Phenanthroquinolizidine Alkaloids from the Roots of *Boehmeria pannosa* Potently Inhibit Hypoxia-Inducible Factor-1 in AGS Human Gastric Cancer Cells

Xing Fu Cai,[†] Xuejun Jin,[†] Dongho Lee,[†] Young Taek Yang,[‡] Kyeong Lee,[†] Young-Soo Hong,[†] Jeong-Hyung Lee,[†] and Jung Joon Lee*[,†]

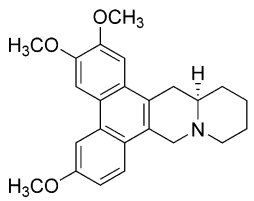
Korea Research Institute of Bioscience and Biotechnology, P.O. Box 115, Yuseong, Daejeon 305-600, Korea, and Jeju-do Agricultural Research and Extension Services, Jeju, Jeju 690-815, Korea

Received February 22, 2006

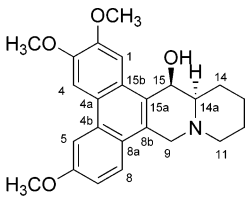
A bioassay-guided phytochemical investigation on the methanol extract of *Boehmeria pannosa*, using a HIF-1-mediated reporter gene assay, led to the isolation of two phenanthroquinolizidine alkaloids, (−)-cryptopleurine (**1**) and (−)-(15*R*)-hydroxycryptopleurine (**2**). The structure of the new compound **2** was determined by spectroscopic methods. Compounds **1** and **2** potently inhibited the hypoxia-induced expression of a reporter gene under the control of a hypoxia response element (HRE) with IC_{50} values of 8.7 and 48.1 nM, respectively. Furthermore, **1** and **2** suppressed the accumulation of HIF-1α protein in a dose-dependent manner, but not the HIF-1β protein and inhibited expression of vascular endothelial growth factor (VEGF) by hypoxia.

Hypoxia is a reduction in the normal level of tissue oxygen tension and is observed during acute and chronic vascular disease, pulmonary disease, and cancer.[1] The occurrence of hypoxic regions within the tumor tissue is important for triggering angiogenesis and thus enables tumors to develop their own blood supply. The cellular response to hypoxia is controlled by hypoxia-inducible factor-1 (HIF-1). HIF-1 consists of an oxygen-regulated α subunit and a constitutively expressed β subunit and activates the transcription of genes that are involved in angiogenesis, glucose metabolism, apoptosis, invasion, and metastasis.[2,3] HIF-1α is overexpressed in a large number of human cancers, and its overexpression correlates with poor prognosis and treatment failure. Manipulation of HIF-1 activity by genetic or pharmacological means has marked effects on tumor growth in preclinical studies. Therefore, HIF-1 could be an important target of cancer chemotherapy, and efforts are underway to identify selective inhibitors of HIF-1.[2,4−7]

In our search for HIF-1 inhibitors of natural origin, a methanol extract of the roots of *Boehmeria pannosa* (Urticaceae) potently inhibited HIF-1 activation induced by hypoxia (80% inhibition at 0.8 μg mL^{-1}) in a HIF-1-mediated reporter gene assay. *B. pannosa* is a perennial herb and is distributed in East Asia.[8,9] In the only previous phytochemical report on *B. pannosa*, the occurrence of anthraquinones, flavonoids, and terpenoids was reported.[10] Bioassay-guided phytochemical investigation on the methanol extract of *B. pannosa* using a HIF-1-mediated reporter gene assay led to the isolation of two phenanthroquinolizidine alkaloids, (−)-cryptopleurine (**1**) and (−)-(15*R*)-hydroxycryptopleurine (**2**). The structure

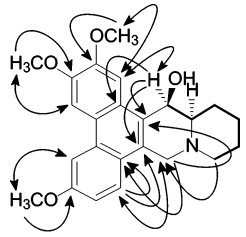


1



2

of the new compound **2** was determined by spectroscopic methods. Compounds **1** and **2** potently inhibited the hypoxia-induced expression of the HRE-reporter gene with IC_{50} values of 8.7 and 48.1 nM, respectively. Compounds **1** and **2** were further evaluated for their potential to inhibit the accumulation of the hypoxia-activated HIF-1α protein and the expression of vascular endothelial growth factor (VEGF), a HIF-1 target gene. The isolation, structure determination, and biological evaluation of these compounds are the subject of this communication.



