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New cytotoxic bisindole alkaloids with protein tyrosine kinase inhibitory activity from a myxomycete *Lycogala epidendrum*

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Abstract—Two new bisindole alkaloids, 6-hydroxystaurosporinone (**1**) and 5,6-dihydroxyarcyriaflavin A (**2**) were isolated from field-collected fruit bodies of a myxomycete *Lycogala epidendrum*, along with eight known bisindoles (**3**–**10**). The structures of these new compounds were determined on the basis of spectroscopic data. Compounds **1** and **2** showed cytotoxicity against HeLa, Jurkat, and vincristine resistant KB/VJ300 cells, and compound **1**, particularly, inhibited protein tyrosine kinase activity.

The myxomycetes (true slime molds) are an unusual group of primitive organisms that may be assigned to one of the lowest classes of eukaryote, and the chemical investigation of the secondary metabolites has been limited so far.[1] During our study on the search for bioactive natural products from myxomycetes,[2,3] we recently investigated a field-collected sample of fruit bodies of *Lycogala epidendrum* (Linnaeus), one of well-known myxomycetes. From the fruit bodies of *L. epidendrum*, a number of bisindoles,[4,5] polyacetylene triglycerides,[6,7] glycosides of polyenoic fatty acid,[8] and polypropionate lactones[9] have ever been isolated. In the present work, we further examined chemical constituents of *L. epidendrum* collected in the year 2004, and isolated two new bisindole alkaloids, 6-hydroxystaurosporinone (**1**) and 5,6-dihydroxyarcyriaflavin A (**2**), along with eight known bisindole alkaloids (**3**–**10**). In this paper, we describe their isolation, structural elucidation, and cytotoxic activity.

The fruit bodies of *L. epidendrum*,[10] collected in Kochi Prefecture in 2004, were extracted with 90% MeOH and 90% acetone, successively, and the combined crude extract was partitioned between H_2O and EtOAc. The EtOAc-soluble fraction was subjected to silica gel, ODS or/and Sephadex LH-20 column chromatography, and further purification by reversed-phase HPLC gave new bisindole alkaloids (**1** and **2**), as well as eight known ones (**3**–**10**).[11] These known compounds were identified as lycogarubin B (**3**),[5] arcyriarubin A (**4**),[4] lycogarubin C (**5**),[5] arcyriaflavin B (**6**),[12] staurosporinone (**7**),[13] lycogaric acid (**8**),[4] arcyriaflavin A (**9**),[4] and lycogarubin A (**10**),[5] on the basis of comparison with their spectral data in the literature.

Compound **1**[14] was obtained as light brown amorphous solid, and its molecular formula was determined as $C_{20}H_{13}O_2N_3$ by the HRFABMS data (*m*/*z* 327.0981, M^+, Δ −2.7 mmu). The IR absorptions were indicative of the presence of hydroxyl group (3282 cm^{-1}) and aromatic ring (1646 and 1583 cm^{-1}), and the UV absorption maxima were observed at 342, 294, and 226 nm, suggesting the presence of conjugating or aromatic system(s). The ^1H NMR spectral data (Table 1) in acetone-d_6 showed the signals for seven aromatic protons, sp^3 methylene protons (δ_H 5.02, 2H, s), and two NH signals (δ_H 11.08 and 10.76). The ^{13}C NMR spectrum (Table 1) displayed signals assignable to 18 aromatic (or olefinic) carbons, one sp^3 methylene carbon (δ_C 46.2) and one carbonyl carbon (δ_C 173.6). These ^1H and ^{13}C

Keywords: Myxomycete; *Lycogala epidendrum*; Bisindole; Cytotoxicity; Tyrosine kinase inhibitor.
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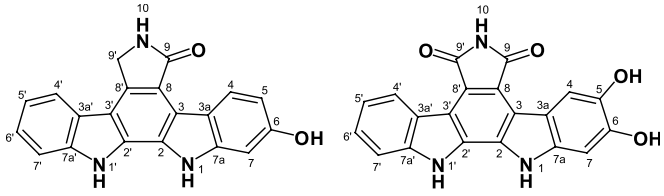

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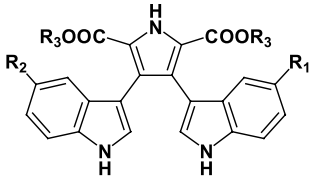
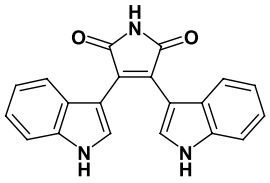


