Supporting Information

Identification and Synthesis of Quinolizidines with Anti-Influenza A Virus Activity

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EXPERIMENTAL SECTION

Material.
Chemicals including compounds and synthetic reagents were purchased from Sigma-Aldrich if unspecified. The (+)- and (-)-sparteine were purchased from AEchem Scientific Corp. and Annova Chem Inc.; respectively. Methyl cytisine was purchased from Santa Cruz Biotechnology. Oseltamivir and amantadine were purchased from Tszchem and LKT laboratories and Sigma-Aldrich, respectively. The IAV VR1679 and MDCK cells were obtained from ATCC and the PR8 (A/Puerto Rico/8/34) virus was kindly provided by Dr. Elizabeth Ramsburg at Duke University.

Methods.
Anti-influenza virus assay. MDCK cells at 5,000 cells/well were cultured in 96-well plates for a day before IAV infection. The infection medium was MEM containing 1% FBS, 1 µg/mL trypsin (Sigma-Aldrich), 1 mM sodium pyruvate, 0.1 mM non-essential amino acid, and 100 U/mL penicillin/streptomycin. Various concentrations of compounds were added to the cell culture. Then the cells were infected with IAV at a multiplicity of infection (MOI) of 1, unless otherwise indicated. After 48 hours of incubation, Promega CellTiter-Glo® reagent was added to each well following the protocol provided by the supplier. The luminescence (RLU) emitted from each well was quantified with a Promega Victor III plate reader. The % protection of MDCK cells from the cytocidal effect of the flu virus was calculated by the following formula: 100 x [(RLUvs - RLUv) / (RLUctr - RLUv)], where RLUvs is the relative luminescence unit (RLU) from the cells cultured with virus and compounds, RLUv is the RLU from the flu virus infected cells only, and RLUctr is the cells cultured in the absence of virus and compounds.
EC₅₀, the concentration required to protect 50% of MDCK cells from the cytocidal effect of flu viruses, was calculated using the software CalcuSyn (Biosoft, Cambridge, UK).

**Immunostaining and confocal microscopy.** MDCK cells cultured in 96-well glass-bottom plates were treated with compounds and infected with IAV (MOI = 1) for 6 hours. The cells were fixed with 4% formaldehyde in PBS for 15 minutes. The cells were then treated with a blocking buffer containing 5% FBS and 0.3% Triton X-100 in PBS for 60 min. Immunostaining was carried out by incubating FITC-conjugated anti-influenza A NP antibody (Thermo Fisher Scientific) with the cells at 4°C overnight. The samples were washed three times in PBS before treated with Prolong® Gold Anti-Fade Reagent with DAPI (Cell Signaling Technology). Confocal images were acquired using a Nikon TE2000-U laser-scanning confocal microscope. Confocal image analysis was performed with NIS-Elements AR 3.0 software.

**Chemistry.**

**General.** Compounds (9 - 16) (all new except 9) were synthesized and analyzed with positive HR-FABMS on a Shimadzu LCMS-IT-TOF or a Joel SX-102 mass spectrometer. ¹H, ¹³C and 2D NMR spectra were measured on a Varian 400 or 800 MHz spectrometer as indicated. Samples were dissolved in CD₃OD unless indicated otherwise. Silica gel chromatography was carried out on an ISCO CombiFlash Rf flash chromatograph system with a pre-packed Redi Sep Rf Si gel column (Teledyne ISCO) and mobile phase of EtOAc/MeOH/NH₄OH in gradient of increased polarity. Compounds were purified on HPLC using a Varian ProStar HPLC system with a PDA detector and Agilent Zorbax C18 columns (5 μM particle size, 4.6 × 250 mm or 9.4 × 250 mm). The mobile phase used for the HPLC was ACN/MeOH/H₂O/TFA in a gradient of decreasing polarity. All synthesized compounds were confirmed a purity of over 95% by HPLC.
**Synthesis of compounds 9 – 14.** To a mixture of **1** (46 mg, 0.2 mmol) and isobutyraldehyde (43 mg, 0.6 mmol) in 2 mL 1, 2-dichloroethane was added sodium triacetoxyborohydride (60 mg, 0.28 mmol) at room temperature. The reaction mixture was stirred under **N₂** for 5 hours and was then diluted with ethyl acetate. The organic layer was washed with saturated NaHCO₃, dried over MgSO₄, and concentrated. The residue was chromatographed with Si-gel chromatography to give **11** with 73% yield. Compound **9, 10, and 12 - 14** were synthesized by same method with 23-72% yield.

