A Bisindole Alkaloid with Hedgehog Signal Inhibitory Activity from the Myxomycete *Perichaena chrysosperma*

Akinori Shintani, Kazufumi Toume, Yusnita Rifai, Midori A. Arai, and Masami Ishibashi*

Graduate School of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263-8522, Japan

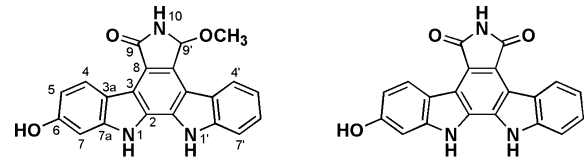
Received May 5, 2010

6-Hydroxy-9′-methoxystaurosporinone (**1**), a new bisindole alkaloid, was isolated from field-collected fruiting bodies of the myxomycete *Perichaena chrysosperma*, together with two known compounds. The structure of the new alkaloid was elucidated from spectral data, and compound **1** was shown to have hedgehog signal inhibitory activity. A related new alkaloid, 6,9′-dihydroxystaurosporinone (**4**), was also isolated from *Arcyria cinerea*.

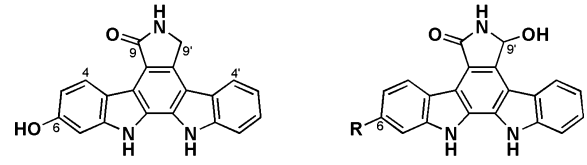
Myxomycetes (true slime molds) are an unusual group of primitive organisms that may be assigned to one of the lowest classes of eukaryotes, and chemical studies of the secondary metabolites of myxomycetes had been limited.[1] During our search for bioactive natural products from myxomycetes,[2] we have studied spore germination experiments of hundreds of field-collected myxomycetes in Japan and also searched for bioactive secondary metabolites from wild or cultured myxomycetes to obtain a number of new compounds, such as a yellow pigment with a chlorinated polyene-pyrone acid structure,[3] a peptide lactone, a derivative of which showed Wnt signaling-inhibitory activity,[4] and a TRAIL (TNF-related apoptosis-inducing ligand) resistance-overcoming cycloanthranilylproline.[5] We recently investigated a field-collected sample of fruiting bodies of *Perichaena chrysosperma* and isolated a new bisindole alkaloid, 6-hydroxy-9′-methoxystaurosporinone (**1**), along with two known bisindole alkaloids, arcyriaflavin B (**2**) and 6-hydroxystaurosporinone (**3**).[6] This paper describes the isolation and structural elucidation of the new compound as well as its hedgehog signal inhibitory activity and also describes the isolation of a related new alkaloid, 6,9′-dihydroxystaurosporinone (**4**), from *Arcyria cinerea*.

Fruiting bodies of *P. chrysosperma*, collected in Tochigi Prefecture in 2009, were extracted with 90% aqueous methanol and 90% aqueous acetone, successively, and the combined crude extract was subjected to silica gel and ODS column chromatography to afford a new bisindole alkaloid (**1**) as well as two known bisindoles (**2** and **3**). The two known alkaloids were identified as arcyriaflavin B (**2**) and 6-hydroxystaurosporinone (**3**), respectively, on the basis of comparison with their spectral data in the literature.[6]

