Inhibition of the ANT(2")-Ia Resistance Enzyme and Rescue of Aminoglycoside Antibiotic Activity by Synthetic α-Hydroxytropolones

Danielle Hirsch,[†,‡] Georgina Cox,[§] Michael D'Erasmo,[†,‡] Tushar Shakya,[§] Christine Meck,[†,‡] Noushad Mohd,[†] Gerard D. Wright,[§] and Ryan P. Murelli[†,‡,*]

[†]*Department of Chemistry, Brooklyn College, The City University of New York, 2900 Bedford Avenue, Brooklyn College 11210, United States*
[‡]*Department of Chemistry, The Graduate Center, The City University of New York, 365 Fifth Avenue, New York, NY 10016, United States*
[§]*M. G. DeGroote Institute for Infectious Disease Research, McMaster University, 1200 Main Street, West Hamilton, ON L8N 4K1,Canada*

Procedures for Synthesis and Biological Studies

Table of Contents

General Information. All starting materials and reagents were purchased from commercially available sources and used without further purification, with exception of CH_2Cl_2, which was purified on a solvent purification system prior to the reaction. ^{1}H NMR shifts are measured using the solvent residual peak as the internal standard ($CHCl_3$ δ 7.26), and reported as follows: chemical shift, multiplicity (s = singlet, bs = broad singlet, d = doublet, t = triplet, dd = doublet of doublet, q = quartet, m = multiplet), coupling constant

(Hz), and integration. ^{13}C NMR shifts are measured using the solvent residual peak as the internal standard ($CHCl_3$ δ 77.20), and reported as chemical shifts. Infrared (IR) spectral bands are characterized as broad (br), strong (s), medium (m), and weak (w). Microwave reactions were performed via the Biotage Initiator 2.5. Column chromatography was performed on the Biotage Isolera Prime, with Biotage SNAP 10g or 25g silica gel cartridges, in a solvent system of ethyl acetate and hexanes.

3-methoxy-5-methyl-6-(naphthalen-1-yl)-8-oxabicyclo[3.2.1]octa-3,6-dien-2-one (**2j**). To a solution of salt **1a** (108.2 mg, 0.373 mmol) and 1-ethynylnapthalene (527 μL, 3.73 mmol) in $CDCl_3$ (746 μL) was added N,N-diisopropylaniline (87 μL, 0.447 mmol). After microwave irradiation at 100 °C for 60 min, the reaction mixture was purified by chromatography (Biotage Isolera Prime, 10 g silica gel column, solvent gradient: 5% EtOAc in hexanes (3 CV); 2-5% EtOAc in hexanes (8 CV); 5-10% EtOAc in hexanes (10 CV); 10-20% EtOAc in hexanes (10 CV); 20-35% EtOAc in hexanes (8 CV)). Product fractions were concentrated to yield **2j** as an orange oil (85.7 mg, 79% yield). Rf= 0.30 in 25% EtOAc in hexanes. **IR** (thin film, KBr) 3057 (w), 2978 (w), 2930 (w), 2836 (w), 1710 (s), 1605 (m) 1345 (w), 1268 (w), 1132 (m), 1117 (w), 989 (w), 778 (w) cm-1. **^{1}H NMR** (200 MHz, $CDCl_3$) δ 8.05 – 7.94 (m, 1H), 7.91 – 7.67 (m, 2H), 7.58 – 7.35 (m, 3H), 7.22 (d, J = 1.2 Hz, 1H), 6.39 (d, J = 2.2 Hz, 1H), 6.16 (s, 1H), 5.18 (d, J = 2.5 Hz, 1H), 3.67 (s, 3H), 1.51 (s, 3H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 190.22 (s), 156.46 (s), 145.93 (s), 134.03 (s), 131.60 (s), 131.56 (s), 128.84 (s), 128.70 (s), 126.76 (s), 126.67 (s), 126.47 (s), 125.67 (s), 125.09 (s), 123.68 (s), 120.19 (s), 88.18 (s), 86.77 (s), 55.02 (s), 21.64 (s). **HRMS (ESI+)** *m/z* calc'd for $C_{19}H_{17}O_3$ (M+H): 293.1172. Found: 293.1175.

3-methoxy-5-methyl-6-(naphthalen-2-yl)-8-oxabicyclo[3.2.1]octa-3,6-dien-2-one (**2k**). To a solution of salt **1a** (17.0 mg, 0.058 mmol) and 2-ethynylnapthalene (88.9 mg, 0.584 mmol) in $CDCl_3$ (117 μL) was added N,N-diisopropylaniline (13.7 μL, 0.070 mmol). After microwave irradiation at 100 °C for 60 min, the reaction mixture was purified by chromatography (Biotage Isolera Prime, 10 g silica gel column, solvent gradient: 5% EtOAc in hexanes (3 CV); 2-5% EtOAc in hexanes (8 CV); 5-10% EtOAc in hexanes (10 CV); 10-20% EtOAc in hexanes (10 CV); 20-35% EtOAc in hexanes (8 CV). Product fractions were concentrated to yield **2k** as a yellow oil (14.4 mg, 84% yield). Rf= 0.28 in 25% EtOAc in hexanes. IR (thin film, KBr) 3056 (w), 2963 (w), 2933 (w), 2837 (w), 1708 (m), 1605 (w), 1174 (w), 1344 (w), 1131 (m), 1101 (w), 867 (w), 694 (w) cm-1. ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.89 – 7.80 (m, 3H), 7.71 (d, J = 1.5 Hz,