3 R_1=OH, R_2=H, R_3=Me
5 R_1=R_2=H, R_3=Me
8 R_1=R_2=R_3=H
10 R_1=R_2=OH, R_3=Me



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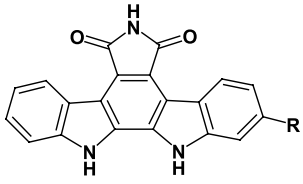

6 R=OH
9 R=H

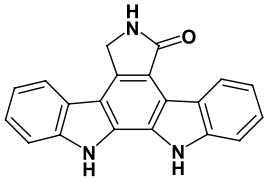

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Table 1. ^{1}H and ^{13}C NMR data for compounds **1** and **2** in acetone-d_6

Position	Compound **1**		Compound **2**	
	δ_H (J, Hz)	δ_C	δ_H (J, Hz)	δ_C
2		126.1		129.8[a]
3		118.2		118.2
3a		117.8		115.5
4	9.17 (1H, d, 8.4)	127.5	8.56 (1H, s)	110.3
5	6.80 (1H, dd, 8.4, 1.8)	109.9		141.4
6		157.3		136.9
7	7.02 (1H, d, 1.8)	97.1	7.12 (1H, s)	98.0
7a		142.3		147.9
8		119.4		120.2[b]
9		173.6		171.6[c]
2′		129.2		130.0[a]
3′		133.6		116.4
3a′		124.2		123.3
4′	8.00 (1H, d, 7.8)	121.7	9.10 (1H, d, 8.0)	125.6
5′	7.27 (1H, t, 7.8)	120.7	7.31 (1H, dd, 7.5, 8.0)	120.9
6′	7.40 (1H, t, 7.8)	125.5	7.48 (1H, dd, 7.5, 8.0)	127.3
7′	7.64 (1H, d, 7.8)	112.7	7.64 (1H, d, 8.0)	112.1
7a′		140.7		141.5
8′		114.8		120.5[b]
9′	5.02 (2H, s)	46.2		171.8[c]
1-NH	11.08 (1H, s)		10.96 (1H, s)	
10-NH			9.62 (1H, s)	
1′-NH	10.76 (1H, s)		10.73 (1H, s)	

[a,b,c] Signals may be reversed.

NMR data were similar to those of staurosporinone (**7**),[13] while compound **1** possessed one more oxygen atom in its molecular formula than **7**. The ^{1}H–^{1}H COSY spectrum showed the connectivity between two aromatic protons of H-4 (δ_H 9.17) and H-5 (δ_H 6.80), and also showed the presence of four consecutive aromatic protons of H-4′ (δ_H 8.00), H-5′ (δ_H 7.27), H-6′ (δ_H 7.40), and H-7′ (δ_H 7.64). The H-5 showed *meta*-coupling (J = 1.8 Hz) with an aromatic proton resonating at δ_H 7.64, which was assignable to H-7. This observation suggested that a hydroxyl group was attached on C-6, which was consistent with the low field resonance of C-6 (δ_C 157.3). These assignments were also suggested by the HMBC correlations observed for H-4/C-2, H-4/C-3, H-4/C-6, H-5/C-3a, H-5/C-7, H-7/C-3a, and H-7/C-5. In addition, the NOESY spectrum showed correlations from the methylene protons on C-9′ (H_2-9′, δ_H 5.02, 2H, s) to H-4′ (δ_H 8.00) and H-5′ (δ_H 7.27), suggesting that the sp^3 methylene (C-9′) was present on the side of the benzene ring without hydroxyl group. Accordingly, the structure of **1** was concluded to be 6-hydroxystaurosporinone.

Compound **2**,[15] obtained as yellow amorphous solid, had the molecular formula $C_{20}H_{11}O_4N_3$ as determined by the HRFABMS data (*m/z* 357.0762, M^+, Δ +1.2 mmu). Analysis of the ^{1}H and ^{13}C NMR spectra of **2** and comparison of these data with those of arcyriaflavin A (**9**)[4] or B (**6**)[12] implied that they had the same bisindole backbone skeleton. Moreover, its molecular weight was 32 amu bigger than those of **9** in FABMS analysis (*m/z* 357 $[M]^+$ for **2** and *m/z* 325 $[M]^+$ for **9**); 32 amu corresponded to two oxygen atoms, thus suggesting that **2** possessed two hydroxyl groups (IR, 3310 cm^{-1}) attached on the bisindole nucleus of **9**. The