2

Figure 1. Selected COSY (—), NOE (curved double arrows), and HMBC (curved single arrows) correlations of **2**.

Compound **1** was obtained as a brownish amorphous powder and was positive to Dragendorff's reagent. The structure of **1** was identified as (−)-cryptopleurine by comparing its spectroscopic data to values reported in the literature.[11,12] Compound **2** also gave an orange-red coloration with Dragendorff's reagent, and its UV spectrum obtained by analytical HPLC using a PDA detector exhibited the same profile as that of (−)-cryptopleurine (**1**). The ^{1}H and ^{13}C NMR spectra of **2** were closely comparable to those of **1**, suggesting that **2** is a modified (−)-cryptopleurine (**1**) and is also a phenanthroquinolizidine alkaloid structure type. In the ^{1}H NMR spectrum of **2**, a set of singlet signals at δ_H 7.60 (1H, s, H-1) and 8.05 (1H, s, H-4), an AMX proton spin system at δ_H 8.04 (1H, d, J = 2.4 Hz, H-5), 7.29 (1H, dd, J = 2.4 and 8.8 Hz, H-7), and 7.83 (1H, d, J = 8.8 Hz, H-8), and three methoxyl signals at δ_H 4.03 (3H, s, OCH_3-6), 4.04 (3H, s, OCH_3-2), and 4.08 (3H, s, OCH_3-3) were apparent. However, additional methine signals at δ_H 5.27 (1H, d, J = 1.2 Hz, H-15) and δ_C 64.6 (C-15) were observed in the ^{1}H and ^{13}C NMR spectra of **2**, respectively. On the basis of these observations and by comparison of its spectroscopic data with those of **1**, compound **2** was suggested to be a hydroxylated cryptopleurine. This was consistent with the molecular formula ($C_{24}H_{27}NO_4$; HRESIMS, m/z 394.2012 [M + H]$^+$) obtained. The positions of the substituents were confirmed as occurring at C-2, C-3, and C-6 (three methoxyls) and C-15 (hydroxylated methine) using 2D NMR experiments (Figure 1). The relative configuration of the hydroxy group at C-15 was determined to be *trans* to H-14a,

* To whom correspondence should be addressed. Tel: +82-42-860-4360. Fax: +82-42-860-4595. E-mail: jjlee@kribb.re.kr.
[†] Korea Research Institute of Bioscience and Biotechnology.
[‡] Jeju-do Agricultural Research and Extension Services.

10.1021/np060081y CCC: $33.50
Published on Web 06/22/2006

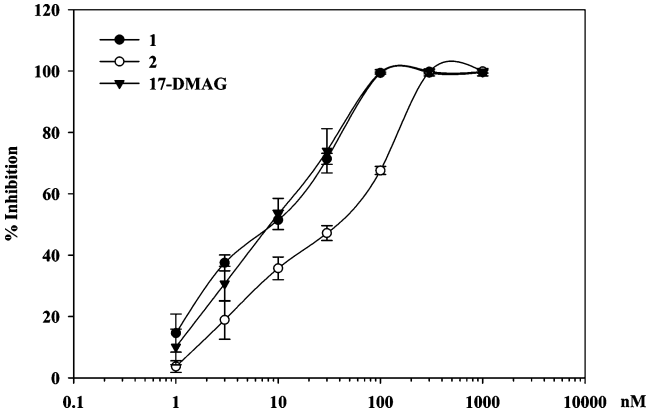


Figure 2. Dose−response curves of **1** and **2** on hypoxia-induced HIF-1 activation in AGS cells in comparison with 17-desmethoxy-17-*N*,*N*-dimethylaminoethylaminogeldanamycin (17-DMAG) as a positive control. Data shown are means from one representative experiment performed in triplicate, and the bars represent standard deviation. Similar results were obtained from separate experiments.

on the basis of the small ^{1}H NMR coupling constant (1.2 Hz). The absolute configuration of H-14a was deduced to be *R* from the CD spectrum, exhibiting a negative Cotton effect at 273 nm.[13] Therefore, the structure of the new compound **2** was elucidated as (−)-(15*R*)-hydroxycryptopleurine.

