# A New Pregnane Glycoside from *Dioscorea collettii* var. *hypoglauca*

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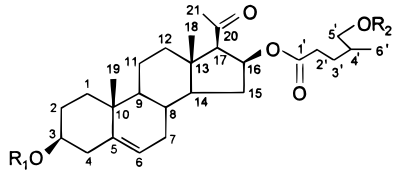
During further bioactivity-guided fractionation, a new pregnane glycoside, hypoglaucin G (**1**), and a known compound, pregna-5,16-dien-3*β*-ol-20-one 3-*O*-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→4)]-*β*-D-glucopyranoside (**2**), were isolated from the EtOH extract of *Dioscorea collettii* var. *hypoglauca* rhizomes, which induced morphological deformation of *Pyricularia oryzae* mycelia with minimum morphological deformation concentration values of 135 *μ*M and 236 *μ*M, respectively. The structure of **1** was established as 16*β*-(4′-methyl-5′-*O*-*β*-D-glucopyranosyl-pentanoxyl)-pregn-5-en-3*β*-ol-20-one 3-*O*-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→4)]-*β*-D-glucopyranoside on the basis of chemical evidence and spectral analysis, especially by 2D NMR techniques.

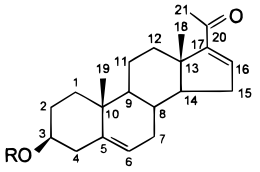
Morphological deformations of mycelia or conidia of microorganisms, such as curling, swelling, hyperdivergence, bead formation, and inhibition of germination, are often induced in the presence of bioactive substances.[1−4] A new screening bioassay detecting deformation of mycelia germinated from conidia of *Pyricularia oryzae* (a common phytopathogenic fungus) was developed for quantitative application to screening antifungal and antineoplastic agents by Kobayashi et al.[5] This bioassay is quick, easy to perform, and has been efficiently used in the preliminary screening of antineoplastic and antifungal agents from fungal metabolites.[6,7]

This bioassay was applied to screening antineoplastic and antifungal agents from traditional Chinese medicines (TCMs) for the first time, and 250 species of TCM plants used in the treatment of cancer have been screened. As a result, the ethanol extract of *Dioscorea collettii* Hook. f. var. *hypoglauca* Pei & Ting (Dioscoreaceae) showed the strongest activity.

The rhizome of *D. collettii* var. *hypoglauca* is used as a TCM "Fen Bei Bi Xie" for the treatment of cervical carcinoma, carcinoma of urinary bladder, and renal tumor in China. It is widely distributed in Southeast China and was included in the 1985, 1990, and 1995 versions of the Pharmacopoeia of the People's Republic of China. Our previous work on this plant resulted in the isolation of 11 bioactive steroidal saponins[8,9] that induced morphological abnormality of *P. oryzae* mycelia and showed cytotoxicity against the cancer cell line of K-562 in vitro. In this paper, further bioactivity-guided isolation has led to a new pregnane glycoside hypoglaucin G (**1**) and a known compound pregna-5,16-dien-3*β*-ol-20-one 3-*O*-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→4)]-*β*-D-glucopyranoside (**2**) from the same source. Compounds **1** and **2** induced morphological deformation of *P. oryzae* mycelia with minimum morphological deformation concentration (MMDC) values of 135 and 236 *μ*M, respectively. The cytotoxic bioassay indicated that **1** and **2** were inactive against six cultured human tumor cell lines of HT-29 (colon), HCT-15 (colon), LNCaP (prostate), PC-3 (prostate), T47D (breast) and MDA-MB-231 (breast), in vitro. Further cytotoxic bioassay of **1** (NSC-698795) is to be done by National Cancer Institute (U.S.A.) with a panel of 60 human cancer cell lines in vitro. The structures of **1** and **2** were elucidated on the basis of chemical evidence and spectral analysis, especially by 2D NMR techniques.




