Molecular-Targeted Antitumor Agents: The *Saururus cernuus* Dineolignans Manassantin B and 4-*O*-Demethylmanassantin B Are Potent Inhibitors of Hypoxia-Activated HIF-1

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The transcription factor hypoxia-inducible factor-1 (HIF-1) is a key regulator of tumor cell adaptation and survival under hypoxic conditions. Selective HIF-1 inhibitors represent an important new class of potential molecular-targeted antitumor therapeutic agents. Extracts of plants and marine organisms were evaluated using a T47D human breast tumor cell-based reporter assay for HIF-1 inhibitors. Bioassay-guided fractionation of the lipid extract of *Saururus cernuus* resulted in the isolation of manassantin B (**1**) and a new compound, 4-*O*-demethylmanassantin B (**2**). The structure of **2** was determined spectroscopically. The absolute configurations of manassantin-type dineolignans have not been previously reported. Therefore, the absolute configurations of the chiral centers in each side chain were deduced from spectroscopic analysis of the Mosher MTPA ester derivatives of **1**. Both **1** and **2** are among the most potent small molecule HIF-1 inhibitors discovered, to date, with IC_{50} values of 3 and 30 nM, respectively. Compounds **1** and **2** selectively inhibited hypoxia-activated HIF-1 in contrast to iron chelator-activated HIF-1. Compounds **1** and **2** also inhibited hypoxic induction of the angiogenic factor VEGF. Further study revealed that **1** selectively blocked the induction of HIF-1α protein, the oxygen regulated HIF-1 subunit that determines HIF-1 activity.

Hypoxic regions are commonly found in solid tumors, and the extent of tumor hypoxia correlates with advanced stages and treatment resistance.[1] Presently, there is only one bioreductive drug (tirapazamine) in clinical trial that selectively kills hypoxic tumor cells, and no hypoxic cytotoxin-based drug is currently approved. Recent results from clinical tirapazamine studies have indicated the significant potential of drugs that target tumor hypoxia.[2,3] The transcription factor hypoxia-inducible factor-1 (HIF-1) is a key regulator of tumor cell adaptation and survival under hypoxic conditions.[4,5] Upon hypoxic induction and activation, HIF-1 binds to the hypoxia response element (HRE) present in the promoters of target genes and activates transcription. Overexpression of the oxygen-regulated HIF-1α subunit correlates positively with poor prognosis and treatment resistance in cancer patients.[6,7] Inhibition of HIF-1 production/function significantly reduces tumor growth in animal models.[8] Low molecular weight compounds that selectively inhibit HIF-1 represent potential anticancer drug leads that target tumor hypoxia.

We have established a cell-based high-throughput reporter assay for HIF-1 functional antagonists in T47D human breast tumor cells. A luciferase reporter gene under the control of HRE from the erythropoietin gene (pTK-HRE3-luc) was employed to monitor HIF-1 activity.[9] Terrestrial and marine organism extracts were examined for HIF-1 inhibitors. The active extract from the aquatic plant *Saururus cernuus* was selected for bioassay-guided fractionation of the constituents responsible for HIF-1 inhibition. *Saururus cernuus* L. (Saururaceae), also known as "lizard's tail", is a native aquatic/wetland plant found throughout the eastern half of the United States.[10] *Saururus cernuus* has a long history of medicinal use by both Native Americans and early colonists, including use in the treatment of tumors.[11] The majority of compounds isolated from *S. cernuus* are lignoid in nature. The dineolignan manassantin A has shown tumor-selective cytotoxicity,[12] the monolignans sauriols A and B have ecologically relevant feeding deterrent activity,[13] and the novel *S. cernuus* triene 12,13-dehydrogeranylgeraniol is a potent antioxidant in cell-based systems.[14]

Results and Discussion

Bioassay-guided chromatographic fractionation of the *S. cernuus* lipid extract led to the isolation of compounds **1** and **2**. Compound **1** was found to be the previously reported *S. cernuus* dineolignan manassantin B (also known as saucernetin 7 or SC-7).[15] In terms of the ^{1}H NMR spectrum, it was determined that **2** was nearly identical to **1**, except that **2** lacked one methoxyl group. An additional exchangeable proton resonance at δ 5.61 in **2** suggested the presence of a phenolic hydroxyl moiety. The HRESIMS of **2** confirmed a molecular formula of $C_{40}H_{46}O_{11}$. In the ^{13}C spectrum of **1**, the methoxylated aromatic carbons at C-3 and C-4 resonate at δ 149.4 and 149.2, respectively. However, in the structure of **2** these signals were shifted upfield to δ 147.0 and 145.9, respectively. Further, the aromatic C-5 resonance at δ 111.2 in **1** was shifted downfield to δ 114.5 in **2**. These ^{13}C shifts are characteristic of neolignans with a 3-methoxy, 4-hydroxy substitution pattern.[16] To confirm this hypothesis, ^{1}H−^{13}C HMBC heteronuclear correlations were used. In **2,** the phenolic proton resonance at δ 5.61 (C-4−O*H*) was coupled to the carbon resonances at δ 147.0 (C-3) and 114.5 (C-5). These couplings indicate that the phenolic hydroxyl group is substituted at the aromatic C-4 position, rather than at the C-3 carbon. The ^{1}H and ^{13}C chemical shifts in **2** are essentially identical with those of **1**, indicating that **2** has the same *cis*−*trans*−*cis* orientation around the tetrahy-

