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Cytotoxic and HIF-1α Inhibitory Compounds from *Crossosoma bigelovii*

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Cytotoxicity-guided fractionation of an organic solvent extract of the plant *Crossosoma bigelovii* led to the discovery of a new strophanthidin glycoside (**1**) and two new 2-methylchromone glycosides (**2** and **3**). Also isolated were the known chromones eugenin and noreugenin, the indole alkaloid ajmalicine, the dibenzylbutane lignan secoisolariciresinol, the dibenzylbutyrolactone lignan matairesinol, and the furanone 5-tetradec-5-enyldihydrofuran-2-one. Further investigation into the biological properties of strophanthidin glycosides revealed a connection between inhibition of HIF-1 activation and the glycosylation of the genin. This work is the first published study of the bioactive phytochemicals of the family Crossosomataceae.

The plant family Crossosomataceae Engl., aligned with Rosidae, is comprised of three genera, *Apacheria*, *Crossosoma,* and *Forsellesia*, represented by a total of eight species, all found in the southwestern United States and Mexico.[1,2] Cytotoxicity-guided isolation from an organic solvent extract of *Crossosoma bigelovii* S. Wats. uncovered three new cytotoxic compounds: a cardenolide, **1**, two chromone glycosides, **2** and **3**, and known structures **4** and **5**.[3] High-throughput *in vitro* screening in a molecular target assay[4] revealed HIF-1 inhibitory activity from an organic solvent extract of the aerial parts of *C. bigelovii*. Bioassay-guided fractionation of this extract led to the identification of **1** as the most prominent HIF-1 inhibitory component, as well as four additional known minor compounds, **6**−**9**. Only a single reference to an investigation of the aromatic aldehydes present in Crossosoma could be found.[5] The findings presented here record for the first time the interesting bioactivities and the high chemical diversity of secondary metabolites produced by *C. bigelovii*.

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Results and Discussion

Elucidation of Structure. The molecular formula of **1** was assigned as $C_{36}H_{54}O_{14}$ by HRTOFMS, which showed an *m*/*z* 733.3434 $[M + Na]^+$. The IR spectrum exhibited absorption bands for a hydroxy group (3448 cm^{-1}, br), an aldehyde group (1735 cm^{-1}), and an α,β-unsaturated lactone (1715 and 1640 cm^{-1}). The UV spectrum in MeOH showed one strong absorption maximum at 208 nm ($\epsilon = 9300$) and one weak absorption maximum at 276 nm ($\epsilon = 502$), indicating the presence of a conjugated system. When sprayed with Kedde reagent and chloramine-trichloroacetic acid reagent on TLC, **1** displayed a violet color and brown fluorescence, respectively. These data provided evidence that **1** was a cardiac glycoside, perhaps a strophanthin glycoside such as k-strophanthin-β, a cytotoxic cardenolide previously isolated from *Apocynum venetum* var. *basikurumon*.[6] k-Strophanthin-β consists of a strophanthidin aglycone bound to a cymarose + glucose disaccharide, a molecule of the same MW as **1**. HPLC analysis of a DTP/NCI

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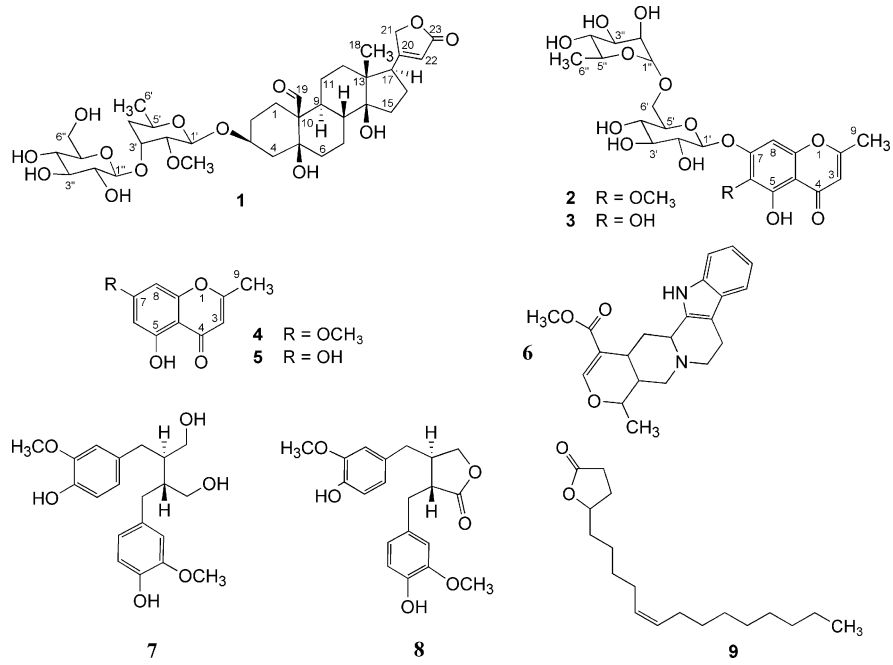

repository standard of k-strophanthin-β (NSC 4320) and **1** showed the compounds had markedly different retention times (8.6 and 11.3 min, respectively) on a cyano bonded-phase HPLC column eluted with CH_3CN-aqueous buffered mobile phase, providing proof that the structures were not equivalent.

Several NMR spectroscopic techniques were applied to elucidate the structural differences between k-strophanthin-β and **1**. The ^1H NMR spectrum of **1** displayed signals for two methylene protons (δ 4.93 and 5.08, 1H each, dd, $J = 20.0$, 1.0 Hz) and an olefinic proton (δ 5.90, br s), giving further evidence for the presence of an α,β-unsaturated lactone. The ^{13}C NMR spectrum showed signals at δ 178.2 (C-20), 177.2 (C-23), 117.9 (C-22), and 75.3 (C-21). A weak HMBC correlation between H-22 (δ 5.90) and C-17 (δ 51.7) verified the attachment of the α,β-unsaturated γ-lactone ring to C-17. In addition, the signals of a singlet methyl group (δ 0.85) and an aldehyde group (δ 10.05) were observed in the ^1H NMR spectrum. The existence of these groups was confirmed by the ^{13}C NMR spectrum with chemical shifts at δ 16.1 (C-18) and 210.0 (C-19). The methyl group was attached to C-13 because the long-range couplings of the methyl protons to C-12 (δ 40.5), C-14 (δ 85.9), and C-17 (δ 51.7) were observed in the HMBC spectrum. The aldehyde group was placed at C-10, because of the HMBC long-range correlation between the aldehyde proton and C-10 (δ 56.2). The remaining ^{13}C NMR signals of the aglycone were unambiguously assigned through systematic analyses of the DEPT, HMQC, and ^{13}C NMR spectra (see Supporting Information). The relative configuration of the fused rings A and B was determined to be cis because of the diagnostic ^{13}C chemical shifts of C-7 (δ 19.0) and C-9 (δ 40.3).[6] All these aglycone ^{13}C NMR data appeared identical to those of the known cardenolide strophanthidin.[6] Thus, differences between **1** and k-strophanthin-β must reside in the disaccharide moiety.

