A New Indanone from the Marine Cyanobacterium *Lyngbya majuscula* That Inhibits Hypoxia-Induced Activation of the VEGF Promoter in Hep3B Cells

Dale G. Nagle,*[†,‡] Yu-Dong Zhou,[†] Peter U. Park,[‡] Valerie J. Paul,[‡] Ira Rajbhandari,[†] Christine J. G. Duncan,[†] and David S. Pasco[†]

Department of Pharmacognosy and National Center for Natural Products Research, Research Institute of Pharmaceutical Sciences, School of Pharmacy, University of Mississippi, University, Mississippi 38677-1848, and The University of Guam Marine Laboratory, U.O.G. Station, Mangilao, Guam 96923

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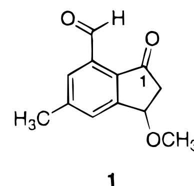
A new indanone (**1**) has been isolated from the filamentous marine cyanobacterium *Lyngbya majuscula*, and its structure determined spectroscopically. Vascular endothelial growth factor (VEGF) is an important regulator of tumor angiogenesis. Compound **1** inhibits hypoxia-induced activation of the VEGF gene promoter in Hep3B human liver tumor cells, *in vitro*.

Benthic filamentous marine cyanobacteria (blue-green algae) often produce biologically active secondary metabolites and have proven to be a rich source of new leads for drug discovery.[1] Recent studies suggest that these metabolites may act as chemical defenses to protect these high-protein organisms from herbivory and may even play a role in algal bloom formation.[1−4] We have recently reported the discovery of dolastatins and related metabolites from collections of cyanobacteria obtained from the reefs of Guam.[5−9] Cyanobacteria may be the original source of these important cytotoxic antitumor agents, previously isolated from the herbivorous sea hare *Dolabella auricularia*. We have developed new gene transcription-based high-throughput assays, as part of our ongoing investigation of aquatic and marine organisms for structurally novel inhibitors of tumor angiogenesis. We have found cyanobacteria and algae produce secondary metabolites that inhibit the transcriptional activation of the gene responsible for the production of vascular endothelial growth factor (VEGF), a critical angiogenic factor produced by tumor cells to promote new blood vessel formation and facilitate tumor growth.[10] Herein, we report the structure of a new indanone metabolite, isolated from the tropical marine cyanobacterium *Lyngbya majuscula* Gomont (Oscillatoriaceae), that inhibits activation of the human VEGF gene promoter in a luciferase reporter assay.

Tufts of a thin, light red to purple-gray variety of *L. majuscula* found growing on living algae, coral, and coral rubble in shallow water (−1 m) of Tumon Bay in Guam were collected and exhaustively extracted with MeOH−CH_2Cl_2 (1:1). Our previous findings indicate that numerous, distinctly different chemotypes of *L. majuscula* can be identified from Guam, throughout Micronesia, and in the Caribbean.[1−3] Therefore, a 2D TLC analysis of the extract was performed to characterize the secondary metabolite profile of this *L. majuscula* collection, using the method recently described by Nagle and Paul to rapidly distinguish distinct cyanobacterial chemotypes.[1] Analysis by 2D TLC revealed this extract to be chromatographically unique among extracts of Guam cyanobacteria. This chemotype of *L. majuscula* was chemically rich with a variety of UV-active secondary metabolites that produced unusual pink, orange, and yellow char reactions upon treatment with ethanolic H_2SO_4 (heat).

A portion of the crude extract (1.6819 g, dark oil) was fractionated by Si gel flash chromatography, followed by Sephadex LH-20 chromatography. The fraction that eluted after that of the chlorophyll bands on LH-20 was shown to contain a UV-active, pink-charring (H_2SO_4, heat) nonpolar secondary metabolite by TLC analysis. Final purification by NP−HPLC provided compound **1** as a pure minor extract component (1.4 mg, 0.083% yield).

