Convenient Synthesis of Toxoflavin that Targets β-Catenin/Tcf4 Signaling Activities

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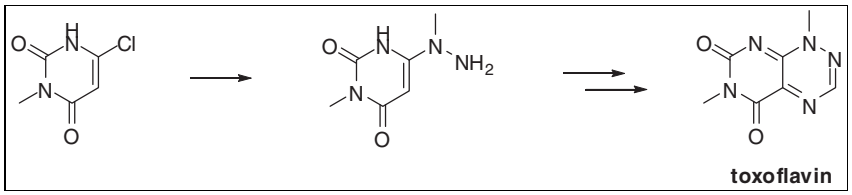

A rapid and improved route for synthesis of toxoflavin, an antibiotic and antitumor agent, is described. The method uses easily obtained materials and simple and practical reactions, including chlorination, condensation, and diazotization to produce toxoflavin in five steps with 14.2% yield and 98.6% purity (HPLC). This synthetic toxoflavin effectively inhibited β-catenin/Tcf4 driven TOP-luciferase activity with an IC_{50} of less than 0.5 μM and induced colon cancer cell death in a dose-dependent manner with an IC_{50} of 0.29 μM.

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INTRODUCTION

Toxoflavin (xanthothricin, PKF118-310, **1**), which possesses the pyrimido[5,4-*e*][1,2,4]triazine ring, is a toxin produced by a variety of bacteria including *Burkholderia gladioli* [1]. It has antibiosis, antiviral, and antitumor properties and other useful bioactivities [2–5]. Recent studies indicated that compound **1** can also act on the β-catenin/Tcf signal pathway that plays critical roles in normal development and tumorigenesis [6]. For example, compound **1** has shown good antitumor activities with IC_{50} of 0.66, 0.36, and 0.98 μM for Huh7, Hep40, and HepG2 hepatoma cell lines, respectively [7,8].

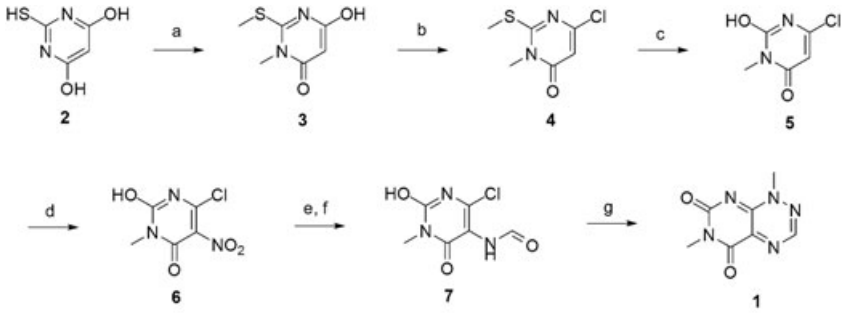
RESULTS AND DISCUSSION

Chemistry. The first complete synthesis of compound **1** was described by Daves *et al.* [9,10] and Black [11], who reported its preparation at the hundreds of grams scale, on the basis of the same route depicted in Scheme 1. Later, Nagamatsu [12–15] and Turbiak [16–18] also developed their methods for preparation of derivatives of compound **1**. The starting material in Daves's route was 2-thiobarbituric acid, which was methylated with excess dimethyl sulfate to give the methylthio product **3** with 35% yield. After chlorination by phosphorus oxychloride, hydrolysis of the methylthio group, and nitrification with fuming nitric acid, the pyrimidinedione **6** was obtained with 19% yield over three steps. Subsequent catalytic hydrogenation over platinum-on-carbon at 10–45 psi gave the amine, which was immediately reacted with excess formyl acetate to afford the formamide **7** with 63% yield. Finally, compound **7** was reacted with methylhydrazine, and the final product **1** was produced with 1.7% yield over seven steps.

In the present article, we reported a new, facile, and practical method for preparation of toxoflavin (Scheme 2). The starting materials methylurea, malonic acid, and acetic anhydride were reacted in one-pot to give the pyrimidinetrione **8** with over 80% yield [19]. This compound was then chlorinated by phosphorus oxychloride, catalyzed by a small amount of water, to obtain the pyrimidinedione **9** with 81% yield [20]. Compound **9** was refluxed with methylhydrazine in ethanol to give the hydrazinylpyrimidinedione **10** with 65% yield after isolation. Product **10** was condensed with a slight excess of 37% formaldehyde in 90% acetic acid to generate the intermediate **11**, which was not isolated but was treated *in situ* with sodium nitrite at 0–5°C to give the product **1**. After recrystallization from 1-propanol, pure product **1** was obtained with 33% yield over the last two steps.