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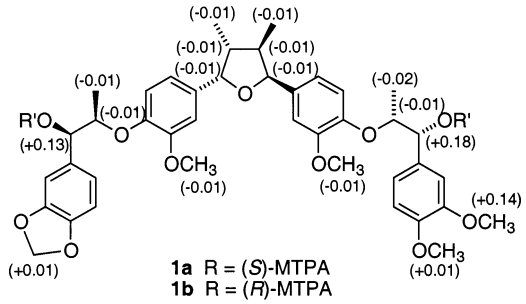
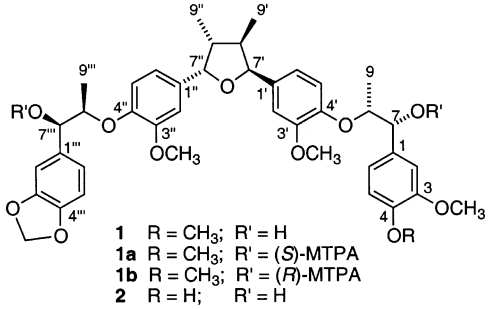


Figure 1. Selected $\Delta\delta$ values [$\Delta\delta$ (in ppm) = $\delta_S - \delta_R$] for (*R*)- and (*S*)-MTPA esters of **1**.

drofuran ring and a *threo* orientation between each of the side chain ethers and the C-7/C-7‴ hydroxyl moieties.[15] The side chain coupling constants (J_{H7-H8} = 8.2 Hz and $J_{H7'''-H8'''}$ = 8.2 Hz) and deshielded ^{13}C δ values for the C-7 (78.8)/C-8 (84.5)/C-9 (17.4) system and C-7‴ (78.9)/C-8‴ (84.6)/C-9‴ (17.5) systems are also indicative of *threo* side chain configurations.[16,17]



Only partial relative configurations of manassantin A, manassantin B (**1**), and other structurally related dineolignans have been previously reported.[15] Therefore, the absolute configurations of the C-7 and C-7‴ were determined using the Mosher ester method.[18] Separate samples of compound **1** were each treated with (*R*)-(+)- and (*S*)-(−)-α-methoxy-α-trifluoromethylphenylacetyl chloride in anhydrous pyridine, respectively, to yield the (*S*)- and (*R*)-MTPA ester derivatives **1a** and **1b**. The ^1H chemical shift values of the (*R*)-MTPA ester (**1b**) were subtracted from the values of the (*S*)-MTPA ester (**1a**) [$\delta\Delta = \delta$ (*S*)-MTPA $- \delta$ (*R*)-MTPA] and are shown in Figure 1. The negative $\Delta\delta$ values for H-8, H-9, H-8‴, H-9‴, H-7′, H-8′, H-9′, H7″, H-8″, H-9″, C-3′-OCH_3, and C-3″-OCH_3 and positive values for the C-3-OCH_3, C-4-OCH_3, and C-3‴-OCH_2O-C-4‴ indicate an *R*-configuration for both C-7 and C-7‴. Since each of the side chain ethers and the C-7/C-7‴ hydroxyl groups are arranged in a *threo* orientation, the C-8 and C-8‴ can, therefore, also be assigned *R*-configurations. The absolute configuration of the tetrahydrofuran ring cannot be deduced by relationship to either side chain and was not determined. Compounds **1** and **2** have nearly identical NMR spectra and both have a negative optical rotation, suggesting both to be of the same absolute configuration. Therefore, according to IUPAC convention for the nomenclature of lignans,[19] manassantin B (**1**) is (7*R*,7‴*R*,8*R*,8‴*R*)-(7′*S**,7″*S**,8′*R**,8″*R**)-7,7‴-dihydroxy-3,3′,3″,4-tetramethoxy-3‴,4‴-methylenedioxy-7′,7″-epoxy-8,4′:4″,8‴-bisoxy-8′,8″-dineolignane and 4-*O*-demethylmanassantin B (**2**) is (7*R*,7‴*R*,8*R*,8‴*R*)-(7′*S**,7″*S**,8′*R**,8″*R**)-4,7,7‴-trihydroxy-3,3′,3″-trimethoxy-3‴,4‴-methylenedioxy-7′,7″-epoxy-8,4′:4″,8‴-bisoxy-8′,8″-dineolignane. These side chain configurations are consistent with the side chain *R*/*R*-configuration recently established for a pair of new sesquineolignins (saurcerneols D and E), isolated from a Korean collection of *S. chinensis*.[20]