1H), 7.55 – 7.48 (s, 1H), 7.44 (d, J = 1.7 Hz, 1H), 7.40 (d, J = 1.8 Hz, 1H), 6.41 (d, J = 2.5 Hz, 1H), 6.30 (s, 1H), 5.05 (d, J = 2.5 Hz, 1H), 3.64 (s, 3H), 1.76 (s, 3H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 190.17 (s), 159.03 (s), 146.36 (s), 133.49 (s), 133.39 (s), 130.68 (s), 128.88 (s), 128.47 (s), 128.10 (s), 127.07 (s), 127.02 (s), 125.04 (s), 124.44 (s), 123.68 (s), 119.45 (s), 86.80 (s), 86.16 (s), 55.12 (s), 22.65 (s). **HRMS (ESI+)** *m/z* calc'd for $C_{19}H_{16}O_3Na$ (M+Na): 315.0992. Found: 315.0997.

6-(4-chlorophenyl)-3-methoxy-5-methyl-8-oxabicyclo[3.2.1]octa-3,6-dien-2-one (**2m**). To a solution of salt **1a** (100.0 mg, 0.344 mmol) and 1-chloro-4-ethynylbenzene (469.83 mg, 3.44 mmol) in $CDCl_3$ (689 μL) was added N,N-diisopropylaniline (80 μL, 0.413 mmol). After microwave irradiation at 100 °C for 60 min, the reaction mixture was purified by chromatography (Biotage Isolera Prime, 10 g silica gel column, solvent gradient: 5% EtOAc in hexanes (3 CV); 2-5% EtOAc in hexanes (8 CV); 5-10% EtOAc in hexanes (10 CV); 10-20% EtOAc in hexanes (10 CV); 20-35% EtOAc in hexanes (8 CV)). Product fractions were concentrated to yield **2m** as a yellow solid (37.8 mg, 40% yield). Melting Point (MP) = 138-141 °C. Rf= 0.27 in 25% EtOAc in hexanes. IR (thin film, KBr) 3066 (w), 2974 (w), 2935 (w), 2839 (w), 1711 (s), 1604 (m), 1490 (w), 1174 (w), 1131 (w), 1092 (w), 864 (w), 827 (w) cm-1. ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.36 (d, J = 8.7 Hz, 2H), 7.21 (d, J = 8.7 Hz, 2H), 6.29 (d, J = 2.3 Hz, 1H), 6.16 (s, 1H), 4.98 (d, J = 2.5 Hz, 1H), 3.60 (s, 3H), 1.66 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 189.89 (s), 157.91 (s), 146.22 (s), 134.83 (s), 131.68 (s), 129.23 (s), 127.52 (s), 123.89 (s), 119.02 (s), 86.52 (s), 85.99 (s), 55.01 (s), 22.27 (s). **HRMS (ESI+)** *m/z* calc'd for $C_{15}H_4ClO_3$ (M+H): 277.0626. Found: 277.0622.

3-methoxy-5-methyl-6-(4-(trifluoromethyl)phenyl)-8-oxabicyclo[3.2.1]octa-3,6-dien-2-one (**2n**). To a solution of salt **1a** (100.2 mg, 0.345 mmol) and 1-ethynyl-4-(trifluoromethyl)benzene (563 μL, 3.45 mmol) in $CDCl_3$ (690 μL) was added N,N-diisopropylaniline (80 μL, 0.414 mmol). After microwave irradiation at 100 °C for 60 min, the reaction mixture was purified by chromatography (Biotage Isolera Prime, 25 g silica gel column, solvent gradient: 5% EtOAc in hexanes (3 CV); 2-5% EtOAc in hexanes (8 CV); 5-10% EtOAc in hexanes (10 CV); 10-20% EtOAc in hexanes (10 CV); 20-35% EtOAc in hexanes (8 CV)). Product fractions were concentrated to yield **2n** as a yellow oil (77.6 mg, 72% yield). Rf= 0.30 in 25% EtOAc in hexanes. IR (thin film, KBr) 3063 (w), 2981 (w), 2938 (w), 2840 (w), 1713 (s), 1606 (m), 1410 (w), 1327 (s), 1129 (s), 1016 (m), 865 (w), 832 (w) cm-1. ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.63 (d, J = 8.1 Hz, 2H), 7.38 (d, J = 8.1 Hz, 2H), 6.40 (d, J = 2.5 Hz, 1H), 6.16 (s, 1H), 5.01 (d, J = 2.5 Hz, 1H), 3.60 (s,

1H), 1.66 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 189.76 (s), 157.84 (s), 146.31 (s), 136.93 (s), 130.89 (q, J = 32.8 Hz), 126.60 (s), 126.03 (q, J = 3.8 Hz), 125.85 (s), 124.18 (q, J = 272.1 Hz), 118.92 (s), 86.65 (s), 86.20 (s), 55.09 (s), 22.27 (s). **HRMS (ESI+)** *m/z* calc'd for $C_{16}H_{14}F_3O_3$ (M+H): 311.0890. Found: 311.0890.