Compound **1** was shown to have the molecular formula $C_{21}H_{14}O_3N_3$ from negative HRESIMS data [*m*/*z* 356.1055 $(M - H)^-$, Δ +1.4 mmu]. The IR absorption bands suggested the presence of hydroxyl (3330 cm^{-1}) and carbonyl (1680 cm^{-1}) groups, and the UV absorption maxima at 331, 301, and 228 nm implied the presence of conjugating or aromatic systems. The ^1H NMR spectrum of **1** in acetone-d_6 (Table 1) showed signals due to a methoxy group [δ_H 3.22 (3H, s)], seven aromatic hydrogens, and one methine proton with low-field resonance (δ_H 6.56). In addition, four signals due to NH or OH hydrogens were observed at δ_H 10.91 (1H, s), 10.66 (1H, s), 8.44 (1H, s), and 7.77 (1H, s). The ^{13}C NMR spectrum of **1** showed 21 signals, 19 of which were assignable to sp^2 carbons, and the remaining two were ascribed to one methoxy group (δ_C 50.6) and one sp^3 methine bearing heteroatoms (δ_C 86.2). The $^1H-^1H$ COSY of **1** (Figure 1) showed connectivities for four successive aromatic protons of H-4′ (δ_H 8.33), H-5′ (δ_H 7.28), H-6′ (δ_H 7.44), and H-7′ (δ_H 7.65) and also suggested the presence of a 1,2,4-trisubstituted benzene ring [δ_H 9.10 (d, J = 8.6 Hz; H-4), 6.84 (dd, J = 8.6 and 2.2 Hz; H-5), and 7.05 (d, J = 2.2 Hz; H-7)].



1 **2**



3 **4** R = OH, **5** R = H

Table 1. ^1H and ^{13}C NMR Spectral Data of Compounds **1** and **4**

	1		**4**	
position	δ_H (J in Hz)[a]	δ_C[a]	δ_H (J in Hz)[a]	δ_C[b]
1-NH	10.66 s[d]		11.44 s[e]	
2		126.1[f]		[c]
3		118.9		[c]
3a		116.7		119.9
4	9.10 d (8.6)	127.3	9.09 d (8.6)	127.2
5	6.84 dd (8.6, 2.2)	109.8	6.83 br d (8.6)	112.2
6		157.0		157.9
7	7.05 d (2.2)	97.3	7.06 br s	97.3
7a		142.0		143.2
8		119.5		[c]
9		170.0		176.3
10-NH	7.77 s		[c]	
1′-NH	10.91 s[d]		11.17 s[e]	
2′		129.3[f]		[c]
3′		131.7		132.4
3a’		123.4		126.0
4′	8.33 d (7.6)	123.2	8.38 d (7.2)	125.0
5′	7.28 t (7.6)	120.3	7.27 t (7.2)	123.5
6′	7.44 t (7.6)	125.5	7.42 t (7.2)	126.5
7′	7.65 d (7.6)	111.7	7.65 d (7.2)	114.8
7a’		140.3		141.6
8′		115.9		[c]
9′	6.56 s	86.2	6.55 s	86.2
6-OH	8.44 s		[c]	
9′-OCH_3	3.22 s (3H)	50.6		

[a] In acetone-d_6. [b] In CD_3OD based on HMQC and HMBC spectra. [c] Not observed. [d–f] Signals may be reversed.

* To whom correspondence should be addressed. Tel: +81-43-290-2913. Fax: +81-43-290-2913. E-mail: mish@p.chiba-u.ac.jp.

10.1021/np1002687
Published on Web 09/14/2010

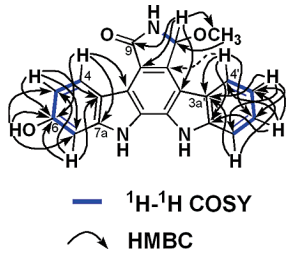


Figure 1. Key ^{1}H−^{1}H COSY and HMBC correlations observed for **1**.Dashed arrow indicates HMBC four-bond correlation.