^{1}H NMR and ^{1}H–^{1}H COSY of **2** showed signals due to four successive aromatic protons [δ_H 9.10 (H-4′), 7.31 (H-5′), 7.48 (H-6′), and 7.64 (H-7′)] and two other aromatic protons observed as singlets [δ_H 8.56 (1H, s) and 7.12 (1H, s)]. These two singlets were assignable to H-4 and H-7, respectively, from the HMBC correlations observed for H-4/C-3, H-4/C-5, H-4/C-6, H-4/C-7a, H-7/C-3a, H-7/C-5, and H-7/C-7a. From these findings, two hydroxyl groups were inferred to be located vicinally at C-5 and C-6 positions, the ^{13}C chemical shifts of which (δ_C 141.4 and 136.9, respectively) were consistent with those of corresponding positions of 5,6-dihydroxyindole (δ_C 142.4 and 140.3, respectively).[16] From these results, the structure of **2** was revealed as 5,6-dihydroxyarcyriaflavin A.

Two new compounds (**1** and **2**) exhibited cytotoxicity against HeLa cells with IC_{50} values of 5.4 and 2.1 μg/mL, respectively.[17] The new compounds (**1** and **2**) as well as known compounds **3**, **4**, and **5** also exhibited cytotoxicity against Jurkat cells[18] as shown in Table 2, while compounds **1** and **2** showed slightly weak cytotoxicity against vincristine resistant KB/VJ300 cells,[19,20] as compared with that on Jurkat cells, and compounds **3**–**5** were almost inactive (Table 2).

Table 2. Cytotoxicity against Jurkat cells and KB/VJ300 cells (IC_{50}, μg/mL)

	Jurkat	KB/VJ300
1	1.34	7.6
2	0.84	25
3	18.0	>25
4	6.3	>25
5	15.3	>25

The effects of these bisindole alkaloids (**1**–**5**) on protein kinase activities were examined using a method that allows detection of activities of several protein kinases simultaneously.[21,22] When compound **1** was added at 1 μg/mL to a postnuclear supernatant of v-Src expressing NIH3T3 cells and phosphorylation was allowed to proceed, the intensities of band of proteins phosphorylated by protein tyrosine kinase (PTK) markedly decreased (Fig. 1). Compound **1**, added at a higher concentration (10 μg/mL), also induced the decrease of the band intensities of proteins phosphorylated by protein kinase C (PKC), protein kinase A (PKA), eukaryotic elongation factor-2 kinase (eEF2K), and vascular endothelial growth factor receptor-1 (VEGFR-1 or Flt-1) kinase (results not shown), while the phosphorylation by epidermal growth-factor receptor (EGFR) kinase was not altered as summarized in Table 3.[23] Compounds **2** and **4** added at 10 μg/mL also induced decrease of the band intensity due to phosphorylation by PTK and PKC, while lycogarubins B (**3**) and C (**5**) added at 10 μg/mL showed almost no effects on phosphorylation by these protein kinases. These results suggested that compound **1** having a γ-lactam structure showed selectively inhibited PTK at lower concentration than bisindoles possessing a maleimide moiety (compounds **2** and **4**). Bisindoles having a 2,5-dimethoxycarbonylpyrrole ring [lycogarubins B (**3**) and C (**5**)] had almost no protein kinase inhibition activity (Fig. 2). The order of the potency of cytotoxicity (Table 2) appears almost

