desalted and dissolved in double deionized water (ddH_2O) and confirmed by MALDI mass spectrometry. All RNA concentrations were determined based on the extinction coefficient of unmodified 19-nt H69 RNA.[1]

Fluorescence experiments. The general protocol for 2AP fluorescence measurements in different pH buffers is as follows. The 2AP-RNA was dissolved in 15 µl renaturing buffer (5 mM K^+-cacodylate, 70 mM NH_4Cl, 30 mM KCl, pH 7.0) and denatured at 80 °C for 3 min, followed by slow cooling to room temperature over 30 min in the dark. Renatured 2AP-RNA was added to 45 µl assay buffer (20 mM K^+-cacodylate, 70 mM NH_4Cl, 30 mM KCl, varying pH) and incubated for 5 min at room temperature before data collection. For ligand binding experiments (magnesium or aminoglycoside), the 2AP-RNA was dissolved in 60 µl assay buffer (20 mM K^+-HEPES, 70 mM NH_4Cl, 30 mM KCl, pH 7.3 at 22 °C). Ligand solutions for the titration experiments were prepared in the same buffer, and fluorescence changes were collected by adding 0.5 µl of ligand at each titration point followed by incubation for 2 min. 2AP-RNA was excited at 315 nm and fluorescence emission spectra were collected from 330 to 430 nm on a Cary Eclipse fluorescence spectrophotometer (Agilent Technology, Santa Clara, CA). All fluorescence spectra data were collected at 22 °C with 1.6 µM RNA concentration unless mentioned otherwise. A buffer background for each pH/temperature/buffer/ligand condition was subtracted from all fluorescence data. Relative fluorescence data that were used to determined K_d values were obtained by using the following equation: $F_r = (F_{min} - F_x)/(F_{min} - F_{max})$, in which F_r is relative fluorescence intensity, F_{min} is the initial or minimum fluorescence intensity at 370 nm, F_{max} is maximum fluorescence intensity (or endpoint of the titration) at 370 nm, and F_x is fluorescence intensity at the individual titration point at 370 nm (all F values were also corrected for volume changes). The titration curves were fit to a quadratic equation to obtain K_d values. It should be noted that the use of assay buffer conditions at each pH with or without 3 mM Mg^{2+} to renature the RNA gave the same results.

RNase A digestion. RNase A digestion experiments were performed as follows. RNA samples were prepared as mentioned above and fluorescence curve for no RNase A data was collected. One µl of RNase A (1 µg/mL; Ambion, Austin, TX) was added after denaturing at 80 °C for 3 min, and then incubated in the dark at 60 °C for 30 min followed by further incubation at 37 °C overnight in the dark to complete RNA digestion. Collection of fluorescence change at different time points and gel analysis revealed that overnight incubation is well enough for the complete digestions of both ΨΨΨ- and UUU-H69 based on the observation of fluorescence saturation. Buffer type, cacodylate buffer or HEPES buffer, did not matter and gave the same data.

Electrospray ionization mass spectrometry (ESI-MS). The ESI-MS experiments were carried out on a Micromass Quattro-LC double quadrupole mass spectrometer (Micromass, Manchester, UK) operated in the negative ion mode as described previously.[2] The spectra were analyzed using Masslynx 4.0 (Micromass). Neomycin and paromomycin stock solutions (50-100 μM) were prepared in water. The modified RNAs (Ψm^{3}ΨΨ, ΨΨΨ, and 2AP-ΨΨΨ) and unmodified RNAs (2AP-UUU and UUU) (100 μM stocks) were gel purified, ethanol precipitated with NH_4OAc, and renatured in 100 mM NH_4OAc before use. RNA (1 μM) was incubated with varying concentrations of aminoglycoside (0-32 μM) at room temperature for 10 min in a 50 μl reaction volume containing 50% 2-propanol and 150 mM NH_4OAc (pH 7.0). Under these conditions the main ion peaks observed corresponded to the 4– and 3– charge states. The bound fraction of RNA (Fr) was calculated as the ratio of peak area of complexed RNA over the peak area of total RNA (the sum of free RNA, including salt adducts and aminoglycoside-complexed RNA). In the case of neomycin and ΨΨΨ, 2AP-ΨΨΨ, 2AP-UUU, and UUU RNAs, the bound fraction of RNA was determined by using the change in peak intensity of the free RNA. This analysis accounts for all RNA adducts (1:1, 1:2, etc.), since non-specific binding is observed with increasing drug concentrations. The dissociation constants were obtained by plotting Fr vs. aminoglycoside concentration and non-linear curve fitting with a quadratic equation.[3]

[1] S. C. Abeysirigunawardena, C. S. Chow, *RNA* **2008**, *14*, 782-792.

[2] M. Li, A.-C. E. Duc, E. Klosi, S. Pattabiraman, M. R. Spaller, C. S. Chow, *Biochemistry* **2009**, *48*, 8299-8311.

[3] K. A. Sannes-Lowery, R. H. Griffey, S. A. Hofstadler, *Anal. Biochem*. **2000**, 280, 264-271.