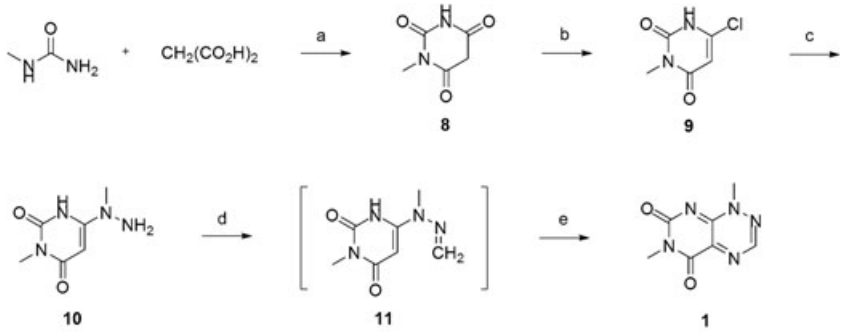
Bioactivity. We tested the biological activity of our newly synthesized toxoflavin for its effects on the β-catenin/Tcf4 signaling using a cell-based TOP/FOP assay. HCT116 colon cancer cells were transfected with a reporter gene TOP-flash harboring the normal Tcf4 binding sites for β-catenin or with a control gene corresponding to FOP-flash without these binding sites. Four hours post-transfection, various concentrations of toxoflavin were added to the cells. Luciferase activities were determined 24 h post-transfection. As shown in

Scheme 1



Reagents and conditions: (a) NaOH, Me_2SO_4, 35%; (b) $POCl_3$, $PhNMe_2$, 51%; (c) HCl, ethanol, 54%; (d) HNO_3, H_2SO_4, 69%; (e) H_2, Pt/C, NH_3/MeOH, 10-45 psi; (f) HCO_2Ac, 63%; (g) $MeNHNH_2$, 41%.

Scheme 2



Reagents and conditions: (a) Ac_2O, 70 °C, 82%; (b) $POCl_3$, H_2O (cat.), 81%; (c) $MeNHNH_2$, ethanol, reflux, 65%; (d) 37%HCHO, HOAc; (e) $NaNO_2$, HOAc, 0-5 °C, 33%.

Figure 1, toxoflavin effectively inhibited the β-catenin/Tcf4 driven TOP-luciferase activity with an IC_{50} value of less than 0.5 μM. We also examined the cytotoxicity effects of this compound on HCT116 cells using a CellTiter-Blue cell viability assay (Promega, Madison, WI). As shown in Figure 2, the synthesized toxoflavin effectively induced cell death in a dose-dependent manner with an IC_{50} value of 0.29 ± 0.6 μM.

In summary, we have developed a quick and improved synthetic route for toxoflavin. The overall yield of compound **1** obtained by this route is around 14.2% with 98.6% purity (HPLC) over five steps. Our biological tests confirm the effectiveness of the final products. The synthetic toxoflavin effectively inhibited the β-catenin/Tcf4 driven TOP-luciferase activity with an IC_{50} value between 0.1 and 0.5 μM and induced cell death in a dose-dependent manner with an IC_{50} of 0.29 ± 0.6 μM. These results

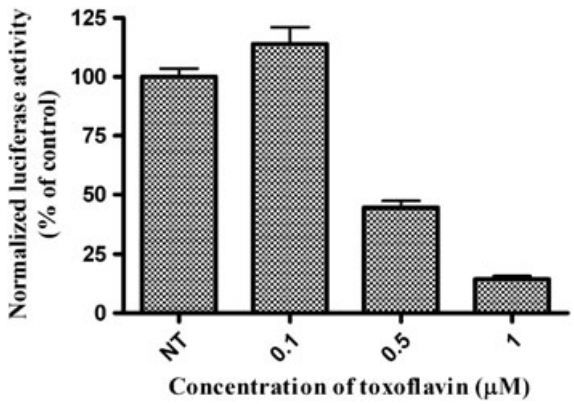


Figure 1. TOP-luciferase reporter gene assay of toxoflavin.

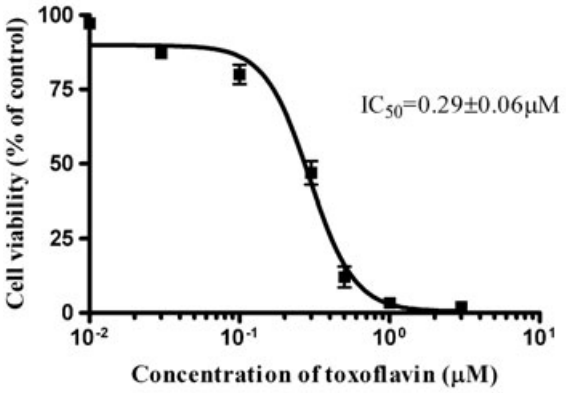


Figure 2. Cell (HCT116) viability assay of toxoflavin.

indicate this to be a new, convenient, and practical method for preparation of compound **1**.

