

# RNA-Ligand Interactions: Affinity and Specificity of Aminoglycoside Dimers and Acridine Conjugates to the HIV-1 Rev Response Element

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## Supporting Information

### **S.1 Synthesis and Characterization of Neo-N-acridine**

### **S.2 Synthesis and Characterization of Neo-C-acridine**

### **S.3 Synthesis and Characterization of Tobra-N-acridine**

### **S.4 Synthesis and Characterization of Kana-N-acridine**

### **S.5 Synthesis and Characterization of Neo-Neo**

Structure, synthesis and characterization of the "linker only" neomycin B conjugate "5"- $\beta$ -mercaptoethylether-neomycin B".

### **S.6 Synthesis and Characterization of Neo-N-Neo**

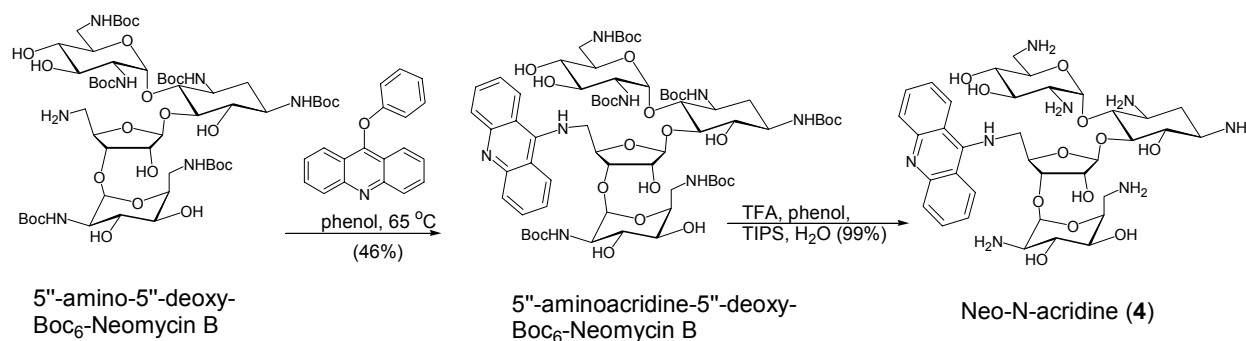
Figure S.1: Comparison of the  $^1\text{H}$  NMR spectra of Neo-N-Neo, Neo-Neo, 5"- $\beta$ -mercaptoethylether-neomycin B, and neomycin B (TFA salts in  $\text{D}_2\text{O}$ ).

### **S.7 Synthesis and Characterization of Tobra-N-Tobra**

### **S.8 Representative Data for the Displacement of RevFI from the Solid-Phase Immobilized RRE**

Figure S.2: Displacement of RevFI by neomycin B

## S.1 Synthesis of Neo-N-acridine (4):



**5''-amino-5''-deoxy-Boc<sub>6</sub>-Neomycin B.** Synthesized as described (Hai Wang Ph. D. Thesis, University of California, San Diego, 1998).

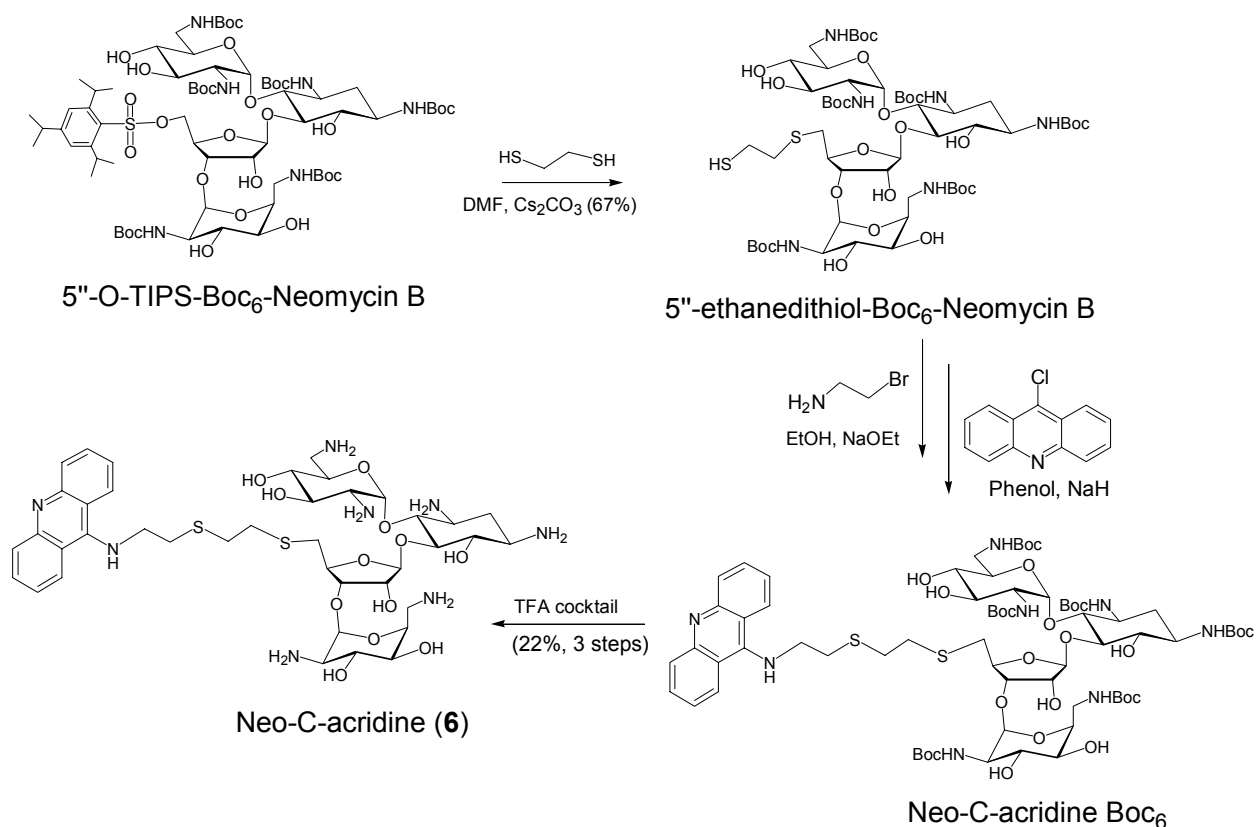
**9-Phenoxyacridine.** Prepared according to Dupre and Robinson (*J. Chem. Soc.* **1945**, 549-551).

**5''-aminoacridine-5''-deoxy-Boc<sub>6</sub>-Neomycin B.** 5''-amino-5''-deoxy-Boc<sub>6</sub>-Neomycin B (12.7 mg, 10.4 μmoles) was combined with phenol (200 mg), DMSO (350 μL), and 9-phenoxyacridine (8.2 mg, 30 μmoles, 2.9 equiv.) and heated, under argon, at 70 °C for 2 h. The reaction was loaded directly onto a silica column and purified using flash chromatography (5-10% MeOH / CH<sub>2</sub>Cl<sub>2</sub>) to afford 6.7 mg of a yellow product (46%). R<sub>f</sub> = .28 (10% MeOH / CH<sub>2</sub>Cl<sub>2</sub>). This product was carried over, without further characterization to the next step.