**1** $R_1$ = -β-D-Glc(2→α-L-Rha, 4→α-L-Rha); $R_2$ = -β-D-Glc




**2** R = -β-D-Glc(2→α-L-Rha, 4→α-L-Rha)

Compound **1** was isolated as white amorphous powder and responded positively to Libermann−Burchardt and Molish tests, indicating its glycosidic nature.[10] The HRMS showed a $[M + Na]^+$ peak at *m*/*z* 1085.5186, corresponding to $C_{51}H_{82}O_{23}Na$. On acid hydrolysis, GC analysis of the pertrimethylsilylated sugars in the hydrolysate of **1** showed rhamnose and glucose to be present in a ratio of 1:1. The precipitate was identified as pregna-5,16-dien-3*β*-ol-20-one by comparison with the authentic compound.

Full assignments of the proton and carbon signals of **1** were listed in Table 1 based on an analysis of 1D and 2D NMR spectral data. The $^1$H NMR spectrum of **1** showed the presence of six methyl groups at *δ* 0.90 (3H, d, *J* = 6.5 Hz, Me-6′), 1.03 (3H, s, Me-18), 1.21 (3H, s, Me-19), 1.62 (3H, d, *J* = 6.0 Hz, Rha Me-6‴′), 1.74 (3H, d, *J* = 6.0 Hz, Rha Me-6‴), and 2.11 (3H, s, Me-21); four anomeric protons

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**Table 1.** The $^1$H and $^{13}$C NMR Spectral Data[a] for **1** and **2** in $C_5D_5N$ ($\delta$ Values)[b]

| | **1** | | **2**[c] | |
|---|---|---|---|---|
| position | H | C | H | C |
| 1 | 0.95, 1.70 (o)[d] | 37.4 | 0.92, 1.67 (o) | 37.3 |
| 2 | 1.83, 2.06 (o) | 30.1 | 1.83, 2.05 (o) | 30.2 |
| 3 | 3.87 (o) | 78.1 | 3.82 (m) | 78.1 |
| 4 | 2.70, 2.78 (m) | 39.0 | 2.71, 2.80 (m) | 39.0 |
| 5 | | 150.9 | | 141.3 |
| 6 | 5.30 (br d) | 121.6 | 5.32 (br d) | 121.5 |
| 7 | 1.86 (o) | 31.9 | 1.53, 1.87 (o) | 31.8 |
| 8 | 1.49 (o) | 31.0 | 1.53 (o) | 30.3 |
| 9 | 0.89 (o) | 50.9 | 0.92 (o) | 50.8 |
| 10 | | 37.0 | | 37.2 |
| 11 | 1.44 (o) | 20.6 | 1.50 (o) | 20.9 |
| 12 | 1.08, 2.16 (o) | 38.1 | 1.37, 2.60 (o) | 35.1 |
| 13 | | 42.3 | | 46.3 |
| 14 | 0.80 (m) | 54.1 | 1.30 (o) | 56.5 |
| 15 | 1.31, 2.40 (o) | 35.5 | 1.90, 2.14 (o) | 32.3 |
| 16 | 5.66 (m) | 74.7 | 6.59 (m) | 144.7 |
| 17 | 2.47 (d, 8.0)[e] | 66.6 | | 155.2 |
| 18 | 1.21 (s) | 13.8 | 0.92 (s) | 15.9 |
| 19 | 1.03 (s) | 19.4 | 1.04 (s) | 19.3 |
| 20 | | 205.4 | | 196.3 |
| 21 | 2.11 (s) | 30.4 | 2.23 (s) | 27.1 |
| 1′ | | 173.3 | | |
| 2′ | 2.40 (o) | 32.2 | | |
| 3′ | 1.53, 1.94 (o) | 29.0 | | |
| 4′ | 1.86 (o) | 33.4 | | |
| 5′ | 3.50, 3.87 (o) | 74.7 | | |
| 6′ | 0.90 (d, 6.5) | 16.9 | | |