Compounds **1** and **2** were evaluated for their potential to inhibit HIF-1 activation induced by hypoxia using a HIF-1-mediated reporter gene assay in AGS human gastric cancer cells (Figure 2). Compounds **1** and **2** inhibited hypoxic activation of HIF-1 with IC_{50} values of 8.7 nM for **1** and 48.1 nM for **2**, as assessed by a HRE-reporter assay. Complete inhibition was observed at 100 nM for **1** and 300 nM for **2**. Cell viability, when measured by the MTT assay, showed that both compounds had no significant cytotoxicity to AGS cells at their effective concentrations for the inhibition of HIF-1 activation (data not shown). To confirm that these compounds inhibit the hypoxic activation of HIF-1 in AGS cells, we evaluated the effect of **1** and **2** on hypoxia-induced accumulation of HIF-1α protein by Western blot analysis. As shown in Figure 3A, both **1** and **2** blocked the accumulation of HIF-1α protein induced by hypoxia in a dose-dependent manner without affecting the expression of HIF-1*β* protein. Expression of several HIF-1 target genes, such as vascular endothelial growth factor (VEGF), is induced by hypoxia in most cell types. VEGF stimulates new blood vessel formation from existing vasculature, increasing tumor oxygenation and, ultimately, tumor growth and metastasis. This makes hypoxic tumors highly pro-angiogenic tumors with a highly aggressive phenotype.[1,2,6] Therefore, the effect of **1** on hypoxia-induced induction of VEGF in AGS cells was tested by RT-PCR analysis. The hypoxia-induced expression of VEGF was suppressed by **1** dose-dependently (Figure 3B).

Since HIF-1 has been known to be the center of most adaptation responses of cancer cells to hypoxia, considerable efforts are underway to identify small molecule HIF-1 inhibitors from chemical libraries and natural products.[14,15] Among the HIF-1 inhibitors discovered from such efforts, the most potent inhibitors are mostly natural products such as lignoid manassantins,[4] a diterpenoid laurenditerpenol,[5] terpenoid tetrahydroquinoline alkaloids,[7] and an isoquinoline alkaloid berberine.[16] Manassantin B is among the most potent inhibitors of HIF-1 activation (IC_{50} 3 nM) reported so far. Compound **1** exhibited a comparable potency to manassantin B. The detailed mechanism of compounds **1** and **2** on the inhibition of hypoxia-induced HIF-1 activation is still unclear. Further studies are needed to elucidate how **1** and **2** inhibit HIF-1 activation.

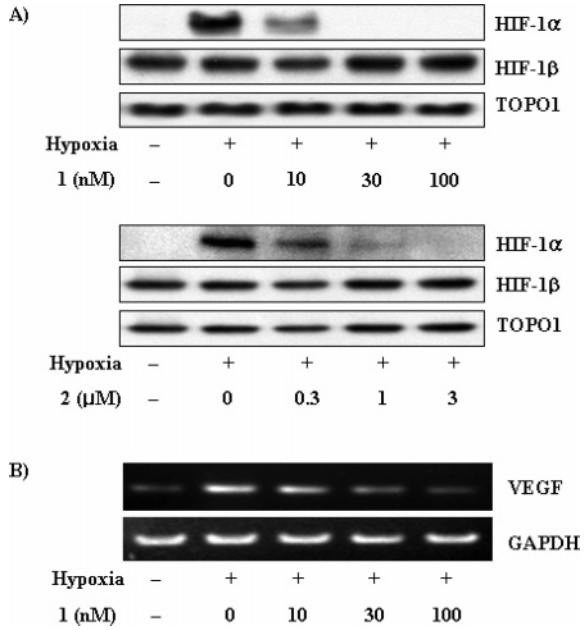


Figure 3. Effect of **1** and **2** on the accumulation of HIF-1α and HIF-1*β* proteins (A) and effect of **1** on VEGF expression (B) in AGS cells under hypoxic condition. For A, AGS cells were exposed to various concentrations of **1** and **2** for 1 h and then incubated for 12 h under hypoxic condition (1% O_2, 94% N_2, and 5% CO_2 at 37 °C). The relative levels of HIF-1α and HIF-1*β* proteins were determined by Western blot using monoclonal anti-HIF-1α and anti-HIF-1*β* antibodies. For B, AGS cells were exposed to various concentrations of **1** for 1 h and then incubated for 16 h under hypoxic condition. Total RNAs were isolated and VEGF RNA was detected by an RT-PCR analysis as described in the Experimental Section.

Experimental Section

General Experimental Procedures. Melting points were determined on a Kofler micro-hotstage without correction. Optical rotations were measured on a JASCO P-1020 polarimeter. UV and CD spectra were measured on a Shimadzu UV-1601 UV−visible and a JASCO J-7200 CD spectrophotometer, respectively. The NMR spectra were recorded on a Varian UNITY 400 FT-NMR spectrometer in CD_3OD. ESIMS and HRESIMS were obtained on a Platform quadrupole and a Mariner mass spectrometer, respectively. HPLC was carried out on a Waters semipreparative system (Waters Delta Prep 3000).