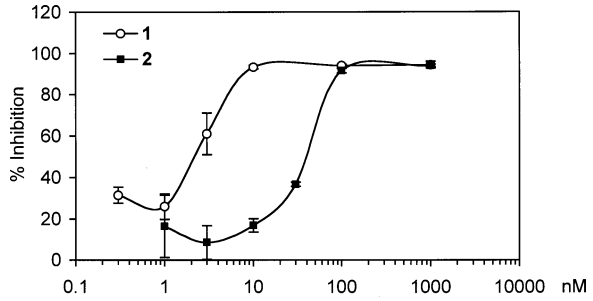


Figure 2. Dose−response curves of **1** and **2** inhibiting hypoxic activation of HIF-1 in T47D cells. Data shown are means from one experiment performed in triplicate, and the bars represent standard deviation. Similar results were obtained from separate experiments.

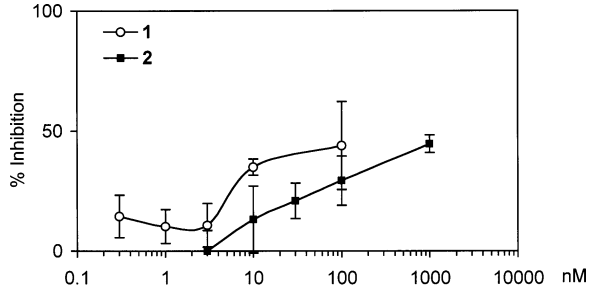


Figure 3. Effects of **1** and **2** on HIF-1 activation by 1,10-phenanthroline in T47D cells. Data shown are means from one representative experiment performed in quadruplicate, and the bars represent standard deviation.

Dose−response studies were performed to determine the effects of **1** and **2** on HIF-1 activity. Exponentially grown T47D cells transfected with the pTK-HRE3-luc reporter construct were exposed to active compounds in a series of dilutions for 30 min, followed by another 16 h incubation under hypoxic conditions (1% O_2/5% CO_2/94% N_2). The cells were harvested and luciferase activities determined using a luciferase assay system. Hypoxic exposure induced a 28-fold activation in HIF-1 activity. As shown in Figure 2, both compounds inhibit hypoxic activation of HIF-1 in T47D cells. The IC_{50} is 3 nM for **1** and 30 nM for **2**. Complete inhibition was observed at 10 nM for **1** and 100 nM for **2**. Statistical analysis (ANOVA) revealed that these inhibitory activities are statistically significant, relative to the hypoxic control ($p < 0.0001$). A similar experiment was performed with a control plasmid pGL3-Control. No statistically significant difference in luciferase expression was observed using the pGL3-Control construct in the presence of either **1** or **2** (data not shown).

Iron chelators and transition metals (such as cobalt and nickel) can activate HIF-1 and have been used as hypoxia mimetics.[21] We found that the Fe^{2+} selective chelator 1,-10-phenanthroline is at least 10 times more potent than the commonly used Fe^{3+} selective chelator DFO in activating HIF-1 (Zhou and Nagle, unpublished observations). The effects of **1** and **2** on HIF-1 activation by 1,10-phenanthroline were examined in T47D cells, and the data are shown in Figure 3. Exposure (16 h) to 1,10-phenanthroline (10 μM) activated HIF-1 by 47-fold. Significantly higher concentrations of both compounds are required to inhibit HIF-1 activation by 1,10-phenathroline. Statistically significant inhibition was observed in the presence of **1** at ≥10 nM and **2** at ≥30 nM. At the concentrations tested, neither compound inhibited more than 50%. In a similar experiment, T47D cells transfected with a control construct

