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Antimitotic activity of glaupalol-related coumarins from *Glaucidium palmatum*

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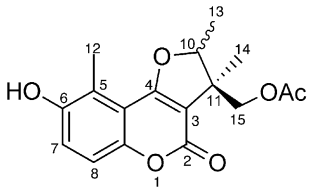
Abstract—Two new coumarins, glaumacidines A (**1**) and B (**2**), and the related coumarins (**3**–**7**) have been isolated from the rhizomes of *Glaucidium palmatum* (Glaucidiaceae). The absolute configurations of **1** and **2** and *trans*- and *cis*-glaupadiols (**3** and **4**, respectively) were elucidated by spectroscopic data, chemical derivatization, and exciton chirality method. Glaupalol (**5**) enhanced the polymerization of tubulin and affected synergistically with paclitaxel on inhibition of KB cell proliferation.

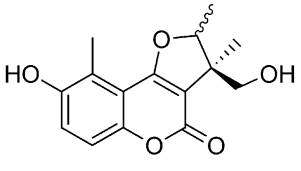
Antimitotic agents that inhibit the microtubule formation and the mitotic arrest of eukaryotic cells, such as paclitaxel and vinblastine, are important components of current anticancer therapy.[1] Paclitaxel is potent inhibitor of cell proliferation and arrest cells in mitosis, but in contrast to vinblastine, promote the polymerization of purified tubulin, causing stabilization and bundling of microtubules.[2] The antimitotic agents have potential applications in drug development. Recently much effort has been directed to the isolation and synthesis of new antimitotic drugs that target the tubulin/microtubule system and display efficacy against drug-refractory carcinomas.[3]

Glaucidium palmatum (Glaucidiaceae) is one of northern plants in Japan. During our search for bioactive compounds targeting the tubulin/microtubules from medicinal plants,[4] we found that the extract from the rhizomes of *G. palmatum* remarkably stabilized the tubulin/microtubule system. Our efforts on identifying new agents that target tubulin resulted in the isolation of two new coumarins, glaumacidines A (**1**) and B (**2**). The stereostructures of **1** and **2** were established by spectroscopic data and chemical means. This paper describes the structures of glaumacidines A (**1**) and B (**2**), and effects of **1** and **2** and its related coumarins (**3**–**7**) on tubulin assembly.

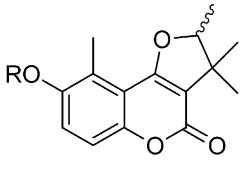
The rhizomes of *G. palmatum* were extracted with MeOH, and the MeOH extract was in turn partitioned with hexane, EtOAc, $CHCl_3$, and *n*-BuOH. EtOAc and $CHCl_3$-soluble materials stabilizing the polymerization of tubulin were subjected to a silica gel column ($CHCl_3$/MeOH, 1:0 → 0:1) followed by a C_{18} column (CH_3CN/



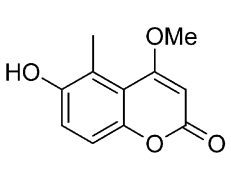
glaumacidine A (**1**): *10S11R*
glaumacidine B (**2**): *10R11R*



trans-glaupadiol (**3**): *10S11R*
cis-glaupadiol (**4**): *10R11R*



glaupalol (**5**): R=H
glaupalol-β-D-glucoside (**6**): R=β-D-Glc



6-Hydroxy-4-methoxy-5-methylcoumarin (**7**)

Keywords: Antimitotic activity; Coumarin; *Glaucidium palmatum*.
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0.1% TFA, 4:1) to afford glaumacidines A (**1**, 0.03% yield)[5] and B (**2**, 0.03% yield)[6] as colorless powder together with *trans*- and *cis*-glaupadiols (**3** and **4**),[7,8] glaupalol (**5**),[9] glaupalol-β-D-glucoside (**6**),[10] and 6-hydroxy-4-methoxy-5-methyl coumarin (**7**).[11]

