Inhibitors of the NF-κB Activation Pathway from *Cryptocarya rugulosa*[¶]

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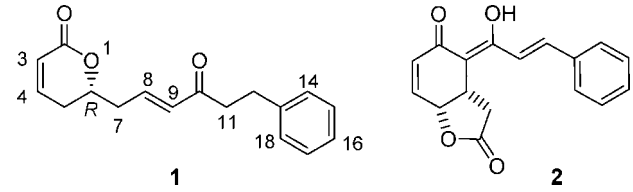
The nuclear factor-κB (NF-κB) signaling pathway is constitutively active in many types of cancers and is a potential therapeutic target. Using a cell-based assay for stability of inhibitor of kappa B (IκB), a critical regulator of NF-κB activity, we found that an organic solvent extract of the plant *Cryptocarya rugulosa* inhibited constitutive NF-κB activity in human lymphoma cell lines. The active components were identified as rugulactone, a new α-pyrone (**1**), and the known cryptocaryone (**2**). Rugulactone was the more active compound, exhibiting up to 5-fold induction of IκB at 25 μg/mL; maximal activity was observed with 10 h exposure of test cells to **1** or **2**.

Transient activation of the nuclear factor-κB (NF-κB) family of transcription factors is essential in cell development, inflammation, and the immune response. NF-κB target genes fall into four functional categories: inflammatory and immunoregulatory genes, antiapoptotic genes, genes that positively regulate the cell cycle, and genes that code for negative regulators of NF-κB. In recent years, it has also been demonstrated that constitutive activation of NF-κB is pathogenic in many cancers, and target genes of all four categories contribute to tumorigenesis.[1] The classical or canonical pathway of NF-κB activation depends on phosphorylation-induced ubiquitination of the Inhibitor of kappa B (IκB) proteins, leading to their degradation. This sequential modification depends on two protein complexes, the IκB kinase (IKK) complex and the $E3^{I\kappa B}$ ubiquitin ligase complex.[2] Pharmaceutical companies have targeted both complexes in developing inhibitors that may be used against cancer and autoimmune diseases.

The basal level of IKK activity is low in normal unstimulated B lymphocytes, but certain types of B-cell lymphoma are among those cancers that exhibit high constitutive NF-κB activity.[3] We have developed a cell-based assay of IκB stability using a reporter protein of IκBα fused to *Photinus* luciferase in a diffuse large B cell lymphoma (DLBCL) cell line (OCI-Ly3) that has high constitutive IKK activity.[4] In high-throughput screening of natural products, we found that an organic solvent extract of the plant *Cryptocarya rugulosa* Hook. f. (Lauraceae) raised levels of the IκBα-luciferase reporter in OCI-Ly3 cells 1.5−2.5-fold compared to the positive control, suggesting inhibition of IKKβ activity.

The plant genus *Cryptocarya* is comprised of a large number of species distributed throughout the tropics and subtropics.[5] The most common secondary metabolites reported from this genus are alkaloids, flavonoids, and α-pyrones.[6−8] The application of a multidetector-HPLC-bioassay/database comparison (dereplication) strategy designed in our laboratory[9−11] to the *C. rugulosa* extract indicated that the active zones in the biogram corresponded to α-pyrone and, possibly, flavonoid chemotypes. The active components were identified after a scale-up isolation that included liquid−liquid partitioning, vacuum−liquid chromatography over C_{18}-bonded phase, gel permeation through Sephadex LH-20, and HPLC using C_{18}- and diol-bonded phase columns. Two active metabolites were identified as rugulactone (**1**), a new α-pyrone, and cryptocaryone (**2**).[12,13]



