Synthesis and Cancer Chemopreventive Activity of Zapotin, a Natural Product from *Casimiroa edulis*

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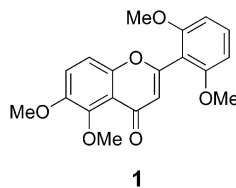
Received July 31, 2006

An efficient method has been developed to synthesize zapotin (5,6,2′,6′-tetramethoxyflavone), a component of the edible fruit *Casimiroa edulis*, on a multigram scale. The synthesis utilizes a regioselective C-acylation of a dilithium dianion derived from a substituted *o*-hydroxyactophenone to afford a β-diketone intermediate that can be cyclized to zapotin in good overall yield, thus avoiding the inefficient Baker−Venkataraman rearrangement pathway. Zapotin was found to induce both cell differentiation and apoptosis with cultured human promyelocytic leukemia cells (HL-60 cells). In addition, the compound inhibits 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced ornithine decarboxylase (ODC) activity with human bladder carcinoma cells (T24 cells), and TPA-induced nuclear factor-kappa B (NF-κB) activity with human hepatocellular liver carcinoma cells (HepG2 cells). These data suggest that zapotin merits further investigation as a potential cancer chemopreventive agent.

Introduction

Cancer is currently the second most common cause of death in the United States, and it is likely to become the most common in the near future.[1] Chemoprevention is the use of either synthetic drugs or natural products to inhibit, reverse, or suppress the development of invasive malignant cancer, either by blocking the DNA damage that initiates carcinogenesis or by arresting or reversing the progression of premalignant cells in which DNA damage has already started. Chemoprevention is one of the most direct ways to reduce cancer-related morbidity and mortality.[2] Several food-based chemopreventive agents have shown promise in clinical trials.[3] The medicinal value of zapote blanco, a fruit of *Casimiroa edulis* Llave and Lex (Rutaceae) that is consumed in many parts of the world, was first discovered by Aztecs, and crude plant extracts of the seeds or leaves of *Casimiroa edulis* were later found to affect blood pressure,[4−6] cardiac activity,[4−6] aortic muscular tone,[7] and to possess anticonvulsant activity.[8,9] Recently, zapotin (**1**), a polymethoxylated flavonoid isolated



from zapote blanco seeds, has been found to be a nontoxic inducer of cellular differentiation with cultured HL-60[a] promyelocytic cells.[10] Zapotin (**1**), therefore, has the potential to inhibit carcinogenesis. The goals of the present investigation were to evaluate the potential of zapotin (**1**) as a chemopreventive agent in various biological systems and to devise and execute a practical synthesis of zapotin (**1**) that would afford gram quantities for more advanced biological testing.

Limited quantities of zapotin (**1**) are available from natural sources as well as from several poor-yielding syntheses that are based on the Baker−Venkataraman rearrangement pathway.[11−14] In general, the Baker−Venkataraman flavone synthesis (Scheme 1) involves the O-acylation of *o*-hydroxyacetophenone **2** with a benzoyl chloride **3** to afford an ester intermediate **4**, which rearranges to a β-diketone **5** under basic conditions. Cyclization of the β-diketone **5** then affords the desired flavone **6**. The main disadvantages of this method of flavone synthesis are that it involves an indirect rearrangement pathway to yield the β-diketone intermediate **5**, and it often results in the formation of 3-aroylflavones instead of the desired 3-unsubstituted flavones.[15] In order to circumvent these difficulties, a direct synthesis of the required β-diketone was investigated in which a dilithium dianion of an appropriately substituted *o*-hydroxyacetophenone would be directly acylated regioselectively on carbon, and the resulting β-diketone would be cyclized to afford zapotin (**1**).[16−18]

Chemistry. In practice, the present synthesis of zapotin (**1**) was executed in a straightforward manner without any unexpected difficulties as outlined in Scheme 2. Commercially available 2-hydroxy-6-methoxyacetophenone (**7**) was subjected to Elbs oxidation[19] using sodium persulfate and aqueous sodium hydroxide to yield the substituted acetophenone **8**, followed by regioselective methylation using anhydrous potassium carbonate and dimethyl sulfate in acetone to afford 6-hydroxy-2,3-dimethoxyacetophenone (**9**) in 53% yield in two steps. Intermediate **9** is a known compound that has been synthesized previously,[19] but the reported reaction conditions were modified as described in the Experimental Section. The yields of both steps increase significantly when the mixtures are stirred for a prolonged period at room temperature; however, over-oxidation of the intermediate hydroquinone **8** was detected if the oxidation was carried out for more than one week. The generation of the dilithium dianion **10** of the acetophenone **9** was ensured by treatment with four equivalents of lithium hexamethyldisilylazide in THF. Treatment of dilithium dianion **10** with com-