These ^{1}H and ^{13}C NMR data were almost identical to those of 6-hydroxystaurosporinone (**3**),[6] which were also consistent with the HMBC correlations observed for **1** (Figure 1). The difference in ^{1}H and ^{13}C NMR spectra of **1** from those of 6-hydroxystaurosporinone (**3**) was the observation of signals for a methoxy group [δ_H 3.22; δ_C 50.6] and an sp^3 methine group [δ_H 6.56; δ_C 86.2]; this methine carbon bore the methoxy group and was suggested to be located at the C-9′ position from the HMBC correlations of CH_3O-9′/C-9′, H-9′/C-9, and H-9′/C-3′ (Figure 1). The difference in ^{1}H NMR chemical shifts between H-4 (δ_H 9.10) and H-4′ (δ_H 8.33) implied that H-4 resonating in the lower field was on the same side as the C-9 carbonyl group and H-4′, with a higher-field resonance, was close to the methoxy group on C-9′; this chemical shift difference was also observed for the same positions of 6-hydroxystaurosporinone (**3**) [H-4 (δ_H 9.17) and H-4′ (δ_H 8.00)].[6] From these results, the structure of compound **1** was revealed to be 6-hydroxy-9′-methoxystaurosporinone.

We recently also isolated a related new bisindole alkaloid from field-collected fruiting bodies of the myxomycete *Arcyria cinerea*,[7] and the structure of this compound was proposed as 6,9′-dihydroxystaurosporinone (**4**)[8,9] on the basis of the comparison of ^{1}H and ^{13}C NMR spectral data (Table 1) with those of 6-hydroxy-9′-methoxystaurosporinone (**1**), together with analysis of the ^{1}H−^{1}H COSY, HMQC, and HMBC spectra of **4**, which showed almost parallel correlations to those of **1** in Figure 1. Compounds **1** and **4** had no optical rotation, thus implying that the C-9′ position bearing the methoxy or hydroxy group is racemic.

During our screening studies on inhibitors of the hedgehog signaling pathway, we found that bisindole alkaloids, such as 6-hydroxystaurosporinone (**3**), exhibited inhibition activity of GLI-mediated transcription in the downstream of the hedgehog signaling pathway (IC_{50} value of **3**, 3.6 μM).[10] We therefore examined the hedgehog signal inhibitory activity of compound **1** by our luciferace assay system using HaCaT-GLI1-Luc cells[10,11] and revealed that compound **1** dose-dependently inhibited GLI-mediated transcriptional activity with an IC_{50} value of 4.6 μM with high cell viability (Figure 2). Compound **1** was evaluated for cytotoxicity against a panel of cells with increased Hh signaling levels (PANC1 and DU145) and without reliance on Hh ligand for survival (C3H10T1/2). It was shown to be cytotoxic against PANC1 and DU145 (IC_{50} values 5.0 and 4.2 μM, respectively), but did not affect normal cell line C3H10T1/2 (IC_{50} value >14.0 μM; Figure 3). The cytotoxicity of **1** against PANC1 and DU145 cells may be related to its inhibition of Hh/GLI transcriptional activity.

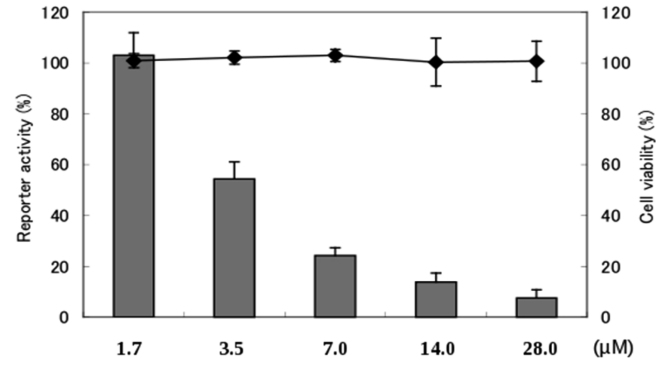


Figure 2. Inhibition of GLI1-mediated transcriptional activity (columns) and cell viability (lines) of compound **1**. HaCaT-GLI1-Luc cells[1112] were seeded onto a 96-well plate (2×10^5 cells per well) then treated with compounds after 12 h tetracycline addition. Cell viability and luciferase activity were determined at the same time. The assays were performed at 0.05% DMSO ($n = 3$). Error bars represent SD.

