Antibiotic Selectivity for Prokaryotic vs. Eukaryotic Decoding Sites
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- Abbreviations used
  - EDC = N-(3-Dimethylaminopropyl)-N’-ethylcarbodiimide
  - DMSO = dimethylsulfoxide
  - PAGE = polyacrylamide gel electrophoresis
  - TFA = trifluoroacetic acid
S.1 – Selectivity Ratio and Average Histopathology Score

![Graph showing selectivity ratio and average histopathology score for neomycin, tobramycin, and paromomycin.]

**Figure S1:** The selectivity ratio and average histopathology score for neomycin, tobramycin, and paromomycin.

S.2 – Synthesis

**General Procedures**

NMR spectra were recorded on a Varian Mercury 400 or 500 MHz spectrometer. Mass spectra were recorded at the UCSD Chemistry and Biochemistry Mass Spectrometry Facility, utilizing either a LCQDECA (Finnigan) ESI with a quadrupole ion trap or a MAT900XL (ThermoFinnigan) FAB double focusing mass spectrometer. UV-Vis spectra were recorded on either a Hewlett Packard 8453 Diode Array Spectrometer or Shimadzu UV-2450. Unless otherwise specified, materials obtained from commercial suppliers were used without further purification. NMR solvents were purchased from Cambridge Isotope Laboratories (Andover, MA).

**Synthetic Schemes**

![Chemical structures showing synthetic scheme for coumarin-labeled-kanamycin A.](image)

**Scheme S1:** Synthesis of coumarin-labeled-kanamycin A.  
(a) 7-((Et$_2$N)coumarin-3-carboxylic acid (1.2 eq.), EDC (1.2 eq.), DMAP (1.2 eq.), iPr$_2$EtN (2.2 eq.), dichloromethane, 87%.  
(b) TFA, triisopropylsilane, CH$_2$Cl$_2$, 72%.
6"′-Amino-6"′-deoxy-(Boc)_4 kanamycin A (4). Synthesis and characterization of precursors previously reported.\textsuperscript{52}

Boc_4-protected coumarin-labeled-kanamycin A (5). Anhydrous N,N-dimethylformamide (300 μL), and 7-(diethylamino)coumarin-3-carboxylic acid (20 mg, 76.6 μMol) were added to 4 (45.2 mg, 51.1 μMol). To this, EDC (11.8 mg, 61.3 μMol), N,N-diisopropylethylamine (22 μL, 127.7 μMol), and 4-(dimethylamino)pyridine (1.9 mg, 15.3 μMol) were added. The reaction was stirred at RT for 18 h. The solvent was removed under reduced pressure and the resulting solid was dissolved in ethyl acetate and washed with water and brine. The organic layer was dried over sodium sulfate and the solvent was removed under reduced pressure. After flash chromatography (5-10% methanol in dichloromethane), a green product (50.1 mg, 44.5 μMol, 87% yield) was isolated.\textsuperscript{1}H-NMR (400 MHz, CD_3OD): δ 8.61 (s, 1H), 7.54 (d, J=12, 1H), 7.31–7.24 (m, 1H), 6.82 (dd, J=4, 10, 1H), 6.56 (d, J=4, 1H), 5.14 (s, 1H), 4.96 (s, 1H), 4.72 (s, 1H), 4.51–4.43 (m, 1H), 3.85–3.81 (m, 1H), 3.65–3.30 (m, 1H), 3.19–3.14 (m, 4H), 3.08 (t, J=10, 1H), 2.06–1.97 (m, 2H), 1.44 (s, 36H), 1.23–1.17 (m, 10H); \textsuperscript{13}C-NMR (125 MHz, DMSO-d_6): δ 174.25, 162.64, 161.80, 161.75, 157.25, 156.17, 154.89, 152.49, 147.79, 131.67, 129.64, 110.20, 107.64, 95.79, 77.85, 77, 71, 77.27, 44.30, 35.13, 31.29, 29.09, 29.03, 28.99, 28.83, 28.69, 28.58, 28.22, 28.16, 27.33, 26.56, 25.09, 22.08, 13.89, 12.30; ESI-MS calculated for C_{52}H_{82}N_{6}O_{21} [M+Na]^+ 1149.33, found 1149.54.
Coumarin-labeled-kanamycin A. Anhydrous dichloromethane (2 mL) and triisopropylsilane (200 μL) were added to 5 (8.64 mg, 7.67 μmol). To this solution, trifluoroacetic acid (2 mL) was added and the reaction was stirred at RT for 15 min. The reaction was diluted with toluene (5 mL) and the solvent was removed under reduced pressure. The resulting solid was dissolved in water and washed with dichloromethane. The aqueous layer was dried concentrated under reduced pressure and further purified by reverse phase HPLC, 15 – 26% acetonitrile (0.1% TFA) in water (0.1% TFA) over 16 min, and eluted at 13.2 min. Product: yellow powder (7.1 mg, 6.0 μmol, 72% yield).