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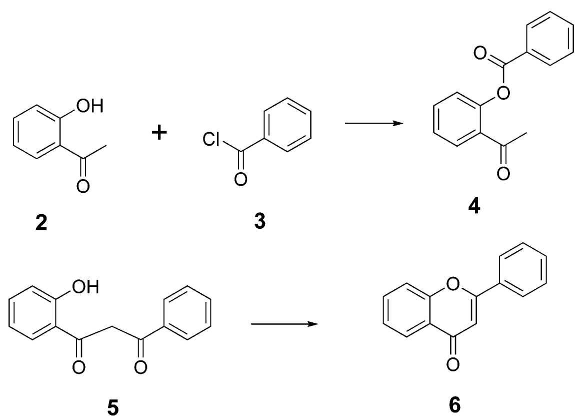
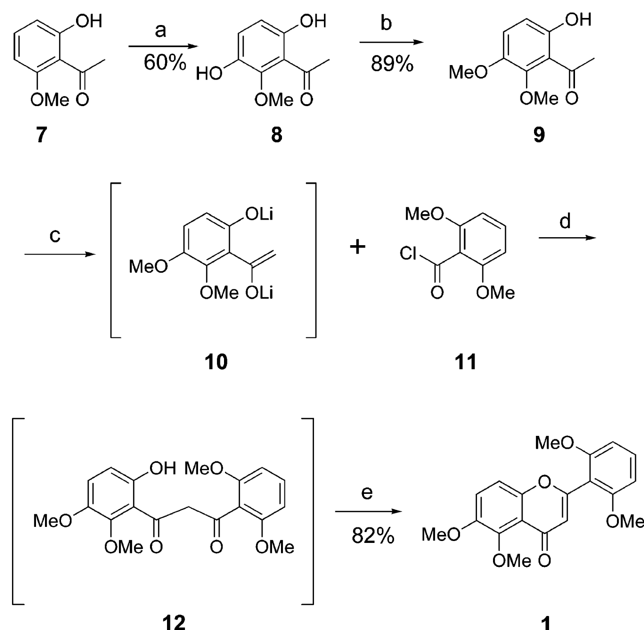
[a] Abbreviations: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; ODC, ornithine decarboxylase; T24 cells, human bladder carcinoma cells; NF-κB, nuclear factor-kappa B; HepG2 cells, human hepatocellular liver carcinoma cells; HL-60 cells, promyelocytic leukemia cells; DAPI, 4′,6-diamidino-2-phenylindole; LiHMDS, lithium hexamethyldisilazide; BrdU, 5-bromo-2-deoxyuridine.

10.1021/jm060915+ CCC: $37.00 © 2007 American Chemical Society
Published on Web 12/23/2006

Scheme 1



Scheme 2[a]



[a] Reagents and conditions: (a) (1) NaOH, $K_2S_2O_8$, 20 °C (7 d), (2) aq HCl; (b) K_2CO_3, Me_2SO_4, Me_2CO (7 d); (c) compound **9**, LiHMDS, THF, −78 °C to −10 °C, (3 h), (1.5 h); (d) Compound **11**, −78 °C to 23 °C (25 h), (2) aq HCl; (e) H_2SO_4, AcOH, 95 °C to 100 °C (3.5 h).

mercially available 2,6-dimethoxybenzoyl chloride (**11**), followed by acidification, afforded the β-diketone intermediate **12**, which was used without purification for cyclization to zapotin (**1**). The crude intermediate **12** was heated at 100 °C in the presence of glacial acetic acid containing 0.5% sulfuric acid for 3.5 h to provide zapotin (**1**) on multigram scale and also in high yield (82%). Thus, a short and economical synthesis of zapotin (**1**) can be executed in 44% overall yield from a commercially available starting material, which compares favorably with the previously reported zapotin (**1**) syntheses that rely on the Baker−Venkataraman rearrangement.[11−14]

Biological Results and Discussion

As a part of a comprehensive program for discovery of cancer chemopreventive agents from natural sources, synthetic zapotin (**1**) was tested in several in vitro systems and found to demonstrate potential chemopreventive activity.

Inhibition of TPA-Induced ODC Activity in T24 Cells. Ornithine decarboxylase (ODC) is the first and apparently the rate-limiting enzyme for the biosynthesis of polyamines in mammalian cells and is highly inducible by growth-promoting stimuli including growth factors, steroid hormones, cAMP-elevating agents, and tumor promoters.[20,21] A number of factors finely tune ODC activity, including the expression, stability, and transcription rate of ODC messenger RNA, the stability and translation rate of the ODC enzyme, and also the post-translational modification of the enzyme.[22] Since ODC activity is essential for proliferation of normal cells, and, on the other hand, is overexpressed in various cancer cell lines, it is now recognized that inhibition of ODC may be a good strategy for cancer chemoprevention and chemotherapy.[23,24] Zapotin (**1**) was therefore tested for inhibition of the induction of ODC activity by TPA using human bladder carcinoma T24 cells and was found to be active, with an IC_{50} of 3.4 ± 1.7 μM. This activity is comparable to standard inhibitors of TPA-induced ODC activity, such as apigenin (IC_{50} 6.0 μM), menadione (IC_{50} 8.3 μM), and deguelin (IC_{50} 0.1 μM).