6-isobutyryl-3-methoxy-5-methyl-8-oxabicyclo[3.2.1]octa-3,6-dien-2-one (**2o**). To a solution of salt **1a** (258.3 mg, 0.890 mmol) and 4-methylpent-1-yn-3-one (427.64 mg, 4.45 mmol) in $CDCl_3$ (3 mL) was added N,N-diisopropylaniline (433 μL, 2.23 mmol). After microwave irradiation at 100°C for 45 min, the reaction mixture was purified by chromatography (Biotage Isolera Prime, 10 g silica gel column, solvent gradient: 2% EtOAc in hexanes (3 CV); 2-5% EtOAc in hexanes (8 CV); 5-10% EtOAc in hexanes (12 CV); 10-15% EtOAc in hexanes (8 CV); 15-25% EtOAc in hexanes (10 CV); 25-35% EtOAc in hexanes (7 CV)). Product fractions were concentrated to yield **2o** as a yellow oil (148.1 mg, 70% yield). Rf= 0.34 in 20% EtOAc in hexanes. IR (thin film, KBr) 3061 (s), 2974 (m), 2936 (w), 1713 (s), 1670 (s), 1610 (s), 1459 (w), 1343 (m), 1291 (m), 1180 (m), 1128 (s), 1048 (m), 873 (w) cm-1. ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.93 (d, J = 2.2 Hz, 1H), 6.06 (s, 1H), 4.98 (d, J = 2.4 Hz, 1H), 3.46 (s, 3H), 3.09 – 2.94 (m, 1H), 1.61 (s, 3H), 1.07 (d, J = 7.1 Hz, 3H), 1.03 (d, J = 6.6 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 201.47 (s), 189.01 (s), 155.67 (s), 145.15 (s), 136.90 (s), 120.03 (s), 86.42 (s), 86.14 (s), 54.81 (s), 38.15 (s), 21.22 (s), 19.76 (s), 17.79 (s). **HRMS (ESI+)** *m/z* calc'd for $C_{13}H_{17}O_4$ (M+H): 237.1121. Found: 237.1124.

6-(cyclohexanecarbonyl)-3-methoxy-5-methyl-8-oxabicyclo[3.2.1]octa-3,6-dien-2-one (**2p**). To a solution of salt **1a** (94 mg, 0.324 mmol) and 1-cyclohexylprop-2-yn-1-one (220.5 mg, 1.62 mmol) in $CDCl_3$ (2 μL) was added N,N-diisopropylaniline (75.7 μL, 0.390 mmol). After microwave irradiation at 100°C for 45 min, the reaction mixture was purified by chromatography (silica gel, 18 cm x 1.8 cm, solvent gradient: 5% EtOAc in hexanes (100 mL); 10% EtOAc in hexanes (100 mL); 20% EtOAc in hexanes (200 mL); 30% EtOAc in hexanes (100 mL)). Product fractions were concentrated to yield **2p** as a yellow oil (78.23 mg, 87% yield). Rf= 0.24 in 15% EtOAc in hexanes. IR (thin film, KBr) 3057 (w), 2933 (s), 2855 (s), 1712 (s), 1667 (s), 1609 (s), 1450 (m), 1341 (m), 1179 (m), 1128 (s), 871 (m), 746 (m) cm-1. ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.91 (d, J = 2.5 Hz, 1H), 6.05 (s, 1H), 4.97 (d, J = 2.6 Hz, 1H), 3.45 (s, 3H), 2.73 (tt, J = 11.2, 3.3 Hz, 1H), 1.59 (s, 3H), 1.84 – 1.05 (m, 10H). ^{13}C N3MR (100 MHz, $CDCl_3$) δ 200.87 (s), 189.01 (s), 155.74 (s), 145.12 (s), 136.64 (s), 120.06 (s), 86.40 (s), 86.12 (s), 54.78 (s), 48.18 (s), 30.12 (s), 27.93 (s), 26.01

(s), 25.86 (s), 25.26 (s), 21.22 (s). **HRMS (ESI+)** *m/z* calc'd for $C_{16}H_{21}O_4$ (M+H): 277.1434. Found: 277.1439.