**Neo-N-acridine · TFA<sub>6</sub> (4).** 5''-amino-5''-deoxy-Boc<sub>6</sub>-Neomycin B (6.7 mg, 4.8 μmoles) was dissolved in a “deprotection cocktail” (4.5 mL trifluoroacetic acid, 0.2 mL phenol, 0.2 mL water, and 87 μL of triisopropylsilane) and mixed for 30 min at RT. The reaction was then diluted into 2% acetic acid/water (30 mL) and washed with diethyl ether (4x15 mL). The aqueous phase was then concentrated to a solid under reduced pressure. The crude product was HPLC purified (R<sub>t</sub> = 7.6 min) on a C-18 semiprep column under isocratic conditions using 11% acetonitrile in water (0.1% TFA) and lyophilized to yield 7.1 mg of a

yellow solid (99% assuming 6 TFA counter ions).  $^1\text{H}$  NMR (300 MHz,  $\text{D}_2\text{O}$ , 25  $^\circ\text{C}$ ):  $\delta$  8.36 (d,  $J$  = 8.7 Hz, 2H),  $\delta$  7.86 (t,  $J$  = 7.8 Hz, 2H),  $\delta$  7.73 (d,  $J$  = 8.4 Hz, 2H),  $\delta$  7.46 (t,  $J$  = 7.8 Hz, 2H),  $\delta$  5.76 (d,  $J$  = 3.9 Hz, 1H),  $\delta$  5.29 (d,  $J$  = 0.9 Hz, 1H),  $\delta$  5.14 (d,  $J$  = 1.2 Hz, 1H),  $\delta$  4.48-4.55 (m, 2H),  $\delta$  4.37 (d,d  $J_1$  = 4.2 Hz,  $J_2$  = 1.5 Hz, 1H),  $\delta$  4.25 (d,d  $J_1$  = 13.8 Hz,  $J_2$  = 8.1 Hz, 1H),  $\delta$  4.1 (t,  $J$  = 4.2 Hz, 1H),  $\delta$  4.03 (t,  $J$  = 3.0 Hz, 1H),  $\delta$  3.74-3.85 (m, 2H),  $\delta$  3.65 (d,  $J$  = 3.0 Hz, 1H),  $\delta$  3.51-3.59 (m, 1H),  $\delta$  3.40-3.47 (m, 3H),  $\delta$  3.27-3.35 (m, 1H),  $\delta$  3.10-3.20 (m, 3H),  $\delta$  2.94 (d,d  $J_1$  = 13.5 Hz,  $J_2$  = 3.6 Hz, 1H),  $\delta$  2.18-2.33 (m, 3H),  $\delta$  1.95 (d,d  $J_1$  = 10.2 Hz,  $J_2$  = 3.9 Hz, 1H),  $\delta$  1.66 (q,  $J$  = 12.6 Hz, 1H). MALDI TOF MS calculated for  $\text{C}_{36}\text{H}_{54}\text{N}_8\text{O}_{12}$ : 790.4, found 813.3  $[\text{M}+\text{Na}]^+$ , found 829.3  $[\text{M}+\text{K}]^+$ . UV-vis (50 mM sodium phosphate pH 7.5):  $\lambda_{\text{max}}$  (nm) and  $\epsilon$  ( $\text{cm}^{-1}\text{M}^{-1}$ ): 222 ( $2.0 \times 10^4$ ), 266 ( $5.6 \times 10^4$ ), 412 ( $10.3 \times 10^3$ ), 434 ( $8.3 \times 10^3$ ).

## S.2 Synthesis of Neo-C-acridine (6):



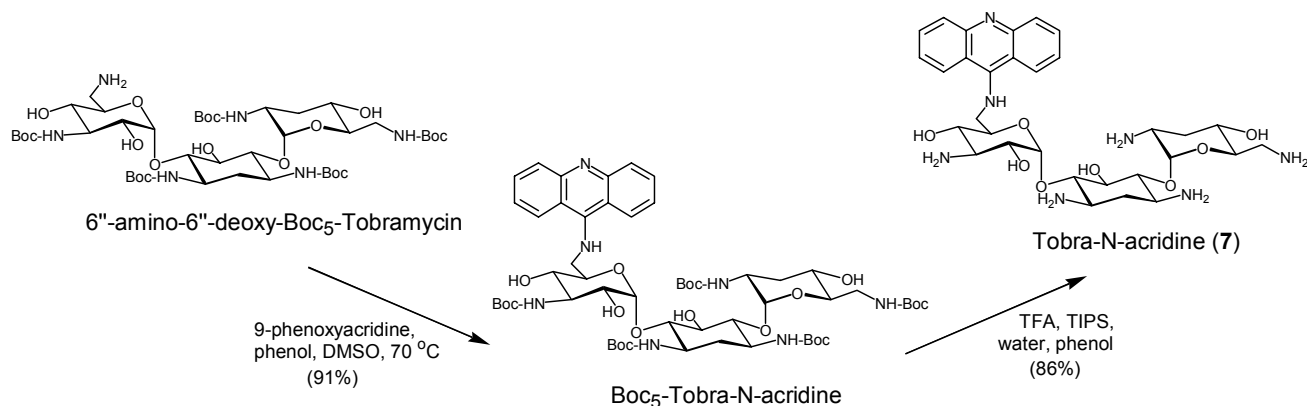
**5''-O-TIPS-Boc<sub>6</sub>-Neomycin B.** Synthesized as described (Hai Wang Ph. D. Thesis, University of California, San Diego, 1998; see also: Michael, K., Wang, H., and Tor, Y. (1999) *Bioorg. Med. Chem.* 7, 1361-1371.)

**5''-ethanedithiol-Boc<sub>6</sub>-Neomycin.** 5''-O-TIPS-Boc<sub>6</sub>-Neomycin B (38 mg, 25.6  $\mu$ moles), cesium carbonate (176 mg, 540  $\mu$ moles, 20 equiv), DMF (2 mL) and 1,2-ethanedithiol (230  $\mu$ L, 2.7 mmoles, 107 equiv) were stirred under argon for 4 h at RT. The reaction was diluted into ethyl acetate (180 mL) and washed with 1M NaH<sub>2</sub>PO<sub>4</sub> (4x80 mL), brine (80 mL) and dried under reduced pressure, then kept under high vacuum for 24 h. The crude product was purified using standard flash chromatography (4-5% MeOH / CH<sub>2</sub>Cl<sub>2</sub>) to yield 22 mg of a white solid (67%). R<sub>f</sub> = 0.5 (10% MeOH / CH<sub>2</sub>Cl<sub>2</sub>) (the same R<sub>f</sub> as the starting material). <sup>1</sup>H-NMR (400 MHz, d<sub>3</sub>-MeOD)  $\delta$  5.41 (s, 1H),  $\delta$  5.13 (s, 1H),  $\delta$  4.92 (s, 1H),  $\delta$  4.21-4.24 (m, 2H),  $\delta$  4.08 (q, J = 5.2 Hz, 1H),  $\delta$  3.85-3.89 (m, 2H),  $\delta$  3.70-3.76 (m, 2H),  $\delta$  3.17-3.57 (m, 12H),  $\delta$  2.86-2.95 (m, 5H),  $\delta$  2.72 (t, J = 7.2 Hz, 2H),  $\delta$  1.92-1.98 (m, 1H),  $\delta$  1.28-1.46 (m, 56H).