Inhibition of TPA-induced NF-κB Activity in HepG2 Cells. Nuclear factor-κB (NF-κB) is an inducible transcription factor for genes involved in cell survival, cell adhesion, inflammation, differentiation, and growth.[25−28] In normal cells, NF-κB present in the cytoplasm binds to the inhibitory IκB proteins, which blocks the nuclear localization sequences of NF-κB.[29] Activation of NF-κB by a variety of stimuli, such as carcinogens, inflammatory agents, tumor promoters including cigarette smoke, phorbol esters, and okadaic acid, promote degradation of IκBα and thus unmasks the nuclear localization sequences, permitting NF-κB to enter the nucleus and bind to a specific sequence in DNA, which in turn results in transcription of targeted genes. Various genes that are involved in tumor cell invasion and angiogenesis have been found to be regulated by NF-κB. During the past few years, several reports have shown that activation of NF-κB promotes cell survival and proliferation, and down regulation of NF-κB sensitizes the cell to apoptosis. Thus, agents that suppress NF-κB activation can abrogate carcinogenesis. This has encouraged a search for specific inhibitors of NF-κB activation from natural sources, which might lead to a good candidate for cancer chemoprevention. Zapotin (**1**) was tested, and it displayed promising inhibition of TPA-induced NF-κB activity in HepG2 cells stably transfected with NF-κB-luciferase plasmid with an IC_{50} value of 7.6 ± 3.3 μM.

Induction of Differentiation by Zapotin (1) in HL-60 Cell Line. The HL-60 cell line is used as a model system to investigate the mechanism of cell differentiation, proliferation, and cell death by inducers. Differentiation-inducing agents suppress cancer cell self-renewal selectivity from normal stem cell renewal by inducing terminal differentiation followed by apoptosis. Inducers of terminal differentiation have shown promising chemopreventive activity as suppressing agents that act during the promotion−progression stages of carcinogenesis. Previous studies performed in our laboratories demonstrated that zapotin (**1**) was able to induce differentiation of HL-60 cells in a concentration-dependent fashion without cytotoxicity.[10] Zapotin (**1**) induced 50% of the cells to differentiate at 0.2 μg/mL (ED_{50} 0.5 μM), compared to 10 μM required by apigenin and 30 μM by genistein to exert the same activity, representing a 20−60 fold increase in potency. In the current study, cells were treated with various concentrations of zapotin (**1**) for 24, 48, 72, or 96 h and harvested after 4 days for evaluation of enzymatic and cell membrane markers of differentiation.

Analysis of NBT (nitroblue tetrazolium)-reduction for evaluation of superoxide formation demonstrated myeloid maturation in HL-60 cells. The irreversibility of zapotin (**1**) effects on growth and differentiation of HL-60 cells was tested using withdrawal assays during a 4-day experiment. Withdrawal of

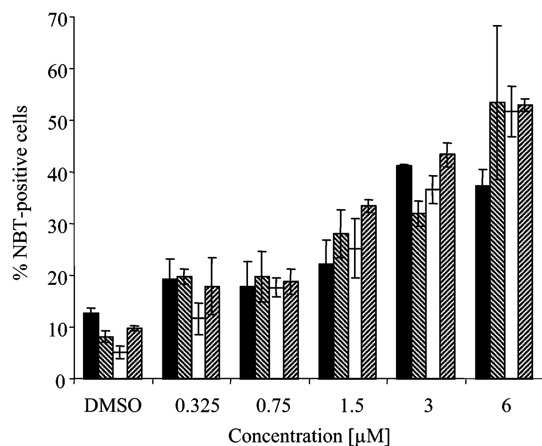


Figure 1. Commitment toward differentiation of HL-60 cells is obtained at 24 h of exposure to zapotin (**1**). In each case, total incubation time was 4 days (96 h), and then cells were analyzed for differentiation marker. Cells were treated with the specified concentrations of zapotin (**1**), which was withdrawn after 24 (■), 48 (diagonal negative slope), 72 (□) or 96 (diagonal positive slope) h. For the 24, 48, and 72 h exposures, cells were resuspended in fresh complete media for the remaining time. Results are shown as the mean of duplicate samples.