6-(2-cyclohexylacetyl)-3-methoxy-5-methyl-8-oxabicyclo[3.2.1]octa-3,6-dien-2-one (**2q**). To a solution of salt **1a** (106 mg, 0.363 mmol) and 1-cyclohexylbut-3-yn-2-one (272.8 mg, 1.82 mmol) in $CDCl_3$ (4 mL) was added triethylamine (60.7 μL, 0.436 mmol). After microwave irradiation at 100 °C for 35 min, the reaction mixture was purified by chromatography (Biotage Isolera Prime, 10 g silica gel column, solvent gradient: 2% EtOAc in hexanes (3 CV); 2-5% EtOAc in hexanes (10 CV); 5-10% EtOAc in hexanes (15 CV); 10-20% EtOAc in hexanes (10 CV); 20-35% EtOAc in hexanes (8 CV)). Product fractions were concentrated to yield **2q** as a yellow/brown oil (67.7 mg, 64% yield). Rf= 0.20 in 15% EtOAc in hexanes. IR (thin film, KBr) 3052 (w), 2924 (s), 2851 (m), 1713 (m), 1668 (m), 1609 (m), 1449 (w), 1178 (w), 1127 (m), 1047 (w), 987 (w), 869 (w), 773 (w) cm-1. ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.96 (d, J = 2.3 Hz, 1H), 6.07 (s, 1H), 5.01 (d, J = 2.4 Hz, 1H), 3.50 (s, 3H), 2.55 (dd, J = 15.3, 6.5 Hz, 1H), 2.44 (dd, J = 15.3, 7.3 Hz, 1H), 1.87 – 1.74 (m, 1H), 1.67 (s, 3H), 1.65 – 1.55 (m, 4H), 1.31 – 0.81 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 197.12 (s), 188.84 (s), 156.63 (s), 144.93 (s), 137.58 (s), 119.68 (s), 86.12 (s), 85.78 (s), 54.60 (s), 47.78 (s), 34.58 (s), 33.31 (s), 33.04 (s), 26.04 (s), 26.00 (s), 25.94 (s), 21.17 (s). **HRMS (ESI+)** *m/z* calc'd for for $C_{17}H_{22}NaO_4$ (M+Na): 313.1410. Found: 313.1419

4-acetyl-2-hydroxy-7-methoxy-5-methylcyclohepta-2,4,6-trienone (**4e**). To a solution of the previously-made corresponding 8-oxabicyclo[3.2.1]octane intermediate **2e** (20.0 mg, 0.0961 mmol) in $CHCl_3$ (960 μL) was added triflic acid (33.92 μL, 0.385 mmol). The reaction was allowed to stir for 30 minutes at rt before quenching with pH 7 phosphate buffer and extracting with CH_2Cl_2. Combined organics were dried over Na_2SO_4, filtered, and concentrated under reduced pressure to yield **4e** as a pale green/brown solid that melts at 164-167°C (3.3 mg, 17% yield). IR (thin film, KBr) 3245 (m), 2998 (w), 1703 (m), 1577 (w), 1559 (s), 1455 (w), 1330 (m), 1296 (m), 1265 (m), 1229 (w), 1201 (w), 1034 (w), 787 (w). ^{1}H NMR (200 MHz, CDCl3) δ 7.26 (s, 1H), 7.02 (s, 1H), 4.02 (s, 3H), 2.53 (s, 3H), 2.43 (s, 3H). ^{13}C NMR (100 MHz, CDCl3) δ 204.36 (s), 171.08 (s), 159.50 (s), 159.42 (s), 140.99 (s), 134.04 (s), 122.46 (s), 115.27 (s), 56.88 (s), 31.02 (s), 25.12 (s). **HRMS (ESI+)** *m/z* calc'd for $C_{11}H_{13}O_4$ (M+H): 209.0808. Found: 209.0816.

2,7-dihydroxy-4-methyl-5-phenylcyclohepta-2,4,6-trienone (**3i**). To a solution of previously-made 8-oxabicyclo[3.2.1]octene intermediate **2i** (13.7 mg, 0.0566 mmol) in $CHCl_3$ (566 μL) was added triflic acid

(20.0 μL, 0.226 mmol). The reaction was allowed to stir for 30 minutes at rt before quenching with sodium acetate (46.43 mg, 0.566 mmol). To this mixture was added 1.87 mL 33% HBr/AcOH solution (twice the solvent volume). The reaction was heated to 130°C for 4 hours before being quenched to pH 5 with 10 mL of pH 7 phosphate buffer. The organic layer was isolated and the aqueous layer was extracted with CH_2Cl_2 (5 x 10 mL). Combined organics were dried over Na_2SO_4, filtered, and concentrated under reduced pressure to yield **3i** as a black oil (13.0 mg, 95% yield). IR (thin film, KBr) 3727 (w), 3702 (w), 3625 (w), 3599 (w), 3247 (br), 2924 (w), 2854 (w), 1527 (m), 1392 (m), 1279 (m), 1129 (w), 768 (w), 669 (w) cm-1. ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.58 (s, 1H), 7.50 (s, 1H), 7.48 – 7.33 (m, 3H), 7.23 (dd, J = 7.6, 1.8 Hz, 2H), 2.26 (s, 3H). ^{13}C NMR (200 MHz, $CDCl_3$) δ 167.11 (s), 157.65 (s), 156.39 (s), 143.74 (s), 143.60 (s), 138.93 (s), 128.59 (s), 128.25 (s), 127.68 (s), 124.27 (s), 124.11 (s), 26.42 (s). **HRMS (ESI+)** *m/z* calc'd for $C_{14}H_{13}O_3$ (M+H): 299.0859. Found: 299.0865.