1 **2**

Results and Discussion

Rugulactone (**1**) was isolated as an optically active compound with a molecular formula of $C_{17}H_{18}O_3$ (HRMS). The ^{1}H NMR spectrum showed a complex five-proton multiplet at δ 7.18−7.30, indicating a monosubstituted benzene ring, which was confirmed by the presence of ^{13}C NMR resonances at δ 141.0, 128.5, 128.4, and 126.1. Furthermore, two spin systems at δ 6.88 and 6.05 (1H, ddd, J = 8.9, 4.4, 3.6 Hz, and 1H, ddd, J = 8.9, 2.0, 1.6 Hz) and δ 6.80 and 6.20 (1H, dt, J = 16.0, 7.2 Hz and 1H, ddd, J = 16.0, 1.6, 1.2 Hz) indicated two disubstituted double bonds with *Z* and *E* configurations, respectively. The chemical shifts of the vinylic protons of each pair suggested that both systems were conjugated. This observation, in combination with UV absorption maxima at 209, 260, and 315 nm and IR bands at 1721 and 1672 cm^{-1}, suggested two α,β-unsaturated carbonyl groups as components of the skeleton. Besides the ^{13}C NMR signals that confirmed the aforementioned functionalities (α,β-unsaturated-ketone: 199.0, 139.8, and 133.4 ppm; α,β-unsaturated-δ-lactone: 163.7, 144.5, and 121.4 ppm), four methylenes and an oxymethine were observed. The spectroscopic data and the known chemotaxonomy of this genus were in accord with a 6-arylalkyl-5,6-dihydro-α-pyrone. The substituents were placed in the alkyl chain following the ^{1}H−^{1}H COSY and HMBC connectivities. The stereochemistry of the only chiral center in the molecule, C-6, was determined using the Snatzke rule, which predicts the configuration as *R* if the CD curve at 254−272 nm is positive.[14]

6-Arylalkyl-5,6-dihydro-α-pyrones have been reported in only two genera of the Lauraceae family, *Aniba* and *Cryptocarya*. Those from *Cryptocarya* characteristically lack substituents on C-4.[8] Cryptocaryone (**2**) was isolated previously from *C. bourdilloni* in 1972, but it was reported as a chalcone.[12] More than 10 years later, an X-ray analysis of **2** led to revision of its structure.[13] This compound was reported as active against the KB, erythroleukemic K562, and doxorubicin-resistant K562 cell lines with IC_{50} values

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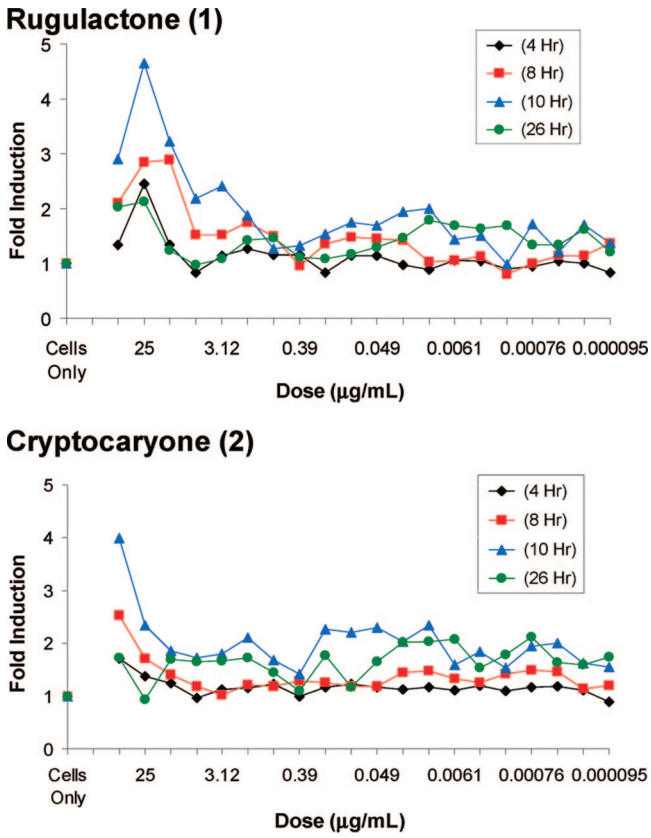

Figure 1. Time-course evaluation of the level of the IκB-luciferase reporter protein in OCI-Ly3 cells at 4, 8, 10, and 26 h of exposure to compounds **1** and **2**.

between 1.8 and 2 μM.[15] A few examples of 6-substituted-5,6-dihydro-α-pyrones were previously evaluated for their cytotoxic activity,[16−18] but no details of secondary biological evaluations, to our knowledge, have appeared in the literature.

The preliminary evaluation of the pure compounds showed that both could produce inductions of IκBα-luciferase reporter up to 4-fold higher than controls, in a dose−response manner, at concentrations over 11 and 16 μM for **1** and **2**, respectively. Time-course assays indicated that the magnitude of the effect was highest after 10 h of exposure of the OCI-Ly3 cells to **1** and **2** (Figure 1). To evaluate the selectivity of these metabolites, additional assays were run in a time-course fashion in multiple DLBCL cell lines with the IκBα-luciferase fusion reporter. Both OCI-Ly3 and OCI-Ly10 possess constitutive IKK activity; OCI-Ly19, lacking constitutive IKK activity, was used as control. Cell lines in these additional assays were also engineered to express a second, unmodified luciferase (from *Renilla*) to serve as a control for cytotoxicity and assay imprecision. The combination of these reporters provides more detailed information about a compound's behavior, as has been documented elsewhere.[4] A pure IKK inhibitor should raise the IκB-*Photinus*/*Renilla* (I-P/R) ratio in IKK-active lines, without substantial change in the *Renilla* reporter value alone during brief (<12 h) incubations. However, compounds may affect both reporters, as we have observed for a large screen using a similar cell-based assay.[4] In particular, compounds may lower the *Renilla* reporter value due to effects such as "off-target" toxicity, while increasing the I-P/R ratio due to IKK inhibition (more than what is mathematically due to the decrease in the *Renilla* reporter value).