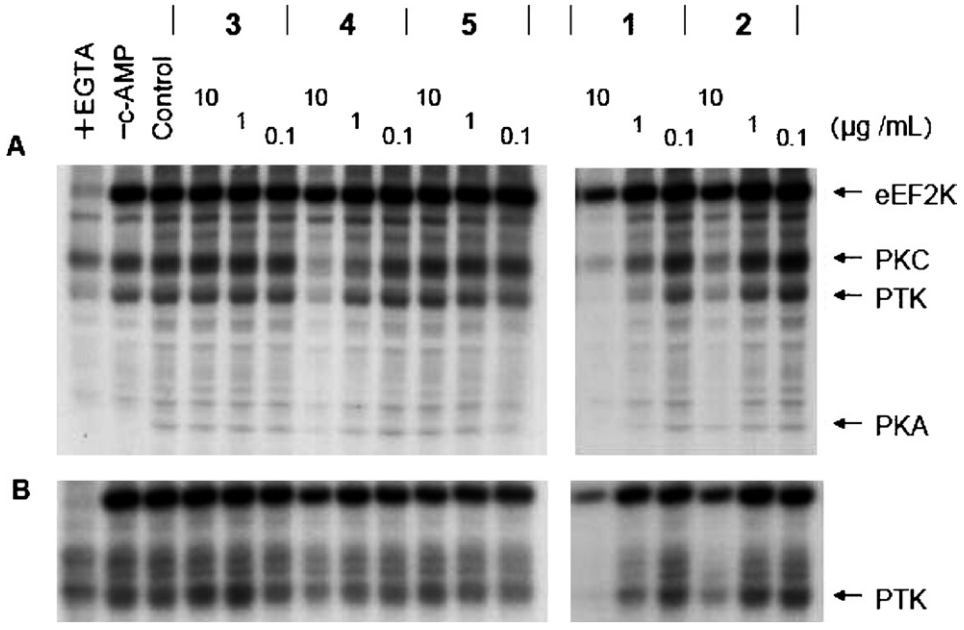

Figure 1. Protein kinase inhibition activities of compounds **1**–**5**. Effects of compounds **1**–**5** on the activities of various protein kinases in a postnuclear supernatant of v-Src expressing NIH3T3 cells. Phosphorylation in the presence of [γ-^{32}P]ATP was allowed to proceed[21] with the indicated additions to reaction mixtures. The final concentrations of EGTA and cAMP were 0.5 mM and 20 μM, respectively. Phosphorylated proteins were analyzed by SDS-PAGE and autoradiography. (A) Bands of proteins that were phosphorylated by various protein kinases are indicated by arrows and were identified by reference to Fukazawa et al.[21] (B) The gel shown in A was exposed to 1 M KOH at 55 °C for 2 h to increase the intensity of signals due to phosphorylated tyrosine.

Table 3. Inhibition specificity of compounds **1**–**5**[a]

Compounds	μg/mL	eEF2K	PKC	PTK	PKA	EGFR	Flt-1
1	10	+	+	++	+	–	+
	1	–	–	+	–	–	–
	0.1	–	–	–	–	–	–
2	10	–	+	+	–	–	+
	1	–	–	–	–	–	–
	0.1	–	–	–	–	–	
3	10	–	–	–	–	–	–
	1	–	–	–	–	–	–
	0.1	–	–	–	–	–	
4	10	–	+	+	+	–	–
	1	–	–	–	–	–	–
	0.1	–	–	–	–	–	
5	10	–	–	–	–	–	–
	1	–	–	–	–	–	–
	0.1	–	–	–	–	–	

[a] The indications '++', '+', and '–' mean >80%, 50–80%, and <50% kinase inhibition in each kinase assay, respectively.

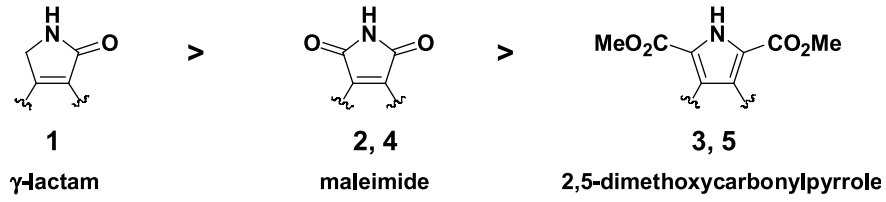

Figure 2. Effectiveness of PTK inhibition activity and central part structures of bisindole alkaloids (**1**–**5**).

parallel to that of protein kinase inhibition activity (Fig. 2 and Table 3).