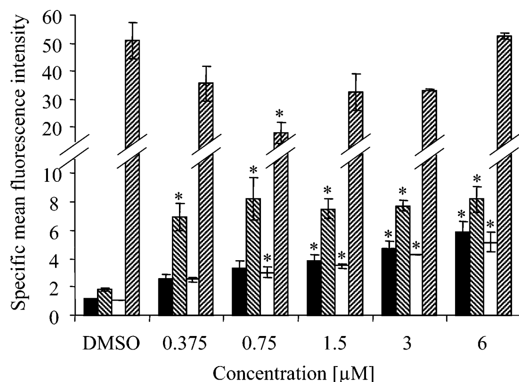


Figure 2. Effect of zapotin (**1**) on the membrane phenotype of HL-60 cells. As described in the Experimental Section, cells were induced to differentiate with the indicated concentrations of zapotin (**1**) using a 4-day protocol and then analyzed for the following membrane markers of differentiation: CD11b (■), CD13 (diagonal negative slope), CD14 (□), and CD15 (diagonal positive slope). Results are expressed as the specific mean fluorescence intensity (ratio of antigen antibody fluorescence over isotype antibody fluorescence) and represent the mean of two independent studies. *Significantly different from control values ($p < 0.05$).

zapotin (**1**) after 24 h of exposure resulted in the differentiation of a similar percentage of cells as without withdrawal (Figure 1), while maintaining a higher cellular viability and density. This indicates that there is no further need for the presence of the compound after 24 h, at which time cells have become committed to differentiate. However, cell density was reduced by increasing the time of exposure to the drug.

Membrane phenotype was also analyzed using flow cytometry with a set of four myeloid markers (CD11b, CD13, CD14, and CD15). Zapotin (**1**) up-regulated CD11b, CD13, and CD14, and down-regulated CD15 in HL-60 cells (Figure 2). Thus, zapotin (**1**) induced a pattern of expression similar to that produced by macrophage inducers, with down-regulation of CD15 (granulocytic marker) and up-regulation of CD13 and CD11b (granulocytic/monocytic markers).[30]

Induction of Apoptosis by Zapotin (1) in HL-60 Cell Line. After a 24 h treatment period, zapotin (**1**) induced dose-dependent increases in apoptosis, as judged by the formation of apoptotic bodies observed with DAPI (4′,6-diamidino-2-phenylindole) staining (Figure 3), which were significant with doses of 3 μM and higher ($p < 0.01$). A time-dependent increase of apoptosis was also observed when cells were treated with 12 μM zapotin (**1**) (data not shown).

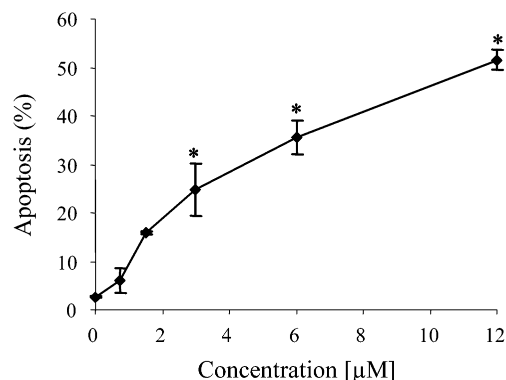


Figure 3. Dose-dependent induction of apoptosis in HL-60 cells treated with zapotin (**1**). Cells were treated with the indicated concentrations for 24 h. Apoptosis was quantified by counting nuclei stained with 4′,6-diamidino-2-phenylindole (DAPI). Results represent the mean of two independent studies. *Significantly different from control values ($p < 0.01$).

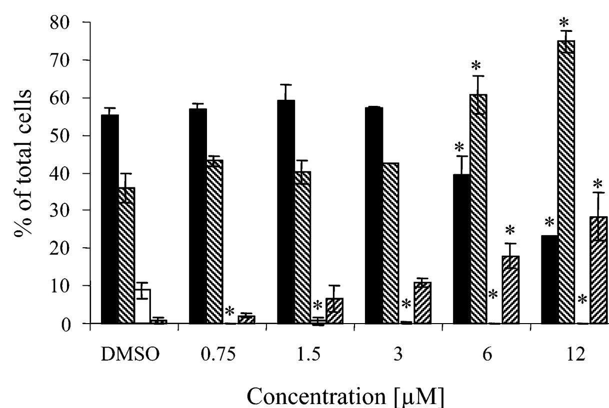


Figure 4. Cell cycle effects of zapotin (**1**) in HL-60 cells. Cells were treated with the indicated concentrations for 24 h, fixed in ethanol, and stained with PI (propidium iodide) for flow cytometric analysis, as described in the Experimental Section. Values are expressed as percentage of total cells and represent the mean ± SD of three determinations, for the following compartments of the cell cycle: G_1 (■), S (diagonal negative slope), G_2/M (□), and apoptotic peak sub G_1 (diagonal positive slope). *Significantly different from control values ($p < 0.01$).

Zapotin (1) Causes Accumulation of HL-60 Cells in S Phase. The impact of zapotin (**1**) on the cell cycle progression of HL-60 cells was determined by flow cytometry. Cell cycle analysis performed after 24-h incubations of HL-60 cells with increasing concentrations of zapotin (**1**) showed that this agent arrested the cells in the S phase of the cell cycle in a dose-dependent manner (Figure 4). In the cells treated with 3 μM zapotin (**1**), 61% of the cells were in S-phase as compared with 36% in the control population. A complete suppression of cells in the G_2/M phase of the cycle could be noted with concentrations of zapotin (**1**) as low as 0.75 μM. Also, a dose-dependent increase of the sub G_1 peak, characteristic of apoptosis, was evident.

