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NEW SYNTHESIS OF PYRVINIUM THAT INHIBITS THE β-CATENIN/TCF4 PATHWAY

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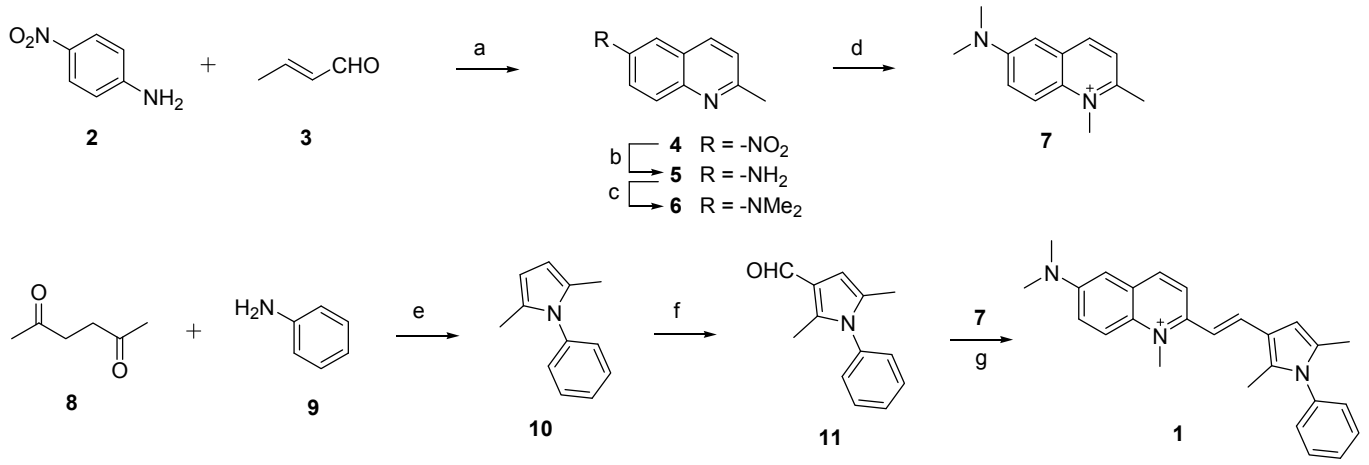
Abstract – A new and converged route is described for synthesis of pyrvinium, an anthelmintic and antitumor agent. This method uses easily obtained materials and simple and practical reactions, including the key Friedländer condensation, to form the quinoline ring. The final product is generated through eight steps, with 23% yield and 96.6% purity (HPLC). This synthetic pyrvinium effectively inhibits β-catenin/Tcf4 driven TOP-luciferase activity, with an IC_{50} value < 1 μM, and induces colon cancer cell death in a dose-dependent manner with an IC_{50} of 0.54 ± 0.06 μM.

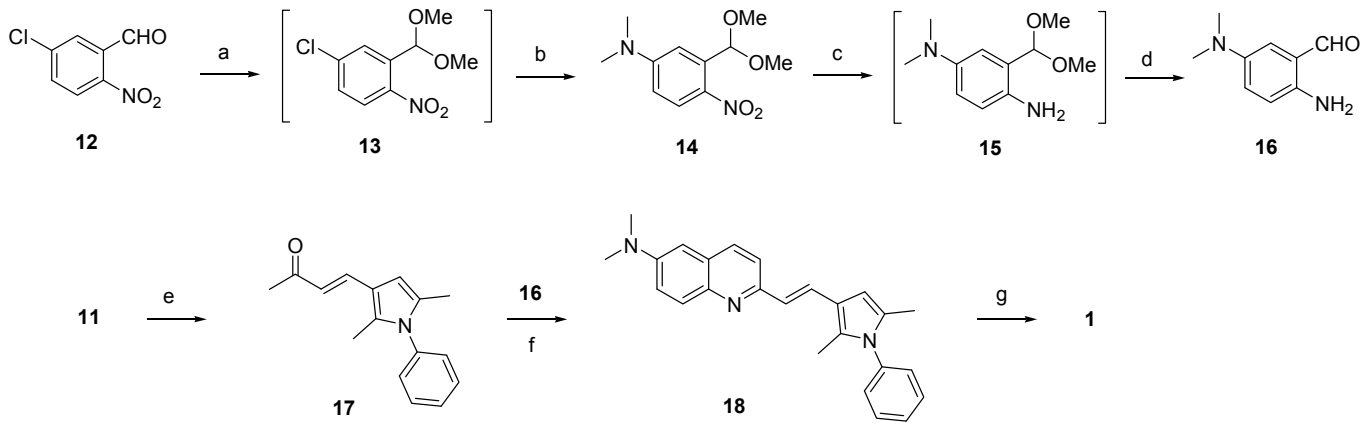
Pyrvinium (**1**, Scheme 1), a FDA-approved drug, is used in the treatment of enterobiasis caused by *Enterobius vermicularis* (pinworm).[1] Recent research has indicated it to be a potent inhibitor of Wnt/β-catenin signaling pathway (EC_{50} ~ 10 nM).[2] This pathway underlies many human cancers through mutations in the APC, axin, and β-catenin genes.[3] Inappropriate activation of the Wnt pathway is believed to be the initial event leading to colorectal cancer, as well as a number of other cancers. As such, this pathway is a therapeutic target of great interest to the field.[4]