2,7-dihydroxy-4-methyl-5-(naphthalen-1-yl)cyclohepta-2,4,6-trienone (**3j**). To a solution of bicycle **2j** (35.6 mg, 0.1218 mmol) in CH_2Cl_2 (1.22 mL) was added triflic acid (43.1 μL, 0.487 mmol). The reaction was allowed to stir for 30 minutes at rt before quenching to pH 7 with pH 7 phosphate buffer and extracting with CH_2Cl_2 (5 x 10 mL). Combined organics were dried over Na_2SO_4, filtered, and concentrated under reduced pressure. To the isolated methoxytropolone (18.2 mg) was added 800 μL 33% HBr/AcOH solution. The reaction was heated to reflux (120 °C) for 7 hours before being quenched to pH 6 with 10 mL of pH 7 phosphate buffer. The organic layer was isolated and the aqueous layer was extracted with CH_2Cl_2 (5 x 10 mL). Combined organics were dried over Na_2SO_4, filtered, and concentrated under reduced pressure to yield **3j** as a brown oil (13.4 mg, 77% yield). IR (thin film, KBr) 3248 (br), 1526 (m), 1447 (w), 1383 (m), 1278 (w), 1239 (w), 1220 (w), 1086 (w), 783 (w), 730 (w) cm-1. ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.92 (dd, J = 7.8, 4.3 Hz, 2H), 7.63 (s, 1H), 7.52 (s, 1H), 7.61 – 7.27 (m, 5H), 2.08 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.87 (s), 158.28 (s), 156.75 (s), 147.44 (s), 142.07 (s), 138.91 (s), 130.97 (s), 130.57 (s), 130.25 (s), 129.23 (s), 129.10 (s), 126.02 (s), 125.68 (s), 124.47 (s), 123.67 (s), 122.98 (s), 29.97 (s), 26.51 (s). **HRMS (ESI+)** *m/z* calc'd for $C_{18}H_{15}O_3$ (M+H): 279.1016. Found: 279.1017.

2,7-dihydroxy-4-methyl-5-(naphthalen-2-yl)cyclohepta-2,4,6-trienone (**3k**). To a solution of bicycle **2k** (42.4 mg, 0.145 mmol) in CH_2Cl_2 (1.45 mL) was added triflic acid (51.3 μL, 0.580 mmol). The reaction was allowed to stir for 30 minutes at rt before quenching to pH 6 with pH 7 phosphate buffer and extracting

with CH_2Cl_2 (5 x 10 mL). Combined organics were dried over Na_2SO_4, filtered, and concentrated under reduced pressure. To the isolated methoxytropolone (15.6 mg) was added 686 μL 33% HBr/AcOH solution. The reaction was heated to reflux (120 °C) for 8 hours before being quenched to pH 6 with 10 mL of pH 7 phosphate buffer. The organic layer was isolated and the aqueous layer was extracted with CH_2Cl_2 (5 x 10 mL). Combined organics were dried over Na_2SO_4, filtered, and concentrated under reduced pressure to yield **3k** as a brown oil (12.5 mg, 85% yield). IR (thin film, KBr) 3248 (br), 1526 (w), 1446 (w), 1392 (w), 1284 (w), 1231 (w), 1205 (w), 1122 (w), 1092 (w), 907 (w), 858 (w) 795 (w), 730 (w) cm-1. ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.89 (m, 3H), 7.71 (s, 1H), 7.61 (s, 1H), 7.59 (s, 1H), 7.55 (dd, J = 2.9 Hz, 1H), 7.55 (d, J = 9.5 Hz, 1H), 7.36 (dd, J = 8.4, 1.6 Hz, 1H), 2.29 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.62 (s), 158.13 (s), 156.79 (s), 143.95 (s), 141.39 (s), 139.43 (s), 133.58 (s), 132.87 (s), 128.64 (s), 128.40 (s), 128.14 (s), 127.52 (s), 127.06 (s), 126.87 (s), 126.76 (s), 124.59 (s), 53.75 (s), 26.82 (s). **HRMS (ESI+)** *m/z* calc'd for $C_{18}H_{15}O_3$ (M+H): 279.1016. Found: 279.1022.