The $^1H-^1H$ COSY spectrum of **1** and its peracetylated derivative revealed the presence of one hexose and one 4,6-dideoxyhexose unit. When **1** was subjected to β-D-glucosidase (from *Caldocellum saccharolyticum*), D-glucose and a monosaccharide were liberated. Through detailed analyses of the $^1H-^1H$ coupling constants and consideration of the negative specific rotation of **1** ($[\alpha]_D = -30.6$),[7,8] the structure of this monosaccharide was determined to be 4,6-dideoxy-β-gulose. Furthermore, the HMBC spectrum of **1** indicated that 2′-*O* was methylated and the β-D-glucosyl group was attached to C-3′, on the basis of the long-range couplings of the methoxy protons (δ 3.36) with C-2′ (δ 80.9) and H-1″ (δ 3.36) with C-3′ (δ 70.8). The HMBC spectrum also demonstrated the long-range coupling between H-1′ and C-3, indicating that the disaccharide was attached to the aglycone through the 3-*O* position. These spectroscopic analyses led to the assignment of structure **1** for the new compound as 3β-[(*O*-β-D-glucopyranosyl-(1→3)-4,6-dideoxy-2-*O*-methyl-β-D-gulopyranosyl)]-5β,14β-dihydroxy-19-oxocard-20(22)enolide.

Compound **2** was obtained as a light yellow, amorphous solid. The pseudomolecular ion at *m/z* 531 ($[M + H]^+$) was detected in FABMS, together with its Na^+ and K^+ adducts at *m/z* 553 and 569, respectively. The molecular formula was then assigned as $C_{23}H_{30}O_{14}$ by HRFABMS. The IR spectrum exhibited absorption bands for a hydroxy group (3401 cm^{-1}) and an α,β-unsaturated carbonyl group (1661 and 1622 cm^{-1}). The UV spectrum in MeOH showed three absorption maxima at 253, 285, and 322 nm, suggesting a polyoxygenated chromone derivative.[9] The UV maxima were not affected by the addition of NaOAc, indicative of the absence of a free 7-hydroxy group.[9] Upon addition of anhydrous $AlCl_3$ or $AlCl_3-HCl$, however, the absorption maximum at 322 nm showed a bathochromic shift of 44 nm, indicating the presence of an intramolecularly hydrogen-bonded 5-hydroxy group. These UV spectroscopic characteristics resembled those of 5-hydroxychromones or 5-hydroxyisoflavones.[9,10] An isoflavone skeleton could be excluded because of the degree of unsaturation of the molecule.

The presence of the 5-hydroxy group was further supported by the detection of an intramolecular hydrogen-bonded phenolic hydroxy peak at δ 12.83 in the ^1H NMR spectrum. The existence of a conjugated carbonyl group was confirmed by a carbon signal at δ 182.4 in its ^{13}C NMR spectrum. The ^1H NMR spectrum also disclosed the existence of one methyl signal (br s) at δ 2.41, which was allylically coupled to an olefinic proton (br s) at δ 6.21 in the $^1H-^1H$ COSY spectrum, suggesting that this methyl group was attached to C-2 of a chromone. This functionality was further substantiated by the long-range coupling of the methyl protons to C-2 (δ 168.9) and C-3 (δ 107.7), and the olefinic proton H-3 to C-2, C-5 (δ 152.6), and 2-CH_3 (C-9, δ 20.0) in the HMBC spectrum. Additionally, one aromatic singlet at δ 6.77 and one methoxy singlet at δ 3.73 were observed in the ^1H NMR spectrum. Both protons were coupled to a quaternary carbon at δ 133.1 as shown by the HMBC spectrum, suggesting that the methoxy was attached to either C-6 or C-8. These NMR spectroscopic analyses led to the assignment of a 7-*O*-substituted 6-methoxy-5-hydroxy-2-methylchromone or 7-*O*-substituted 8-methoxy-5-hydroxy-2-methylchromone as the basic skeleton for compound **2**. These two

Table 1. Cytotoxicity (GI_{50}, μM) of Compounds Isolated from *C. bigelovii*

#	human tumor cell line		
	A-549	MCF-7	HT-29
1	0.008	0.01	0.07
aglycone of **2**	225	>450	180
2	0.4	0.8	6
3	0.4	0.8	6
4	14	10	25
5	>50	>50	>50
adriamycin	0.007	0.1	0.06

structures could be distinguished only if the C-13 resonances for C-6 and C-8 could be assigned unambiguously based on the long-range $^1H-^{13}C$ coupling between 5-OH and C-6. When dissolved in DMSO-d_6, this correlation between 5-OH and C-6 was not observed, due to the interference of residual water in the solvent. In order to enhance solubility in $CDCl_3$ for further $^1H-^{13}C$ coupling analysis, **2** was digested with naringinase, an enzyme produced by *Penicillium decumbens* having β-glucosidase specificity, to yield the aglycone. The HMBC spectrum of this aglycone clearly revealed the long-range couplings between the 5-OH and C-6 (δ 133.1). Therefore, it was concluded that the methoxy group was attached at C-6, rather than C-8.

The $^1H-^1H$ COSY spectrum of **2** also indicated the presence of one hexose and one 6-deoxyhexose moiety. Digestion with naringinase released two monosaccharides, in addition to the aglycone. These monosaccharides had the same TLC R_f values as authentic samples of D-glucose and L-rhamnose, which are the components of the disaccharide rutinose, commonly found in flavones. Flavone rutinosides possess negative $[\alpha]_D$,[11] in agreement with our data, and thereby supporting the assignment of the disaccharide substituent as being rutinose. The $^{13}C-^1H$ coupling constant of the anomeric carbons ($J_{C1'-H1'} = 167$ Hz, $J_{C1''-H1''} = 160$ Hz) suggested the presence of a β-anomer of D-glucose and an α-anomer of L-rhamnose.[12] The L-rhamnosyl moiety linkage to C-6′ of the glucose was established due to the downfield shift of the C-6′ signal (δ 66.6) in the ^{13}C NMR spectrum.[13] This linkage was further confirmed by the correlation between H-1″ and C-6′ in the HMBC spectrum. Finally, the linkage between the disaccharide group and the chromone aglycone was indicated by the coupling between H-1′ and C-7 in the HMBC spectrum. This led to the assignment of structure **2** for the new compound as 5-hydroxy-6-methoxy-2-methylchromone-7-*O*-rutinoside.

Compound **3** was obtained as a light yellow, amorphous solid. The pseudomolecular ion at *m/z* 501.8 was detected by FABMS. The molecular formula was assigned as $C_{22}H_{28}O_{13}$ by HRFABMS. The IR and UV spectra of **3** were similar to compound **2**. No change in the UV maximum occurred in the presence of NaOAc. Upon addition of anhydrous $AlCl_3$ or $AlCl_3-HCl$, however, the absorption maximum at 313 nm showed a bathochromic shift of 52 nm. Thus, this compound must be a closely related analogue of **2**.