As can be seen in Figure 2, both compounds **1** and **2** elevated the I-P/R ratio in OCI-Ly3 and OCI-Ly10 cell lines at points in the spectrum of tested concentrations, more than that which could be attributed to decreased *Renilla* reporter values also observed at higher concentrations and longer incubations. In agreement with the primary assay, rugulactone (**1**) is more potent than **2** in both

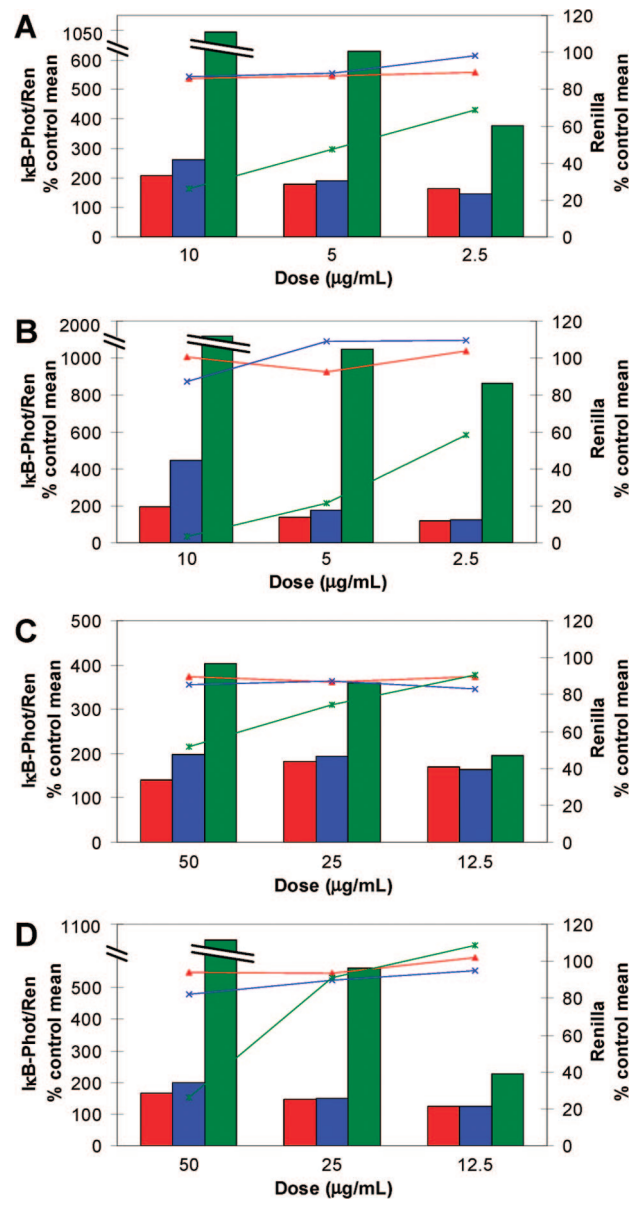

Figure 2. Time-course evaluation of dual-reporter IKK assay: IκB-*Photinus*/*Renilla* ratio (bars), *Renilla* reporter (lines) at 2.5 (red), 5 (blue), and 10 (green) h. Rugulactone (**1**) was evaluated at 10, 5, and 2.5 μg/mL in OCI-Ly3 (A) and OCI-Ly10 (B) cells. Cryptocaryone (**2**) was tested at 50, 25, and 12.5 μg/mL in OCI-Ly3 (C) and OCI-Ly10 (D) cells.

cell lines, showing a clear concentration−response effect when the activity is compared at the same time of exposure (bars with same color). Although rugulactone (**1**) was more active in OCI-Ly10 cells, the depression of *Renilla* values was also more pronounced at 10 h for all concentrations (green lines). However, after 10 h incubation with OCI-Ly3 cells, **1** depressed *Renilla* values only at the two higher concentrations. When both metabolites were tested in OCI-Ly19 cells at the chosen highest concentrations (data in Supporting Information), no significant effect in the I-P/R ratio was observed. After 10 h exposure of OCI-Ly19 cells to both compounds, a slight increase of the I-P/R ratio could be observed, attributable only to the reduction of the *Renilla* reporter value. These final data suggest that effects on *Renilla* values may be due to off-target toxicity, and more exhaustive analysis should be performed to understand the mechanism(s) of action of **1** and **2**.