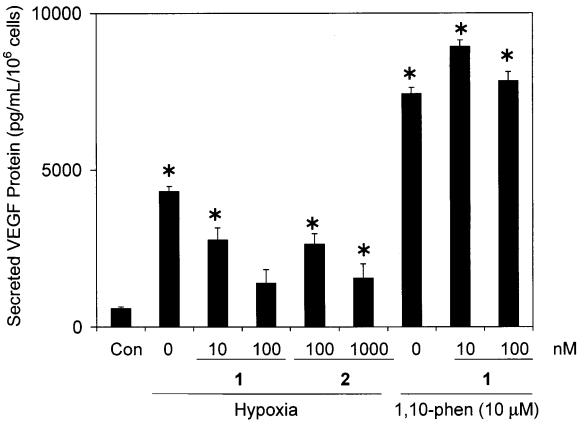


Figure 4. Compounds **1** and **2** selectively inhibit hypoxic induction of secreted VEGF protein in T47D cells. Data shown are averages from a representative experiment performed in quadruplicate; the bars represent standard error of the mean. An asterisk (*) indicates a significance of $p < 0.05$ relative to the control (Con), and lack of asterisk indicates no statistical difference (ANOVA and Fisher's PLSD post hoc test).

pGL3-Control were exposed to compounds **1** and **2**, followed by another 16 h incubation with 1,10-phenanthroline (10 μM). No statistically significant differences in luciferase activities were observed across treatments (data not shown). In summary, these results indicate that **1** and **2** selectively inhibit HIF-1 activation by hypoxia, relative to the activation produced by the iron chelator. This suggests that **1** and **2** are likely to inhibit HIF-1 activation in tumor cells through mechanisms unique to hypoxia.

Over 60 genes have been identified to be direct targets of HIF-1.[4] Most of these genes are induced by hypoxia in a manner specific to each individual type of cell. One gene that is induced in most cells is the angiogenic factor vascular endothelial growth factor (VEGF). In cancer patients, VEGF overexpression correlates positively with high microvessel density, advanced stage diseases, and poor prognosis.[22] Agents that inhibit VEGF production and function are currently in clinical trials for cancer treatments.[23] Since secreted VEGF protein is the bioactive form, compounds that can reduce the level of secreted VEGF protein represent potential tumor angiogenesis inhibitors. The effects of compounds **1** and **2** on hypoxic induction of secreted VEGF protein were examined in T47D cells. Exponentially grown T47D cells were exposed to compounds **1** and **2** at the indicated concentrations for 30 min, followed by another 16 h incubation under hypoxic conditions. The concentration of secreted VEGF protein in the conditioned media was determined (by ELISA), and the data were normalized to the number of viable cells. As shown in Figure 4, hypoxia induces secreted VEGF protein production by 7.4-fold in T47D cells. Both compounds **1** and **2** inhibited hypoxic induction of VEGF protein at the concentrations tested. Compound **1** is at least 10 times more potent than **2**. The effects of **1** on VEGF induction by 1,10-phenanthroline were also examined in T47D cells and the data presented in Figure 4. Although **1** blocked hypoxic induction of secreted VEGF protein at the concentration of 100 nM, **1** did not exert statistically significant inhibition on the induction of secreted VEGF protein by 1,10-phenanthroline (10 μM). These results correlate with earlier observations that **1** is more selective at inhibiting HIF-1 activation by hypoxia.

Under normoxic conditions, HIF-1α protein is post-translationally modified and rapidly degraded.[24] Hypoxic exposure stabilizes HIF-1α protein and activates HIF-1. To investigate the mechanism of action for HIF-1 inhibition

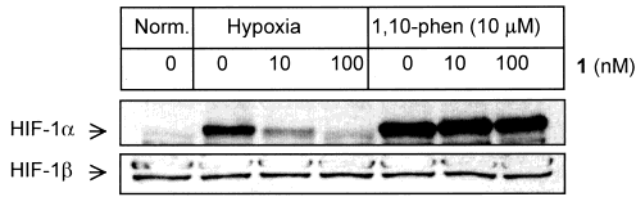


Figure 5. Western blot analysis of nuclear HIF-1α and HIF-1β proteins following treatment of T47D cells with **1** and subjecting the cells to either physiological hypoxia or chemical hypoxia (1,10-phenanthroline). Expression of HIF1α and HIF1β subunits under normoxic (Norm.) conditions is shown.

by compounds **1** and **2**, the effect of **1** on HIF-1α protein stabilization was examined. Exponentially grown T47D cells were exposed to **1** at 10 and 100 nM for 30 min, followed by another 4 h incubation under hypoxic conditions or in the presence of 1,10-phenanthroline (10 μM). Nuclear extracts were prepared from control and treated cells. The nuclear HIF-1α proteins were detected by Western blot, and the results are shown in Figure 5. Both hypoxic exposure and treatment with 1,10-phenanthroline increase nuclear HIF-1α protein. Compound **1** blocks hypoxic induction of nuclear HIF-1α protein without affecting 1,10-phenanthroline induction. HIF-1 is a heterodimer of the bHLH-PAS proteins HIF-1α and HIF-1β/ARNT. HIF-1α protein is regulated by oxygen, while HIF-1β protein is constitutively expressed. As shown in Figure 5, nuclear HIF-1β protein levels remain constant across treatments. These data suggest that certain HIF-1 inhibitors (such as **1**) suppress hypoxic activation of HIF-1 by blocking the induction of nuclear HIF-1α proteins.