**Synthesis of compound 15:** To a mixture of **1** (116 mg, 0.5 mmol) in 5 mL acetonitrile was added 2-(Boc-amino) ethyl bromide (112 mg, 0.5 mmol) and K₂CO₃ (210 mg, 1.5 mmol). The mixture was heated to 110 °C by microwave (Biotage Initiator) for 1 hour. After the solvent was removed under vacuum, the resultant residue was diluted with ethyl acetate, which was then washed with water and brine, dried over MgSO₄, and concentrated. The residue was chromatographed with Si-gel chromatography to give **1a** (35% yield).

To **1a** (38 mg, 0.1 mmol) was added 55%TFA/DCM (1 mL). The mixture was stirred at rt for 20 minutes. After the solvent was removed in vacuum, the resultant residue was chromatographed with Si-gel chromatography to give **1b** (87% yield).

To **1b** (19 mg, 0.069 mmol) and 2-fluorobenzoic acid (21 mg, 0.15 mmol) in 3 mL THF was added EDC (30 mg, 0.15 mmol) and DIEA (52 µL, 0.3 mmol) at room temperature. The mixture was stirred under **N₂** overnight. After the solvent was removed in vacuum, the resultant residue was dissolved in ethyl acetate, washed with water and brine, dried over MgSO₄, and concentrated to give a solid. The residue was chromatographed with Si-gel and then with HPLC to give **15** (36% yield).
Synthesis of compound 16: To a mixture of 1 (24 mg, 0.1 mmol) and thiophene-2,5-dicarboxylic acid (26 mg, 0.15 mmol) in 3 mL THF was added EDC (30 mg, 0.15 mmol) and DIEA (52 µL, 0.3 mmol) at room temperature. The mixture was stirred under N₂ for overnight. After the solvent was removed in vacuum, the resultant residue was diluted with ethyl acetate, washed with water and brine, dried over MgSO₄, and concentrated to give a solid. The residue was chromatographed with Si-gel and further purified with HPLC to give 16 (5% yield).

Synthesis of compounds 17 and 18: To a mixture of 9 (180 mg, 0.73 mmol) in 10 mL methanol was added 10% Pd/C (36 mg, 20% w/w) at room temperature. The mixture was stirred under H₂ for 5 hours. The catalyst was removed by filtration while the liquid part was concentrated to give a crude product, which was subsequently chromatographed with HPLC to give separated 17 (38% yield) and 18 (55% yield). The cis and trans configuration of decahydroquinoline substructure (C7-8-9-11-12-13-14-15-16-17) in 17 and 18 were determined based on NMR data. From the proton NMR data, the H11 in cis configuration (H11/H16) appeared more deshielding than in that in trans, suggesting 17 with H11 at δ 3.19 ppm has cis, while 18 at 3.03 ppm has trans configuration, respectively (1). In addition, the multiplicity of H11 resonance in 18 is consistent with trans decahydroquinoline model, with vicinal proton (H16) oriented in 180 degree in dihedral angle. The H11 signal in 18 located at the 3.03 ppm is a multiplicity with large coupling constants, consistent with coupling patterns of trans configuration. On the other hand, H11 (axial) resonance in 17 is a slightly broad singlet, consistent with cis decahydroquinoline with vicinal proton (H16 in equat.) oriented approximately 90 degree (2). In ¹³C NMR spectra, the chemical shifts of C16 and C11 are located at δ 31.8 and 72.5 ppm for 18, and at 27.1 and
67.5 ppm for 17, which agree with reported decahydroquinoline models that the chemical shifts of these two conjunction carbons appeared at lower fields in trans than in cis isomers (3).