**Neo-C-acridine · TFA<sub>6</sub> (6).** Under argon, sodium ethoxide (14 mg, 206  $\mu$ moles) argon-sparged ethanol (2 mL), and 1-bromo-2-ammonium ethane chloride (14 mg, 68  $\mu$ moles) were stirred for 5 min at RT. To this solution, 5''-ethanedithiol-Boc<sub>6</sub>-neomycin B (5 mg, 3.8 mmoles) (pre-dissolved in 1 mL of argon-sparged ethanol) was added and allowed to react for 2.5 hr at RT. The reaction was then diluted into ethyl acetate (150 mL) and washed with 0.1M citric acid (4x40 mL), 0.1M Na<sub>2</sub>HPO<sub>4</sub> (50 mL), and brine (50 mL), dried over sodium sulfate and concentrated to a solid under reduced pressure. The crude product (R<sub>f</sub> = 0.1 (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>)) was taken, directly, into the next step. 9-phenoxy acridine was generated *in situ* by melting phenol (1 g), under argon, at 70 °C and adding sodium hydride (10 mg, 60% dispersion in wax, 250  $\mu$ moles), followed by 9-chloroacridine (20 mg, 47  $\mu$ moles) and mixing, under argon for 5 min. The neomycin-containing starting material (pre-dissolved in 1 mL of DMSO) was then added to the acridine solution and stirred at 70°C under argon for 14 hr. The reaction was then diluted into ethyl acetate (150 mL) and washed with 1M Na<sub>2</sub>CO<sub>3</sub> (4x40 mL), and brine (50 mL), then dried over sodium sulfate and

concentrated to an oil under reduced pressure. The crude product was purified on silica gel, using standard flash chromatography (0-9% MeOH / CH<sub>2</sub>Cl<sub>2</sub>) to afford a yellow solid R<sub>f</sub> = 0.2 (10% MeOH / CH<sub>2</sub>Cl<sub>2</sub>). All of this product was then taken into the next step without further characterization. Deprotection was conducted by dissolving the product in a deprotection cocktail (5 mL of trifluoroacetic acid, containing 1% ea (v/v) of triisopropyl silane, and 1,2-ethanedithiol) and stirring, RT, for 20 min. The reaction was then diluted into water (100 mL) and washed with CHCl<sub>3</sub> (2x50 mL), diethyl ether (2x50 mL), and concentrated to a yellow solid under reduced pressure. The crude product was HPLC purified (R<sub>t</sub> = 7.4 min) on C-18 semiprep column using isocratic conditions with 15% acetonitrile in water (0.1% TFA) and lyophilized to yield 1.3 mg of a yellow solid (22% yielded for three steps). <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O, 25 °C): δ 8.22 (d, *J* = 8.8 Hz, 2H), δ 7.78 (t, *J* = 7.6 Hz, 2H), δ 7.63 (d, *J* = 8.8 Hz, 2H), δ 7.39 (t, *J* = 7.2 Hz, 2H), δ 5.86 (d, *J* = 4.0 Hz, 1H), δ 5.22 (s, 1H), δ 5.08 (s, 1H), δ 4.23 (t, *J* = 6.0 Hz, 1H), δ 4.18 (s, 1H), δ 4.12-4.16 (m, 1H), δ 4.07 (t, *J* = 4.8 Hz, 1H), δ 4.03 (t, *J* = 2.4 Hz, 1H), δ 3.91 (t, *J* = 10 Hz, 1H), δ 3.82 (t, *J* = 9.6 Hz, 1H), δ 3.73 (t, *J* = 8.6 Hz, 2H), δ 3.62 (s, 1H), δ 3.52 (t, *J* = 10 Hz, 1H), δ 3.16-3.40 (m, 8H), δ 3.04 (d,d *J*<sub>1</sub> = 14 Hz, *J*<sub>2</sub> = 6.8 Hz, 1H), δ 2.98 (t, *J* = 6.4 Hz, 2H), δ 2.90 (d,d *J*<sub>1</sub> = 14 Hz, *J*<sub>2</sub> = 4.4 Hz, 1H), δ 2.61-2.67 (m, 6H), δ 2.31 (d t, *J*<sub>1</sub> = 12.4 Hz, *J*<sub>2</sub> = 4.4 Hz, 1H), δ 1.73 (q, *J* = 12 Hz, 1H). MALDI TOF MS calculated for C<sub>40</sub>H<sub>62</sub>N<sub>8</sub>O<sub>12</sub>S<sub>2</sub>: 910.4, found 911.3 [M+H]<sup>+</sup>, found 933.3 [M+Na]<sup>+</sup>, found 949.3 [M+K]<sup>+</sup>. UV-vis (50 mM sodium phosphate pH 7.5): λ<sub>max</sub> (nm) and ε (cm<sup>-1</sup>M<sup>-1</sup>): 222 (1.95x10<sup>4</sup>), 266 (5.0x10<sup>4</sup>), 412 (9.85x10<sup>3</sup>), 434 (8.3x10<sup>3</sup>).

### S.3 Synthesis and Characterization of Tobra-N-acridine (7):



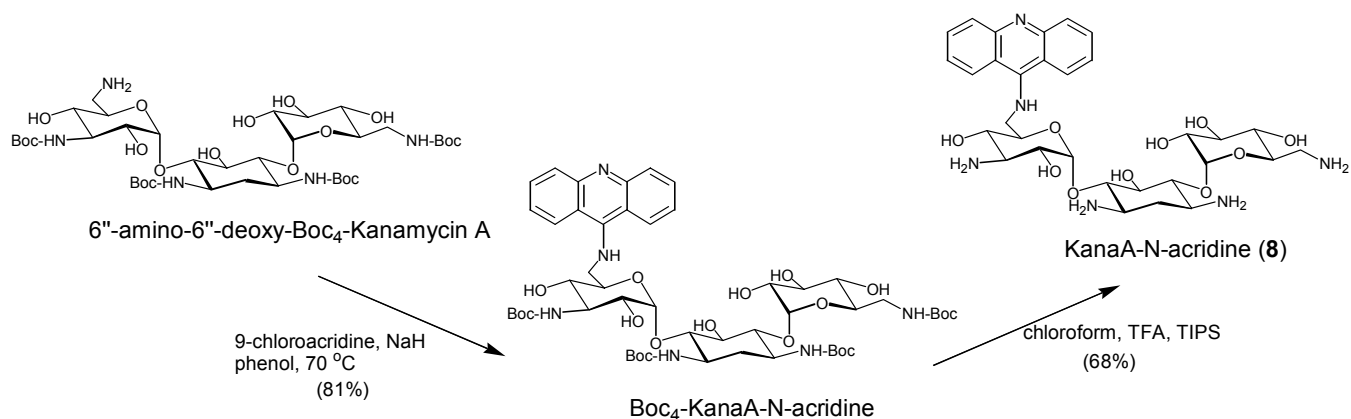
**6''-amino-6''-deoxy-Boc<sub>5</sub>-Tobramycin.** Synthesized as described (Hai Wang Ph. D. Thesis, University of California, San Diego, 1998).