Using a similar luciferase reported-based screening system, researchers at the National Cancer Institute have recently evaluated the NCI "diversity set" (a set of about 2000 pure compounds of structurally diverse chemical nature) for inhibitors of HIF-1 activation.[25] The most potent compounds found to inhibit HIF-1 activation were the DNA topoisomerase I inhibitor topotecan and related camptothecin analogues. However, the EC_{50} of topotecan was reported to be ≥50 nM in each of the tumor cell lines evaluated, and the cytotoxic properties of camptothecins are not selective to tumor cells. Therefore, manassantin B (**1**) is among the most potent inhibitors of tumor cell HIF-1 activation discovered (IC_{50} = 3 nM). Compounds **1** and **2** are highly selective in their inhibition of physiological hypoxia-induced HIF-1 activation, relative to iron chelator-induced HIF-1 activation.

Experimental Section

General Experimental Procedures. Optical rotation was measured on a RUDLPH Research Autopol IV automatic polarimeter. The IR spectrum was obtained using an AATI Mattson Genesis Series FTIR. The ^{1}H NMR, ^{1}H−^{1}H COSY, NOESY, and HMBC spectra were recorded on a Bruker AV 400 spectrometer. The ^{13}C NMR and HMQC spectra were recorded on a Bruker DRX 400 spectrometer. Both NMR spectrometers were operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C, respectively. The NMR spectra were recorded running gradients and using residual solvent peaks (δ 7.26 s) as internal references. The HRESIMS and ESIMS data were acquired on a Bruker BioAPEX 30es mass spectrometer. TLC were run on Merck TLC plates precoated with Si_{60} F_{254} or Si_{60} RP18 F_{254} with visualization by spraying with 1:1 H_2SO_4 in EtOH and heating. HPLC was carried out on a Waters Millennium system with a 996 photodiode array detector.

Plant Material. *Saururus cernuus* was collected from Benton County, Mississippi, near Michigan City (GPS 34°58′3″ N; 89°15′42″ W) during the summer of 1998. The sample was

stored at −20 °C prior to extraction. A voucher specimen (DN.075) was placed on file with the University of Mississippi Herbarium (Department of Biology, University of Mississippi).

Extraction and Isolation. Lyophilized *S. cernuus* stems and leaves (172 g) were extracted with 50% CH_2Cl_2 in MeOH (v/v). A portion (11.0 g) of the crude extract (16.57 g total) was fractionated by VLC (Si gel, 32−63 μm, hexanes−EtOAc−MeOH, step gradient). The fraction that eluted with 100% EtOAc (862.5 mg) was further fractionated by Sephadex LH-20 chromatography (100% MeOH). A subfraction from the LH-20 separation (34.5 mg) was chosen for further purification. Before applying to HPLC, the fraction was passed through a C_{18} SepPack minicolumn (80% CH_3CN−20% H_2O and rinsed with CH_3CN and MeOH). The 80% CH_3CN−20% H_2O subfraction (33.0 mg) was subjected to RP-HPLC (Prodigy ODS-3, 5 μm, 21.2 × 250 mm, 50% H_2O−CH_3CN (v/v)−100% CH_3CN gradient, 7.0 mL min^{-1}, photodiode-array detection monitored at 254 nm) to obtain **1** (11.7 mg, 0.11% yield) and **2** (4.5 mg, 0.04% yield).

Manassantin B (1), (7*R*,7‴*R*,8*R*,8‴*R*)-(7′*S,7″*S**,8′*R**, 8″*R**)-7,7‴-dihydroxy-3,3′,3″,4-tetramethoxy-3‴,4‴-methylenedioxy-7′,7″-epoxy-8,4′:4″,8‴-bisoxy-8′,8″-dineolignane:** clear amorphous solid; $[\alpha]^{25}_D$ −81° (*c* 0.33, $CHCl_3$); HRESIMS *m*/*z* 739.3031 (calcd for $C_{41}H_{48}O_{11}Na$ $[M + Na]^+$ 739.3094). The IR, UV, and ^1H and ^{13}C NMR spectroscopic data are essentially identical to previously published values.[15c]