**N-Methylaloperine (9):** $^1$H NMR (400 MHz) (CDCl$_3$) δ 5.52 (dd, 1H, $J = 8.4$ Hz, $J = 2.0$ Hz), 2.57-2.88 (m, 4H), 2.32-2.43 (m, 2H), 2.24 (s, 3H), 2.18 (dd, 1H, $J = 12.4$ Hz, $J = 5.6$ Hz), 1.91-2.11 (m, 4H), 1.74 (m, 1H), 1.65-1.74 (m, 2H), 1.53-1.63 (m, 4H), 1.37-1.44 (m, 2H), 1.26-1.35 (m, 1H), 1.21 (dd, 1H, $J = 15.6$ Hz, $J = 4.8$ Hz). Calcd for C$_{16}$H$_{27}$N$_2$ (M+H)$^+$: 247.2. Found: 247.1.

**N-Ethylaloperine (10):** $^1$H NMR (400 MHz) (CDCl$_3$) δ 5.52 (dd, 1H, $J = 6.0$ Hz, $J = 2.0$ Hz), 2.60-2.95 (m, 6H), 2.42 (d, 1H, $J = 9.6$ Hz), 2.38 (d, 1H, $J = 9.6$ Hz), 1.18-2.25 (m, 16H), 0.99 (t, 3H, $J = 9.2$ Hz). Calcd for C$_{17}$H$_{29}$N$_2$ (M+H)$^+$: 261.2325. Found: 261.2328.

**N-Isobutylaloperine (11):** $^1$H NMR (400 MHz) δ 5.95 (d, 1H, $J = 6.4$ Hz), 4.20 (d, 1H, $J = 4.4$ Hz), 3.89 (d, 2H, $J = 10.8$ Hz), 3.49 (d, 1H, $J = 12.4$ Hz), 3.14-3.31 (m, 4H), 3.08 (dt, 1H, $J = 12.8$ Hz, $J = 3.2$ Hz), 2.93-2.99 (m, 2H), 2.29-2.52 (m, 4H), 2.16 (ddd, 1H, $J = 13.2$ Hz, $J = 12.4$ Hz, $J = 13.2$ Hz, $J = 13.2$ Hz, $J = 13.2$ Hz, $J = 13.2$ Hz, $J = 12.4$ Hz, $J = 13.2$ Hz), 1.76-2.05 (m, 8H), 1.60 (ddd, 1H, $J = 13.2$ Hz, $J = 13.2$ Hz, $J = 12.4$ Hz, $J = 13.2$ Hz, $J = 12.4$ Hz, $J = 13.2$ Hz, $J = 13.2$ Hz, $J = 13.2$ Hz), 1.28 (d, 3H, $J = 6.0$ Hz), 1.08 (d, 3H, $J = 6.0$ Hz). Calcd for C$_{19}$H$_{33}$N$_2$ (M+H)$^+$: 289.2638. Found: 289.2636.

**N-Cyclopropanemethylaloperine (12):** $^1$H NMR (400 MHz) δ 5.52 (d, 1H, $J = 6.4$ Hz), 3.26 (m, 1H), 2.92-3.01 (m, 2H), 2.60-2.78 (m, 5H), 2.31-2.50 (m, 3H), 2.10-2.21 (m, 3H), 1.84-2.02 (m, 5H), 1.19-1.76 (m, 10H), 0.91 (m, 1H), 0.43-0.48 (m, 2H), 0.11 (m, 2H). Calcd for C$_{19}$H$_{31}$N$_2$ (M+H)$^+$: 287.2482. Found: 287.2486.