**Boc<sub>5</sub>-Tobra-N-acridine.** 6''-amino-6''-deoxy-Boc<sub>5</sub>-Tobramycin (38 mg, 40 μmoles), 9-phenoxyacridine (50 mg, 190 μmoles), phenol (0.5 g), and DMSO (1 mL), were heated under Ar for 2 hr at 70 °C. Volatiles were then removed, under vacuum, for 14 hr. The crude product was purified on ~100 mL of silica gel using standard flash chromatography using an 8 – 13% methanol/CH<sub>2</sub>Cl<sub>2</sub> gradient, yielding a yellow solid (41 mg, 91%). R<sub>f</sub> = .23 (10% MeOH / CH<sub>2</sub>Cl<sub>2</sub>). <sup>1</sup>H NMR (300 MHz, d<sub>3</sub>-MeOD, 25 °C): δ 8.55 (d, *J* = 8.7 Hz, 2H), δ 7.98 (t, *J* = 7.5 Hz, 2H), δ 7.81 (d, *J* = 7.8 Hz, 2H), δ 7.63 (t, *J* = 7.5 Hz, 2H), δ 5.13 (d, *J* = 3.3 Hz, 1H), δ 4.94 (d, *J* = 3.3 Hz, 1H), δ 4.60-4.67 (m, 3H), δ 4.09-4.17 (m, 1H), δ 3.30-3.78 (m, 18H), δ 1.85-1.98 (m, 2H), δ 1.15-1.62 (m, 47H). MALDI FTMS calculated for C<sub>56</sub>H<sub>85</sub>N<sub>7</sub>O<sub>8</sub>: 1143.60, found 1144.60 [M+H]<sup>+</sup>.

**Tobra-N-acridine · TFA<sub>5</sub> (7).** Boc<sub>5</sub>-Tobra-N-acridine (11 mg, 9.6 μmoles) was dissolved in a “deprotection cocktail” (9 mL trifluoroacetic acid, 0.4 mL phenol, 0.4 mL water, and 300 μL of triisopropylsilane) and mixed for 30 min at RT. The reaction was then diluted into 2% acetic acid/water (30 mL) and washed with diethyl ether (4x15 mL). The aqueous phase was concentrated to a solid under reduced pressure and HPLC purified on C-18 semiprep column under isocratic conditions (11% acetonitrile in water and 0.1% TFA) (R<sub>t</sub> = 13.3 min) and lyophilized to yield 10 mg of a yellow solid (86% assuming 5 TFA counter ions). <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O, 25 °C): δ 8.29 (d, *J* = 8.8 Hz, 2H), δ 7.85 (t, *J* = 7.8 Hz, 2H), δ 7.70 (d, *J* = 8.8 Hz, 2H), δ 7.43 (t, *J* = 7.8 Hz, 2H), δ 5.47 (d, *J* = 3.6 Hz, 1H), δ 5.09 (d, *J* = 3.6 Hz, 1H), δ 4.46-4.50 (m, 1H), δ 4.24-4.28 (m, 2H), δ 3.94 (d,d *J*<sub>1</sub> = 10.4 Hz, *J*<sub>2</sub> = 3.2 Hz, 1H), δ 3.87 (t, *J* = 9.4 Hz, 1H), δ 3.56-3.75 (m, 4H), δ 3.36-3.94 (m, 3H), δ 3.30 (d,d *J*<sub>1</sub> = 13.6 Hz, *J*<sub>2</sub> = 4.0 Hz, 1H), δ 3.22 (d,t *J*<sub>1</sub> = 12.0 Hz, *J*<sub>2</sub> = 2.0 Hz, 1H), δ 3.09 (d,d *J*<sub>1</sub> = 13.6 Hz, *J*<sub>2</sub> = 7.6 Hz, 1H), δ 2.41 (d,t *J*<sub>1</sub> = 12.8 Hz, *J*<sub>2</sub> = 4.4 Hz, 1H), δ 2.08 (d,t *J*<sub>1</sub> = 13.2 Hz, *J*<sub>2</sub> = 4.8 Hz, 1H), δ 1.83 (q, *J* = 12.8 Hz, 1H), δ 1.81 (q, *J* = 12.8 Hz, 1H). MALDI FTMS

calculated for  $C_{31}H_{45}N_7O_8$ : 643.34, found 644.34  $[M+H]^+$ . (50 mM Tris·HCl pH 7.5):  $\lambda_{max}$  (nm) and  $\epsilon$  ( $cm^{-1}M^{-1}$ ): 222 ( $1.7 \times 10^4$ ), 266 ( $5.0 \times 10^4$ ), 412 ( $8.9 \times 10^3$ ), 434 ( $7.4 \times 10^3$ ).

#### S.4 Synthesis and Characterization of KanaA-N-acridine (8):

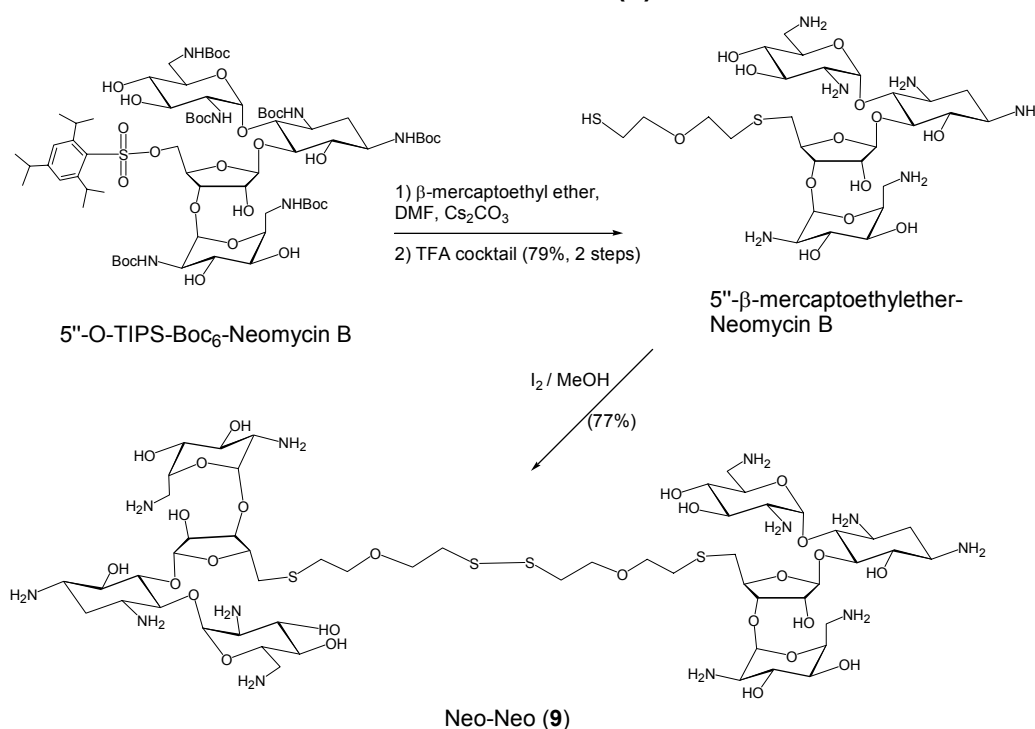


**6''-amino-6''-deoxy-Boc<sub>4</sub>-Kanamycin A.** Synthesized as described (Hai Wang Ph. D. Thesis, University of California, San Diego, 1998).