This report demonstrates the application of a new molecular targeted screen to a natural product extract library, the isolation

and identification of a new α-pyrone, and the identification of a new chemotype of inhibitors of the NF-κB activation pathway.

Experimental Section

General Experimental Procedures. UV measurements were conducted on a Perkin-Elmer Lambda 20 UV/vis spectrometer. The IR spectrum was obtained with a Perkin-Elmer Spectrum 2000 FT-IR spectrometer. A CD spectrum was recorded with a JASCO J-720 spectropolarimeter. ^{1}H and ^{13}C NMR analyses, including 2D experiments, were conducted on a Varian 500 MHz INOVA instrument. Preparative HPLC was performed with a Waters 600 pump controlled by MassLynx software. Post-column detection was accomplished by a parallel arrangement of a Micromass ZMD electrospray ionization (ESI) mass spectrometer (cone voltage = 30), a Waters 996 photodiode array (PDA), and a Sedex 75 evaporative laser light scattering detector (ELSD).

Plant Material. *Cryptocarya rugulosa* Hook. f. was collected by Dr. D. D. Soejarto of the University of Illinois at Chicago, under contract to the NCI, in the Pasoh Forest Reserve, Malaysia, in August 1992. Voucher specimens (U44Z-2846) have been deposited at the Smithsonian Institution and at the DTP Repository in Frederick, MD.

Extraction and Isolation. The dried leaves and twigs were finely ground in a hammer mill (725 g), placed into a 3 L borosilicate glass percolator, and steeped in CH_2Cl_2−MeOH (1:1) for 16 h at rt. After removal of the extraction solvents, the marc was covered briefly with MeOH, which was then drained and combined with the CH_2Cl_2−MeOH extract. Solvent was removed by rotary evaporation at <40 °C, followed by high-vacuum drying, resulting in a yield of 13.2 g. Crude extract (4.31 g) was suspended in 200 mL of MeOH−H_2O (9:1) and extracted with an equal volume of hexane (3×); after evaporation of the MeOH from the polar phase under reduced pressure, H_2O was added to 200 mL total volume and partitioned with CH_2Cl_2 (3 × 200 mL). The CH_2Cl_2 extract (1.29 g) was processed by VLC using C_{18}-bonded phase, affording five fractions upon elution with MeOH−H_2O (1:4), MeOH−H_2O (1:1), MeOH−H_2O (9:1), MeOH, and MeOH−CH_2Cl_2 (3:7). The third fraction (479.6 mg) displayed activation of the luciferase reporter and was further subjected to gel permeation chromatography on Sephadex LH-20 with CH_2Cl_2−MeOH (1:1), yielding 15 fractions (A−O). Fractions F through I exhibited IκB activity. Fractions F and G were combined (252.0 mg) and purified by preparative HPLC [C_{18} Dynamax 60 Å, 8 μm, 2.1 × 25 cm, MeCN−20 mM NH_4OAc pH 4, gradient 40 min (2:3 to 7:3), flow rate 15 mL/min] to obtain a fraction highly enriched with **1**. A final purification step, using normal-phase preparative HPLC [Varian diol, hexane−CH_2Cl_2, 0−15 min isocratic at 13:7, 15−25 min gradient to 0:1, flow rate 10 mL/min], yielded 7.0 mg of **1**. Fractions H and I (79.8 mg) were processed in similar conditions to those detailed above, but in the last step using hexane−EtOAc/0.3% acetic acid as mobile phase (0−10 min isocratic at 7:3, 10−15 min gradient to 1:1, and isocratic to 20 min), affording **2** (2.7 mg).