Plant Material. The roots of *Boehmeria pannosa* Nakai & Satake were collected in Jeju, Korea, in September 2004, identified by Dr. Joong Ku Lee, a plant taxonomist, Korea Research Institute of Bioscience and Biotechnology, and air-dried. A voucher specimen (No. 041002) has been deposited in the Korea Research Institute of Bioscience and Biotechnology.

Extraction and Isolation. The dried and powdered root (2.0 kg) was extracted three times with hot MeOH. The combined extracts were evaporated under reduced pressure to give the MeOH extract (90 g), which was suspended in water and then partitioned with $CHCl_3$ to give a $CHCl_3$-soluble extract (28 g). The $CHCl_3$-soluble extract exhibited potent inhibition of HIF-1 activation by hypoxia (80% inhibition at 0.8 μg mL^{-1}). The $CHCl_3$-soluble extract was chromatographed on a silica gel column eluted with gradients of hexanes−EtOAc (20:1 → 1:1) to afford four fractions (F01−F04). Of these, F04 showed the most significant HIF-1 inhibitory activity (99% inhibition at 0.8 μg mL^{-1}). Thus, F04 (12.0 g) was further purified on silica gel with $CHCl_3$−MeOH (10:1), producing five subfractions (F05−F09). F05 (99% inhibition at 0.8 μg mL^{-1}) was purified using HPLC [YMC Pro C_{18}, 250 × 20 mm i.d., MeCN−H_2O (0.05% TFA) gradient, 10 mL min^{-1}], leading to the isolation of (−)-cryptopleurine (**1**, 19 mg, 0.00095% of dry weight) and (−)-(15*R*)-hydroxycryptopleurine (**2**, 3.8 mg, 0.00019% of dry weight).

(−)-Cryptopleurine (1): brownish, amorphous powder; mp 198−201 °C [lit.[12] 196−197 °C]; $[\alpha]_D^{22}$ −63.8 (*c* 0.05, MeOH) [lit.[12] $[\alpha]_D^{25}$

−96.7 (*c* 0.40, $CHCl_3$)]; UV (MeOH) λ_{max} (log ϵ) 261 (1.44) nm; CD (MeOH) λ_{max} ($\Delta\epsilon$) 230 (+12.6), 273 (−11.7) nm; ESIMS *m*/*z* 378.6 $[M + H]^+$, 755.8 $[2M + H]^+$.

(−)-(15*R*)-Hydroxycryptopleurine (2): yellow powder; mp 201−203 °C; $[\alpha]_D^{22}$ −56.4 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ϵ) 262 (1.34) nm; CD (MeOH) λ_{max} ($\Delta\epsilon$) 229 (+20.4), 273 (−14.0) nm; ^{1}H NMR (CD_3OD, 400 MHz) δ 1.77 (1H, m, H-12), 1.86 (1H, m, H-13), 2.08 (1H, m, H-13), 2.09 (1H, m, H-12), 2.17 (1H, m, H-14), 2.25 (1H, m, H-14), 3.34 (1H, m, H-11), 3.55 (1H, dt, J = 11.6, 1.2 Hz, H-14a), 3.85 (1H, m, H-11), 4.03 (3H, s, OMe-6), 4.04 (3H, s, OMe-2), 4.08 (3H, s, OMe-3), 4.61 (1H, d, J = 16.4 Hz, H-9), 5.01 (1H, d, J = 16.4 Hz, H-9), 5.27 (1H, d, J = 1.2 Hz, H-15), 7.29 (1H, dd, J = 8.8, 2.4 Hz, H-7), 7.60 (1H, s, H-1), 7.83 (1H, d, J = 8.8 Hz, H-8), 8.04 (1H, d, J = 2.4 Hz, H-5), 8.05 (1H, s, H-4); ^{13}C NMR (CD_3OD, 100 MHz) δ 23.1 (t, C-12), 24.8 (t, C-13), 27.1 (t, C-14), 55.1 (t, C-9), 56.2 (q, OMe-6), 56.6 (q, OMe-2), 56.8 (q, OMe-3), 57.0 (t, C-11), 64.3 (d, C-14a), 64.6 (d, C-15), 105.6 (d, C-4), 105.9 (d, C-5), 106.1 (d, C-1), 117.6 (d, C-7), 121.3 (s, C-8b), 122.8 (s, C-8a), 125.6 (d, C-8), 126.2 (s, C-4a), 126.3 (s, C-15b), 127.2 (s, C-15a), 133.2 (s, C-4b), 151.2 (s, C-3), 151.7 (s, C-2), 160.7 (s, C-6); ESIMS *m*/*z* 394.5 $[M + H]^+$, 787.8 $[2M + H]^+$; HRESIMS *m*/*z* 394.2012 $[M + H]^+$ (calcd 394.2018 for $C_{24}H_{28}NO_4$).