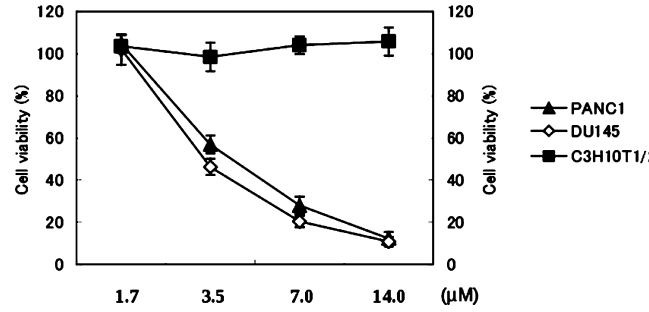


Figure 3. Cytotoxicity of compound **1** against PANC1, DU145, and C3H10T1/2 cells. The assays were performed at 0.05% DMSO ($n = 3$). Error bars represent SD.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a JASCO P-1020 polarimeter. IR spectra were measured on ATR (attenuated total reflection) on a JASCO FT-IR 230 spectrophotometer. UV spectra were measured on a Shimadzu UV mini-1240 spectrometer. NMR spectra were recorded on a JEOL JNM-ECP600 spectrometer with a deuterated solvent, the chemical shift of which was used as an internal standard. High-resolution electrospray ionization mass spectra (ESIMS) were obtained on an Exactive (Thermo Scientific).

Organism. Fruiting bodies of *Perichaena chrysosperma* were collected in Yumoto, Nasu-cho, Tochigi Prefecture, Japan, in November 2009 and identified by Jun Matsumoto of Fukui Botanical Garden, where a voucher specimen (09-01) is maintained. Fruiting bodies of *Arcyria cinerea* were collected in Kochi Prefecture, Japan, in July 2004 and identified by Yukinori Yamamoto of Yamamoto Laboratory, Ohtsuko, Kochi, where a voucher specimen (#26270) is maintained.

Extraction and Isolation. Wild fruiting bodies of *P. chrysosperma* (6.1 g) were extracted with 90% aqueous MeOH (200 mL × 2) and 90% aqueous acetone (100 mL × 2). The combined MeOH and acetone extracts (162 mg) were subjected to silica gel column chromatography (18 × 310 mm) eluted with 10−100% MeOH in $CHCl_3$. The fraction (3.9 mg) eluted with 25% MeOH in $CHCl_3$ was further separated by ODS column chromatography (15 × 300 mm, MeOH/H_2O, 3:1) to afford arcyriaflavin B (**2**, 0.3 mg) and 6-hydroxystaurosporinone (**3**, 0.8 mg), while the fraction of the silica gel column eluted with 50% MeOH in $CHCl_3$ afforded 6-hydroxy-9′-methoxystaurosporinone (**1**, 3.6 mg).

Wild fruiting bodies of *A. cinerea* (1.0 g) were extracted with 90% MeOH (35 mL × 2) and 90% acetone (20 mL × 2). The combined MeOH and acetone extracts (128 mg) were subjected to silica gel column chromatography (18 × 310 mm) eluted with 0−100% MeOH in $CHCl_3$. The fraction (5.6 mg) eluted with 9% MeOH in $CHCl_3$ was further purified with reversed-phase HPLC (YMC-Pack ODS-AM, 250 × 10 mm; flow rate, 2.0 mL/min) with 70% MeOH in H_2O to give arcyriaflavin B (**2**, 0.5 mg) and 6,9′-dihydroxystaurosporinone (**4**, 2.3 mg).

6-Hydroxy-9′-methoxystaurosporinone (1): UV(MeOH) λ_{max} 301 nm (23000); IR (ATR) ν_{max} 3330, 1720, 1680, and 1580 cm^{-1}; ^{1}H and ^{13}C NMR (Table 1); (+)-ESIMS *m*/*z* 380 $(M + Na)^+$ and 326 $(M - OCH_3)^+$; (−)-HRESIMS *m*/*z* 356.1055 [calcd for $C_{21}H_{14}O_3N_3$, $(M - H)^-$, 356.1041].