Glaumacidine A {**1**, $[\alpha]_D$ +30 (*c* 0.2, CH_3OH)} was revealed to have the molecular formula, $C_{17}H_{18}O_6$, by HRFABMS [*m/z* 319.1165 $(M+H)^+$, Δ −1.8 mmu]. The IR absorption implied the presence of hydroxyl (3400 cm^{-1}) and ester (1741 cm^{-1}) group(s), and UV absorptions [335 (ε 4400), 308 (11,000), 295 (12,000), and 212 (35,000)] characteristic of coumarins were observed. Analysis of ^1H and ^{13}C NMR data and the HMQC spectrum provided evidences that **1** possessed 17 carbon signals including nine quaternary carbons ($sp^2\times8$ and $sp^3\times1$), 3 methines ($sp^2\times2$ and $sp^3\times1$), and 1 methylene. Among them, three quaternary carbons (δ_C 150.0, 150.5, and 160.0), one methine (δ_C 91.2), and one methylene (δ_C 64.7) were ascribed to those bearing an oxygen atom. The remaining carbons were assigned as two ester carbonyl carbons (δ_C 168.0 and 170.5). HMBC correlations as shown in Figure 1 suggested that **1** was a glaupadiol-related coumarin with dihydrofurane ring at C-3 and C-4. HMBC correlations for H_2-15 and H_3-17 to C-16 (δ_C 170.5) were indicative of position of the acetyl group at C-15. Thus, the gross structure of glaumacidine A was assigned as **1**. The NOESY spectrum of **1** showed cross-peaks as shown in computer-generated 3D drawing (Fig. 1). NOESY correlations of H_3-13 to H_2-15 and H-10 to H_3-14 indicated that both H_3-13 and H_3-14 were *trans*-oriented. Acetylation of *trans*-glaupadiol (**3**)[8] with Ac_2O/BF_3 gave an monoacetate of **3**,[12] whose spectral data were identical with those of **1**. Thus, glaumacidine A (**1**) was assigned as 15-*O*-acetate of *trans*-glaupadiol (**3**).

Glaumacidine B {**2**, $[\alpha]_D$ −52 (*c* 0.3, CH_3OH)} had the same molecular formula, $C_{17}H_{18}O_6$, as that of **1** by HRFABMS [*m/z* 319.1176 $(M+H)^+$, Δ −0.7 mmu]. IR absorptions at 3400 and 1743 cm^{-1} indicated the presence of hydroxyl and ester group(s). ^1H and ^{13}C NMR data of **2** were very close to those of **1**. Detailed analysis of 2D NMR including the ^1H–^1H COSY, HOHAHA, HMQC, and HMBC spectra indicated that **2** was a stereoisomer at C-10 and/or C-11 of **1**. The *cis* relation between H_3-13 and H_3-14 was assigned by NOESY correlations of H_3-13 to H_3-14 and H-10 to H_2-15. Acetylation of *cis*-glaupadiol (**4**)[8] with Ac_2O/BF_3 gave an monoacetate of **4**, whose spectral data were identical with those of **2**. Thus, the relative stereostructure of glaumacidine B (**2**) was assigned as 15-*O*-acetate of *cis*-glaupadiol (**4**) as shown in Figure 1.

The absolute stereochemistry of **1**–**4** was elucidated by applying exciton chirality method.[13] Each hydroxyl group at C-6 of the *trans*- and *cis*-glaupadiols (**3** and **4**, respectively)[8] was methylated and then *p*-methoxycinnamoyl chromophore was introduced into each hydroxyl group at C-15 to give **8**[14] and **9**,[14] respectively. The signs of the first Cotton effects of **8** and **9** were both negative [**8**, λ_{max} 330 nm ($\Delta\varepsilon$ −14.0); **9**, λ_{max} 330 nm ($\Delta\varepsilon$ −6.0)], while those of the second one [**8**, λ_{max} 280 nm ($\Delta\varepsilon$ +12.0); **9**, λ_{max} 280 nm ($\Delta\varepsilon$ +4.0)] were positive (Fig. 2), indicating that the chirality between the *p*-methoxycinnamoyloxy group at C-15 and the coumarin ring in **8** and **9** was as shown in Figure 2 (left-handed screw). Conformational calculations of **8** and **9** by Monte Carlo simulation[15] suggested that in the most stable conformer, calculated screw senses between the two longitudinal electric transition moments of binaphthyl groups were in good accordance with those expected from their exciton chiralities (Fig. 2). Thus, the absolute configurations at C-10 and C-11 were assigned as *S* and *R* for **1**, **3**, and **8**, respectively, and *R* and *R* for **2**, **4**, and **9**, respectively.