4-*O*-Demethylmanassantin B (2), (7*R*,7‴*R*,8*R*,8‴*R*)-(7′*S,7″*S**,8′*R**,8″*R**)-4,7,7‴-trihydroxy-3,3′,3″-trimethoxy-3‴,4‴-methylenedioxy-7′,7″-epoxy-8,4′:4″,8‴-bisoxy-8′,8″-dineolignane:** clear powder; $[\alpha]^{25}_D$ −45° (*c* 0.08, $CHCl_3$); UV (MeOH) γ_{max} (log ϵ) 208 (4.78), 234 (4.35), 282 (3.36) nm; IR (film) λ_{max} 3450, 2970, 2930, 1610, 1590, 1510, 1460, 1420, 1275, 1255, 1235, 1140, 1040, 935, 860, 820, 760 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 6.99 (1H, d, *J* = 8.0 Hz, H-6″), 6.98 (1H, d, *J* = 8.2 Hz, H-6′), 6.92−6.82 (5H, m, H-5, H-6, H-5′, H-5″, H-6‴), 6.91 (1H, s, H-2‴), 6.88 (3H, s, H-2, H-2′, H-2″), 6.78 (1H, d, *J* = 8.2 Hz, H-5‴), 5.95 (2H, s, OCH_2O), 5.61 (1H, s, OH), 5.46 (2H, d, *J* = 5.7 Hz, H-7′, H-7″), 4.63 (1H, d, *J* = 8.2 Hz, H-7), 4.61 (1H, d, *J* = 8.2 Hz, H-7‴), 4.12 (1H, m, H-8), 4.10 (1H, m, H-8‴), 3.92 (6H, s, OCH_3), 3.90 (3H, s, OCH_3), 2.29 (2H, m, H-8′ and H-8″), 1.17 (3H, d, *J* = 4.6 Hz, H-9‴), 1.15 (3H, d, *J* = 4.6 Hz, H-9), 0.72 (6H, d, *J* = 5.7 Hz, H-9′, H-9″); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 151.0 (C, C-4′, C-4″), 148.2 (C, C-3‴), 147.8 (C, C-4‴), 147.0 (C, C-3), 146.9 (C, C-3″), 146.7 (C, C-3′), 145.9 (C, C-4), 137.0 (C, C-1″), 136.9 (C, C-1′), 134.4 (C, C-1‴), 132.4 (C, C-1), 121.5 (CH, C-6), 121.1 (CH, C-6‴), 119.3 (CH, C-5′), 119.1 (CH, C-6“, C-5”, C-6′), 114.5 (CH, C-5), 110.5 (CH, C-2″), 110.0 (CH, C-2′), 109.8 (CH, C-2), 108.5 (CH, C-5‴), 108.0 (CH, C-2‴), 101.4 (CH_2, OCH_2O), 84.6 (CH, C-8‴) 84.5 (CH, C-8), 83.8 (CH, C-7′, C-7″), 78.9 (CH, C-7‴), 78.8 (CH, C-7), 56.4 (CH_3, OCH_3), 56.3 (CH_3, OCH_3), 44.6 (CH, C-8′, C-8″), 17.5 (CH_3, C-9‴), 17.4 (CH_3, C-9), 15.3 (CH_3, C-9′, C-9″); HRESIMS *m*/*z* 701.2978 (calcd for $C_{40}H_{45}O_{11}$ $[M - H]^-$ 701.2962).

(*R*)- and (*S*)-MTPA Esters of Manassantin B (1). Compound **1** (4 mg) was dissolved in pyridine (180 μL) and treated with 10 μL (13.5 mg) of (*R*)-(−)-MTPA chloride at room temperature for 12 h. MeOH (500 μL) was added, and the solvent was removed in vacuo. The residue was purified by preparative Si gel TLC using 1% MeOH in CH_2Cl_2 to yield the (*S*)-MTPA manassantin B ester **1a** (2 mg). The (*R*)-MTPA manassantin B ester (**1b**) was prepared from the (*S*)-(+)-MTPA chloride using the same procedure as described for **1a**.

Manassantin B, bis-(*S*)-MTPA ester (1a): ^1H NMR ($CDCl_3$, 400 MHz) δ 7.26−7.22 (10H, MTPA-Ar-H), 7.00−6.75 (12H, Ar−H), 6.21 (1H, d, *J* = 8 Hz, H-7, H-7‴), 5.99 (2H, d, *J* = 4 Hz, OCH_2O), 5.43 (2H, d, *J* = 4 Hz, H-7′, H-7″), 4.62 (2H, m, H-8, H-8‴), 3.90 (3H, C-4-OCH_3), 3.86 (3H, C-3-OCH_3), 3.81 (6H, C-3′-OCH_3, C-3″-OCH_3), 3.44 (6H, s, MTPA-OCH_3), 2.27 (2H, m,. H-8′, H-8″), 1.08 (3H, d, *J* = 8 Hz, H-9‴), 1.07 (3H, d, *J* = 4 Hz, H-9), 0.70 (6H, d, *J* = 8 Hz, H-9′, H-9″); HRESIMS *m*/*z* 1171.3899 $[M + Na]^+$ (calcd for $C_{61}H_{62}O_{15}F_6Na$, $[M + Na]^+$, 1171.3891).