Cell Culture. Human gastric cancer AGS cells (American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 (Invitrogen, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (Hyclone, Logan, UT), penicillin, and streptomycin in a humidified 5% CO_2 atmosphere at 37 °C. Hypoxic culture was kept in a gas-controlled chamber (Thermo Electron Corp., Marietta, OH) maintained at 1% O_2, 94% N_2, and 5% CO_2 at 37 °C.

Reporter Assay. The ability of compounds **1** and **2** to inhibit hypoxia-inducible factor-1 was determined by a reporter assay. Exponentially grown AGS cells (5×10^6 cells) at 75−90% confluence were transiently cotransfected with the vectors for pGL3-HRE-Luc plasmid,[17] which contains six copies of HREs derived from the human VEGF gene and pRL-CMV (Promega, Madison, WI) using Lipofectamine plus reagent according to the manufacturer's instructions (Invitrogen). The transfected cells were plated at a density of 4×10^4 cells per well into 96-well plates. After a 48 h incubation, the cells were treated with various concentrations of the tested compounds and incubated for 16 h in hypoxia (1% O_2, 94% N_2, and 5% CO_2). The luciferase assay was performed using a Dual-luciferase reporter assay system according to the instructions of the manufacturer (Promega). Luciferase activity was determined in a Microlumat Plus luminometer (EG&G Berthold, Bad Wildbad, Germany) by injecting 100 μL of assay buffer containing luciferin and measuring light emission for 10 s. The results were normalized to the activity of renilla luciferase expressed by the cotransfected *Rluc* gene under the control of a constitutive promoter. 17-Desmethoxy-17-*N*,*N*-dimethylaminoethylaminogeldanamycin (17-DMAG; Calbiochem, La Jolla, CA) was used as a positive control.

Western Blot Analysis. Exponentially grown AGS cells were plated at a density of 1.5×10^5 cells per cm^2 growth area and incubated at 37 °C overnight. Various concentrations of test compounds were added and incubated for 1 h and then further incubated for 12 h under hypoxic conditions. At the end of incubation, the cells were harvested by scraping, followed by centrifugation at 1000*g* for 5 min at 4 °C, and washed twice with ice-cold phosphate-buffered saline. To analyze the level of HIF-1α and HIF-1β proteins, total cell lysates were prepared from cells using a lysis buffer (50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg mL^{-1} leupeptin, 150 mM NaCl). Total cell lysates (50 μg) were separated by 6% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a Hybond-C membrane (Amersham Biosciences, Piscataway, NJ). The membrane was blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 (TTBS) at room temperature for 1 h and then incubated overnight at 4 °C with an anti-HIF-1α monoclonal antibody (1:200 dilution in 5% nonfat milk in TTBS; R&D systems, Minneapolis, MN) or anti-HIF-1β monoclonal antibody (1:250 dilution in 5% nonfat milk in TTBS; Santa Cruz Biotechnology, Santa Cruz, CA). Horseradish peroxidase-conjugated anti-rabbit antiserum (Amersham Biosciences, Piscataway, NJ) was used as a secondary antibody (1:2500 dilution in 5% nonfat milk in TTBS, 2 h incubation), and the antigen−antibody complexes were visualized by enhanced chemiluminescence according to the instructions of the manufacturer (Amersham Bioscience, Piscataway, NJ). Protein loading was controlled by probing the membranes for TOPO 1 protein with an anti-TOPO I antibody (1:500 dilution in 5% nonfat milk in TTBS; Santa Cruz Biotechnology).