To firmly establish this point, control and zapotin (**1**)-treated cells were allowed to incorporate BrdU for 30 min prior to harvest, and the level of incorporation due to new DNA synthesis was determined by staining with fluorescently labeled antibody to BrdU prior to analysis by flow cytometry. In Figure 5, total DNA content is presented along the *x*-axis and BrdU

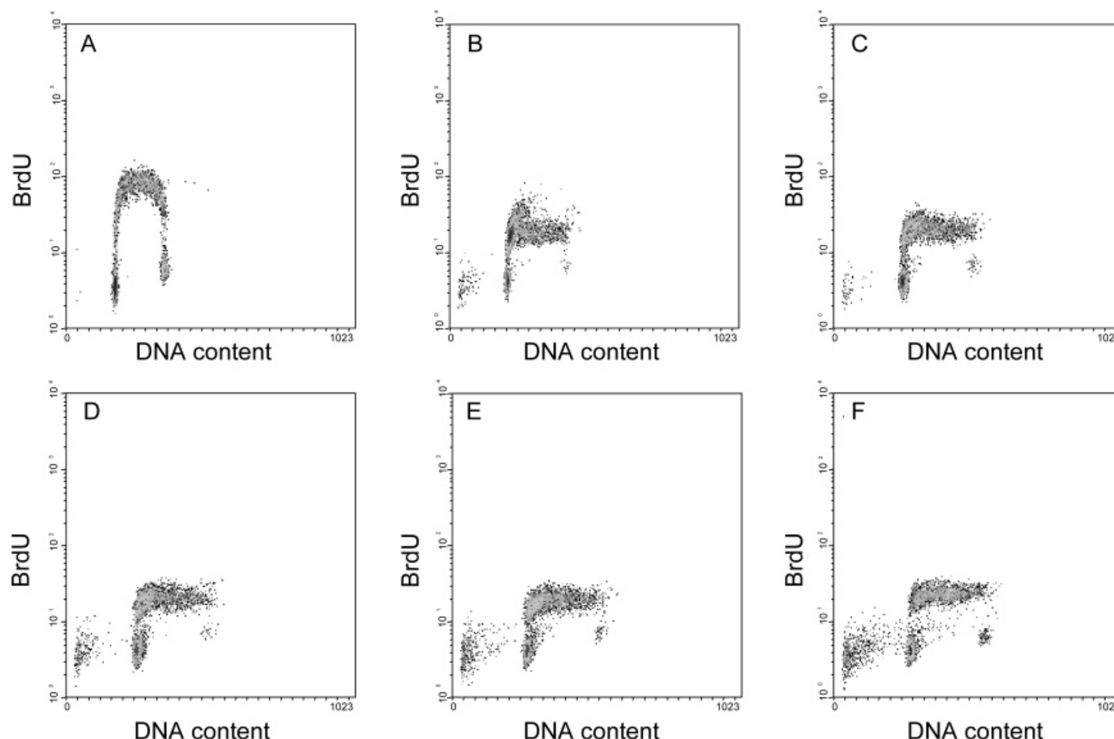


Figure 5. Effects of zapotin (**1**) on BrdU incorporation in HL-60 cells. Cells were treated with DMSO (A), 0.75 μM (B), 1.5 μM (C), 3 μM (D), 6 μM (E), or 12 μM (F) of zapotin (**1**) and harvested for cell cycle analysis 24 h later. For 30 min prior to harvest, cells synthesizing DNA were allowed to incorporate BrdU. Cells synthesizing DNA during this period were then labeled using fluorescein-modified antibody to BrdU and were analyzed by flow cytometry.

staining along the *y*-axis. After treatment with zapotin (**1**), cells did continue to enter the early stages of S phase confirming that zapotin (**1**) induces an S phase arrest.

Conclusion

Zapotin (**1**) was tested for various biological activities and found to be capable of mediating responses of sufficient magnitude or selectivity to demonstrate preliminary chemopreventive activity. To help facilitate more advanced biological investigations, a novel, efficient, multigram synthesis of zapotin (**1**) has been devised, which relies of the regioselective C-acylation of a dilithium dianion generated from an *o*-hydroxyacetophenone.

Experimental Section

NMR spectra were obtained at 300 MHz (^{1}H) and 75 MHz (^{13}C) in $CDCl_3$ using $CHCl_3$ as internal standard. Flash chromatography was performed with 230−400 mesh silica gel. TLC was carried out using commercially available precoated glass silica gel plates of 2.5 mm thickness. Melting points are uncorrected. Unless otherwise stated, chemicals and solvents were of reagent grade and used as obtained from commercial sources without further purification. Tetrahydrofuran (THF) was freshly distilled from sodium/benzophenone ketyl radical prior to use. Acetone was freshly distilled from potassium carbonate prior to use.