The main differences between the ^1H NMR spectra of **2** and **3** were the appearance of two aromatic doublets with a small coupling constant (δ 6.63 and 6.37, $J = 2$ Hz), instead of one singlet aromatic proton as in **2**, and the absence of the methoxy substituent at δ 3.73. Digestion of **3** with naranginase released rhamnose, glucose, and an aglycone that was identified as noreugenin (**4**) based on spectroscopic analyses. COSY, HMQC, and HMBC analyses confirmed that the glycosyl linkages and structures were identical to those of **2**. Therefore, this new compound **3** was assigned as the 6-demethoxy analogue of **2**.

Cytotoxicity. Compounds **1**−**5** and the aglycone of **2** were tested for cytotoxicity against A-549 human lung carcinoma, MCF-7 human breast adenocarcinoma, and HT-29 human colon adenocarcinoma cell lines (Table 1). The cardiac glycoside **1** showed the highest toxicity toward all cell lines. Chromone glycosides **2** and **3** showed differential growth inhibitory activity ($GI_{50} = 0.4-0.8$ μM) against the A-549 and MCF-7 cell lines. Compounds **2** and **3** were 10-fold less active ($GI_{50} = 6$ μM) against the HT-29 cell line. Eugenin (**4**) showed marginal toxicity against all three cell lines ($GI_{50} = 10-25$ μM), while noreugenin (**5**) and the aglycone of **2** were inactive. These results suggest that the alkyl substituent at C-7 is important for chromone-induced cytotoxicity, while a more potent effect is exerted by C-7 glycosyl substitution.

Isolation of the HIF-1 Inhibitor and SAR Study. Natural products have become a reliable source for the discovery of inhibitors of HIF-1 activation.[14−18] The NCI primary HIF-1 screen relies on the binding of the HIF-1 complex to a hypoxic response element (HRE) located upstream of a luciferase reporter gene engineered into U251 glioma cells.[4] As a counter screen for false positives, U251 cells containing the luciferase reporter gene but lacking the HRE are treated with drug and assayed for luciferase expression. In high-throughput screening for inhibitors of HIF-1 activation, a crude extract of *C. bigelovii* was identified as having potent inhibitory activity and was subjected to dereplication, resulting in the collection of 88 fractions (see Supporting Information).[15] In the major zone of HIF-1 inhibition from wells 20−25, analysis by ESIMS showed both H^+ and Na^+ adducts of 711 and 733, respectively, as well as a fragment ion of m/z^+ 531 and a UV absorption maximum of ~220 nm. Scale-up chromatography led to the isolation of the strophanthidin glycoside **1**, confirmed by NMR and comparison with spectra found in ref 3, as the HIF-1 inhibitory component.

In order to explore the HIF-1 activity of the strophanthidin class of cardenolides, additional compounds (Figure 1) were obtained from the DTP/NCI repository. These were purified to >95% by LC with UV detection, and the MW confirmed by LC/MS. Besides showing the correct MW, an $[m/z]^+$ 405.1 signal, diagnostic of the strophanthidin genin, was found for compounds **1** and **10**−**13**, providing additional proof of structure. The aglycone strophanthidin (**10**) exhibited very weak HIF-1 inhibition ($EC_{50} = 2$ μM). However, the 3-*O*-β-L-rhamnosyl analogue of **10**, convallatoxin (**11**), showed a startling increase in potency ($EC_{50} = 0.02$ μM). It appears that the presence of attached glycosides (**1**, **11**−**13**) is critical for HIF-1 inhibition, although a clear correlation relating the number of carbohydrate residues to HIF-1 potency cannot be drawn. Of lesser activity were **13** (k-strophanthoside, $EC_{50} = 0.04$ μM), **12** (k-strophanthin-β, $EC_{50} = 0.12$ μM), and **1** ($EC_{50} = 0.6$ μM). For all compounds tested, luciferase expression in the pGL3 cells exceeded that of the HRE cells (Figure 1), suggesting a degree of specificity. Cell growth experiments were also conducted to determine if the observed HIF-1 inhibition was a consequence of toxicity of the drugs to the cells. U251-HRE and U251-pGL3 cell lines were treated with compounds **1** and **10**−**13** for 24 h. There was no inhibition of cell growth over this period (Figure 2).

To further probe the observed inhibition of HIF-1 activation by cardenolides, compound **11**, having the lowest EC_{50} in the HIF-1 primary screen, was selected for further biological evaluation. Confirmation of HIF-1 inhibitory activity was measured by luciferase expression in U251-HRE and U251-pGL3 stable cell lines under normoxic (21% O_2) and hypoxic (1% O_2) conditions (Figure 3). Reduction in luciferase expression of 77% and 50% is seen in the U251-HRE cells versus U251-pGL3 control cells, respectively, under hypoxic conditions when treated with 0.062 μM **11**. These data suggest moderate, but not total, selectivity toward the cells possessing the HRE construct. In further experiments, U251 wild-type cells transiently transfected with pGL2TK-HRE plasmid were exposed to **11** in concentrations ranging from 0.1 to 0.005 μM (Figure 4). Under hypoxic conditions, a decrease in luciferase production greater than that effected by topotecan was seen at 0.05 μM drug.

To determine whether the decrease in luciferase production in the presence of **11** was the result of a reduced concentration of cellular HIF-1α, protein expression studies were performed. After 8 and 16 h of exposure to **11**, cells subjected to hypoxic conditions showed complete loss of HIF-1α expression at 0.025 μM drug (Figure 5). That this effect is exerted through a specific mechanism of action is reinforced by recent studies with the cardiac glycoside digoxin,[19] in

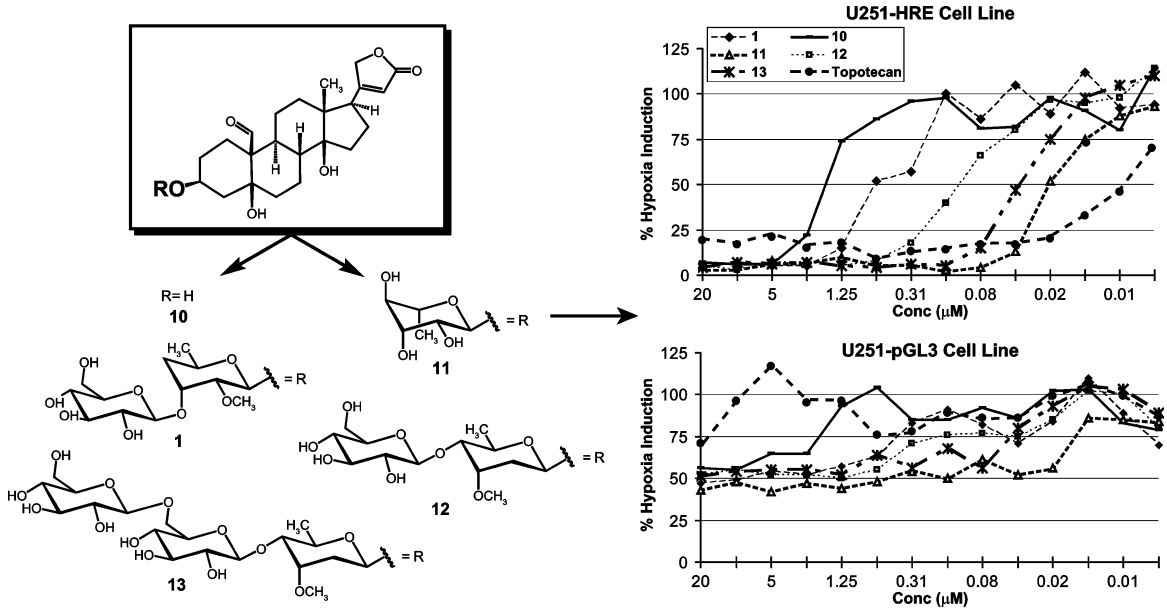

Figure 1. Structure−activity relationship of strophanthidin and strophanthidin glycosides in the HIF-1 primary screen.