The previous synthetic route of **1** is depicted in Scheme 1.[5] The quinoline intermediate **6** was prepared using a Skraup synthesis in ~ 20% yield over three steps.[6] The pyrrole-aldehyde intermediate **11** was synthesized through the Paal-Knorr and Vilsmeier-Haack reactions respectively in 60% yield. The final product **1** was obtained by reaction of **11** with the methylquinolinium **7** in a piperidine condition, with 40% yield.

Here, we report a new and converged synthesis method for compound **1** (Scheme 2). The *o*-aminobenzaldehyde compound **16** was prepared from the commercially available material **12** through simple reactions, including protection with acetal, substitution with dimethylamine, and reduction at H_2/Ni condition, with an overall yield of 85%.[7] The α,β-unsaturated ketone **17** was prepared through the aldol condensation of acetone with the pyrrole-aldehyde **11**, which in turn was prepared through an improved method as described in the experimental section.[8] The pyrrol-vinyl-quinoline compound **18** was then obtained through the Friedländer condensation[9] by reaction of **16** and **17** under sodium ethoxide-ethanol conditions, with a resulting 68% yield after recrystallization from ethanol-water. The final product **1** was produced by methylation of **18** with methyl triflate at room temperature,[10] with 52% overall yield after recrystallization from $CHCl_3$ / hexane.



Scheme 1. Reagents and conditions: (a) 6 N HCl, toluene, reflux, 14 h, 81%; (b) $SnCl_2{\cdot}2H_2O$, 1 N HCl, 100 ºC, 68%; (c) NaH, MeI, DMF, 40 ºC, 39%; (d) MeOTf, CH_2Cl_2, rt, 16 h, 80%; (e) I_2 (cat.), rt, 16 h, 90%; (f) $POCl_3$, DMF, 110 ºC, 73%; (g) piperidine, MeOH, 80 ºC, 12 h, 40%.



Scheme 2. Reagents and conditions: (a) $CH(OMe)_3$, MeOH, HCl (cat.), rt; (b) $Me_2NH{\cdot}HCl$, Et_3N, EtOH, 100 ºC, 94%; (c) H_2, Raney Ni, MeOH, rt, 6 h; (d) HCl, H_2O, 91%; (e) acetone, NaOH, MeOH, H_2O, rt, 48 h, 92%; (f) NaOEt, EtOH, reflux, 6 h, 68%; (g) MeOTf, CH_2Cl_2, 0 ºC – rt, 14 h, 52%.

We tested the biological activity of our newly synthesized pyrvinium (**1**) for its effects on the β-catenin/Tcf4 signaling using a cell-based TOP/FOP assay. HCT116 colon cancer cells were transfected with a reporter gene TOP-flash harboring the normal Tcf4 binding sites for β-catenin, or with a control gene corresponding to FOP-flash without these binding sites. Four hours post-transfection, various concentrations (0.1 – 10 μM) of pyrvinium were added to the cells. Luciferase activities were determined 24 h post-transfection. As shown in Figure 1, pyrvinium effectively inhibited the β-catenin/Tcf4 driven TOP-luciferase activity with an IC_{50} value < 1 μM. We also examined the cytotoxicity effects of this compound on HCT116 cells using a CellTiter-Blue cell viability assay (Promega). As shown in Figure 2, the synthesized pyrvinium effectively induced cell death in a dose-dependent manner, with an IC_{50} value of 0.54 ± 0.06 μM.