$^1$H-NMR (500 MHz, D$_2$O): δ 8.67 (s, 1H), 7.65 (d, $J = 9.3$, 1H), 6.95 (dd, $J = 2.4$, 9.3, 1H), 6.72 (d, $J = 2.0$, 1H), 5.37 (d, $J = 3.9$, 1H), 5.10 (d, $J = 3.5$, 1H), 4.13 – 4.08 (m, 1H), 4.01 (dd, $J = 3.9$, 9.2, 1H), 3.91 (dd, $J = 3$, 14.4, 1H), 3.85 (t, $J = 8.75$, 1H), 3.79 – 3.48 (m, 22H), 3.36 (dd, $J = 2.5$, 13.2, 1H), 3.21 (dd, $J = 7.3$, 14.9, 2H), 3.10 (dd, $J = 6.6$, 11.1, 1H), 2.94 (t, $J = 9.8$, 1H), 2.79 (dd, $J = 9.3$, 13.5, 1H), 2.50 – 2.45 (m, 1H), 1.88 – 1.80 (m, 1H), 1.29 (t, $J = 7.3$, 4H), 1.25 (t, $J = 7.1$, 6H); $^{13}$C-NMR (125 MHz, D$_2$O): δ 166.22, 164.36, 163.68 (q, $J_1 = 42$ Hz, $J_2 = 85.5$), 158.09, 153.21, 149.42, 132.70, 117.04 (q, $J_1 = 343.5$ Hz, $J_2 = 694.5$), 112.85, 109.91, 101.49, 98.09, 96.55, 84.71, 77.20, 73.83, 72.24, 69.36, 68.72, 68.24, 55.39, 50.44, 49.04, 47.35, 46.62, 41.30, 40.82, 28.27, 12.27, 8.91; ESI-MS calculated for C$_{32}$H$_{30}$N$_6$O$_{13}$ [M+2H]$^{2+}$ 364.18, [M+H]$^+$ 727.35, and [M+Na]$^+$ 749.33, found 364.22, 727.14, and 749.29, respectively.

S.3 – Absorption and Emission Spectra of F1, F2, and F3.

Figure S2: Absorption (---) and emission (—) spectra of F1 (blue), F2 (black), and F3 (red) in water. Conditions: F1 (1.0 × 10$^{-5}$ M), F2 (1.0 × 10$^{-5}$ M), and F3 (1 × 10$^{-5}$ M).
S.4 – Oligonucleotide Purification

The Dy547 18S A-site RNA construct was purchased from Thermo Scientific. The modified 16S oligonucleotide was synthesized and characterized according to previously reported procedures.\textsuperscript{53}

S.5 – Antibiotic Titrations

All titrations were performed with working solutions of 0.5 \( \mu \text{M} \) 16S and 18S A-site RNAs in 20mM calcodylate buffer (pH 7.0, 100 mM NaCl, 0.5 mM EDTA). Separate solutions of each RNA were heated to 75 °C for 5 min, cooled to room temperature over 2 h, and placed on ice for 30 min prior to titrations. Then an equimolar amount of the two annealed RNA constructs were combined with a two mole equivalent of coumarin-labeled-kanamycin A to give a final concentration of 0.5 \( \mu \text{M} \) for each of the RNA components and 2 \( \mu \text{M} \) for coumarin-labeled-kanamycin. The solutions were placed in a 0.125 mL quartz fluorescence cell with a path length of 1.0 cm (Hellma GmbH & Co KG, Müllheim, Germany). Steady state fluorescence experiments were carried out at ambient temperature (21°C) on a Jobin Yvon Horiba FluoroMax-3 luminescence spectrometer with excitation and emission slit-widths of 8 nm. A background spectrum was subtracted from each sample. For binding studies, the 16S A-site was excited at 320 nm and changes in emission upon titration with antibiotics were monitored at 395 nm and 473 nm. For the 18S A-site, antibiotics were titrated and the system was excited at 400 nm. Changes in emission were monitored at 561 nm. Errors were generated from three sets of measurements.