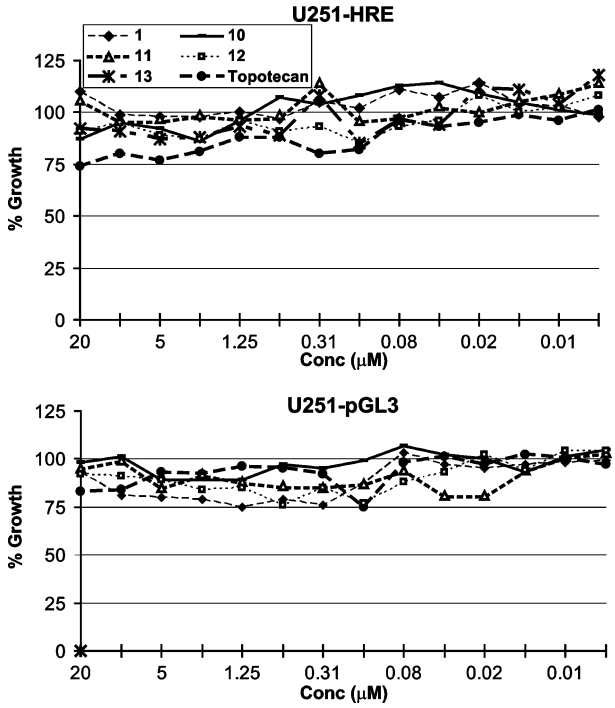

Figure 2. Growth in U251-HRE and U251-pGL3 cell lines over 24 h in the presence of cardenolides **1** and **11**−**13**.

which *de novo* HIF-1α protein synthesis was reduced to a far greater extent than overall protein synthesis.

HIF-1 is known to be a master regulator of the expression of more than 100 genes and their gene products.[4] Thus, loss of HIF-1 function should be reflected in the expression of genes under its control. To demonstrate this effect, the levels of vascular endothelial growth factor (VEGF) mRNA and carbonic anhydrase (CA9) mRNA, the gene products that are known to be upregulated in response to hypoxic conditions, were monitored in U251 wild-type cells in response to **11** (Figure 6). VEGF mRNA was reduced to an extent similar to topotecan-treated cells[4] after 8 h, although the effect was significantly less at 16 h. CA9 mRNA also experiences a decline at both 8 and 16 h at concentrations as low as 0.1 μM drug.

Strophanthin, in one of its several forms, has seen clinical use as a cardiotonic for perhaps 100 years.[20] The presence of strophanthidin in Apocynaceae (i.e., k-strophanthin and others), Liliaceae (i.e., convallatoxin), Moraceae (i.e., antiaris toxin),[21] and others is known, but finding it in this unrelated family was unexpected. Mono- and diglycosides comprised of rhamnose, cymarose, digitoxose, and glucose linked to strophanthidin are likewise known,[22] but a glycoside of strophanthidin containing the uncommon monosaccharide gulose is new. The identification of these bioactive cardenolides plus molecules of four additional chemotypes shows the diversity of secondary metabolites contained in *Crossosoma* and suggests that further investigation of the chemistry of members of Crossosomataceae may result in the identification of additional new compounds.

Experimental Section

General Experimental Procedures. Optical rotation was determined with a PerkinElmer 241 polarimeter, UV spectra were acquired by a Beckman DU-7, and IR spectra were obtained with a Perkin-Elmer 1600 FTIR spectrometer. ^{1}H and ^{13}C NMR spectra were acquired with either a Bruker ARX-300 or a Varian VXR-500 in $CDCl_3$, CD_3OD, or DMSO-d_6 as solvent. Chemical shift values were reported in δ values (ppm) relative to an internal reference of ^{1}H [δ 7.24 ($CHCl_3$), δ 3.30 (CD_2HOD), δ 2.49 (DMSO-d_6)] and ^{13}C [δ 77.0 ($CDCl_3$), δ 49.5 (CD_3OD), δ 39.5 (DMSO-d_6)], respectively. Low- and high-resolution FABMS were recorded on a Kratos MS50 spectrometer or a Waters LCT Premier TOF mass spectrometer. Silica gel (230−400 mesh, E. Merck) and LRP-2 C_{18} gel (Whatman) were used for column chromatography. NMR spectra were recorded with a Varian 400 MHz INOVA spectrometer in methanol-d_4 or DMSO-d_6 with TMS as internal standard. HPLC-MS hardware consisted of a Waters 600 pump, a Micromass ZQ electrospray mass spectrometer (cone voltage = 30), a Waters 996 photodiode array spectrometer, and a Sedex 75 evaporative laser light scattering detector. C_{18} HPLC was used to fractionate crude organic extracts in the initial dereplication experiment. Scale-up purification was accomplished on Sephadex LH-20 (Pharmacia), phenyl bonded-phase silica (Waters or Varian), and cyano bonded-phase silica (Varian) by HPLC.

Plant Material. The flowers, fruits, leaves, and twigs of *Crossosoma bigelovii* S. Wats. (Crossosomataceae) were collected near Cataviña, Baja California Norte, Mexico, in April 1990, by R. Spjut of World Botanical Associates. Herbarium vouchers were deposited at the U.S. National Herbarium of the Smithsonian Institution in Washington, DC as SPJ-11942, and a representative sample is maintained in the DTP Repository in Frederick, MD, as 627C0130.

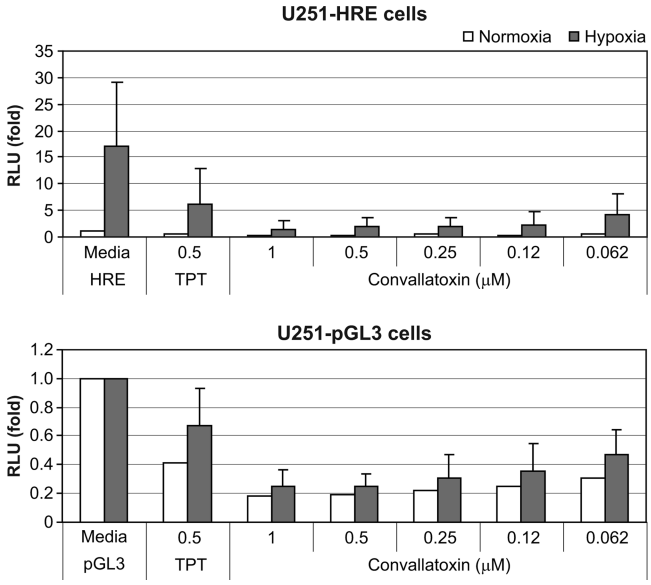

Figure 3. Luciferase expression in stably transfected U251-HRE and U251-pGL3 cell lines averaged over two experiments. Topotecan (TPT), a known HIF-1 inhibitor,[4] was included as a positive control. Normoxia = 21% O_2. Hypoxia =1% O_2.