4-(4-bromophenyl)-2,7-dihydroxy-5-methylcyclohepta-2,4,6-trienone (**3l**). To a solution of previously-made 8-oxabicyclo[3.2.1]octene intermediate **2l** (17.6 mg, 0.0548 mmol) in CH_2Cl_2 (548 μL) was added triflic acid (19.4 μL, 0.219 mmol). The reaction was allowed to stir for 30 minutes at rt before quenching to pH 6 with pH 7 phosphate buffer and extracting with CH_2Cl_2 (5 x 10 mL). Combined organics were dried over Na_2SO_4, filtered, and concentrated under reduced pressure. To the isolated methoxytropolone (12.5 mg) was added 550 μL 33% HBr/AcOH solution. The reaction was heated to reflux (120 °C) for 4 hours before being quenched to pH 5 with 10 mL of pH 7 phosphate buffer. The organic layer was isolated and the aqueous layer was extracted with CH_2Cl_2 (5 x 10 mL). Combined organics were dried over Na_2SO_4, filtered, and concentrated under reduced pressure to yield **3l** as an orange/brown oil (10.3 mg, 86% yield). IR (thin film, KBr) 3248 (br), 1528 (m), 1487 (w), 1447 (w), 1388 (w), 1279 (w), 1208 (w), 1128 (w), 1092 (w), 1072 (w), 1012 (w), 814 (w), 669 (w) cm-1. ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.58 (d, J = 6.8 Hz, 2H), 7.43 (s, 1H), 7.26 (s, 1H), 7.12 (d, J = 8.4 Hz, 2H), 2.23 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.61 (s), 158.35 (s), 156.55 (s), 142.76 (s), 142.52 (s), 139.09 (s), 132.21 (s), 130.36 (s), 124.54 (s), 124.00 (s), 122.43 (s), 26.79 (s). **HRMS (ESI+)** *m/z* calc'd for $C_{14}H_{12}BrO_3$ (M+H): 306.9964. Found: 306.9966

4-(4-chlorophenyl)-2,7-dihydroxy-5-methylcyclohepta-2,4,6-trienone (**3m**). To a solution of bicycle **2m** (19.2 mg, 0.0694 mmol) in CH_2Cl_2 (694 μL) was added triflic acid (24.5 μL, 0.278 mmol). The reaction

was allowed to stir for 30 minutes at rt before quenching with sodium acetate (56.9 mg, 0.693 mmol). After stirring for an additional 15 minutes, the solution was concentrated under reduced pressure. To the mixture was added 696 μL AcOH and 151 μL 33% HBr/AcOH solution. The reaction was heated to 90 °C for 4 hours before being quenched to pH 5 with 10 mL of pH 7 phosphate buffer. The organic layer was isolated and the aqueous layer was extracted with CH_2Cl_2 (5 x 10 mL). Combined organics were dried over Na_2SO_4, filtered, and concentrated under reduced pressure. Upon finding clean conversion to the methoxytropolone, the product was resubjected to the HBr/AcOH reflux conditions (847 μL 33% HBr/AcOH solution, 120 °C) for two hours to yield **3m** as a black oil (16.5 mg, 90% yield). IR (thin film, KBr) 3066 (w), 2974 (w), 2935 (w), 2839 (w), 1711 (s), 1604 (m), 1490 (w), 1174 (w), 1131 (w), 1092 (w), 864 (w), 827 (w) cm-1. ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.56 (s, 1H), 7.43 (s, 1H), 7.42 (d, J = 8.3 Hz, 2H), 7.18 (d, J = 8.4 Hz, 2H), 2.24 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.62 (s), 158.11 (s), 156.78 (s), 142.55 (s), 139.09 (s), 134.04 (s), 130.14 (s), 129.31 (s), 124.55 (s), 124.04 (s), 29.99 (s), 26.83 (s). **HRMS (ESI+)** *m/z* calc'd for $C_{14}H_{12}ClO_3$ (M+H): 263.0469. Found: 263.0474.

2,7-dihydroxy-4-methyl-5-(4-(trifluoromethyl)phenyl)cyclohepta-2,4,6-trienone (**3n**). To a solution of bicycle **2n** (27.3 mg, 0.0880 mmol) in CH_2Cl_2 (880 μL) was added triflic acid (31.1 μL, 0.352 mmol). The reaction was allowed to stir for 30 minutes at rt before quenching to pH 7 with pH 7 phosphate buffer and extracting with CH_2Cl_2 (5 x 10 mL). Combined organics were dried over Na_2SO_4, filtered, and concentrated under reduced pressure. To the isolated methoxytropolone (10.5 mg) was added 461 μL 33% HBr/AcOH solution. The reaction was heated to reflux (120 °C) for 7 hours before being quenched to pH 5 with 10 mL of pH 7 phosphate buffer. The organic layer was isolated and the aqueous layer was extracted with CH_2Cl_2 (5 x 10 mL). Combined organics were dried over Na_2SO_4, filtered, and concentrated under reduced pressure to yield **3n** as a brown oil (6.3 mg, 63% yield). IR (thin film, KBr) 3247 (br), 1617 (w), 1530 (m), 1392 (w), 1324 (s), 1281 (w), 1166 (w), 1126 (m), 1068 (m), 1017 (w), 822 (w) cm-1. ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.72 (d, J = 8.5 Hz, 2H), 7.58 (s, 1H), 7.41 (s, 1H), 7.38 (d, J = 8.5 Hz, 2H), 2.24 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.82 (s), 158.28 (s), 156.75 (s), 147.43 (s), 142.07 (s), 138.91 (s), 130.41 (q, J = 32.7 Hz), 129.14 (s), 126.04 (q, J = 3.7 Hz), 124.47 (s), 124.33 (q, J = 270.0 Hz) 123.67 (s), 26.66 (s). **HRMS (ESI+)** *m/z* calc'd for $C_{15}H_{12}F_3O_3$ (M+H): 297.0733. Found: 297.0739.