**Boc<sub>4</sub>-KanaA-N-acridine.** To a solution of 9-chloroacridine (20 mg, 94  $\mu$ moles) in 500 mg phenol at 70 °C, NaH (3.8 mg, 94  $\mu$ moles, 60% suspended in mineral oil) was added and stirred for 15 minutes. To this solution, 6''-amino-6''-deoxy-Boc<sub>4</sub>-Kanamycin A (15 mg, 17  $\mu$ moles) was added and heated under Ar for 1 hr at 70 °C. The resulting solution was cooled to room temperature and then diluted with  $CH_2Cl_2$  (50 mL). The organic phase was washed with saturated  $NaHCO_3$  (2x50 mL) and then concentrated under reduced pressure. The crude product was purified on ~40 mL of silica gel using standard flash chromatography and a 5 – 10% methanol/ $CH_2Cl_2$  gradient, yielding a yellow solid (8 mg, 81%).  $R_f = .67$  (15% MeOH /  $CH_2Cl_2$ ).  $^1H$  NMR (300 MHz,  $d_3$ -MeOD, 25 °C):  $\delta$  8.58 (d,  $J = 8.4$  Hz, 2H),  $\delta$  7.97 (t,  $J = 7.5$  Hz, 2H),  $\delta$  7.82 (d,  $J = 8.1$  Hz, 2H),  $\delta$  7.62 (t,  $J = 7.8$  Hz, 2H),  $\delta$  5.08 (d,  $J = 3.6$  Hz, 1H),  $\delta$  4.82 (d,  $J = 3.3$  Hz, 1H),  $\delta$  4.69-4.63 (m, 1H),  $\delta$  4.52 (d,  $J = 7.8$  Hz, 1H),  $\delta$  4.13-4.03 (m, 1H),  $\delta$  3.70-3.30 (m, 13H),  $\delta$  3.12-3.08 (m, 1H),  $\delta$  2.98-2.90 (m, 1H),  $\delta$  2.03 (m, 1H),  $\delta$  148-1.42 (m, 36H).

**KanaA-N-acridine · TFA<sub>4</sub> (8).** Boc<sub>4</sub>-KanaA-N-acridine (7 mg, 6.6 μmoles) was dissolved in a “deprotection cocktail” (1.5 mL trifluoroacetic acid, 1.5 mL chloroform, and 100 μL of triisopropyl silane) and mixed for 30 min at RT. The reaction was then diluted into 2% acetic acid/water (30 mL) and washed with diethyl ether (3x20 mL). The aqueous phase was concentrated to a solid under reduced pressure and HPLC purified on C-18 semiprep column under isocratic conditions (12% acetonitrile in water and 0.1% TFA) ( $R_t = 9.5$  min) and lyophilized to yield 5 mg of a yellow solid (68% assuming 4 TFA counter ions). <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O, 25 °C): δ 8.25 (d,  $J = 9.2$  Hz, 2H), δ 7.79 (t,  $J = 8.4$  Hz, 2H), δ 7.64 (d,  $J = 8.8$  Hz, 2H), δ 7.38 (t,  $J = 8.4$  Hz, 2H), δ 5.01 (d,  $J = 3.6$  Hz, 1H), δ 4.99 (d,  $J = 3.6$  Hz, 1H), δ 4.51-4.48 (m, 1H), δ 4.32-4.27 (m, 1H), δ 4.13-4.07 (m, 1H), δ 3.90-3.86 (m, 1H), δ 3.63-3.57 (m, 5H), δ 3.42-3.37 (m, 3H), δ 3.28-3.21 (m, 1H), δ 3.14-3.05 (m, 2H), δ 2.98-2.93 (m, 1H), δ 2.88-2.85 (m, 1H), δ 2.31-2.29 (m, 1H), δ 1.67 (q,  $J = 13.2$  Hz, 1H). ESI Mass calculated for C<sub>31</sub>H<sub>44</sub>N<sub>6</sub>O<sub>10</sub>: 660.3 found 661.0 [M+H]<sup>+</sup>.

### S.5 Synthesis and Characterization of Neo-Neo (9):



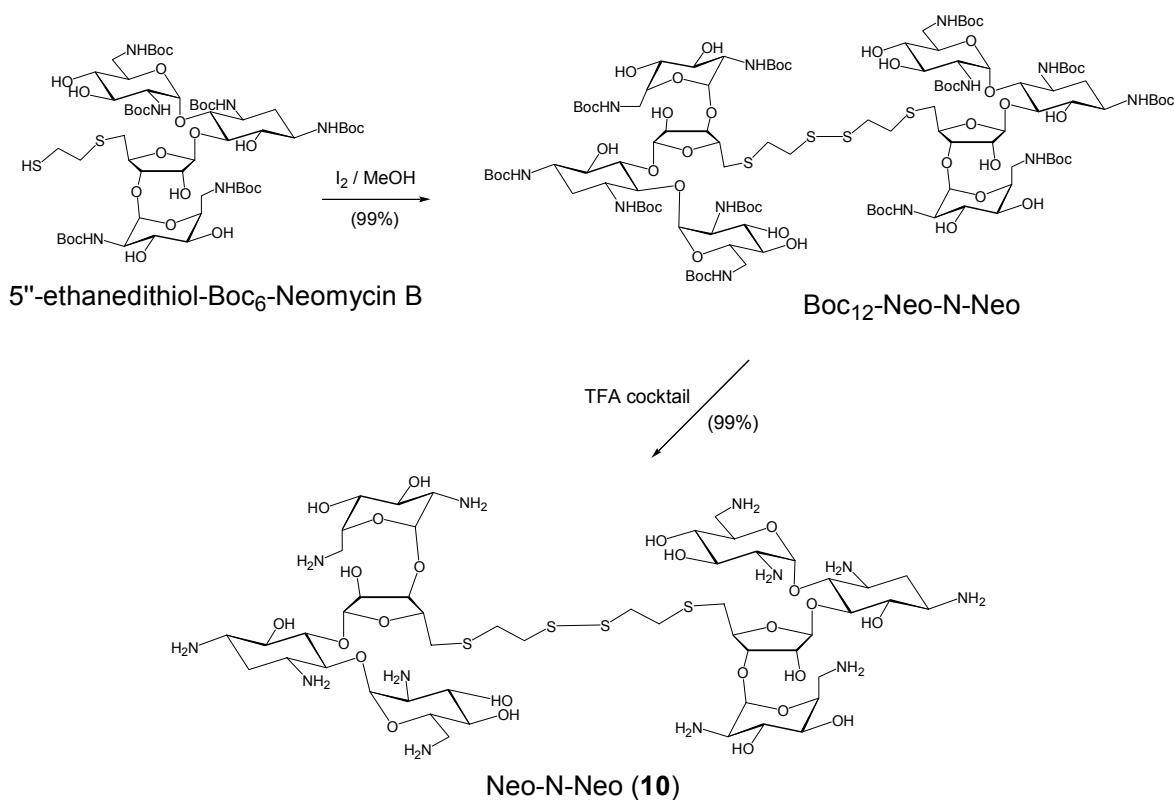
**5''  $\beta$ -mercaptoethylether-Neomycin  $\cdot$  TFA<sub>6</sub>:** 5''-O-TIPS-Boc<sub>6</sub>-Neomycin B (section S.2) (40 mg, 27  $\mu$ moles) was dissolved in DMF (1.5 mL) and treated with Cs<sub>2</sub>CO<sub>3</sub> (100 mg, 307  $\mu$ moles), and 2-mercaptoethyl ether (125  $\mu$ L, 1 mmoles, 37 equiv). The reaction was kept under argon for 7h at 30 °C, diluted into ethyl acetate (150 mL), washed with 0.1 M citric acid (50 mL), water (3x50 mL), brine (50 mL) and dried over sodium sulfate. The organic layer was then concentrated under reduced pressure, and kept under a high vacuum overnight. The crude product was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (4 mL) and treated with 1,2-ethanedithiol (20  $\mu$ L), triisopropylsilane (20  $\mu$ L), and trifluoroacetic acid (5 mL) for 15 min. at RT. The reaction was diluted into toluene (50 mL) and concentrated under vacuum at 50 °C (2x) and kept under high-vacuum for 6 h. The solid was then dissolved in 0.1% TFA in water (3 mL), filtered through glass wool and lyophilized to afford 30 mg of a white solid (79% yield, two steps). <sup>1</sup>H-NMR (400 MHz, D<sub>2</sub>O)  $\delta$  5.88 (d,  $J$  = 4.0 Hz, 1H),  $\delta$  5.23 (d,  $J$  = 3.2 Hz, 1H),  $\delta$  5.12 (s, 1H),  $\delta$  4.21-4.24 (m, 2H),  $\delta$  4.13-4.18 (m, 2H),  $\delta$  4.04 (s, 1H),  $\delta$  3.91 (t,  $J$  = 10 Hz, 1H),  $\delta$  3.82 (t,  $J$  = 9.6 Hz, 1H),  $\delta$  3.71-3.75 (m, 2H),  $\delta$  3.64 (s, 1H),  $\delta$  3.48-3.57 (m, 4H),  $\delta$  3.17-3.41 (m, 8H),  $\delta$  3.11 (d,d  $J_1$  = 13.6 Hz,  $J_2$  = 9.6 Hz, 1H),  $\delta$  2.97 (d,d  $J_1$  = 13.2 Hz,  $J_2$  = 3.6 Hz, 1H),  $\delta$  2.65-2.74 (m, 4H),  $\delta$  2.55 (t,  $J$  = 6.0 Hz, 2H),  $\delta$  2.30 (d, t  $J_1$  = 12.4 Hz,  $J_2$  = 4.4 Hz, 1H),  $\delta$  1.71 (q,  $J$  = 12.4 Hz, 1H). ESI MS calculated for C<sub>27</sub>H<sub>54</sub>N<sub>6</sub>O<sub>13</sub>S<sub>2</sub>: 734.3, found 735.3 [M+H]<sup>+</sup>.