Extraction and Isolation. The fractionation and isolation of active compounds were directed by cytotoxicity against human A-498 (kidney), MCF-7 (breast), and HT-29 (colon) tumor cells using the MTT assay. The dried, ground stems of *C. bigelovii* (3.8 kg) were extracted with 95% EtOH (38 L), from which **4** and **5** were isolated. Sequential extraction with EtOH−H_2O (1:1) (38 L) yielded an extract from which **1**−**3** were isolated.

The EtOH−H_2O (1:1) extract (50 g) was redissolved in EtOH−H_2O (3:7) and further partitioned with *n*-hexane, EtOAc, and *n*-BuOH sequentially. The *n*-BuOH fraction showed the greatest toxicity and was subjected to C_2 reversed-phase chromatography with MeOH−H_2O (1:9, 2:8, 5:5, 8:2, and 1:0) to afford five fractions. After bioassay, a 1.1 g aliquot of the MeOH−H_2O (5:5) elutable material was further purified through a silica gel (75 g) gravity flow column, eluted with $CHCl_3$−MeOH−H_2O (12:3:0.3). The first of the active fractions was subjected to two silica gel chromatography steps, (1) CH_2Cl_2−MeOH−H_2O (100:0:0, 95:5:0, 85:15:1, 75:25:2, 50:50:3), followed by (2) CH_2Cl_2−MeOH (99:1, 97:3, 94:6, 90:10, 85:15), to afford 9.8 mg of pure **1**, which was eluted by CH_2Cl_2−MeOH (90:10). The second of the active fractions was chromatographed on a reversed-phase C_2 gel column, eluted with MeOH−H_2O (2:3). A fraction of intermediate toxicity was selected for further purification on silica gel, which was eluted with CH_2Cl_2−MeOH−H_2O (12:3:0.3) to yield pure compounds **2** and **3**.

The 95% EtOH extract (175 g) was subjected to sequential partitioning, first between 3 L of CH_2Cl_2−H_2O (1:1), with the CH_2Cl_2-soluble fraction (48 g) further partitioned between 1 L of 90% MeOH−*n*-hexane (1:1). The 90% MeOH fraction was chromatographed on a C_2 reversed-phase column. The most active fraction was subjected to a second C_2 gel purification step, eluted with 40% MeOH in H_2O. Further purification of two of the active fractions with *n*-hexane−CH_2Cl_2−MeOH (50:50:0, 50:70:2, 50:70:4, 50:70:8) on silica gel yielded the noncytotoxic compounds **4** (noreugenin, 8 mg) and **5** (eugenin, 19 mg).

The dried, ground specimen of *C. bigelovii* flowers, fruits, leaves, and twigs (600 g) had been stored in a non-temperature-controlled warehouse until extraction at NCI-Frederick by percolation with CH_2Cl_2−MeOH (1:1) for 16 h, the solvent was removed by rotary evaporation, and residue was dried under high vacuum, yielding 3.64 g of crude extract. A published dereplication method[15] was used, which revealed six distinct zones of HIF-1α inhibitory activity (see Supporting Information). Because the UV and MS data of the active zones suggested the presence of compounds similar to **1**, differing in the number and/or arrangement of attached sugar residues, it was decided to focus on the elucidation of the major component only. Scale-up isolation was undertaken utilizing 2 g of crude organic extract, which was adsorbed to C_{18} silica, then subjected to VLC using increasing

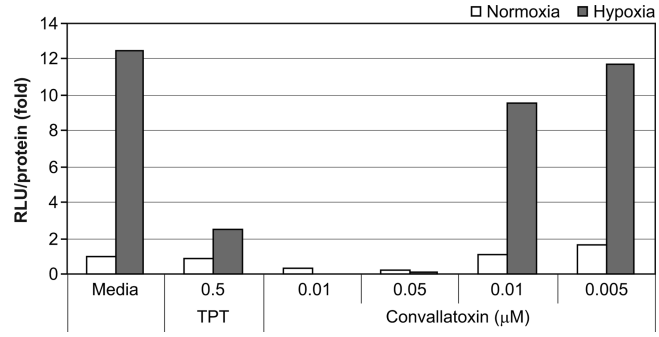

Figure 4. U251 wild-type cells were transiently transfected with pGL2TK-HRE plasmid for 16 h prior to treatment with drug. Transient transfection yields a cell line that produces more luminescence than a stably transfected cell line, allowing greater sensitivity in the presence of HRE-binding compounds. Normoxia = 21% O_2. Hypoxia = 1% O_2.

amounts of CH_3CN in H_2O. The 50% CH_3CN fraction (430 mg) inhibited HIF-1α levels as measured by luciferase expression.[3] Further chromatography of 200 mg of this fraction on a C_{18} Michel-Miller glass column in a CH_3CN−20 mM NH_4OAc (pH 4.0) mobile phase gradient, resulted in the isolation of **1** and **6**−**9**, as follows: the active material from the C_{18} column (36 mg) was further purified by phenyl bonded-phase semipreparative HPLC (CH_3CN−20 mM NH_4OAc pH 4, 0−20 min = 25:75, 30−60 min = 50:50, 15 mL min^{-1}). A major peak eluting from the column at 30.1 min showed potent HIF-1α inhibitory activity. This was subjected to a final polishing step on Sephadex LH-20 eluted with MeOH, resulting in 1.3 mg of **1** (0.14% yield).

In order to gain greater knowledge of the chemotaxonomy of the unstudied plant, several noncardenolide compounds were also isolated. Compounds **6** and **8** were obtained in a single fraction produced from the same phenyl HPLC step that resulted in enriched **1**. Sephadex LH-20 gel permeation chromatography in MeOH resulted in a 0.032% yield of both **6** and **8**. The structure of **6** (ajmalicine) was confirmed via HPLC-MS and NMR comparison to a certified standard, while 1D- and 2D-NMR analyses were used to confirm the identity of **8** (matairesinol).[23] Compound **7** was obtained in impure form from a polar HIF-active C_{18} Michel-Miller column fraction. This material (20 mg) was chromatographed by phenyl bonded-phase preparative HPLC in CH_3CN−20 mM NH_4OAc pH 4 (0−20 min = 25:75, 30−60 min = 50:50, 15 mL min^{-1}), producing 0.9 mg of an enriched fraction containing a major component of MW 362. Sephadex LH-20 purification of this material resulted in 0.4 mg of secoisolariciresinol (**7**)[24] (0.043% yield).

Compound **9** was isolated from the CH_2Cl_2−MeOH (1:3) flush of the C_{18}-adsorbed crude extract. From this fraction, 50 mg was chromatographed on the X-bridge phenyl bonded-phase HPLC column (Waters) under a mobile phase gradient (CH_3CN−20 mM NH_4OAc, pH 4, 0−5 min = 55:45, 45−60 min = 100:0, 15 mL min^{-1}). Compound **9** eluted at 22.7 min. ^1H NMR was used in conjunction with the LC/MS data in a dereplication analysis to determine the structure of this sample. Compounds **6**−**9** showed no inhibition of HIF-1α expression.