Generally antimitotic agents bind to either the colchicine binding site or the vinca alkaloid binding site. On the other hand, paclitaxel promotes the polymerization of tubulin by binding to and stabilizing the resulting microtubule polymer, which differs from those of colchicines, podophyllotoxin, and the vinca alkaloids. Microtubules polymerized in the presence of paclitaxel are resistant to depolymerization by Ca^{2+} ions.

Microtubule proteins were polymerized under normal polymerization conditions in the absence and the presence of paclitaxel or glaupalol (**5**), and after 10 min incubation, $CaCl_2$ was added. Microtubule polymerization and depolymerization were monitored by the increase and the decrease in turbidity. The results were shown in Figure 3 as the changes in the relative absorbance at 400 nm. The $CaCl_2$-induced depolymerization of microtubules was completely inhibited by 5 μM of paclitaxel. On the other hand, glaupalol (**5**) remarkably

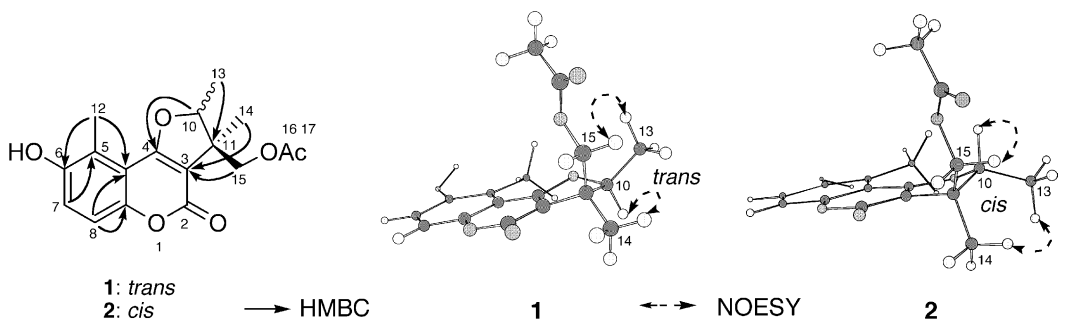

Figure 1. Selected HMBC and NOESY correlations of glaumacidine A (**1**) and B (**2**).