**N-Cyclobutanemethylaloperine (13):** $^1$H NMR (400 MHz) δ 5.94 (d, 1H, $J = 5.6$ Hz), 4.18 (d, 1H, $J = 6.8$ Hz), 3.86 (dd, 1H, $J = 5.6$ Hz, $J = 19.2$ Hz), 3.66 (d, 1H, $J = 16.0$), 3.27-3.47 (m,
4H), 3.03-3.14 (m, 2H), 2.88-2.96 (m, 2H), 1.52-2.50 (m, 20H). Calcd for C_{20}H_{33}N_2 (M+H)^+: 301.2638. Found: 301.2645.

**N-Cyclohexanemethylaloperine (14):** $^1$H NMR (400 MHz) $\delta$ 5.95 (d, 1H, $J = 6.0$ Hz), 4.19 (d, 1H, $J = 4.8$ Hz), 3.90 (d, 2H, $J = 14.0$ Hz), 3.49 (d, 1H, $J = 12.4$ Hz), 3.17-3.31 (m, 4H), 3.08 (dt, 1H, $J = 12.8$ Hz, $J = 12.4$ Hz, $J = 3.2$ Hz), 2.98 (wide s, 1H), 2.87 (dd, 1H, $J = 12.4$ Hz, $J = 3.2$ Hz), 1.55-2.52 (m, 18H), 1.02-1.145 (m, 6H). Calcd for C_{22}H_{37}N_2 (M+H)^+: 329.2951. Found: 329.2951.

**2-Fluoro-N-[2-(N'-aloperine-yl)ethyl]benzamide (15):** $^1$H NMR (400 MHz) $\delta$ 7.78 (dt, 1H, $J = 10.0$ Hz, $J = 2.0$ Hz), 7.49-7.57 (m, 1H), 7.26 (dt, 1H, $J = 10.0$ Hz, $J = 1.6$ Hz), 7.18 (ddd, 1H, $J = 15.2$ Hz, $J = 11.2$ Hz, $J = 1.2$ Hz), 5.91 (d, 1H, $J = 6.8$ Hz), 4.26 (d, 1H, $J = 8.8$ Hz), 3.80-3.93 (m, 4H), 3.67 (m, 1H), 2.97-3.45 (m, 8H), 1.48-2.53 (m, 13H). Calcd for C_{24}H_{33}FN_3O (M+H)^+: 398.2602. Found: 398.2602.

**N,N'-Di-aloperine-yl thiophene-2,5-dicaboximide (16):** $^1$H NMR (400 MHz) $\delta$ 7.47 (s, 2H), 5.47 (wide s, 2H.), 4.18 (dd, 2H, $J = 16.8$ Hz, $J = 6.4$ Hz), 3.83 (d, 1H, $J = 7.9$ Hz), 3.79 (d, 1H, $J = 6.8$ Hz), 3.20-3.45 (m, 12H), 2.87 (m, 2H), 2.32-2.58 (m, 6H), 2.14-2.30 (m, 4H), 1.55-2.10 (m, 16H). Calcd for C_{36}H_{49}N_4O_2S (M+H)^+: 601.3576. Found: 601.3573.