Manassantin B, bis-(*R*)-MTPA ester (1b): ^1H NMR ($CDCl_3$, 400 MHz) δ 7.22−7.26 (10H, MTPA-Ar−H), 6.91−6.70 (12H, Ar-H), 6.08 (1H, d, *J* = 8 Hz, H-7‴), 6.03 (1H, d, *J* = 8 Hz, H-7), 5.98 (2H, d, *J* = 1.5 Hz, OCH_2O), 5.45 (2H, d, *J* = 4 Hz, H-7′, H-7″), 4.63 (2H, m, H-8, H-8‴), 3.89 (3H, s, C-4-OCH_3), 3.82 (6H, s, C-3′-OCH_3, C-3″-OCH_3), 3.72 (3H, s, C-3-OCH_3), 3.50 (3H, s, MTPA-OCH_3), 3.48 (3H, s, MTPA-OCH_3), 2.28 (2H, m, H-8′, H-8″), 1.09 (6H, d, *J* = 8 Hz, H-9, H-9‴), 0.71 (6H, d, *J* = 4 Hz, H-9′, H-9″); HRESIMS *m*/*z* 1171.3891 $[M + Na]^+$ (calcd for $C_{61}H_{62}O_{15}F_6Na$, $[M + Na]^+$, 1171.3891).

Reporter Assay for HIF-1 Activity. T47D cells (American Type Culture Collection) were maintained in DMEM/F12 medium (JRH Biosciences), supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone), 50 U/mL penicillin G sodium, and 50 μg/mL streptomycin (GIBCO BRL) in a humidified atmosphere (5% CO_2 and 95% air) at 37 °C. Exponentially grown T47D cells (1 × 10^7 cells) were transfected with the pTK-HRE3-luc reporter[9] or the control construct pGL3-Control (Promega) by electroporation using an ECM830 square wave electroporation system (BTX Inc) at 140 V for 70 ms (1 pulse). The transfected cells were plated at 4.5 × 10^4 cells per well into 96-well plates in a volume of 100 μL of DMEM/F12 medium supplemented with 10% FBS and antibiotics. After 24 h, test compounds were diluted in DMEM/F12 medium with antibiotics, added in a volume of 100 μL per well, and the incubation continued for another 30 min at 37 °C. The cells were exposed to hypoxic (1% O_2/5% CO_2/94% N_2) or normoxic (5% CO_2/95% air) conditions or hypoxia mimetic (10 μM 1,10-phenanthroline, Sigma) at 37 °C for 16 h. Hypoxic conditions were achieved, as previously described.[26] The cells were then lysed and luciferase activities determined following manufacturer's instructions (Promega) using a TopCount microplate scintillation counter (Packard).

ELISA for VEGF Protein. Exponentially grown T47D cells were plated at the density of 3 × 10^4 cells per well into 96-well plates in a volume of 100 μL DMEM/F12 medium supplemented with 10% FBS and antibiotics. Compound treatment and hypoxic exposure were achieved as that described in the reporter assay section. Following 16 h incubation, the plates were centrifuged at 100*g* for 2 min, and the conditioned media transferred to a new plate and stored at −80 °C. Cell viability in the control and treated samples was determined using the Neutral Red method.[27] Briefly, fresh DMEM/F12 medium with 10% FBS, antibiotics, and Neutral Red (0.15 mg mL^{-1}) was added at 100 μL per well. After 2 h incubation at 37 °C, the medium was removed and the cells were washed once with saline solution (0.9% NaCl) and lysed with 0.04 N HCl in 2-propanol (100 μL per well). Absorbance at 540 nm was measured and background absorbance (630 nm) subtracted using a EL312e plate reader (Bio-Tek Instruments). A standard curve of OD_{540} versus cell number was generated from wells plated at different densities (1.5 × 10^4, 3 × 10^4, and 6 × 10^4 cells per well into 96-well plates). The number of viable cells in the standard well was determined using a hemocytometer following trypsin treatment. The standard curve was used to calculate the viable cell number in the control and treated samples.