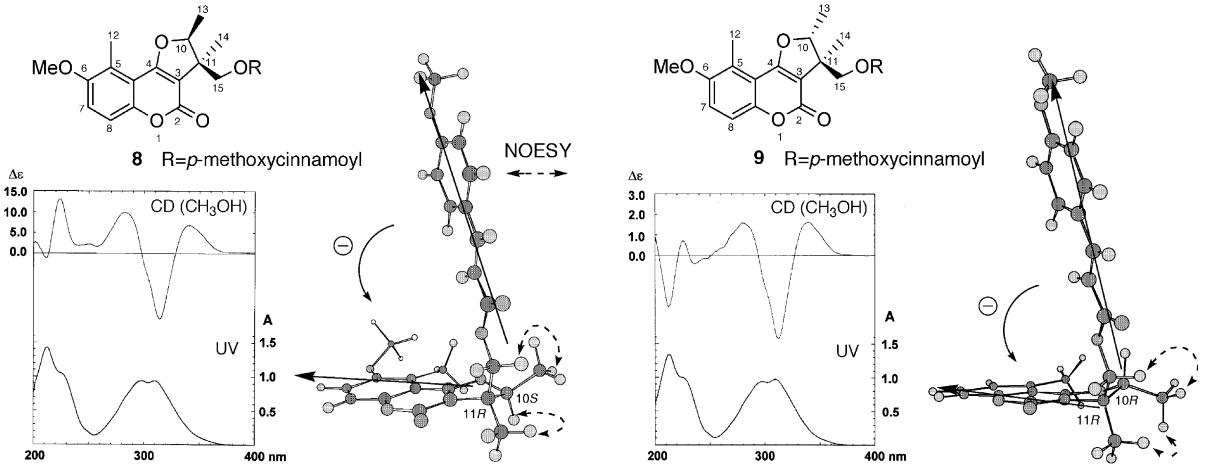

Figure 2. CD and UV spectra and selected NOESY correlations of 12-*O*-*p*-methoxycinnamate of *trans*- and *cis*-glaupadiols (**8** and **9**).

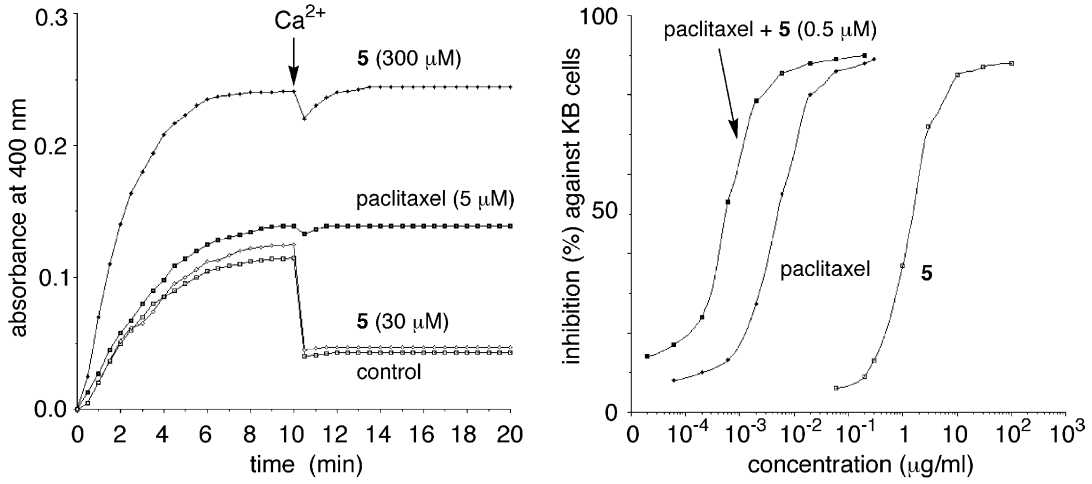

Figure 3. Effects of glaupalol (**5**) and paclitaxel on the polymerization of tubulin and KB cells. Left: glaupalol (**5**) at various concentrations was mixed with tubulin (1.5 mg/mL) at 0 °C and incubated at 37 °C. The absorbance at 400 nm was measured. Right: cytotoxicity against KB cells. Dose inhibition curve of KB cells in the presence of paclitaxel was shifted to the left hand by addition of glaupalol (**5**, 0.5 μM).

Table 1. ^{1}H and ^{13}C NMR data of glaumacidines A (**1**) and B (**2**) in $CDCl_3$