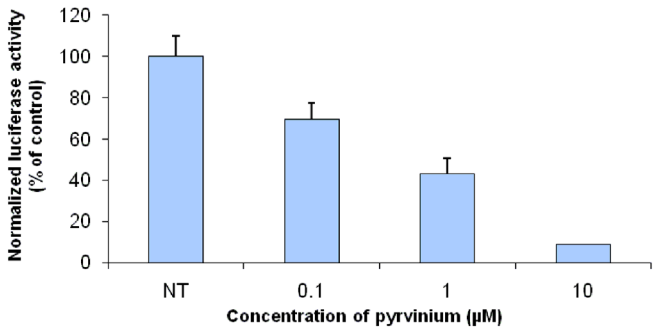


Figure 1

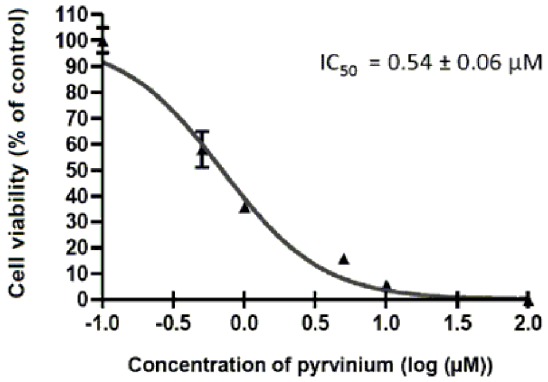


Figure 2

In summary, we have developed a new and improved synthetic route for pyrvinium (**1**) through a key Friedländer condensation reaction. The overall yield of compound **1** obtained from this converged route is around 23% over eight steps, which has 96.6% purity (HPLC) after recrystallization from $CHCl_3$ / hexane. Our biological tests confirmed the effectiveness of the final product. The synthetic pyrvinium effectively inhibited the β-catenin/Tcf4 driven TOP-luciferase activity, with an IC_{50} value < 1 μM, and induced cell death in a dose-dependent manner with an IC_{50} of 0.54 ± 0.06 μM. These results indicate this to be a new and convenient method for preparation of pyrvinium.

EXPERIMENTAL

All commercially available chemicals and solvents were purchased and used as received without further purification. ^1H and ^{13}C NMR spectra were recorded with a Bruker-BioSpin 300/600 MHz spectrometer, using TMS as an internal standard. The mass spectra were obtained using a Thermo Q-Tof micro™

spectrometer. The HPLC results were generated using a Waters 2489 UV/Visible Detector and Waters 1525 Binary HPLC Pump. Conditions: Waters XBridge BEH130 C18 4.6 mm × 250 mm × 5 μm; Detection: 254 nm; Flow rate: 1.0 mL/min; Temperature: 25 °C; Injection load: 10 μL; Concentration: 0.5 mg/mL; Run time: 25 min; Mobile phase A: water (0.01% TFA); Mobile phase B: 90% acetonitrile/water (0.01% TFA); Gradient program: time (min): 0 18 20 25; % of mobile phase A: 100, 0, 100, 100; % of mobile phase B: 0, 100, 0, 0.

The HCT116 colon cancer cell line was obtained from American Type Culture Collection (ATCC) and cultured in RPMI 1640 (Hyclone, Thermal Scientific) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100U/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained in a humidified 5% CO_2 atmosphere at 37 °C.

3-(Dimethoxymethyl)-*N,N*-dimethyl-4-nitrobenzenamine (14).[11] 5-Chloro-2-nitrobenzaldehyde **12** (18.5 g, 0.1 mol), trimethyl orthoformate (13.1 mL, 0.12 mol), and concentrated HCl (0.8 mL, 0.01 mol) were mixed with MeOH (150 mL) and stirred at room temperature overnight. The volatile materials were removed to give a faint yellow oil 4-chloro-2-(dimethoxymethyl)-1-nitrobenzene **13** (23.5 g, 100%), which was used directly at the next step. ^{1}H NMR (DMSO-d_6, 600 MHz): δ 3.32 (s, 6H), 5.80 (s, 1H), 7.67 (d, 1H, J = 2.4 Hz), 7.71 (dd, 1H, J = 2.4, 8.4 Hz), 7.97 (d, 1H, J = 8.4 Hz).