3,6-Dihydroxy-2-methoxyacetophenone (**8**). A solution of 2-hydroxy-6-methoxyacetophenone (5.0 g, 30.1 mmol) in 10% sodium hydroxide (57.5 g in 575 mL H_2O) was added dropwise at room temperature to an aqueous solution of potassium persulfate (8.2 g, 30.3 mmol) in water (350 mL) with stirring for 7 d at 20 °C. The mixture was cooled in an ice bath, acidified to pH 5−6 with concd HCl, and left overnight at room temperature. The unreacted starting material was removed by extraction with ethyl acetate, and the aqueous solution was further acidified to pH 2, and then heated for 4 h on a water bath after addition of solid sodium sulfite (5.8 g, 46.0 mmol). The cooled solution was extracted with chloroform (3 × 100 mL). The organic extract was evaporated under reduced pressure, and the residue was purified by chromatography on silica gel (eluent: $CHCl_3$) to give 3,6-dihydroxy-2-methoxyacetophenone (**8**) as a pale greenish-yellow solid (3.2 g, 60%): mp 95 °C (lit 94−97 °C).[19] R_f = 0.25 (SiO_2, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 12.0 (s, 1 H), 7.12 (d, J = 9.2 Hz, 1 H), 6.67 (d, J = 9.2 Hz, 1 H), 5.08 (s, 1 H), 3.81 (s, 3 H), 2.70 (s, 3 H). All other data are the same as reported.[19]

6-Hydroxy-2,3-dimethoxyacetophenone (9). A mixture of 3,6-dihydroxy-2-methoxyacetophenone (1.3 g, 7.1 mmol) and oven-dried potassium carbonate (1.0 g, 7.6 mmol) in dry acetone was stirred for 10 min. Dimethyl sulfate (0.6 mL, 7.1 mmol) was added dropwise to the reaction mixture, and the mixture was stirred for 7 d at room temperature. The solvent was evaporated under reduced pressure, and water (25 mL) was added to the residue. The mixture was extracted with chloroform (3 × 100 mL), and the organic phase was dried over Na_2SO_4 and evaporated under reduced pressure. The residue was column chromatographed on silica gel eluting with $CHCl_3$ to afford compound **9** (1.26 g, 89% yield). R_f = 0.75 (SiO_2, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.10 (d, J = 9.3 Hz, 1 H), 6.66 (d, J = 9.3 Hz, 1 H), 3.92 (s, 3 H), 3.81 (s, 3 H), 2.70 (s, 3 H). All other data are the same as reported.[19]

Zapotin (1). A solution of LiHMDS in THF (1 M, 20 mL, 20 mmol) was added to a well-stirred solution of acetophenone **9** (1.0 g, 5.1 mmol) in THF (10 mL) under argon at −78 °C over 15 min. The reaction mixture was stirred at −78 °C for 1 h and at −10 °C for 2 h and was cooled again to −78 °C, and a solution of 2,6-dimethoxybenzoyl chloride **11** (1.38 g, 90% tech. grade, 6.2 mmol) in THF (10 mL) was added in one portion. Stirring was continued at −78 °C for 1 h and at room temperature for 24 h (until the disappearance of the starting material by TLC). The reaction mixture was poured into a mixture of ice (50 g) and concd HCl (5.4 mL) and extracted with $CHCl_3$ (3 × 100 mL). The solvent was evaporated from the dried (Na_2SO_4) extracts, and the residue was dried under vacuum for 24 h. A small portion of the crude product was taken out and purified by column chromatography on silica gel (eluent EtOAc−hexanes 3:1) to give compound **12**: mp 119−120 °C. R_f = 0.55 (SiO_2, EtOAc−hexanes 3:1); IR (neat) 2939, 2838, 1610, 1593, 1574, 1474, 1269, 1254, 1112 cm^{-1}; ^{1}H NMR