EXPERIMENTAL

All commercially available chemicals and solvents were purchased and used as received without further purification. ^{1}H NMR and ^{13}C NMR spectra were recorded with a Bruker-BioSpin 300/600 MHz spectrometer (Bruker BioSpin Corporation, Billerica, MA) using TMS as an internal standard. The mass spectra were obtained from a Thermo Q-Tof micro spectrometer (Thermo Fisher Scientific, Waltham, MA). The HPLC results were generated using a Waters 2489 UV/Visible Detector and Waters 1525 Binary HPLC Pump (Waters Corporation, Milford, MA). The colon cancer cell line HCT116 was obtained from American Type Culture Collection and cultured in RPMI 1640 (Hyclone, Thermal Scientific) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained in a humidified 5% CO_2 atmosphere at 37°C.

1-Methyl-2,4,6(1*H*,3*H*,5*H*)-pyrimidinetrione (8). Methylurea (148 g, 2 mol), malonic acid (208 g, 2 mol), and acetic anhydride (380 mL, 4.1 mol) were mixed and stirred at 70°C for 2 h. The resulting acetic acid was removed under reduced pressure, and 400 mL ethanol was added to the residue. The mixture was then stirred and cooled in ice-water bath. The resulting precipitate was filtered off and washed with 100 mL ethanol. Obtained pale solid **8** (233 g, 82%). ^{1}H NMR (DMSO-d_6, 300 MHz): δ 3.03 (s, 3H), 3.56 (s, 2H), 11.31 (s, 1H); ms: *m/z* 143.06 (M + 1).

6-Chloro-3-methyl-2,4(1*H*,3*H*)-pyrimidinedione (9). To a stirred mixture of the pyrimidinetrione **8** (213 g, 1.5 mol) in phosphorus oxychloride (600 mL, 6.4 mol) at 0°C, water (10 mL, 0.55 mol) was added slowly, and the mixture was then stirred at 80°C for 4 h. The excess phosphorus oxychloride was removed under reduced pressure, and the residue was poured into ice water. The resulting precipitate was collected by suction filtration and washed with 100 mL water. Gave **9** (195 g, 81%) as a white solid. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 3.07 (s, 3H), 5.87 (s, 1H), 12.35 (s, 1H); ms: *m/z* 161.03 (M + 1).

3-Methyl-6-(1-methylhydrazinyl)-2,4(1*H*,3*H*)pyrimidinedione (10). A mixture of the pyrimidinedione **9** (80 g, 0.5 mol) was suspended in ethanol (1 L) plus methylhydrazine (59 mL, 1.1 mol) and heated at reflux for 2 h. After cooling the solution to room temperature, the resulting precipitate was filtered and dried to give the faint yellow solid product **10** (56 g, 65%). ^{1}H NMR (DMSO-d_6, 300 MHz): δ 3.00 (s, 3H), 3.02 (s, 3H), 4.64 (s, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 26.428, 40.716, 72.695, 150.816, 154.266, 163.336; ms: *m/z* 171.09 (M + 1).

1,6-Dimethyl-pyrimido[5,4-*e*]-1,2,4-triazine-5,7(1*H*,6*H*)-dione (1). The compound **10** (34 g, 0.2 mol) was suspended in 300 mL acetic acid and 30 mL water. The mixture was cooled to 0–5°C, and 37% aqueous formaldehyde (18 mL, 0.22 mol) was added slowly to the mixture, and the solution was stirred at this temperature for 30 min to give the intermediate **11** monitored by TLC. Saturate sodium nitrite (15.2 g, 0.22 mol) aqueous solution was added slowly to the mixture, keeping the temperature below 5°C. The mixture was slowly warmed to room temperature and stirred overnight. Diethyl ether (800 mL) was added to the mixture, and the resulting precipitate was filtered and dried to afford the crude product **1**, which was recrystallized twice from 1-propanol (300 mL) to afford the bright yellow solid toxoflavin (**1**) (13 g, 33%), with 98.6% purity (HPLC). ^{1}H NMR (DMSO-d_6, 600 MHz): δ 3.23 (s, 3H), 3.95 (s, 3H), 8.97 (s, 1H); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 28.144, 42.328, 144.971, 146.375, 150.821, 154.059, 158.924; ms: *m/z* 194.09 (M + 1). HPLC conditions: Waters XBridge BEH130 C18 4.6 × 250 × 5 mm; Detection: 254 nm; Flow rate: 1.0 mL/min; Temperature: 25°C; Injection load: 5 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; Retention time of **1**: 8.715 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, respectively. Four hours post-transfection, compound was added, and luciferase activities were determined after 24 h using "Firefly Luciferase Assay Kit" (Biotium, Inc. Hayward, CA).

Cell viability assay. HCT116 cells were plated at 1×10^4/well in 96-well plate and incubated overnight at 37°C. Cells were treated with different concentrations of taxoflavin and incubated for 72 h. Cell viability was measured by a CellTiter-Blue Cell Vability assay (Promega).

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