Compound **1** was a nonpolar, UV-absorbing (λ_{max} 214, 250), optically active, clear oil. Analysis of **1** by ^{13}C NMR and HREIMS provided a molecular formula for $C_{12}H_{12}O_3$ that indicated seven degrees of unsaturation. Six degrees of unsaturation could be accounted for by the presence of two carbonyl ^{13}C NMR signals (a ketone resonance at 202.76 ppm and an aldehyde at 190.71) and four sp^2 resonances of a tetrasubstituted aromatic ring. Therefore, the one remaining degree of unsaturation was in the form of a ring. The structure of compound **1**, as a substituted methoxy-indanone was readily deduced from backbone structural couplings and substituent correlations from analysis of the $^1H-^{13}C$ HMBC spectrum (Table 1). The stereochemistry at C3 was not assigned and further stereochemical studies were discontinued due to the lack of sufficient material following pharmacological evaluation. However, compound **1** may be nearly racemic, as the observed $[\alpha]_D$ for **1** is only +1.3°, while the $[\alpha]_D$ reported for the synthetic chiral standard of (−)-(3*R*)-3-hydroxyindan-1-one is −101° ($CHCl_3$).[11] The occurrence of **1** in marine cyanobacteria is rather remarkable, in that structurally similar phytotoxic methylated indane-aldehydes have been isolated from phytopathogenic fungi (*Botrytis squamosa* and *Botrytis cinerosa*).[12,13]



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We have established gene transcription-based high-throughput assays for inhibitors of VEGF gene expression in human tumor cell systems. These primary assays monitor VEGF gene expression through the use of a

* To whom correspondence should be addressed. Tel.: (662) 915-7143. Fax: (662) 915-7026. E-mail: dnagle@olemiss.edu.

† Department of Pharmacognosy and National Center for Natural Products Research.

‡ University of Guam Marine Laboratory.

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Table 1. NMR Spectral Data for Compound **1** in $CDCl_3$

position	^{13}C δ	^1H δ^a	$^1H-^{13}C$ HMBC^b
1	202.76		
2	44.02	a) 2.73 dd 18.4, 3.0	C1, C3, C9
		b) 3.06 dd 18.4, 6.6	C1, C3, C8, C9
3	76.38	5.01 dd 6.6, 3.0	C1, C3-OCH_3, C8, C9
4	137.06	7.74 s	C3, C5-CH_3, C6, C8
5	146.21		
6	128.28	7.86 s	C4, C5-CH_3, C7^c, C7-CHO, C8^c
7	133.9^c		
8	133.9^c		
9	154.65		
3-OCH_3	57.37	3.52 s	C3
5-CH_3	21.92	2.52 s	C4, C5-CH_3, C6
7-CHO	190.71	11.01 s	C7^c, C8^c

^{a} Data presented as δ in ppm, multiplicity, J in Hz. Assignments based on $^1H-^{13}C$ HMQC spectra. ^{b} $^1H-^{13}C$ HMBC optimized for J_{CH} = 7 Hz. ^{c} Assignments are interchangeable.

luciferase reporter gene that is under the control of the human VEGF gene promoter,[14] transiently transfected into human hepatocellular carcinoma Hep3B cells. Incubation of the transfected Hep3B cells under hypoxia, a known physiological stimulus of VEGF gene expression, results in increased expression of the luciferase reporter that can be detected in a luciferase assay. Hypoxic incubation (18 h) gave rise to an 8-fold increase in luciferase activity. Natural products that potently inhibit the hypoxic induction of VEGF gene expression (greater than 50% inhibition at 100 μg/mL) are selected for further evaluation. Compound **1** showed activity in the primary assay (extrapolated $IC_{50} = 25$ μM), yet did not inhibit either Hep3B cell growth or luciferase expression from the pGL3-Control plasmid (Promega). The impact of **1** on the secretion of the bioactive $VEGF_{165}$ isoform was further evaluated using human $VEGF_{165}$ quantitative colorimetric sandwich ELISA. Hypoxic incubation (18 h) led to a 2.5-fold increase in secreted $VEGF_{165}$ protein, yet treatment with compound **1** failed to produce any significant inhibition under test conditions. Therefore, **1** was not selected for further evaluation.

Experimental Section

General Experimental Procedures. ^1H and ^{13}C NMR spectra of **1** were recorded in $CDCl_3$ on a GE Omega 500 spectrometer operating at 500 MHz for ^1H and 125 MHz for ^{13}C, using residual solvent peaks as internal references.

Cyanobacterial Material. Tufts of cyanobacteria, found overgrowing coral rubble and living organisms on the shallow (−1 m) sand bottom of Tumon Bay of Guam in February and March 1995, were collected. A voucher specimen (GTR 6 III 95−1) was placed on file with Professor William Gerwick's herbarium (Oregon State University College of Pharmacy), and a specimen is preserved at the University of Guam Marine Laboratory.