(300 MHz, $CDCl_3$) δ 7.30 (t, $J = 8.7$ Hz, 1 H), 7.02 (d, $J = 8.7$ Hz, 1 H), 6.87 (s, 1 H), 6.67 (d, $J = 8.7$ Hz, 1 H), 6.58 (d, $J = 8.4$ Hz, 2 H), 3.80 (s, 6 H), 3.78 (s, 6 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 193.6, 178.2, 158.0, 155.9, 145.5, 131.5, 120.4, 114.1, 112.7, 105.8, 104.0, 61.5, 57.1, 56.0; EIMS (*m/z*, relative intensity) 360 (M^+, 6), 329 (3), 222 (2), 180 (7), 165 (100), 150 (9), 137 (5), 122 (5), 107 (7); HRMS *m/z* calcd for ($C_{19}H_{20}O_7$) 360.1209, found 360.1213. Anal. ($C_{19}H_{22}O_7$) C, H. The rest of the residue was mixed with glacial acetic acid (20.0 mL) and sulfuric acid (0.1 mL) and heated at 95−100 °C under argon atmosphere for 3.5 h. Solvent was removed under reduced pressure, and the residue was poured into water (100 mL). The mixture was extracted with chloroform (3 × 100 mL) and dried with Na_2SO_4, and the residue was chromatographed with silica gel (eluent ethyl acetate−hexane, 3:1) to yield pure zapotin (**1**, 1.4 g, 82%): mp 146−147 °C (lit.[31] 147−148 °C). $R_f = 0.25$ (SiO_2, EtOAc-hexane 3:1); IR (neat) 2939, 2840, 1650, 1592, 1475, 1417, 1357, 1281, 1255, 1111 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.35 (t, $J = 8.7$ Hz, 1 H), 7.25 (d, $J = 9.3$ Hz, 1 H), 7.16 (d, $J = 9.3$ Hz, 1 H), 6.59 (d, $J = 8.4$ Hz, 2 H), 6.26 (s, 1 H), 3.94 (s, 3 H), 3.88 (s, 3 H), 3.75 (s, 6 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 177.9, 158.7, 158.2, 152.2, 149.3, 147.4, 131.8, 119.0, 118.5, 114.9, 113.5, 110.9, 103.6, 61.5, 56.8, 55.7; EIMS (*m/z*, relative intensity) 342 (M^+, 50), 327 (100), 311 (7), 283 (5), 253 (8), 237 (3), 197 (3), 182 (5), 165 (37), 137 (83), 109 (26), 91 (18), 69 (19), 53 (14); HRMS *m/z* calcd for ($C_{19}H_{18}O_6$) 342.1103, found 342.1107. Anal. ($C_{19}H_{18}O_6$) C, H.

Cell Culture. T24 and HL-60 cells were obtained from the American Type Culture Collection (Rockville, MD). T24 cells were cultured in MEM medium (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum, non-essential amino acids, 1 mM sodium pyruvate (BioWhittaker, Walkersville, MD), 100 units penicillin/mL, 100 μg streptomycin/mL, and 250 ng amphotericin B/mL (Gibco Invitrogen, Grand Island, NY). The HL-60 cell line was maintained in suspension culture using RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum, 100 units penicillin/mL, and 100 μg streptomycin/mL. HepG2 human hepatoma cells stably transfected with NF-κB-luciferase plasmid[32] were maintained in Ham's F12 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin G sodium, 100 μg/mL streptomycin sulfate, 1% MEM amino acid, and 0.1% insulin. The cell lines were maintained in a 5% CO_2 atmosphere at 37 °C and were routinely tested for mycoplasma contamination.

Determination of TPA-Induced ODC Activity in T24 Cells. T24 cells were treated with various concentrations of zapotin (**1**) and determination of ODC activity was performed as described previously.[33] In brief, cells were plated at an initial density of 2 × 10^5 cells per well in 24-well plates. After an 18 h preincubation, a solution of zapotin (**1**) in DMSO was added in duplicate (5 μL, 0.5% final concentration) before the induction of ODC activity with TPA (200 nM final concentration). After an additional 6 h incubation, plates were washed twice with PBS and kept at −85 °C until tested. ODC activity was directly assayed by measuring the release of [^{14}C]CO_2 from L-[1-^{14}C]-ornithine HCl in the presence of 190 μM nonradioactive ornithine HCl. The amount of radioactivity captured in NaOH-impregnated filter discs was determined by scintillation counting in 24-well plates using a Wallac 1450 Microbeta liquid scintillation counter. Protein was determined according to the Lowry procedure. Interfering dithiothreitol contained in the reaction mixture was destroyed by adding chloramine T (50 μL, 8 mg/mL) to each well (30 min incubation at RT), followed by NaOH (50 μM, 5.7 M) to solubilize the protein. The protein was measured in 96-well plates using an aliquot of the reaction mixture and bovine serum albumin as a standard. The optical density was measured at 660 nm using a BT2000 Microkinetic Reader. The results were calculated as nmol [^{14}C]CO_2/h/mg protein and expressed as a percentage in comparison with a control treated with DMSO and TPA. Dose−response curves were prepared, and the results were expressed as IC_{50} values in micromolar concentrations. IC_{50} values were generated from the results of four serial dilutions of zapotin (**1**) tested in duplicate.