Neo-neo (chloride salt) was first synthesized by Hai Wang (*Bioorg. Med. Chem. Lett.*, **1997**, 7, 1951-1956.). It has more recently been synthesized using this simplified method:

**Neo-Neo  $\cdot$  TFA<sub>12</sub> (9).** 5''- $\beta$ -mercaptoethylether-Neomycin B  $\cdot$  TFA<sub>6</sub> (9 mg, 6.4  $\mu$ moles) was dissolved in methanol (3 mL) and, while stirring, a dilute solution of I<sub>2</sub> (in methanol) was added drop-wise two drops past the end-point (where the reaction no longer decolorized the I<sub>2</sub> solution) and stirred and additional 15 min at RT. The methanol was then removed under reduced pressure and the solid was washed with acetonitrile (0.1 % TFA) (3x2 mL) to remove the remaining traces of I<sub>2</sub>. The white solid was then lyophilized from water (0.1% TFA) to afford 6.9 mg of a white solid (77%). <sup>1</sup>H-NMR (300 MHz, D<sub>2</sub>O)  $\delta$  5.90 (d,  $J$  = 4.0

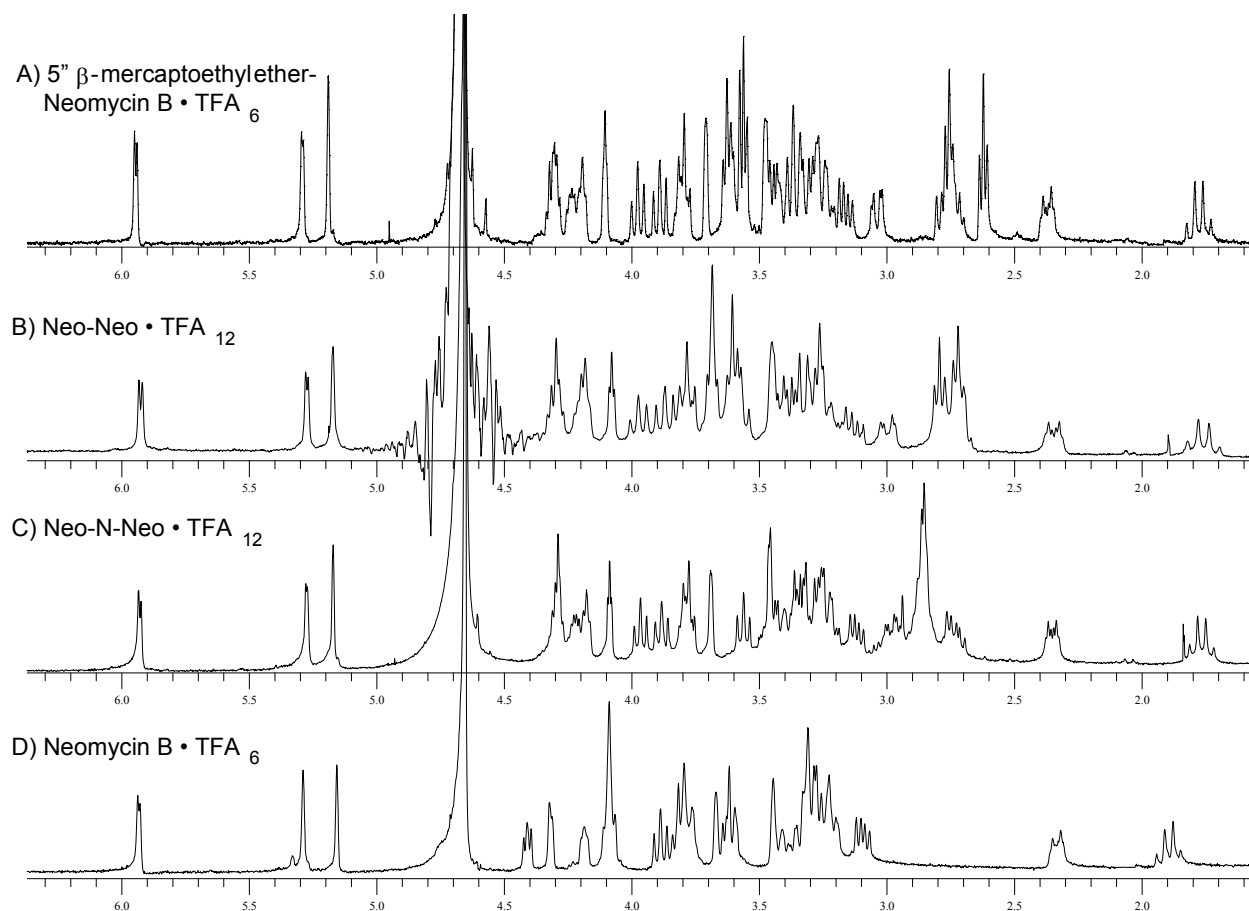
Hz, 2H),  $\delta$  5.25 (d,  $J = 3.2$  Hz, 2H),  $\delta$  5.15 (s, 2H),  $\delta$  4.25-4.29 (m, 4H),  $\delta$  4.16-4.17 (m, 4H),  $\delta$  4.06 (t,  $J = 2.8$  Hz, 2H),  $\delta$  3.95 (t,  $J = 9.8$  Hz, 2H),  $\delta$  3.85 (t,  $J = 9.9$  Hz, 2H),  $\delta$  3.73-3.79 (m, 4H),  $\delta$  3.52-3.68 (m, 10H),  $\delta$  3.20-3.43 (m, 16H),  $\delta$  3.14 (d,d  $J_1 = 13.5$  Hz,  $J_2 = 6.9$  Hz, 2H),  $\delta$  2.97 (d,d  $J_1 = 13.5$  Hz,  $J_2 = 3.6$  Hz, 2H),  $\delta$  2.68-2.79 (m, 12H),  $\delta$  2.32 (d,t  $J_1 = 12.9$  Hz,  $J_2 = 4.2$  Hz, 2H),  $\delta$  1.73 (q,  $J = 12.6$  Hz, 2H). MALDI TOF MS calculated for  $C_{54}H_{106}N_{12}O_{26}S_4$ : 1466.6, observed: 1489.7  $[M+Na]^+$