3*β*-[(*O*-*β*-D-Glucopyranosyl-(1→3)-4,6-dideoxy-2-*O*-methyl-*β*-D-gulopyranosyl)]-5*β*,14*β*-dihydroxy-19-oxocard-20(22)enolide (1): colorless solid; $[\alpha]^{20}_D$ −30.6 (*c* 0.327, MeOH); UV (MeOH) λ_{max} (log ϵ) 208 (3.97), 276 (2.70) nm; IR (KBr) ν_{max} 3448, 1735, 1715, 1540 cm^{-1};^1H NMR (DMSO-d_6, 500 MHz) δ 10.05 (1H, s, H-19), 5.90 (1H, br, H-22), 5.08 (1H, dd, J = 20.1 Hz, H-21a), 4.93 (1H, dd, J = 20.1 Hz, H-21b), 4.86 (1H, d, J = 8.0 Hz, H-1′), 4.53 (1H, ddd, J = 3.5, 3.5, 3.0 Hz, H-3′), 4.50 (1H, d, J = 8.0 Hz, H-1″), 4.17 (1H, m, H-3), 4.08 (1H, dqd, J = 10.0, 5.5, 2.0 H-5′), 3.85 (1H, dd, J = 13.3, 2.5 Hz, H-6a″), 3.64 (1H, dd, J = 13.3, 5.0 Hz, H-6b″), 3.36 (3H, s, 2′-OMe), 3.35 (1H, dd, J = 10.0, 10.0 Hz, H-3″), 3.27 (1H, dd, J = 10.0, 10.0 Hz, H-4″), 3.26 (1H, ddd, J = 10.0, 5.0, 2.5 Hz, H-5″), 3.24 (1H, dd, J = 10.0, 8.0 Hz, H-2″), 3.06 (1H, dd, J = 8.0, 3.5 Hz, H-2′), 2.82 (1H, m, H-17), 1.55 (2H, m, H-4′), 1.18 (3H, d, J = 5.5 Hz, H-6′), 0.85 (3H, s, H-18); ^{13}C NMR (DMSO, 125 MHz) δ 210.0 (CO, C-19), 178.2 (C, C-20), 177.2 (C, C-23), 117.9 (CH, C-22), 101.5 (CH, C-1″), 98.2 (CH, C-1′), 85.9 (C, C-14), 80.9 (CH, C-2′), 78.1 (CH, C-3″), 78.1 (CH, C-5″), 75.3 (CH_2, C-21), 74.9 (C, C-5), 74.7 (CH, C-2″), 74.3 (CH, C-3), 71.7 (CH, C-4″), 70.8 (CH, C-3′), 67.7 (CH, C-5′),

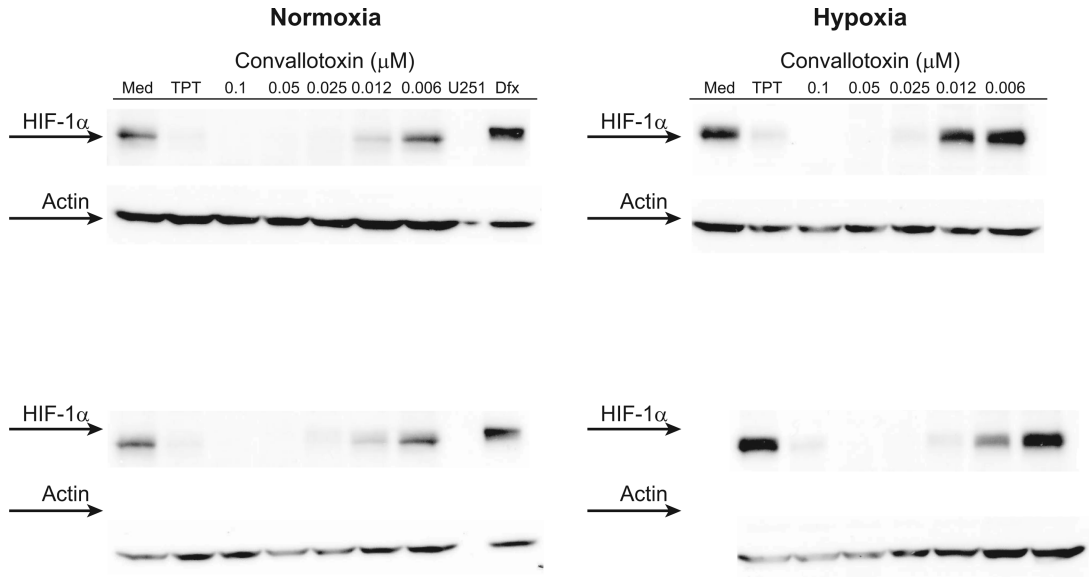

Figure 5. Effect of convallatoxin (**11**) on HIF-1α protein expression in U251 wt cells was tested under normoxic and hypoxic conditions. Topotecan (TPT) was included as a positive control. Actin expression is not regulated by HIF-1α and serves as a negative control in the presence of HIF inhibitors. Dfx = desferroxamine (a compound that mimics hypoxia). Normoxia = 21% O_2. Hypoxia = 1% O_2. Drug concentrations are in μM units.