| | **1** | | | **2** | |
|---|---|---|---|---|---|
| position | H | C | position | H | C |
| Glc (inner) | | | Glc (inner) | | |
| 1″ | 4.93 (d, 7.8) | 100.3 | 1′ | 4.95 (d, 7.8) | 100.3 |
| 2″ | 4.20 (o) | 77.7 | 2′ | 4.22 (o) | 77.8 |
| 3″ | 4.20 (o) | 78.0 | 3′ | 4.22 (o) | 78.0 |
| 4″ | 4.38 (o) | 78.6 | 4′ | 4.40 (o) | 78.6 |
| 5″ | 3.63 (m) | 77.0 | 5′ | 3.63 (m) | 77.0 |
| 6″ | 4.08, 4.21 (o) | 61.3 | 6′ | 4.08, 4.21 (o) | 61.3 |
| Rha (1→2) | | | Rha (1→2) | | |
| 1‴ | 6.40 (d, 1.0) | 102.0 | 1″ | 6.41 (d, 1.0) | 102.0 |
| 2‴ | 4.82 (dd, 1.0, 3.5) | 72.5 | 2″ | 4.84 (dd, 1.0, 3.5) | 72.6 |
| 3‴ | 4.61 (dd, 3.5, 9.5) | 72.8 | 3″ | 4.63 (dd, 3.5, 9.0) | 72.9 |
| 4‴ | 4.36 (o) | 74.1 | 4″ | 4.36 (o) | 74.1 |
| 5‴ | 4.95 (o) | 69.5 | 5″ | 4.95 (o) | 69.5 |
| 6‴ | 1.74 (d, 6.0) | 18.6 | 6″ | 1.76 (d, 6.0) | 18.6 |
| Rha (1→4) | | | Rha (1→4) | | |
| 1⁗ | 5.85 (d, 1.0) | 102.9 | 1‴ | 5.86 (d, 1.0) | 102.9 |
| 2⁗ | 4.67 (dd, 1.0, 3.5) | 72.6 | 2‴ | 4.68 (dd, 1.0, 3.5) | 72.6 |
| 3⁗ | 4.54 (o) | 72.7 | 3‴ | 4.54 (dd, 3.5, 9.0) | 72.7 |
| 4⁗ | 4.32 (o) | 73.9 | 4‴ | 4.34 (o) | 73.9 |
| 5⁗ | 4.92 (o) | 70.4 | 5‴ | 4.94 (o) | 70.4 |
| 6⁗ | 1.62 (d, 6.0) | 18.5 | 6‴ | 1.63 (d, 6.0) | 18.5 |
| Glc | | | | | |
| 1⁗′ | 4.78 (d, 8.0) | 104.9 | | | |
| 2⁗′ | 4.00 (o) | 75.2 | | | |
| 3⁗′ | 4.20 (o) | 78.5 | | | |
| 4⁗′ | 4.20 (o) | 71.7 | | | |
| 5⁗′ | 3.93 (o) | 78.5 | | | |
| 6⁗′ | 4.38, 4.55 (o) | 62.8 | | | |

[a] Recorded on a JNM Alpha-500 ($^1$H 500 MHz, $^{13}$C 125 MHz) spectrometer in $C_5D_5N$. [b] All the signals were assigned by 1D and 2D NMR spectra. [c] The data of **2** are in agreement with those of Yu et al.[13] [d] Overlapped signals are indicated by "(o)". [e] *J* values (in parentheses) are reported in Hz.

at $\delta$ 6.40 (1H, d, $J$ = 1.0 Hz, Rha H-1‴), 5.85 (1H, d, $J$ = 1.0 Hz, Rha H-1⁗), 4.93 (1H, d, $J$ = 7.8 Hz, Glc H-1″), and 4.78 (1H, d, $J$ = 8.0 Hz, Glc H-1⁗′); and an olefinic proton at $\delta$ 5.30 (1H, br d, H-6). The fully decoupled $^{13}$C and DEPT NMR spectra of **1** exhibited 51 carbon signals, which consisted of six methyls, 12 methylenes, 28 methines, and five quaternary carbons. Two carbonyl carbons at $\delta$ 205.4 (C-20) and 173.3 (C-1′); two olefinic carbons at $\delta$ 150.9 (C-5) and 121.6 (C-6); four anomeric carbons at 100.3 (Glc C-1″), 102.0 (Rha C-1‴), 102.9 (Rha C-1⁗), and 104.9 (Glc C-1⁗′); and six methyl groups at $\delta$ 13.8 (C-18), 19.4 (C-19), 30.4 (C-21), 16.9 (C-1′), 18.6 (Rha C-6‴), and 18.5 (Rha C-6⁗) were also confirmed in the $^{13}$C NMR spectra. The $J$ value of 8.0 Hz between H-16 and H-17 suggested the $\beta$-configuration of the side chain attached to C-16. The $\beta$-configuration of the anomeric carbon of two glucopyranosyl units was determined by $J_{H1-H2}$ values (>7.0 Hz). The α-configuration of the anomeric carbons of two rhamnopyranosyl units was confirmed by comparison of the chemical shift values of carbons 3 and 5 with those of the corresponding carbons of methyl α- and $\beta$-rhamnopyranoside.[11,12]