Rugulactone (1): UV (MeOH) λ_{max} (log ϵ) 209 (4.26), 260 (2.85), 315 (1.95) nm; CD (MeOH) $[\theta]_{206}$ −5.3, $[\theta]_{212}$ 0, $[\theta]_{228}$ 18.0, $[\theta]_{256}$ 29.5, $[\theta]_{310}$ 0; IR (NaCl) ν_{max} 2922, 1721, 1672, 1630, 1494, 1452, 1384, 1245, 1042, 979, 700 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.27 (2H, m, H-15/H-17), 7.20 (1H, m, H-16), 7.20 (2H, m, H-14/H-18), 6.88 (1H, ddd, J = 8.9, 4.4, 3.6 Hz, H-4), 6.80 (1H, dt, J = 16.0, 7.2 Hz, H-8), 6.20 (1H, ddd, J = 16.0, 1.6, 1.2 Hz, H-9), 6.05 (1H, ddd, J = 8.9, 2.0, 1.6 Hz, H-3), 4.55 (1H, tdd, J = 8.0, 6.8, 5.6 Hz, H-6), 2.93 (2H, m, H_2-12), 2.91 (2H, m, H_2-11), 2.64 (2H, m, H_2-7), 2.33 (2H, m, H_2-5); ^{13}C NMR ($CDCl_3$) δ 199.0 (C, C-10), 163.7 (C, C-2), 144.5 (CH, C-4), 141.0 (C, C-13), 139.8 (CH, C-8), 133.4 (CH, C-9), 128.5 (CH, C-15/C-17), 128.4 (CH, C-14/C-18), 126.1 (CH, C-16), 121.4 (CH, C-3), 75.8 (CH, C-6), 41.6 (CH_2, C-11), 37.3 (CH_2, C-7), 29.7 (CH_2, C-12), 28.7 (CH_2, C-5); HRFABMS *m/z* 271.1333 $[M + H]^+$ (calcd for $C_{17}H_{19}O_3$, 271.1334).

Screening Cell-Based Assays. The OCI-Ly3 cell line was engineered to express the IκB-*Photinus* fusion reporter as previously described[19] and maintained in Iscoves modified Dulbecco phenol red-free medium with 20% supplemented calf serum (catalog no. SH 30541. Thermo Fisher Scientific) and standard concentrations of 2-mercaptoethanol, penicillin, and streptomycin. To perform the screening assay, cells were pelleted, resuspended in fresh medium, and dispensed into 384-well plates at 30 000 cells in 25 μL/well. The plates were then incubated under 5% CO_2 at 37 °C for 24 h before addition of test compounds and during the subsequent incubation. Using robotic assisted plate preparation, compounds **1**, **2**, and a positive control (PS341, bortezomib) were added to the plates in a serial dilution mode, creating final test concentrations from 50 to 9.5 × 10^{-5} μg/mL. An untreated (negative) control was also employed. Readings were taken at 4, 8, 10, and 26 h post drug addition for viability and IκB-*Photinus* level. To measure the IκB-*Photinus* level, plates were removed from the incubator and equilibrated for 1 h at 24−26 °C in a 5% CO_2 incubator. To each well was added 25 μL of luciferase substrate (Britelite, Perkin-Elmer); then plates were left at rt in a laminar flow hood for at least 3 min before being read in sequential order with a WALLAC 1420 reader in luminescence mode. Results are reported as fold induction, relative to the positive control (e.g., 2.1-fold induction means 210% of the response of PS341).

Confirmatory Cell-Based Assay. Three cell lines (OCI-Ly3, OCI-Ly10, and OCI-Ly19) were engineered to stably express the IκB-*Photinus* fusion reporter as previously described,[19] as well as unmodified *Renilla* luciferase (Promega) as a normalization control. Cells were maintained in Iscoves modified Dulbecco phenol red-free medium with 20% heparinized human plasma and standard concentrations of 2-mercaptoethanol, penicillin, and streptomycin. To perform the assay, cell suspensions were dispensed in 96-well V-bottom plates, inoculated with various concentrations of test compounds, and harvested for analysis 4 h later, after being returned to 5% CO_2 incubators at 37 °C. For analysis, cells were pelleted by centrifugation of plates, the medium was removed with a multichannel aspirator, pellets were lysed by addition of 50 μL/well buffer (Glo-Lysis, Promega), and then 50 μL/well of substrates for the two luciferases (Dual-Glo, Promega) was added for successive readings of luminescence. Results for each well were calculated as the IκB-*Photinus*/*Renilla* luciferase ratio or the value of *Renilla* luciferase alone, after normalization to the untreated control mean, as respective measures of IKK activity and viability.

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Supporting Information Available: ^{1}H, HSQC, and HMBC NMR spectra of rugulactone (**1**); dereplication fingerprint of *C. rugulosa*; toxicity data of **1** and **2**; time-course assay data of **1** and **2** in OCI-Ly19 cells; and experimental conditions for toxicity assays are provided free of charge via the Internet at http://pubs.acs.org/jnp.

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