	1		**2**	
	δ_H [int. mult, *J* (Hz)]	δ_C	δ_H	δ_C
2		168.0		167.5
3		106.7		106.9
4		160.0		160.7
4a		120.5		120.5
5		112.7		112.8
6		150.5		150.4
7	7.05 (1H, d, 8.0)	115.5	7.04 (1H, d, 8.0)	115.4
8	7.10 (1H, d, 8.0)	120.0	7.11 (1H, d, 8.0)	120.0
8a		150.0		150.0
10	4.71 (1H, q, 5.6)	91.2	4.93 (1H, q, 6.3)	88.0
11		46.1		46.7
12	2.57 (3H, s)	12.7	2.57 (3H, s)	12.7
13	1.54 (3H, d, 5.6)	14.6	1.49 (3H, d, 6.3)	15.7
14	1.50 (3H, s)	20.8	1.34 (3H, s)	15.8
15	4.19 (2H, s)	64.7	4.37 (2H, s)	67.9
16		170.5		170.9
17	1.99 (3H, s)	20.9	2.05 (3H, s)	20.9

promotes the polymerization of tubulin at high concentration (300 μM) and reduced the depolymerization process induced by Ca^{2+} addition, suggesting that glaupalol (**5**) has paclitaxel-like activity to microtubule systems. Whereas glaumacidines A (**1**) and B (**2**) inhibited the polymerization of tubulin. Glaumacidine A (**1**: IC_{50} 10.0 μM) with *trans* methyl groups showed three times more potent than glaumacidine B (**2**: IC_{50} 30.0 μM).

On the other hand, these coumarins showed moderate cytotoxicity against KB cells (IC_{50}: **1**, 23.0; **2**, 17.0; **3**, 5.7; **4**, 4.3; **5**, 1.5; **6**, 6.7; **7**, 4.8 μg/mL). Interestingly, cytotoxicity of paclitaxel was synergistically enhanced in addition of glaupalol (**5**, 0.5 μM). It is noted that the glaupalol-related coumarins with small substituents at C-15 showed more potent cytotoxicity and those with *cis* geometry at C-10 and C-11 such as **2** and **4** showed more potent than those with *trans* ones such as **1** and **3**. These results suggest that glaupalol (**5**) is a new class of microtubule stabilizing natural product and coumarin ring system with a dihydrofuran ring may be important for the activity.