\( \text{IC}_{50} \) values were calculated using OriginPro 8 software by fitting a dose response curve (eq 1) to the fractional fluorescence saturation \((F_s)\) plotted against the log of antibiotic \((A)\) concentration.

\[
F_s = F_0 + \frac{(F_x[A]^n)/([IC_{50}]^n + [A]^n)}{F_0 + (F_x[A]^n)/([IC_{50}]^n + [A]^n)} \quad (1)
\]

\( F_i \) is the fluorescence intensity at each titration point. \( F_0 \) and \( F_x \) are the fluorescence intensity in the absence of aminoglycoside or at saturation, respectively, and \( n \) is the Hill coefficient or degree of cooperativity associated with the binding.
**Figure S3:** Fractional fluorescence saturation of the donor F1 (■) in the labeled 16S A-site and the emissive fluorophore F2 (●) tagged to kanamycin A in studying the binding of different antibiotics. Conditions: 16S RNA (5 × 10⁻⁷ M), 18S RNA (5 × 10⁻⁷ M), coumarin-labeled-kanamycin A (2.2 × 10⁻⁶ M), cacodylate buffer pH 7.0 (2.0 × 10⁻⁲ M), NaCl (1.0 × 10⁻³ M).
**Figure S4:** Fractional fluorescence saturation of the emissive acceptor F3 (■) of the 18S A-site in studying the binding of different antibiotics. Conditions: 16S RNA (5 × 10⁻⁷ M), 18S RNA (5 × 10⁻⁷ M), coumarin-labeled-kanamycin A (2.2 × 10⁻⁶ M), cacodylate buffer pH 7.0 (2.0 × 10⁻² M), NaCl (1.0 × 10⁻¹ M).

S.6 – Independent Titration Data

To determine if the placement of an internal fluorescent nucleoside in the A-site construct would inherently produce a difference in affinity as compared to end labeling the RNA, an independent experiment was conducted. A 16S A-site construct was labeled at the 5’ end with Dy547 (Figure S5), which is how the 18S construct was labeled for the double FRET experiments. IC₅₀ values (Table S1) were determined for unlabeled antibiotics by monitoring the displacement of coumarin-labeled-kanamycin A from the 16S-Dy547 A-site RNA. The IC₅₀ values obtained were in good agreement and within experimental error for the values obtained in the 16S A-site that was labeled with an isosteric fluorescent nucleoside analogue at position 1406.

In addition, to determine if the system would be sensitive to a possible 16S RNA/18S RNA interaction, the 5’ end Dy547 labeled 18S A-site construct was used alone to monitor the displacement of coumarin-labeled-kanamycin A by unlabeled neomycin (IC₅₀ = 5.0 ± 0.4) and tobramycin (IC₅₀ = 21 ± 2). The data is within error to the values obtained from the orthogonal FRET system, indicating that a 16S RNA/18S RNA interaction is unlikely to occur.
Figure S5: Secondary structures for the 27-base RNA models of the internally- and end-labeled 16S A-sites.

Table S1. IC\textsubscript{50} Values of Antibiotics for the 16S and 16S-Dy547 A-sites.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>16S A-Site (10\textsuperscript{-6} M)</th>
<th>16S-Dy547 A-Site (10\textsuperscript{-6} M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neo	extsubscript{mycin} B</td>
<td>2.8 (± 0.3)</td>
<td>2.6 (± 0.4)</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>20.2 (± 0.4)</td>
<td>18.5 (± 0.5)</td>
</tr>
<tr>
<td>Paromomycin</td>
<td>9 (± 1)</td>
<td>10 (± 1)</td>
</tr>
<tr>
<td>Kanamycin A</td>
<td>75 (± 3)</td>
<td>80 (± 4)</td>
</tr>
<tr>
<td>Amino-Tobramycin</td>
<td>4.2 (± 0.4)</td>
<td>3.5 (± 0.6)</td>
</tr>
<tr>
<td>Amino-Kanamycin A</td>
<td>11.9 (± 0.4)</td>
<td>11 (± 1)</td>
</tr>
<tr>
<td>Negamycin</td>
<td>62 (± 5)</td>
<td>60 (± 4)</td>
</tr>
<tr>
<td>Neamine</td>
<td>18 (± 2)</td>
<td>19 (± 1)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>1880 (± 10)</td>
<td>—</td>
</tr>
<tr>
<td>Lincomycin</td>
<td>&gt; 8.5 \times 10\textsuperscript{9}</td>
<td>—</td>
</tr>
<tr>
<td>Linezolid</td>
<td>&gt; 9.6 \times 10\textsuperscript{3}</td>
<td>—</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Conditions: 16S RNA (5 × 10\textsuperscript{-7} M), 16S-Dy547 RNA (5 × 10\textsuperscript{-7} M), coumarin-labeled-kanamycin A (2.2 × 10\textsuperscript{-6} M), cacodylate buffer pH 7.0 (2.0 × 10\textsuperscript{-2} M), NaCl (1.0 × 10\textsuperscript{-1} M).

S.7 – References