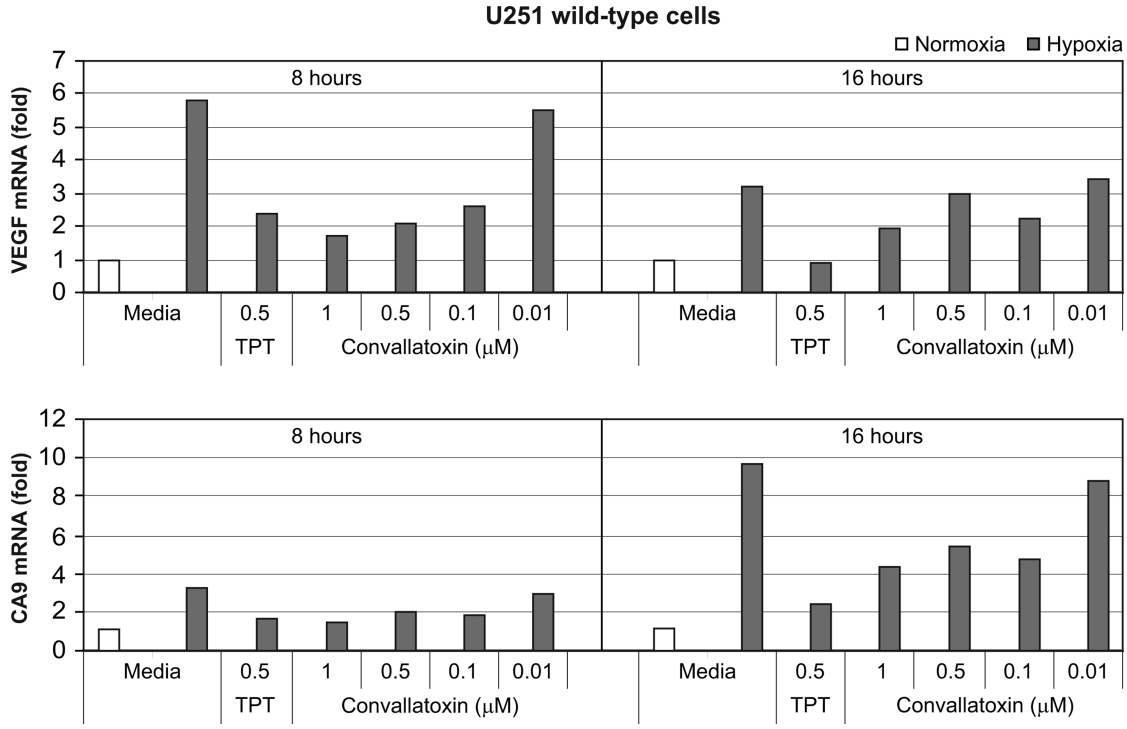

Figure 6. Effect of **11** on the expression of vascular endothelial growth factor (VEGF) mRNA and carbonic anhydrase (CA9) mRNA was measured in U251 wild-type cells. Panels below show effects at 8 and 16 h on the left and right sides, respectively. Normoxia = 21% O_2. Hypoxia = 1% O_2.

62.9 (CH_2, C-6″), 56.5 (OCH_3, C-2′), 56.2 (CH, C-10), 51.7 (CH, C-17), 50.7 (C, C-13), 42.5 (CH, C-8), 40.5 (CH_2, C-12), 40.3 (CH, C-9), 37.8 (CH_2, C-4′), 36.9 (CH_2, C-6), 35.3 (CH_2, C-4), 32.4 (CH_2, C-15), 27.9 (CH_2, C-16), 26.1 (CH_2, C-2), 25.3 (CH_2, C-1), 23.2 (CH_2, C-11), 21.0 (CH_2, C-6′), 19.0 (CH_2, C-7), 16.1 (CH_3, C-18); FABMS *m/z* 733 $[M + Na]^+$ (21), 711 $[M + H]^+$ (8), 531 (8), 405 (11), 307 (100); HRFABMS *m/z* 733.3434 $[M + Na]^+$ (calcd for $C_{36}H_{54}O_{14}$ + Na, 733.3411).

5-Hydroxy-6-methoxy-2-methylchromone-7-*O*-rutinoside (2): amorphous, light yellow solid; $[\alpha]^{20}_D$ −67.1 (*c* 0.136, MeOH); UV (MeOH) λ_{max} (log ϵ) 253 (3.91), 285 (3.58), 322 (3.27) nm; + NaOAc 252, 281, 322 nm; + $AlCl_3$ 256, 264, 306, 366 nm; + $AlCl_3$/HCl 256, 266, 305, 366 nm; IR (KBr) ν_{max} 3401, 1661, 1622, 1070 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 12.83 (1H, s, 5-OH), 6.77 (1H, s, H-8), 6.21 (1H, s, H-3), 5.01 (1H, d, J = 7.5 Hz, H-1′), 4.50 (1H, d, J = 1.0 Hz, H-1″), 3.87 (1H, dd, J = 11.0, 1.2 Hz, H-6′a), 3.73 (3H, m, H-10), 3.65 (1H, H-2″), 3.59 (1H, ddd, J = 9.0, 9.0, 1.2 Hz, H-5′), 3.46 (1H, dd, J = 9.0, 1.5, H-3″), 3.40 (1H, dd, J = 9.5, 6.5 Hz, H-5″), 3.36 (1H, dd, J = 11.0, 9.0 Hz, H-6′b), 3.28 (1H, H-2′), 3.27 (1H, H-3′), 3.14 (1H, dd, J = 9.5, 9.0 Hz, H-4″), 3.10 (1H, dd, J = 9.0, 9.0 Hz, H-4′), 2.41 (3H, s, H-9), 1.08 (3H, d, J = 6.5 Hz, H-6″); ^{13}C NMR (DMSO, 125 MHz) δ 182.4 (C, C-4), 168.9 (C, C-2), 156.3 (C, C-7), 152.6 (C, C-5), 152.4 (C, C-8a), 133.1 (C, C-6), 107.7 (CH, C-3), 105.2 (C, C-4a), 100.8 (CH, C-1″), 100.3 (CH, C-1′), 94.3 (CH, C-8), 76.7

(CH, C-3′), 75.7 (CH, C-5′), 73.1 (CH, C-2′), 72.1 (CH, C-4″), 70.7 (CH, C-5″), 70.3 (CH, C-2″), 70.0 (CH, C-4′), 68.4 (CH, C-3″), 66.6 (CH_2, C-6′), 60.1 (CH_3, C-10), 20.0 (CH_3, C-9), 17.9 (CH_3, C-6″); FABMS *m/z* 569 $[M + K]^+$ (7), 553 $[M + Na]^+$ (15), 531 $[M + H]^+$ (50), 385 (11), 339 (13), 223 (100); HRFABMS *m/z* 531.1713 $[MH]^+$ (calcd for $C_{23}H_{31}O_{14}$, 531.1714).

5-Hydroxy-2-methylchromone-7-*O*-rutinoside (3): amorphous, light yellow solid; $[\alpha]^{20}_D$ −54.5 (*c* 0.149, MeOH); UV (MeOH) λ_{max} (log ϵ) 249 (3.97), 255 (3.97), 284 (3.51), 313 (2.92) nm; + NaOAc 248, 256, 283, 313 nm; + $AlCl_3$ 257, 302, 360 nm; + $AlCl_3$/HCl 256, 302, 365 nm; IR (KBr) ν_{max} 3404 (br), 1663, 1625, 1070 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 12.78 (1H, s, 5-OH), 6.63 (1H, d, J = 2.0 Hz, H-6), 6.37 (1H, d, J = 2.0 Hz, H-3), 6.24 (1H, s, H-3), 5.00 (1H, d, J = 7.0 Hz, H-1′), 4.50 (1H, s, H-1″), 3.85 (1H, d, J = 10.0 Hz, H-6′a), 3.64 (1H, H-2″), 3.58 (1H, dd, J = 9.0, 9.0 Hz, H-5′), 3.46 (1H, dd, J = 7.5, 2.5, H-3″), 3.40 (1H, dd, J = 9.5, 6.5 Hz, H-5″), 3.33 (1H, H-6′b), 3.28 (1H, H-3′), 3.27 (1H, H-2′), 3.14 (1H, dd, J = 9.5, 7.5 Hz, H-4″), 3.09 (1H, dd, J = 9.0, 9.0 Hz, H-4′), 2.41 (3H, s, H-9), 1.09 (3H, d, J = 6.5 Hz, H-6″); ^{13}C NMR (DMSO, 125 MHz) δ 182.0 (C, C-4), 169.0 (C, C-2), 162.5 (C, C-7), 161.0 (C, C-5), 157.5 (C, C-8a), 108.2 (C, C-3), 105.2 (CH, C-4a), 100.7 (C, C-1″), 99.8 (CH, C-1′), 99.6 (CH, C-6), 94.4 (CH, C-8), 76.4 (CH, C-3′), 75.7 (CH, C-5′), 73.0 (CH, C-2′), 72.1 (CH, C-4″), 70.7 (CH, C-5″), 70.3 (CH, C-2″), 69.9 (CH, C-4′), 68.4 (CH, C-3″), 66.5 (CH_2, C-6′), 20.2 (CH_3, C-9), 17.8 (CH_3, C-6″); FABMS *m/z* 523 $[M + Na]^+$ (28), 501 $[M + H]^+$ (13), 355 (26), 193 (100); HRFABMS *m/z* 501.1613 $[MH]^+$ (calcd for $C_{22}H_{29}O_{13}$, 501.1608).