Acknowledgements

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5. Glaumacidine A (**1**): colorless plates; $[\alpha]_D$ +30 (*c* 0.2, CH_3OH); IR (neat) ν_{max} 3400, 1741, 1684, and 1570 cm^{-1}; UV (CH_3OH) λ_{max} 335 (4400), 308 (11,000), 295 (12,000), and 212 (35,000); ^1H NMR and ^{13}C NMR (Table 1); FABMS *m/z* 319 $(M+H)^+$; HRFABMS *m/z* 319.1165 (M+H; calcd for $C_{17}H_{19}O_6$, 319.1183).
6. Glaumacidine B (**2**): colorless plates; $[\alpha]_D$ −52 (*c* 0.3, CH_3OH); IR (neat) ν_{max} 3400, 1743, 1690, and 1568 cm^{-1}; UV (CH_3OH) λ_{max} 308 (ε 8400), 295 (9600), and 212 (29,000); ^1H NMR and ^{13}C NMR (Table 1); FABMS *m/z* 319 $(M+H)^+$; HRFABMS *m/z* 319.1176 (M+H; calcd for $C_{17}H_{19}O_6$, 319.1183).
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8. *trans*-Glaupadiol (**3**): colorless plates; $[\alpha]_D$ +40 (*c* 0.2, CH_3OH); IR (neat) ν_{max} 3206 and 1673 cm^{-1}; UV (CH_3OH) λ_{max} 310 (1300), 297 (1400), and 211 (6200); ^1H NMR ($CDCl_3/CD_3OD$, 1:1) δ 7.04 (1H, d, 9.0, H-7), 7.04 (1H, d, 9.0, H-8), 4.74 (1H, q, 6.7, H-10), 2.52 (3H, s, H-12), 1.58 (3H, d, 7, H-13), 1.37 (3H, s, H-14), 3.74 (1H, s, H-15); ^{13}C NMR ($CDCl_3/CD_3OD$, 1:1) δ 170.8 (C-2), 108.3 (C-3), 163.6 (C-4), 122.6 (C-4a), 114.3 (C-5), 153.2 (C-6), 116.1 (C-7), 121.2 (C-8), 151.1 (C-8a), 93.5 (C-10), 49.9 (C-11), 13.8 (C-12), 21.8 (C-13), 15.5 (C-14), 64.6 (C-15); FABMS *m/z* 299 $(M+Na)^+$; HRFABMS *m/z* 299.0902 (M+Na; calcd for $C_{15}H_{16}O_5Na$, 299.0895). *cis*-Glaupadiol (**4**): colorless plates; $[\alpha]_D$ −44 (*c* 0.4, CH_3OH); IR (neat) ν_{max} 3210 and 1664 cm^{-1}; UV (CH_3OH) λ_{max} 309 (4800), 297 (5300), and 213 (16,000); ^1H NMR ($CDCl_3/CD_3OD$, 1:1) δ 7.03 (1H, d, 9.0, H-7), 7.04 (1H, d, 9.0, H-8), 5.04 (1H, q, 6.7, H-10), 2.52 (3H, s, H-12), 1.48 (3H, d, 6.7, H-13), 1.24 (3H, s, H-14), 3.81 (1H, d, 11.0, H-15a), 3.64 (1H, d, 11.0, H-15b); ^{13}C NMR ($CDCl_3/CD_3OD$, 1:1) δ 169.5 (C-2), 107.9 (C-3), 162.7 (C-4), 121.8 (C-4a), 113.4 (C-5), 152.4 (C-6), 115.3 (C-7), 120.4 (C-8), 150.2 (C-8a), 88.7 (C-10), 49.1 (C-11), 13.0 (C-12), 15.9 (C-13), 15.7 (C-14), 67.0 (C-15); FABMS *m/z* 277 $(M+H)^+$; HRFABMS *m/z* 277.1070 (M+H; calcd for $C_{15}H_{17}O_5$, 277.1076).
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14. *trans*-Methylglaupadiol *p*-methoxycinnamate (**8**): ^1H NMR ($CDCl_3$) δ 7.10 (1H, d, 9.0, H-7), 7.20 (1H, d, 9.0, H-8), 4.74 (1H, q, 6.7, H-10), 2.57 (3H, s, H-12), 1.58 (3H, d, 6.7, H-13), 1.55 (3H, s, H-14), 4.41 (1H, d, 11.6, H-15a), 4.36 (1H, d, 11.6, H-15b), 6.86 (1H, d, 8.6, H-2′), 7.35 (1H, d, 8.6, H-3′), 7.35 (1H, d, 8.6, H-5′), 6.86 (1H, d, 8.6, H-6′), 6.19 (1H, d, 16.0, H-3′), 7.49 (1H, d, 16.0, H-8′), 3.86 (3H, s, H-6OMe), 3.83 (3H, s, H-1′OMe); FABMS *m/z* 451 $(M+H)^+$; HRFABMS *m/z* 451.1776 (M+H; calcd for $C_{26}H_{27}O_7$, 451.1757). *cis*-Methylglaupadiol *p*-methoxycinnamate (**9**): ^1H NMR ($CDCl_3$) δ 7.11 (1H, d, 9.0, H-7), 7.21 (1H, d, 9.0, H-8), 5.01 (1H, q, 6.6, H-10), 2.56 (3H, s, H-12), 1.51 (3H, d, 6.6, H-13), 1.39 (3H, s, H-14), 4.50 (2H, s, H-15), 6.88 (1H, d, 8.6, H-2′), 7.44 (1H, d, 8.6, H-3′), 7.44 (1H, d, 8.6, H-5′), 6.88 (1H, d, 8.6, H-6′), 6.27 (1H, d, 16.0, H-3′), 7.61 (1H, d, 16.0, H-8′), 3.86 (3H, s, H-6OMe), 3.83 (3H, s, H-1′OMe); FABMS *m/z* 451 $(M+H)^+$; HRFABMS *m/z* 451.1759 (M+H; calcd for $C_{26}H_{27}O_7$, 451.1757).
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