VEGF protein levels in the conditioned media were determined using an ELISA assay following manufacturer's instructions (R&D Systems) with the following modifications. The capture antibody anti-human VEGF antibody was used at 0.4 μg mL^{-1}, and the detection antibody biotinylated anti-human VEGF antibody at 0.1 μg mL^{-1}. The conditioned media samples were thawed on ice, and 100 μL samples were used. The peroxidase-conjugated strepavidin (Jackson ImmunoResearch Laboratories) was added at 1 μg mL^{-1}. After the addition of the peroxidase-conjugated strepavidin and wash, 50 μL of TMB liquid substrate system for ELISA (Sigma) was added and incubated at room temperature for 10 min. The reaction was stopped with the addition of 50 μL of 1 N H_2SO_4. The optical density at 450 nm was measured with correction wavelength at 540 nm on a EL312e plate reader (Bio-Tek Instruments). Sample VEGF protein concentration was calculated using a formula derived from the VEGF standard curve within the linear detection range. The level of secreted VEGF protein was normalized with the number of viable cells.

Nuclear Extract Preparation, Western Blot Analysis of HIF-1α and HIF-1β Protein. Ten million exponentially grown T47D cells were seeded at a density of 1.7×10^5 cells per cm^2 growth area and incubated at 37 °C overnight. Test compounds were added and incubated for another 30 min at 37 °C. The cells were exposed to hypoxic or normoxic conditions or iron chelators at 37 °C for another 4 h. At the end of incubation, the cells were harvested by scraping, followed by centrifugation at 150*g* for 5 min at 4 °C. After washing three times with ice-cold 1× PBS, the cell pellet was resuspended in 800 μL of Tris-buffered KCl solution (20 mM Tris-HCl, pH 7.8, 50 mM KCl) supplemented with 1 mM dithiothreitol and a cocktail of protease and phosphatase inhibitors (2 mM AEBSF, 1 mM EDTA, 130 μM bestatin, 14 μM E-64, 1 μM leupeptin, 0.3 μM aprotinin, 50 mM NaF, and 1 mM Na_3VO_4). Nonidet P-40 (Calbiochem) was added at 0.6% (v/v) final concentration, and the cell suspension was vortexed for 10 s and centrifuged at 12 000*g* for 1 min at 4 °C to obtain the nuclei pellet. The nuclei were resuspended in 30 μL ice-cold Tris-buffered high-KCl solution (20 mM Tris-HCl, pH 7.8, 500 mM KCl) supplemented with 20% (v/v) glycerol, 1 mM dithiothreitol, and the cocktail of protease and phosphatase inhibitors. After 1 h incubation on ice, the nuclear lysate was centrifuged at 12 000*g* for 20 min at 4 °C, and the supernatant (nuclear extract) was mixed with 3× SDS/PAGE sample buffer (50 mM Tris, pH 7.4, 4% SDS, 10% glycerol, 4% 2-mercaptoethanol, and 0.05 mg mL^{-1} bromophenol blue) at 2:1 ratio (v/v), boiled for 5 min, and stored at −80 °C.

For HIF-1α Western blot analysis, the nuclear extract was separated on a 7% SDS/PAGE gel and the separated proteins were transferred to a Hybond-C membrane (Amersham Biosciences). The membrane was stained with Ponceau-S solution (Sigma) to ensure equal loading and transferring before blocking with TTBS (20 mM Tris-HCl, pH 6.8, 150 mM NaCl, and 0.05% Tween 20) supplemented with 5% nonfat milk (Bio-Rad) at room temperature for 1 h. The anti-HIF-1α monoclonal antibody (Novus Biologicals) was diluted at 1:500 in 1% BSA/TTBS solution and incubated with the membrane at 4 °C overnight. After washing with TTBS at room temperature, the membrane was incubated with the secondary antibody biotinylated anti-mouse immunoglobulin G (Vector Laboratories) diluted at 1:1000 in 1% BSA/TTBS solution at 4 °C for 4 h. The membrane was then incubated with Vectastain ABC reagent (Vector Laboratories) according to the vendor's protocol at room temperature for 30 min and developed using enhanced chemiluminescence (ECL) reagents (Amersham Biosciences).

For HIF-1β Western blot analysis, the membrane (after HIF-1α detection) was stripped by incubating at 80 °C for 30 min in the stripping buffer (62 mM Tris-HCl, pH 6.7, 2% SDS, and 100 mM 2-mercaptoethanol), washed in TTBS, and blocked in 5% nonfat milk/TTBS at room temperature for 1 h. The HIF-1β monoclonal antibody (Novus Biologicals) was diluted at 1:1500 in 1% BSA/TTBS solution and incubated with the membrane at 4 °C overnight. Incubations with the secondary antibody and the Vectastain ABC reagent were the same as that described for HIF-1α. After washing, the membrane was developed using the ECL reagents.

Statistic Analysis. Data were compared using ANOVA and post hoc analyses using Fisher's PLSD (StatView software version 5.01, SAS Institute Inc).

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