4-acetyl-2,7-dihydroxy-5-methylcyclohepta-2,4,6-trienone (**3o**). To a solution of bicycle **2o** (30.0 mg, 0.127 mmol) in CH_2Cl_2 (86.7 μL) was added triflic acid (45.0 μL, 0.507 mmol). The reaction was allowed to stir for 30 minutes at rt. To this mixture was added 3.07 mL AcOH and 834 μL of 33% HBr/AcOH solution. The reaction was heated to 90°C for 4 hours before being quenched with pH 7 phosphate buffer and washed 3x with buffer. Combined organics were dried over Na_2SO_4, filtered, and concentrated under reduced pressure to yield **3o** as a brown oil (21.7 mg, 77% yield). IR (thin film, KBr) 3261 (br), 2971 (w), 2933 (w), 2873 (w), 1701 (m), 1538 (m), 1397 (w), 1282 (m), 1189 (w), 1147 (w), 1092 (w), 904 (w), 669 (w) cm-1. ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.47 (s, 1H), 7.28 (s, 1H), 3.19 – 3.04 (m, 1H), 2.38 (s, 3H), 1.21 (d, J = 6.9 Hz, 6H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 210.15 (s), 168.03 (s), 158.66 (s), 157.14 (s), 141.39 (s), 137.70 (s), 124.64 (s), 117.96 (s), 40.57 (s), 24.50 (s), 18.05 (s). **HRMS (ESI+)** *m/z* calc'd for $C_{12}H_{15}O_4$ (M+H): 223.0965. Found: 223.0965.

4-(cyclohexanecarbonyl)-2,7-dihydroxy-5-methylcyclohepta-2,4,6-trienone (**3p**). To a solution of bicycle **2p** (33.55 mg, 0.121 mmol) in CH_2Cl_2 (95.3 μL) was added triflic acid (43.0 μL, 0.486 mmol). The reaction was allowed to stir for 30 minutes at rt. To this mixture was added 482 μL AcOH and 91.58 μL 33% HBr/AcOH solution. The reaction was heated to 90 °C for 3 hours before being quenched with pH 7 phosphate buffer and washed 3x with buffer. Combined organics were dried over Na_2SO_4, filtered, and concentrated under reduced pressure to yield **3p** as a black oil (26.6 mg, 84% yield). IR (thin film, KBr) 3262 (br), 2930 (m), 2854 (w), 1698 (m), 1538 (s), 1395 (m), 1281 (s), 1193 (w), 1160 (w), 1089 (w), 906 (w), 731 (w), 648 (w) cm-1. ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.46 (s, 1H), 7.28 (s, 1H), 2.83 (t, J = 11.2 Hz, 1H), 2.37 (s, 3H), 1.98 – 1.13 (m, 10H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 209.07 (s), 158.23 (s), 156.71 (s), 141.23 (s), 137.29 (s), 124.21 (s), 117.56 (s), 76.36 (s), 50.10 (s), 28.10 (s), 25.35 (s), 25.29 (s), 24.15 (s). **HRMS (ESI+)** *m/z* calc'd for $C_{15}H_{19}O_4$ (M+H): 263.1278. Found: 263.1283.

4-(2-cyclohexylacetyl)-2,7-dihydroxy-5-methylcyclohepta-2,4,6-trienone (**3q**). To a solution of bicycle **2q** (30.0 mg, 0.103 mmol) in CH_2Cl_2 (900 μL) was added triflic acid (37.0 μL, 0.413 mmol). The reaction was allowed to stir for 30 minutes at rt before quenching with sodium acetate (85 mg, 1.03 mmol). After stirring for an additional 15 minutes, the solution was concentrated under reduced pressure. To this mixture was added 900 μL AcOH and 1.1 mL 33% HBr/AcOH solution. The reaction was heated to 90 °C for 4 hours before being quenched with pH 7 phosphate buffer and washed 3x with buffer. Combined organics

were allowed to stir with solid potassium carbonate (72 mg, 0.515 mmol) for 10 minutes before washing with an equivalent volume of water. Aqueous layer was acidified with HCl dropwise to pH 5 and extracted with CH_2Cl_2. Combined organics were dried over Na_2SO_4, filtered, and concentrated under reduced pressure to yield **3q** as brown oil (23.3 mg, 82% yield). IR (thin film, KBr) 3256 (br), 2924 (s), 2852 (m), 1704 (w), 1538 (w), 1447 (w), 1397 (w), 1280 (s), 1156 (m), 1090 (w), 906 (w), 788 (w), 733 (w) cm-1. ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.46 (s, 1H), 7.27 (s, 1H), 2.67 (d, J = 6.7 Hz, 2H), 2.40 (s, 3H), 1.99 (m, 1H), 1.85 – 1.62 (m, 5H), 1.39 – 0.91 (m, 5H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 205.95 (s), 167.92 (s), 158.66 (s), 157.26 (s), 142.10 (s), 137.27 (s), 124.89 (s), 117.91 (s), 50.75 (s), 33.49 (s), 33.20 (s), 26.10 (s), 26.03 (s), 24.22 (s). **HRMS (ESI+)** *m/z* calc'd for $C_{16}H_{21}O_4$ (M+H): 277.1434. Found: 277.1441.