6,9′-Dihydroxystaurosporinone (4): UV(MeOH) λ_{max} 282 nm; IR (ATR) ν_{max} 3310, 1670, and 1580 cm^{-1}; ^{1}H NMR (acetone-d_6) (Table 1); ^{1}H NMR (CD_3OD) δ_H 8.90 (1H, d, $J = 8.7$ Hz; H-4), 6.78 (1H, br d, $J = 8.7$ Hz; H-5), 6.99 (1H, br s; H-7), 8.28 (1H, d, $J = 7.9$ Hz; H-4′), 7.28 (1H, t, $J = 7.9$ Hz; H-5′), 7.45 (1H, t, $J = 7.9$ Hz; H-6′),

7.62 (1H, d, $J = 7.9$ Hz; H-7′), and 6.48 (1H, s, H-9′); ^{13}C NMR (CD_3OD) (Table 1).[7,10]

GLI-Mediated Transcriptional Activity Assay. Detailed procedures were described previously.[10,11]

Cytotoxicity Test. Cancer cells (PANC1 and DU145) or normal cells (C3H10T1/2) were seeded onto 96-well black plates in 100 μL of RPMI medium containing 10% FBS at 1×10^4 cells per well and preincubated at 37 °C for 24 h. The medium was then replaced with fresh RPMI + 10% FBS containing different concentrations of each compound. After incubation for 24 h, the medium was removed, and cell proliferation was determined by FMCA using a fluorescence plate reader (Thermo).

Acknowledgment. We thank members of the Japanese Society of Myxomycetology (Y. Yamamoto, H. Hagiwara, J. Matsumoto, and T. Tominaga) for the collection of wild samples of myxomycetes. This study was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS).

Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Steglich, W. *Pure Appl. Chem.* **1989**, *61*, 281–288.
(2) Ishibashi, M. *Yakugaku Zasshi* **2007**, *127*, 1369–1381.
(3) Shintani, A.; Ohtsuki, T.; Yamamoto, Y.; Hakamatsuka, T.; Kawahara, N.; Goda, Y.; Ishibashi, M. *Tetrahedron Lett.* **2009**, *50*, 3189–3190.
(4) Nakatani, S.; Kamata, K.; Sato, M.; Onuki, H.; Hirota, H.; Matsumoto, J.; Ishibashi, M. *Tetrahedron Lett.* **2005**, *46*, 267–271.
(5) Nakatani, S.; Yamamoto, Y.; Hayashi, M.; Komiyama, K.; Ishibashi, M. *Chem. Pharm. Bull.* **2004**, *52*, 368–370.
(6) Hosoya, T.; Yamamoto, Y.; Uehara, Y.; Hayashi, M.; Komiyama, K.; Ishibashi, M. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 2776–2780.
(7) Shintani, A.; Ishibashi, M.; Yamamoto, Y. 52th Annual Meeting of the Kanto Branch, the Pharmaceutical Society of Japan, 2008, Noda, Abstract, p 85.
(8) A related alkaloid, 9′-hydroxystaurosporinone (**5**), was previously prepared by synthesis. Harris, W.; Hill, C. H.; Keech, E.; Malsher, P. *Tetrahedron Lett.* **1993**, *51*, 8361–8364.
(9) 6,9′-Dihydroxystaurosporinone (**4**) obtained in a small quantity from *A. cinerea* was unstable and was probably susceptible to conversion to arcyriaflavin B (**2**) in air; unfortunately, its MS data, including high-resolution analysis data, were not determined.
(10) Hosoya, T.; Arai, M. A.; Koyano, T.; Kowithayakorn, T.; Ishibashi, M. *ChemBioChem* **2008**, *9*, 1082–1092.
(11) Arai, M. A.; Tateno, C.; Hosoya, T.; Koyano, T.; Kowithayakorn, T.; Ishibashi, M. *Bioorg. Med. Chem.* **2008**, *16*, 9420–9424.

NP1002687