Cytotoxicity Testing. The human tumor cytotoxicity assays were performed at the Purdue University Cell Culture Laboratory, Purdue Cancer Center, using the standard MTT protocol.[25] The following human tumor cell lines were employed: A-549 lung carcinoma; MCF-7, breast adenocarcinoma; and HT-29, colon adenocarcinoma. Adriamycin was used as a reference compound in all the assays. Cells were maintained in MEM supplemented with 10% fetal calf serum and 0.1 g L^{-1} penicillin G + 0.1 g L^{-1} streptomycin sulfate. The cells were transferred into 96-well plates one day prior to the addition of the assayed compounds, and incubated overnight at 37 °C. Compounds were dissolved in DMSO, diluted with medium at different concentrations, and incubated with the cells for six days. Cell survival was determined colorimetrically via MTT, a tetrazolium salt, at 547 nm.

Cell Lines and Reagents. U251 human glioma cells are routinely maintained in RPMI 1640 (Whittaker Bioproducts, Walkersville, MD) supplemented with 5% heat-inactivated fetal bovine serum (Whittaker), penicillin (50 IU mL^{-1}), streptomycin (50 g mL^{-1}), and 2 mM glutamine (all purchased from Invitrogen-Life Technologies, Inc., Carlsbad, CA).

U251-HRE and U251-pGL3 control stable cells were maintained in RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum, penicillin (50 IU mL^{-1}), streptomycin (50 g mL^{-1}), 2 mM glutamine, and G418 at 100 μg mL^{-1}. Cells were maintained at 37 °C in a humidified incubator containing 21% O_2, 5% CO_2 in air (referred to as normoxic conditions). Hypoxia treatment was performed by placing cells in a modular incubator chamber (Billupus-Rothemberg Inc., Del Mar, CA) and then flushing with a mixture of 1% O_2, 5% CO_2, and 94% N_2 for 20 min. The chamber was then placed at 37 °C. Hypoxic conditions were also achieved in an Invivo2 400 hypoxic workstation (Ruskinn Technologies, Cincinnati, OH) set to deliver 1% O_2 in 5% CO_2 at 37 °C.

Plasmids. The pGL2-TK-HRE plasmid was generated by subcloning three copies of the HRE (5′-GTGACTACGTGCTGCCTAG-3′) from the inducible nitric oxide synthase promoter into the pGL2-TK promoter vector. Plasmids were sequenced at the Molecular Technology Laboratory, SAIC-Frederick, Inc. The pGL3-control (Promega) contains the firefly luciferase coding sequence under control of the SV40 promoter and enhancer sequences.

Transient Transfection. U251 cells were transfected with pGL2-TK-HRE plasmid using Effectene Transfection Reagents (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions. After 16 h of transfection, the medium was changed and treated with convallatoxin from 1 μM to 0.005 μM for 16 h, and luciferase expression was measured using Steady Glo luciferase assay reagents.

Stable Transfection and Engineered Cell Lines. DNA plasmids were prepared using a commercially available kit (Endofree Maxi-Prep; Qiagen,Inc.). Transfections were performed using Effectene Transfection Reagents (Qiagen, Inc.) according to the manufacturer's instructions. Stably transfected cells (U251-HRE, U251-pGL3) were generated by cotransfection of the specific reporter plasmid (pGL2-TK-HRE and pGL3-control, respectively) with an expression vector carrying the neomycin resistance gene for selection in mammalian cells (ratio 100:1). Twenty-four hours after transfection, reagents were removed, and cells were allowed to recover for 24 h before the addition of selection medium containing the antibiotic G418 at 500 μg/mL (Invitrogen-Life Technologies, Inc.). Stably transfected cells were seeded at a concentration of 10^4 cells/well in 96-well optiplates (Packard Instrument, Inc., Meriden, CT) the day before treatment and routinely treated for 16−24 h. Luciferase reporter assays were performed in 96-well optiplates using Bright Lite Plus luciferase assay reagents (Perkin Elmer, Waltham, MA).

Total Extract Preparation and Immunoblotting (Western Blot). Cells were washed with phosphate-buffered saline and lysed with RIPA lysis buffer [1 mM NaF, 1 mM sodium vanadate] (Pierce Chemicals, Rockford, IL) containing a cocktail of protease inhibitors (Roche, Mannheim, Germany). Using mechanical scraping, cell lysates then were collected and rotated at 4 °C for 15 min, and cellular debris was pelleted by centrifugation at 15000*g* for 15 min at 4 °C. Protein concentration was measured by Bradford assay, using bovine serum albumin as a standard. Protein (100 μg) was separated on a 4−20% Tris-Glycine gel (Invitrogen, Carlsbad, CA), electroblotted on a polyvinylidene difluoride membrane (Invitrogen), and subjected to immunoblot analysis. Monoclonal anti-HIF-1α antibody was purchased from BD-Transduction Laboratories (Lexington, KY) and used at a 1:300 dilution. Horseradish peroxidase-conjugated antimouse IgG (1:10 000 dilution) and enhanced chemiluminescence reagents were obtained from Amersham Pharmacia Biotech (Piscataway, NJ).

Real-Time PCR. Total RNA from U251 cells was obtained using RNA Mini Kit (Qiagen, Inc.). A total of 500 ng of RNA was used to perform RT-PCR using RT-PCR kit (PE Biosystems, Foster City, CA). The conditions used for RT-PCR were as follows: 10 min at 25 °C, 30 min at 48 °C, and 5 min at 95 °C.

To measure human VEGF and CA9 expression, real-time PCR was performed using an ABI-Prism 7700 sequence detector (Applied Biosystems, Foster City, CA). Typically 5 ng of reverse-transcribed cDNA per sample was used to perform real-time PCR in triplicate samples. Real-time PCR cycles started with 2 min at 50 °C, 10 min at 95 °C, and then 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Primers and specific probes were obtained from Applied Biosystems and are available upon request. [The following primers and probes were used: human VEGF forward, 5′-TACCTCCACCATGCCAAGTG-3′; human VEGF reverse, 5′-ATGATTCTGCCCTCCTCCTTC-3′; probe, 5′-FAM-TCCCAGGCTGCACCCATGGC-TAMRA-3′]. Detection of VEGF and 18S rRNA was performed using TaqMan Universal PCR Master Mix (Applied Biosystems), and CA9 detection was performed using Sybr Green PCR Master Mix (Applied Biosystems).

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Supporting Information Available: 1D- and 2D-NMR spectra of **1**−**3** and the HPLC biogram of the organic extract of *C. bigelovii* are available free of charge via the Internet at http://pubs.acs.org.

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