Determination of TPA-Induced NF-κB Activity in HepG2 Cells. HepG2 cells stably transfected with NF-κB-luciferase plasmid were treated with various concentrations of zapotin (**1**), and determination of luciferase activity was performed as described previously.[34] In brief, transfected cells were incubated for 48 h in 96-well plates. After 6 h incubation with TPA (100 nM) and zapotin (**1**), cells were analyzed for luciferase activity. Cells were washed with PBS and lysed using 50 μL 1X Reporter Lysis Buffer (Promega, Madison, WI) for 10 min, and the luciferase determination was performed according to the manufacturer's protocol. Data were expressed as the concentration required to inhibit activation by 50% (IC_{50} value). Tumor necrosis factor (TNF)-α was used as a standard inhibitor (IC_{50} 15−25 ng/mL).[33] IC_{50} values were generated from the results of four serial dilutions of zapotin (**1**) tested in duplicate. With the experimental conditions used, no signs of overt cellular toxicity were observed.

Cell Differentiation Assays. HL-60 cells were tested using a 4-day protocol.[35] In brief, cells in log phase (approximately 10^6 cells/mL) were diluted to 10^5 cells/ mL and preincubated overnight (18 h) in 24-well plates to allow cell growth recovery. Then, samples dissolved in DMSO were added, keeping the final DMSO concentration at 0.1% (v/v). Control cultures were treated with the same concentration of DMSO. After 4 days of incubation, cells were analyzed to determine the percentage exhibiting functional nitroblue tetrazolium (NBT) reduction, and cell surface markers of differentiated cells, as described below.

Nitroblue Tetrazolium (NBT) Reduction. Evaluation of NBT reduction was used to assess the ability of sample-treated cells to produce superoxide when challenged with TPA. A 1:1 (v/v) mixture of a cell suspension (10^6 cells) and TPA/ NBT solution [2 mg/mL NBT and 1 μg/mL TPA in phosphate buffer saline (PBS)] was incubated for 1 h at 37 °C. Then cells were smeared on glass slides and counterstained with 0.3% (w/v) safranin O in methanol. Positive cells reduce NBT yielding intracellular black-blue formazan deposits and were quantified by microscopic examination of >200 cells. Results were expressed as a percentage of positive cells.

Determination of Cell Surface Antigens. Cells (10^6), prewashed with PBS, were resuspended in 100 μL diluent (PBS with 0.1% sodium azide and 1% BSA) and incubated for 30 min at room temperature with the monoclonal antibodies anti-CD-11b (Sigma, St. Louis, MO), anti-CD13 (Caltag, Burlingame, CA), anti-CD14 (Sigma), and anti-CD15 (Caltag), conjugated with FITC. Cells were washed with 20 volumes of diluent and resuspended in 0.5 mL of 2% paraformaldehyde for flow cytometry evaluation. Identical samples were prepared using isotype antibodies to correct fluorescence due to nonspecific binding.

Quantification of Apoptosis. Cells were treated with various concentrations of zapotin (**1**) for 24 h, or with 12 μM zapotin (**1**) for various time intervals, washed with PBS, and fixed with methanol−acetic acid 1:1 for 30 min at room temperature. Cells were then treated with 4′,6-diamidino-2-phenylindole (DAPI, 1 μg/mL) for 15 min at room temperature. DAPI staining of the nucleus was observed by fluorescence microscopy. At least 100 cells were counted for each sample. Dose−response curves showing the percentage of apoptosis at different doses and times were constructed.

Cell Cycle Analysis. Cells (3 × 10^6) were treated with various concentrations of zapotin (**1**) for 24 h and washed with PBS. Cells were resuspended in 1 mL PBS + 9 mL ice-cold 70% EtOH and stored at −20 °C. Just before analysis, samples were centrifuged and cell pellets were resuspended in 2 mL of propidium iodide solution (2 μg/mL propidium iodide, 100 μg/mL ribonuclease A in PBS). Solutions were incubated at 37 °C for 1 h, placed on ice, and analyzed by flow cytometry. At least 10 000 cells were counted for each sample. The percentage of apoptotic cells was calculated by measuring the area under the subdiploid (DNA < 2 N) peak in the plot of cell number against cellular DNA content.

Alternatively, cells were exposed to 5-bromo-2-deoxyuridine (BrdU) for 30 min prior to trypsinization to specifically label S-phase cells. After fixation, cells were stained with fluorescein-conjugated antibody to BrdU and counterstained with propidium

iodide following the manufacturer's protocol (Phoenix Flow Systems, San Diego, CA). Cell suspensions were analyzed by flow cytometry, and data were collected using appropriate electronic gating to remove background debris and aggregates.

Statistical Analysis. Data were expressed as means ± SD and analyzed through one-way analysis of variance (ANOVA), followed by pairwise comparisons made with Dunnett's test, using the SAS statistical package (SAS Institute, Cary, NC). All of the tests were two-sided, and, unless otherwise specified, a p value of less than 0.01 was considered to be significant.

Acknowledgment. This work was supported by program project P01 CA48112 awarded by the National Cancer Institute.

Supporting Information Available: ^{1}H NMR, ^{13}C NMR, and elemental analysis results for compound **12** and zapotin (**1**). This material is available free of charge via the Internet at http://pubs.acs.org.

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JM060915+