### S.6 Synthesis and Characterization of Neo-N-Neo (10):



**Boc<sub>12</sub>-Neo-N-Neo.** 5''-ethanedithiol-Boc<sub>6</sub>-Neomycin B (section S.2) (9 mg, 7  $\mu$ moles) was dissolved in 1:1  $CHCl_3$ /methanol (4 mL) and triethylamine (10  $\mu$ L, 70  $\mu$ moles, 10 equiv). A dilute  $I_2$  solution ( $\sim 3$ mg/mL in  $CHCl_3$ ) was titrated until two drops past the end-point (where the reaction no longer decolorized the  $I_2$  solution) and stirred an additional 45 min at RT. The reaction was then diluted into  $CHCl_3$  (40 mL) and washed with 1M  $NaH_2PO_4$  (2x15 mL), 1M  $Na_2CO_3$ , (2x15 mL), brine (20 mL) dried over sodium sulfate, and concentrated to a white solid under reduced pressure (9 mg, 99%).  $R_f = 0.45$  (10 % MeOH /  $CH_2Cl_2$ ).  $^1H$ -

NMR (300 MHz,  $d_3$ -MeOD)  $\delta$  5.38 (s, 2H),  $\delta$  5.15 (s, 2H),  $\delta$  4.95 (s, 2H),  $\delta$  4.24-4.28 (m, 4H),  $\delta$  4.07-4.12 (m, 2H),  $\delta$  3.89-3.41 (m, 4H),  $\delta$  3.72-3.78 (m, 4H),  $\delta$  3.17-3.57 (m, 24H),  $\delta$  2.89-2.95 (m, 14H),  $\delta$  1.93-1.99 (m, 2H),  $\delta$  1.27-1.48 (m, 112H).

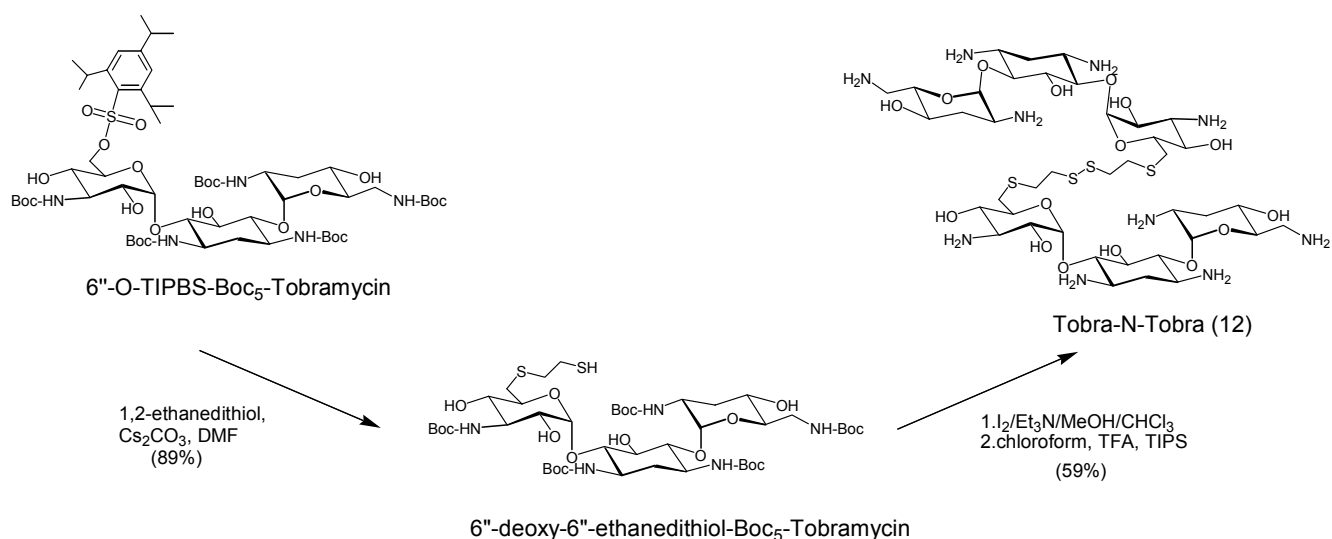


**Figure S.1:**  $^1\text{H}$  NMR of 5''  $\beta$ -mercaptoethylether-neomycin B before and after dimerization (**A** and **B**, respectively). Notice that the only significant changes are in the “linker region” at 2.6 and 3.6 ppm.  $^1\text{H}$  NMR of Neo-N-Neo (**C**); again the only significant differences between Neo-Neo and Neo-N-Neo are in the “linker region”. The  $^1\text{H}$  NMR spectrum of neomycin B  $\cdot$   $\text{TFA}_6$  is also shown (**D**). All spectra were taken in  $\text{D}_2\text{O}$  on either a 300 or 400 MHz Varian NMR.

**Neo-N-Neo  $\cdot$   $\text{TFA}_{12}$  (10).**  $\text{Boc}_6$ -Neo-N-Neo (9 mg, 7  $\mu\text{moles}$ ) was dissolved in  $\text{CHCl}_3$  (1.5 mL), tiisopropylsilane (100  $\mu\text{L}$ ), TFA (1.5 mL), and stirred RT for 10 min. The reaction was then partitioned into water (40 mL) and diethyl ether (20 mL), the ether was discarded and the aqueous phase was washed with 1:30 methanol / diethyl ether (3x20 mL). The

aqueous phase was then concentrated to a solid under vacuum. The solid was dissolved in 4 mL 0.1% TFA / water and lyophilized to yield a white solid (9 mg, 99%).  $^1\text{H-NMR}$  (400 MHz,  $\text{D}_2\text{O}$ )  $\delta$  5.92 (d,  $J = 4.0$  Hz, 2H),  $\delta$  5.27 (d,  $J = 2.0$  Hz, 2H),  $\delta$  5.17 (s, 2H),  $\delta$  4.27-4.31 (m, 4H),  $\delta$  4.21 (q,  $J = 4.0$  Hz, 2H),  $\delta$  4.17 (t,  $J = 4.8$  Hz, 2H),  $\delta$  4.08 (t,  $J = 2.4$  Hz, 2H),  $\delta$  3.96 (t,  $J = 9.6$  Hz, 2H),  $\delta$  3.88 (t,  $J = 10$  Hz, 2H),  $\delta$  3.75-3.80 (m, 4H),  $\delta$  3.68 (d,  $J = 2.4$  Hz, 2H),  $\delta$  3.56 (t,  $J = 9.8$  Hz, 2H),  $\delta$  3.18-3.46 (m, 14H),  $\delta$  3.11 (d,d  $J_1 = 9.2$  Hz,  $J_2 = 7.6$  Hz, 2H),  $\delta$  2.98 (d,d  $J_1 = 13.6$  Hz,  $J_2 = 4.0$  Hz, 2H),  $\delta$  2.84-3.02 (m, 10H),  $\delta$  2.73 (d,d  $J_1 = 13.2$  Hz,  $J_2 = 6.0$  Hz, 2H),  $\delta$  2.35 (d,t  $J_1 = 12.8$  Hz,  $J_2 = 4.0$  Hz, 2H),  $\delta$  1.76 (q,  $J = 12.4$  Hz, 2H). MALDI TOF MS calculated for  $\text{C}_{50}\text{H}_{98}\text{N}_{12}\text{O}_{24}\text{S}_4$ :1378.6, observed: 1401.7  $[\text{M}+\text{Na}]^+$ .