**N-Methyl-11,16-cis-dihydroaloperine (17):** $^1$H NMR (800 MHz) $\delta$ 3.66 (dd, 1H, $J = 13.9$ Hz, $J = 11.4$ Hz, H10a), 3.53 (dt, 1H, $J = 12.0$ Hz, $J = 1.8$ Hz, $J = 1.8$ Hz, H13a), 3.45 (dt, 1H, $J = 12.0$ Hz, $J = 1.8$ Hz, H2a), 3.19 (s, 1H, H11), 3.11 (d, 1H, $J = 13.0$ Hz, H6), 3.10 (td, 1H, $J = 12.7$ Hz, $J = 12.7$ Hz, $J = 4.8$ Hz, H13b), 3.06 (t, 1H, $J = 12.0$ Hz, H2b), 3.05 (dd, 1H, $J = 12.0$ Hz, $J = 3.0$ Hz, H16), 2.91 (s, 3H, NCH$_3$), 2.87 (dd, 1H, $J = 14.1$ Hz, $J = 3.1$ Hz, H10b), 2.68 (dd, 1H, $J = 10.2$ Hz, $J = 2.9$ Hz, H9), 2.08 (s, 1H, H7), 1.76-2.00 (m, 9H, H3b, H4a, H4b, H5a, H8a, H14a, H14b, H15a, H17a), 1.73 (d, 1H, $J = 13.0$ Hz, H5b), 1.71 (d, 1H, $J = 14.0$ Hz,
H15b), 1.66 (d, 1H, J = 15.4 Hz, H8b), 1.62 (ddt, 1H, J = 13.0 Hz, J = 13.0 Hz, J = 3.7 Hz, J = 3.7 Hz, H3b), 1.49 (d, 1H, J = 13.9 Hz, H17b). $^{13}$C NMR (200 MHz) δ 67.5 (C11), 65.9(C6), 59.0(C13), 56.0(C2), 52.9(C10), 42.0(NMe), 34.0(C7), 33.0(C5), 30.3(C17), 28.3(C15), 27.1(16), 26.3(C9), 24.2(C4), 23.6(3), 20.3(14), 18.6(C8). Calcd for $\text{C}_{16}\text{H}_{29}\text{N}_{2}$ (M+H)$^+$: 249.2325. Found: 249.2328.

*N-Methyl-11,16-trans-dihydroaloperine (18):* $^1$H NMR (800 MHz) δ 3.62 (dd, 1H, J = 13.6 Hz, J = 10.4 Hz, H10a), 3.56 (d, 1H, J = 12.0 Hz, H13a), 3.45 (d, 1H, J = 13.6 Hz, H2a), 3.24 (dd, 1H, J = 14.4 Hz, J = 2.4 Hz, H10b), 3.10 (td, 1H, J = 12.2 Hz, J = 12.2 Hz, J = 4.7 Hz, H13b), 3.03-3.08 (m, 3H, H2b, H6, H9), 3.03 (dd, 1H, J = 12.0 Hz, J = 2.9 Hz, H11), 2.84 (s, 3H, NCH$_3$), 2.24 (ddt, 1H, J = 11.7 Hz, J = 11.4 Hz, J = 11.4 Hz, J = 3.6 Hz, J = 3.6 Hz, H16), 2.19 (dd, 1H, J = 14.8 Hz, J = 2.8 Hz, H8a), 1.96 (d, 1H, J = 15.0 Hz, H5a), 1.92-1.96 (m, 5H, H4a, H4b, H7, H14a, H14b), 1.87 (d, 1H, J = 15.8 Hz, H3a), 1.83 (dd, 1H, J = 14.4 Hz, J = 3.1 Hz, H15a), 1.81 (dd, 1H, J = 11.2 Hz, J = 4.1 Hz, H17a), 1.70 (ddd, 1H, J = 13.3 Hz, J = 13.3 Hz, J = 2.9 Hz, H5b), 1.64 (ddt, 1H, J = 13.0 Hz, J = 13.0 Hz, J = 3.5 Hz, J = 3.4 Hz, H3b), 1.59 (d, 1H, J = 14.8 Hz, H8b), 1.42 (dq, 1H, J = 11.2 Hz, J = 12.5 Hz, J = 12.2 Hz, J = 5.9 Hz, H15b), 1.35 (dt, 1H, J = 13.0 Hz, J = 13.0 Hz, J = 3.3 Hz, H17b). $^{13}$C NMR (200 MHz) δ 72.5(C11), 66.2(C6), 58.7(C13), 56.1(C2), 50.5(C10), 40.8(NMe), 36.9(C17), 33.8(C7), 31.8(C16), 33.0(C5), 30.4(C15), 27.1(C9), 25.6(C8), 24.3(C4), 24.1(C14), 23.5 (C3). Calcd for $\text{C}_{16}\text{H}_{29}\text{N}_{2}$ (M+H)$^+$: 249.2325. Found: 249.2328.
References.


NMR profiles of synthesized quinolizidines.