Characterization of inhibitor activity

Over-expression and purification of ANT(2'')Ia. The *aadB* gene[1] (GenBank: ACX70191.1) was cloned into a modified pDEST15 vector (Life Technologies, Burlington ON) encoding an engineered Tobacco Etch Virus (TEV) protease cleavage site. ANT(2'')-Ia was over-expressed in *E. coli* BL21 (λDE3) using auto-induction,[2] cells were harvested by centrifugation at 6,000 *x g* and resuspended in buffer A (20 mM Tris [pH 8.0], 300 mM NaCl). Cells were lysed using a cell disrupter (Constant Systems Limited, Daventry UK) and the lysate cleared by centrifugation at 30,000 x g. The cleared lysate was incubated with Glutathione Agarose (Pierce, Thermo Scientific, Rockford USA) that was pre-equilibrated with buffer A for 1 h at 4°C. The agarose mixture was then applied to a chromatography column (30 cm x 1 cm) (Bio-Rad, Mississauga ON) and washed with 4 column volumes of buffer A. The GST fusion-tag was removed by on column TEV protease cleavage at 12°C, cleaved fusion protein was eluted with buffer A. DTT was added to a final concentration of 1 mM and $MgCl_2$ to 10 mM, and the protein was concentrated to 35 mg/mL.

Inhibition kinetics. The continuous EnzChek pyrophosphate assay (Life Technologies) was used to measure inhibition of ANT(2'')-Ia according to the manufacturer's suggested guidelines. The reaction was followed at 360 nm using a SpectraMax Plus384 (Molecular Devices) microtitre plate reader. For IC_{50} value determination, the EnzChek pyrophosphate assay was performed in duplicate, in flat bottom 96-well plates (NUNC, ThermoScientific) with a final volume of 100 μL and 50 mM HEPES [pH 7.5], 40 mM KCl, 10 mM $MgCl_2$. Per reaction, 5 μg of ANT(2'')-Ia was added, inhibitors were added to a final concentration of 1% (v/v) DMSO, kanamycin B (Sigma Aldrich, Oakville ON) was kept at 5 times the K_m (200 μM) and ATP (Sigma Aldrich) kept at K_m (25 μM). The reaction was incubated with shaking, for 3 minutes at room temperature, followed by initiation with nucleotide and monitored for 10 minutes. Inhibitor K_i values against ATP were conducted in duplicate, data were fit to different models of enzyme inhibition and the most favorable fit was assessed using software in GraFit 5.0.13 (Erithacus Software).

***In vivo* activity of inhibitors.** To determine whether inhibitors potentiate kanamycin B *in vivo*, susceptibility testing was performed in a hyper-permeable strain of *E. coli* BW25113 (due to deletion of the gene encoding Δ*bamB*)[3] that is also deficient in efflux (deletion of the gene encoding Δ*tolC*)[4]. The *aadB*

gene[1] was cloned into the pGDP4 vector (an in house derivative of the pBR322 vector; New England BioLabs) and as a negative control, the gene encoding the aminoglycoside phosphotransferase APH(2'')-Id (GenBank: AY743255.1) was cloned into the pGDP3 vector (an in house derivative of the pBR322 vector). All susceptibility testing was performed in Mueller-Hinton II broth (cation adjusted) (Becton, Dickinson and Co, Franklin Lakes NJ). Synergy was assessed using the broth microdilution checkerboard method[5] to determine the FIC index (FICI), according to CLSI guidelines (M7-A9).[6] Inhibitors were added to a final concentration of 1% (v/v) DMSO.

---

[1] Wright, E.; Serpersu, E. H. "Isolation of aminoglycoside nucleotidyltransferase (2")-Ia from inclusion bodies as active, monomeric enzyme" *Protein Expr. Purif.* **2004**, *35*, 373-380.
[2] Studier, F. W. "Protein production by auto-induction in high density shaking cultures" *Protein Expr. Purif.* **2005**; *41*, 207-234.
[3] Baba, T.; Ara, T.; Hasegawa, M.; Takai, Y.; Okumura, T.; Baba, M.; Datsenko, K. A.; Tomita, M.; Wannter, B. L.; Mori, H. "Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection" *Mol. Syst. Biol.* **2006**, *2*, 2006.0008.
[4] Cox, G.; Koteva, K.; Wright, G. D. "An unusual class of anthracyclines potentiate Gram-positive antibiotics in intrinsically resistant Gram-negative bacteria" *J. Antimicrob. Chemother.* **2014**. (DOI: 10.1093/jac/dku057)
5. Pillai, S.; Moellering, R.; Eliopoulos, G. *Antimicrobial combinations*. Baltimore MD: Williams and Wilkins, 2005.
[6] Wayne, P. "Methods for dilution antimicrobial susceptibility testing of bacteria that grow aerobically." *CLSI,* **2012**.