Extraction and Isolation. Algal material (collected 6 March 1995) was extracted four times with 50% CH_2Cl_2 in MeOH (v/v) and dried under vacuum to yield 2.35 g, dark oil. The marc contained a high sand content, precluding an accurate direct determination of mass. Therefore, an adjusted combustible organic mass (80 g) was calculated by placing the marc (863 g) in a furnace at 500 °C for 24 h, to determine the content of noncombustible inorganic debris in the sample. A portion of the crude extract (1.68 g, dark oil) was fractionated by Si gel flash chromatography with a gradient from CH_2Cl_2 to MeOH. The second nonpolar fraction (165 mg) that eluted with 100% CH_2Cl_2 was separated by Sephadex LH-20 chromatography [50% CH_2Cl_2 in MeOH (v/v)] and a fraction (124 mg) that eluted after that of the chlorophyll bands was shown to contain a UV-active, pink-charring (H_2SO_4, heat) nonpolar secondary metabolite by TLC analysis. Final purification by NP−HPLC (Econosil Si, 10 μM, 10 × 250 mm, 40% (v/v) EtOAc in hexanes, 3 mL/min, detection at 250 nm) provided compound **1** (1.4 mg, 0.083% yield).

Compound 1: clear oil, $[\alpha]_D$ +1.3° (*c* 1.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 214 (4.2), 250 (3.9) nm; IR (film) ν_{max} 2920, 2851, 2359, 2336, 1740, 1730, 668 cm^{-1}; NMR, see Table 1; EIMS *m/z* 204 $[M]^+$ (37), 189 (15), 176 (99), 161 (56), 145 (25), 133 (100), 115 (78), 106 (15), 103 (14), 89 (26), 77 (21).; HREIMS *m/z* 204.07919 (calcd for $C_{12}H_{12}O_3$, 204.0786).

Assay for Inhibitors of VEGF Promoter Activation. Hep3B cells (American Type Culture Collection) were grown in DMEM/F12 medium (JRH Biosciences), supplemented with 10% (v/v) fetal bovine serum (FBS) (Atlanta Biologicals), 10 000 U/mL penicillin G sodium, 10 000 μg/mL streptomycin, and 1.25 μg/mL Fungizone (Life Biotechnologies), and maintained in a humidified atmosphere (5% CO_2 and 95% air) at 37 °C. Hep3B cells were transiently transfected with the pVEGF−KpnI luciferase reporter[14] (kindly provided by Dr. Gregg L. Semenza) by electroporation. Exponentially grown Hep3B cells (10 × 10^6) re-suspended in 2.5% (v/v) FBS DMEM/F12 medium were mixed with the pVEGF−KpnI luciferase reporter (50 μg/mL), and electroporated in 4-mm gap cuvettes under 160 V for 1 pulse lasting 70 ms, using BTX Electro Square Porator T820 (BTX). The transfected cells were plated out at 50 000 cells/well in 96-well CulturPlate plates (Packard), and incubated under 5% CO_2−95% air (37 °C, 24 h) to recover. Compound **1** was then added at 1 μM, 3 μM, 10 μM, 30 μM, and 100 μM. The incubation continued for an additional 18 h under hypoxic (1% O_2) conditions. The hypoxic conditions were achieved by culturing the cells in a humidified modular incubator chamber (Billups-Rothenberg), which was flushed with CO_2−O_2−N_2 (5:1:94), sealed, and placed at 37 °C. Cells were lysed and luciferase activity determined according to the manufacturer's instructions using the LucLite assay system (Packard).

$VEGF_{165}$ Protein Quantitation. Natural product extract treatments of the Hep3B cells were similar to that described in the VEGF gene expression assay, except that nonelectroporated Hep3B cells were plated at 40 000 cells/well into 96-well culture plates (Corning). At the end of hypoxic incubation, the culture media that contained the secreted VEGF protein were transferred to a microcentrifuge tube. The cell debris in the media was removed by a short centrifugation, and the supernatant transferred to a new tube. The amount of $VEGF_{165}$ protein in the supernatant (equivalent to the amount of secreted $VEGF_{165}$ protein) was determined using a human $VEGF_{165}$ quantitative colorimetric sandwich ELISA kit (R & D Systems), following the manufacturer's instructions.

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