Acknowledgements

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10. Organism: The fruit bodies of *Lycogala epidendrum* were collected and identified by Y.Y. at Ohtsu, Kochi-shi in Kochi Prefecture, Japan, February 2004. Voucher specimens (#25630-25632) are maintained by Y.Y. (Ohtsu-ko, Kochi).
11. Extraction and isolation: The field-collected fruit bodies of *Lycogala epidendrum* (177.6 g) were extracted with 90% MeOH (2.5 L × 3) and 90% acetone (2.5 L × 1), successively, at room temperature. The combined extracts (46.4 g) were partitioned between EtOAc (1 L × 3) and H_2O (1 L). The EtOAc-soluble fraction (24.8 g) was subjected to a silica gel column (column A, 50 × 500 mm) and eluted with hexane/$CHCl_3$ mixture (3:7), $CHCl_3$, $CHCl_3$/MeOH (100:1, 50:1, 10:1, 9:1, 4:1 7:3, and 1:1), and MeOH. A fraction (14.3 g) of column A eluted with $CHCl_3$/MeOH (50:1–10:1) was partially (4.2 g) separated by silica gel column (column B, 40 × 400 mm) with $CHCl_3$/MeOH gradient solvent system (1:0, 50:1, 10:1, 4:1, 1:1, and 0:1), and a fraction (1.3 g) of column B eluted with $CHCl_3$/MeOH (50:1) were further purified by Sephadex LH-20 column (20 × 600 mm) to give **5** (83.0 mg, TLC on ODS plate; solvent, 80% MeOH; R_f, 0.58). A fraction (650 mg) of column A eluted with $CHCl_3$/MeOH (9:1) was dissolved in 50% MeOH, and the soluble part was placed on ODS column (25 × 400 mm) eluted with a H_2O/MeOH gradient system (1:1, 2:3, 1:4, and 0:1) to give **3** (200.6 mg) and **4** (7.5 mg) (TLC on ODS plate; solvent, 80% MeOH; R_f, 0.68 for **3** and 0.61 for **4**). Another fraction (2.1 g) of column A eluted with $CHCl_3$/MeOH (9:1) was separated

by ODS column chromatography (column C, 35 × 400 mm) eluted with a H_2O/MeOH gradient system (1:1, 2:3, 3:7, 1:4, and 0:1) to give **6** (280 mg, TLC on ODS plate; solvent, 70% MeOH; R_f, 0.35) in the fraction eluted with H_2O/MeOH (3:7). A fraction (460 mg) of column C eluted with H_2O/MeOH (3:7) was further purified with ODS HPLC (Inertsil ODS-3, 10 × 250 mm; eluent, 60% MeOH; flow rate, 2.0 mL/min) to afford **1** (6.0 mg, t_R 45 min). Another fraction (90 mg) of column C eluted with H_2O/MeOH (3:17) was partially (30 mg) separated by ODS HPLC (CAPCELLPAK C18 MG II, 10 × 250 mm; eluent, 70% MeOH; flow rate, 1.8 mL/min) to give **7** (2.1 mg, t_R 26 min). A fraction (3.2 g) of column A eluted with $CHCl_3$/MeOH (4:1) was separated by ODS column chromatography (column D, 50 × 250 mm) using a gradient system of H_2O/MeOH (4:1, 3:2, 2:3, 1:4, and 0:1) to give **8** (493 mg, TLC on ODS plate; solvent, 30% MeOH; R_f, 0.38) in the fraction eluted with H_2O/MeOH (2:3). A fraction (286 mg) of column D eluted with H_2O/MeOH (3:2) afforded **10** (2.1 mg, t_R 12 min) when purified by ODS HPLC (Inertsil ODS-3, 10 × 250 mm; eluent, 50% MeOH; flow rate, 2.0 mL/min). Another fraction (195 mg) of column D eluted with H_2O/MeOH (1:4) was separated by ODS HPLC (Inertsil ODS-3, 10 × 250 mm; eluent, 68% MeOH; flow rate, 2.0 mL/min) to give **1** (2.6 mg, t_R 22 min), **2** (5.6 mg, t_R 27 min), and crude fraction mainly containing compound **9**, which was subsequently purified by ODS HPLC (Inertsil ODS-3, 10 × 250 mm; eluent, 78% MeOH; flow rate, 2.0 mL/min) to give **9** (0.45 mg, t_R 34 min).

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14. 6-Hydroxystaurosporinone (**1**): amorphous solid; UV (MeOH) λ_{max} nm (log ε) 342 (9.8), 294 (10.9), and 226 (10.2); IR (ATR) ν_{max} 3282, 1646, 1583, 1453, 1402, and 1327 cm^{-1}; ^1H and ^{13}C NMR (Table 1); EIMS *m/z* 327 $[M]^+$; HRFABMS *m/z* 327.0981 (calcd for $C_{20}H_{13}O_2N_3$, 327.1008).
15. 5,6-Dihydroxyarcyriaflavin A (**2**): amorphous solid; UV (MeOH) λ_{max} nm (log ε) 330 (10.5), 284 (9.8), and 230 (10.5); IR (ATR) ν_{max} 3310, 1733, 1637, 1565, 1475, 1404, and 1320 cm^{-1}; ^1H and ^{13}C NMR (Table 1); FABMS *m/z* 357 $[M]^+$; HRFABMS *m/z* 357.0762 (calcd for $C_{20}H_{11}O_4N_3$, 357.0750).
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