### S.7 Synthesis and Characterization of Tobra-N-Tobra (12):



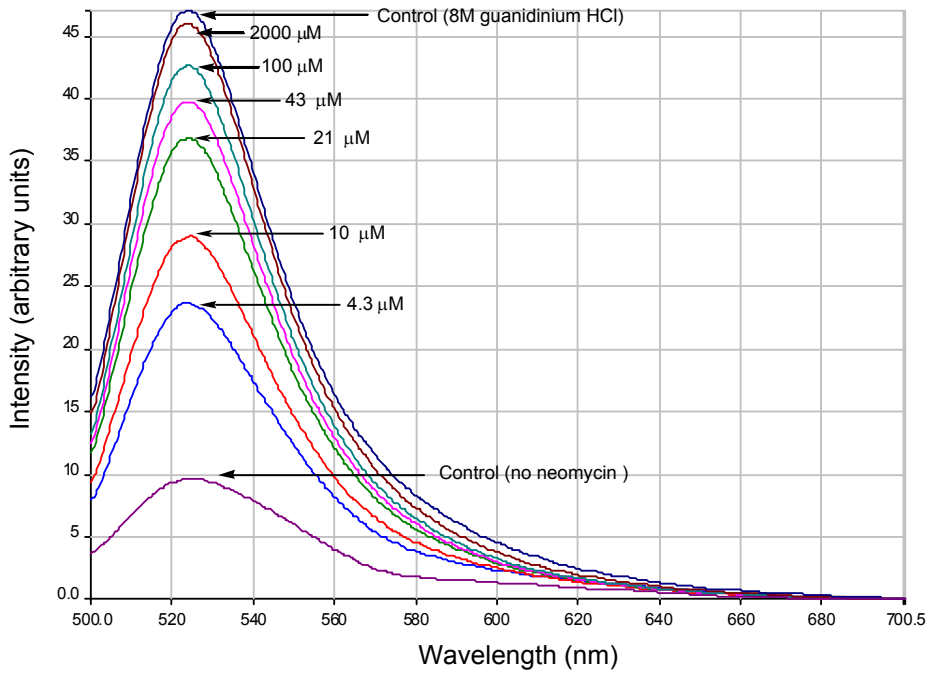
**6''-O-TIPBS-Boc<sub>5</sub>-Tobramycin.** Synthesized as described (Hai Wang Ph. D. Thesis, University of California, San Diego, 1998).

**6''-deoxyethanedithiol-Boc<sub>5</sub> Tobramycin.**  $\text{Cs}_2\text{CO}_3$  (277 mg, 851  $\mu\text{moles}$ ) and 6''-O-TIPBS-Boc<sub>5</sub>-Tobramycin (50 mg, 40.5  $\mu\text{moles}$ ) were dissolved in 2 mL dry DMF in a 10 mL flask. To this solution, 1,2-ethanedithiol (373  $\mu\text{L}$ , 4.45  $\text{mmoles}$ ) was added under argon. After stirring at room temperature for 6 hours, the reaction was diluted into 100 mL EtOAc.

The organic phase was washed with saturated NaHCO<sub>3</sub> (2x50 mL), H<sub>2</sub>O (50 mL), brine (50 mL) then concentrated under reduced pressure. The crude product was purified on ~50 mL of silica gel using standard flash chromatography and a 1– 5% methanol/CH<sub>2</sub>Cl<sub>2</sub> gradient, yielding a white solid (40 mg, 89%). R<sub>f</sub> = 0.7 (10% MeOH / CH<sub>2</sub>Cl<sub>2</sub>). <sup>1</sup>H NMR (400 MHz, d<sub>3</sub>-MeOD, 25 °C): δ 5.10 (s, 1H), δ 5.04 (s, 1H), δ 4.09 (m, 1H), δ 3.61 (m, 2H), δ 3.49-3.35 (m, 12H), δ 3.02 (m, 1H), δ 2.91(t, *J* = 7.2 Hz, 2H), δ 2.80 (m, 1H), δ 2.69 (t, *J* = 7.6 Hz, 2H), δ 2.18-2.11 (m, 1H), δ 2.01 (m, 1H), δ 1.65-1.60 (m, 1H), δ 1.46-1.44 (m, 45H).

**Tobra-N-Tobra · TFA<sub>10</sub> (12).** 6"-deoxyethanedithiol-Boc<sub>5</sub>-Tobramycin (20 mg, 19.2 μmoles) and triethylamine (28 μL, 192 μmoles) were dissolved in 3 mL CHCl<sub>3</sub> and 3 mL MeOH. To this solution, a solution of I<sub>2</sub> (3 mg I<sub>2</sub>/mL CHCl<sub>3</sub>) was added dropwise until no the solution no longer decolorized the I<sub>2</sub> solution. After stirring at room temperature for 45 minutes the reaction was diluted into 50 mL CHCl<sub>3</sub> and washed with 50 mL saturated NaHCO<sub>3</sub> and 50 mL brine. The organic phase was dried over Na<sub>2</sub>SO<sub>4</sub> and concentrated to a white solid under reduced pressure. The crude product was then dissolved in a "deprotection cocktail" (2 mL trifluoroacetic acid, 2 mL chloroform, and 100 μL of triisopropylsilane) and stirred for 30 min at RT. The reaction was then diluted into 2% acetic acid/water (40 mL) and washed with diethyl ether (3x20 mL). The aqueous phase was concentrated to a solid under reduced pressure and HPLC purified on C-18 semiprep column under isocratic conditions (11% acetonitrile in water and 0.1% TFA) (R<sub>t</sub> = 10 min) and lyophilized to yield 12.8 mg of a white solid (59% assuming 10 TFA counter ions). <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O, 25 °C): δ 5.61 (s, 2H), δ 4.94 (s, 2H), δ 3.87-3.25 (m, 26H), δ 3.10(m, 2H), δ 3.02(t, *J* = 7.2 Hz, 4H), δ 2.94-2.90 (m, 2H), δ 2.80 (m, 4H), δ 2.69 (m, 2H), δ 2.41-2.39 (m, 2H), δ 2.15-2.13 (m, 2H), δ 1.89-1.78 (m, 4H). ESI Mass calculated for C<sub>40</sub>H<sub>80</sub>N<sub>10</sub>O<sub>16</sub>S<sub>4</sub>: 1084.46 found 1086.0 [M+H]<sup>+</sup>.

### S.8 Representative Data for the Displacement of RevFI from the Solid-Phase Immobilized RRE:



**Figure S.2:** Raw data from 8 solid-phase displacement experiments showing the fluorescence emission of the supernatant (diluted into "quantification buffer") as a function of neomycin B concentration. To reduce light scattering at 500 nm, excitation of RevFI is at 470nm. Excitation at 490nm also yields the same IC<sub>50</sub> values.