RT-PCR Analysis. After AGS cells (1.5×10^5 cells per cm^2 growth area) were pretreated with various concentrations of **1** for 1 h, the cells were further incubated for 16 h under hypoxic conditions. Total RNA from cells was obtained using an RNA mini kit (Qiagen, Valencia, CA). Total RNA (2 μg) was used to perform reverse transcription-PCR (RT-PCR) using an RT-PCR kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The PCR primers for VEGF were 5′-GCTCTACCTCCACCATGCCAA-3′ (sense) and 5′-TGGAAGATGTCCACCAGGGTC-3′ (antisense), while the PCR primers for GAPDH were 5′-ACCACAGTCCATGCCATCAC-3′ (sense) and 5′-TCCACCACCCTGTTGCTGTA-3′ (antisense). The PCR conditions for both primer sets were as follows: denaturation at 94 °C for 5 min followed by 25 amplification cycles, each consisting of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min, and a final extension step at 72 °C for 7 min. PCR products were separated on 2% agarose gels and visualized by staining with ethidium bromide. The oligonucleotide sequences of the reaction products were confirmed by sequencing.

Statistical Analysis. Each experiment was performed at least three times, and representative data are shown. Data in the graph are given as mean values ± standard error of the mean. Means were checked for statistical differences by using the Student's *t*-test with error probabilities of $p < 0.05$.

Acknowledgment. This study was supported in part by a grant from Korea Research Institute of Bioscience and Biotechnology Research Initiative Program and a research grant (PF0320701-00) from Plant Diversity Research Center of 21st Frontier Research Program funded by the Korean Ministry of Science and Technology. We are grateful to Korea Basic Science Institute for the provision of certain spectroscopic instruments used in this investigation.

References and Notes

(1) Harris, A. L. *Nat. Rev. Cancer* **2002**, *2*, 38−47.
(2) Giaccia, A.; Siim, B. G.; Johnson, R. S. *Nat. Rev. Drug Discovery* **2003**, *2*, 803−811.
(3) Semenza, G. L. *Nat. Rev. Cancer* **2003**, *3*, 721−732.
(4) Hodges, T. W.; Hossain, C. F.; Kim, Y. P.; Zhou, Y. D.; Nagle, D. G. *J. Nat. Prod.* **2004**, *67*, 767−771.
(5) Mohammed, K. A.; Hossain, C. F.; Zhang, L.; Bruick, R. K.; Zhou, Y. D.; Nagle, D. G. *J. Nat. Prod.* **2004**, *67*, 2002−2007.
(6) Welsh, S. J.; Powis, G. *Curr. Cancer Drug Targets* **2003**, *3*, 391−405.
(7) Zhou, Y. D.; Kim, Y. P.; Mohammed, K. A.; Jones, D. K.; Muhammad, I.; Dunbar, D. C.; Nagle, D. G. *J. Nat. Prod.* **2005**, *68*, 947−950.
(8) Lee, C. B. *Illustrated Flora of Korea*; HyangmoonSa: Seoul, 1993; p 294.
(9) Lee, Y. N. *Flora of Korea*; Kyo-Hak Publishing Co.: Seoul, 1996; p 89.
(10) Takemoto, T.; Miyasi, T.; Kusano, G. *Phytochemistry* **1975**, *14*, 1890−1891.
(11) Kim, S.; Lee, T.; Lee, E.; Lee, J.; Fan, G.-J.; Lee, S. K.; Kim, D. *J. Org. Chem.* **2004**, *69*, 3144−3149.
(12) Suzuki, H.; Aoyagi, S.; Kibayashi, C. *J. Org. Chem.* **1995**, *60*, 6114−6122.
(13) Buckley, T. F., III; Rapport, H. *J. Org. Chem.* **1983**, *48*, 4222−4232.
(14) Rapisarda, A.; Uranchimeg, B.; Scudiero, D. A.; Selby, M.; Sausville, E. A.; Shoemaker, R. H.; Melillo, G. *Cancer Res.* **2002**, *62*, 4316−4324.
(15) Nagle, D. G.; Zhou, Y.-D. *Curr. Drug Targets* **2006**, *7*, 355−369.
(16) Lin, S.; Tsai, S.-C.; Lee, C.-C.; Wang, B.-W.; Liou, J.-Y.; Shyu, K.-G. *Mol. Pharmacol.* **2004**, *66*, 612−619.
(17) Maxwell, P. H.; Wiesener, M. S.; Chang, G.-W.; Clifford, S. C.; Vaux, E. C.; Cockman, M. E.; Wykoff. C. C.; Pugh. C. W.; Maher, E. R.; Ratcliffe, P. J. *Nature* **1999**, *399*, 271−275.

NP060081Y