The above product **13** (23.5 g, 0.1 mol), dimethylamine hydrochloride (41 g, 0.5 mol), triethylamine (83 mL, 0.6 mol), and EtOH (300 mL) were mixed in a high-pressure reactor. The reaction mixture was stirred and heated to 100 ºC for 12 h. After cooling, the white suspension was poured into 600 mL water, extracted with CH_2Cl_2 (2 × 300 mL), the organic layers were combined, and washed with water. The solvent was removed to give the product **14** (22.5 g, 94%). ^{1}H NMR (DMSO-d_6, 600 MHz): δ 3.07 (s, 6H), 3.34 (s, 6H), 5.97 (s, 1H), 6.74 (dd, 1H, J = 3.0, 9.6 Hz), 6.88 (d, 1H, J = 3.0 Hz), 7.96 (d, 1H, J = 9.6 Hz); ESI-MS m/z 241.2 [M+1].

2-Amino-5-(dimethylamino)benzaldehyde (16).[12] The *N,N*-dimethyl benzenamine **14** (20 g, 0.083 mol) and Raney Ni (~ 10 g, wet) were suspended in MeOH (300 mL). The reaction mixture was stirred at room temperature for 6 h under hydrogen atmosphere. The suspension was then filtered through a celite pad to produce a dark-red filtrate. Water (30 mL) and concentrated HCl (20 mL) were added to the filtrate and the reaction solution was stirred at room temperature overnight. The MeOH was removed to obtain a dark suspension, which was then diluted with CH_2Cl_2 (400 mL), and washed with water and brine, and dried over anhydrous Na_2SO_4. The solvent was removed to produce a dark-blue solid **16** (12.4 g, 91%), which was used directly in the next step. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 2.83 (s, 6H), 5.6 (br, 2H), 6.86 (d, 1H, J = 3.0 Hz), 6.98 (dd, 1H, J = 3.0, 9.6 Hz), 7.15 (d, 1H, J = 9.6 Hz), 9.86 (s, 1H); ESI-MS m/z 165.1

[M+1].

2,5-Dimethyl-1-phenyl-1*H*-pyrrole-3-carbaldehyde (11).[8] 2,5-Hexanedione (11.7 mL, 0.1 mol), aniline (9.1 mL, 0.1 mol) and acetic acid (0.86 mL, 0.015 mol) were mixed in EtOH (30 mL). The reaction solution was stirred at room temperature for 2 h and a white suspension was generated. The suspension was poured into ice-water (100 mL), stirred, and collected the solid by suction filtration. The solid was dried under reduced pressure to give a tan solid 2,5-dimethyl-1-phenyl-1*H*-pyrrole **10** (15.2, 89%). ^{1}H NMR ($CDCl_3$, 600 MHz): δ 2.05 (s, 6H), 5.82 (br, 2H), 7.24–7.26 (m, 2H), 7.42–7.44 (m, 1H), 7.48–7.51 (m, 2H); ESI-MS *m*/*z* 172.2 [M+1].

Phosphorus oxychloride (8.7 mL, 0.095 mol) was added dropwise to a cooled solution of DMF (50 mL), keeping the reaction temperature below 5 ºC. After the addition, compound **10** (15 g, 0.087 mol) was added to the reaction mixture in several portions. The dark-brown suspension was then stirred and heated to 110 ºC for 6 h to produce a dark-red solution. After cooling, the DMF solution was poured onto 200 g ice, stirred, and 40% aqueous NaOH (35 mL) was slowly added to adjust the pH ~ 10. The resulting solid was collected by suction filtration and dried under reduced pressure to obtain a grey solid **11** (15.8, 91%). ^{1}H NMR ($CDCl_3$, 600 MHz): δ 1.98 (s, 3H), 2.28 (s, 3H), 6.39 (s, 1H), 7.17–7.19 (m, 2H), 7.47–7.54 (m, 3H), 9.82 (s, 1H); ESI-MS *m*/*z* 200.2 [M+1].

4-(2,5-Dimethyl-1-phenyl-1*H*-pyrrol-3-yl)-3-buten-2-one (17). NaOH (15 g, 0.38 mol) was dissolved in water (30 mL), MeOH (60 mL), and acetone (28 mL, 0.38 mol), and then cooled to room temperature. Pyrrole-carbaldehyde **11** (15 g, 0.075 mol) was added and the mixture was stirred at room temperature for 48 h, resulting in a faint yellow solid. The mixture was cooled in an ice bath for 2 h, then the resulting solid was collected by suction filtration, washed with 50% MeOH-water, and dried under reduced pressure to give a faint yellow solid **17** (16.5, 92%). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 2.02 (s, 3H), 2.13 (s, 3H), 2.33 (s, 3H), 6.23 (s, 1H), 6.42 (d, 1H, J = 15.6 Hz), 7.18–7.21 (m, 2H), 7.48–7.54 (m, 3H), 7.58 (d, 1H, J = 15.6 Hz); ^{13}C NMR ($CDCl_3$, 150 MHz): δ 12.4, 14.4, 28.8, 105.5, 118.3, 123.1, 129.6, 130.0, 130.9, 132.7, 135.4, 138.5, 139.5, 199.9; ESI-MS *m*/*z* 240.2 [M+1]. Anal. Calcd for $C_{16}H_{17}NO$: C, 80.30; H, 7.16; N, 5.85. Found: C, 80.55; H, 7.31; N, 5.64.

***N,N*-Dimethyl-2-[2-(2,5-dimethyl-1-phenyl-1*H*-pyrrol-3-yl)ethenyl]-6-quinolinamine (18).**[6] Sodium (1.7 g, 0.075 mol) was added to anhydrous EtOH (200 mL). After the sodium had almost completely dissolved, *o*-aminobenzaldehyde **16** (8.2 g, 0.05 mol) and α,β-unsaturated ketone **17** (12 g, 0.05 mol) were added to the solution and the reaction mixture was refluxed for 6 h, to produce a dark-red solution. After cooling, the EtOH solution was poured into water (500 mL) and stirred. The resulting brown solid

was collected by suction filtration, washed with 50% EtOH-water, and dried under reduced pressure to obtain the crude product, which was recrystallized from EtOH-water to afford product **18** (12.5 g, 68%). ^{1}H NMR (DMSO-d_6, 600 MHz): δ 1.99 (s, 3H), 2.21 (s, 3H), 3.08 (s, 6H), 6.34 (s, 1H), 7.14 (m, 1H), 7.31–7.35 (m, 2H), 7.50–7.63 (m, 5H), 7.76–7.82 (m, 2H), 8.29 (d, 1H, J = 9.6 Hz), 8.54 (d, 1H, J = 9.6 Hz); ESI-MS m/z 368.2 [M+1]; HPLC t_R: 18.732 min.

6-(Dimethylamino)-2-[2-(2,5-dimethyl-1-phenyl-1*H*-pyrrol-3-yl)ethenyl]-1-methyl-quinolinium (1). A solution of the quinolinamine **18** (3.6 g, 0.01 mol) in CH_2Cl_2 (70 mL) was stirred and cooled in an ice bath. Methyl triflate (1.1 mL, 0.01 mol) was added slowly and the reaction solution was stirred at 0 ºC for another 2 h then at room temperature overnight. The resulting red solid was collected by suction filtration, washed with CH_2Cl_2, and dried under reduced pressure. The crude product **1** was purified by recrystallization from $CHCl_3$/hexane (~ 1/1) to obtain the final product **1** (2.6 g, 52%), with 96.6% purity (HPLC). ^{1}H NMR ($CDCl_3$, 600 MHz): δ 2.00 (s, 3H), 2.23 (s, 3H), 3.10 (s, 6H), 4.46 (s, 1H), 6.48 (s, 1H), 6.99 (d, 1H, J = 15.0 Hz), 7.18 (m, 2H), 7.48–7.57 (m, 5H), 7.81 (d, 1H, J = 15.0 Hz), 8.05 (m, 1H), 8.24 (m, 1H), 8.35 (m, 1H); ESI-MS m/z 382.6 [M+1]; HPLC t_R: 12.523 min.

Luciferase reporter gene assay. HCT116 cells (1×10^5) were transfected with TOP-FLASH containing Tcf4 binding sites, or FOP-FLASH harboring mutant Tcf4 binding sites. Four hours post-transfection, pyrvinium was added and luciferase activities were determined after 24 h using a "Firefly Luciferase Assay Kit" (Biotium, Inc. Hayward, CA).

Cell viability assay. The HCT116 cell line was grown in RPMI 1640 + 10% FBS + penicillin/streptomycin. Cells were trypsinized and plated at 1×10^4 cells/well in opaque 96-well plates. Pyrvinium was added 8 h later, so that concentration curves could be generated starting at 10 nM with 7 subsequent 2-fold dilutions. The plates were incubated with pyrvinium for 3 days, at which time the cells were lysed with Promega's Cell Titer-blue reagent. The resulting luminescence was read on a Synergy 2 reader.

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