The pregnane skeleton and the C-16 side chain of the aglycon were determined by $^1$H−$^1$H COSY, HMQC, and HMBC spectra. A combination of $^1$H−$^1$H COSY and HMQC experiments showed the following connectivities C-1−C-2−C-3−C-4, C-6−C-7−C-8−C-9−C-11−C-12, C-14−C-15−C-16−C-17 and C-2′−C-3′−C-4′−C-5′−C-6′, starting from the well-resolved signals at $\delta$ 2.70 and 2.78 ($H_2$-4), 5.30 (H-6), 5.66 (H-16), and 0.90 (Me-6′). The HMBC spectrum especially showed significant cross peaks, due to $^2J_{C-H}$ and $^3J_{C-H}$ correlations, between $H_3$-18 ($\delta$ 1.21) and C-12, C-13, C-14, and C-17; between $H_3$-19 ($\delta$ 1.03) and C-1, C-5, C-9, and C-10; between $H_3$-21 ($\delta$ 2.11) and C-17 and C-20; between H-16 and C-1′ and C-13; and between H-17 ($\delta$ 2.47) and C-12, C-13, C-14, C-16, C-18, and C-20. The structure of the side chain bound to C-16 was also established by long-range signals in the HMBC spectrum, that is, between $H_2$-2′ ($\delta$ 2.40) and C-1′, C-3′, and C-4′; between $H_2$-3′ ($\delta$ 1.53 and 1.94) and C-1′, C-2′, C-5′, and C-6′; between H-4′ and C-2′, C-3′, C-5′, and C-6′; between $H_2$-5′ ($\delta$ 3.50 and 3.87) and C-3′, C-4′, C-6′, and Glc C-1⁗′; and between H-6′ ($\delta$ 0.90) and C-3′, C-4′, and C-5′.

The assignments of the sugar moieties were interpreted similarly as well. A combination of $^1$H−$^1$H COSY and HMQC experiments showed the connectivities from C-1 to C-6 of the sugar units, starting from the well-resolved signals at $\delta$ 4.93 (Glc H-1″), 6.40 (Rha H-1‴), 5.85 (Rha H-1⁗), and 4.78 (Glc H-1⁗′). The sequence of the trisaccharide chain at C-3 of the aglycon was determined by the correlations observed in the HMBC spectrum between Glc H-1″ ($\delta$ 4.83) and C-3 ($\delta$ 78.1), between Rha H-1‴ ($\delta$ 6.40) and Glc C-2″ ($\delta$ 77.7), and between Rha H-1⁗ ($\delta$ 5.85) and Glc C-4″ ($\delta$ 78.6).

Based on all the above data, the structure of compound **1** was established as 16$\beta$-(4′-methyl-5′-*O*-$\beta$-D-glucopyranosyl-pentanoxyl)-pregn-5-en-3$\beta$-ol-20-one 3-*O*-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→4)]-$\beta$-D-glucopyranoside, namely hypoglaucin G.

By similar means, compound **2** was identified as pregna-5,16-dien-3$\beta$-ol-20-one 3-*O*-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→4)]-$\beta$-D-glucopyranoside by comparison with the published data.[13] Here is the first report on the isolation of **2** from plants of the family Dioscoreaceae.

## Experimental Section

**General Experimental Procedures.** Melting points were determined on Yanaco MP−$S_3$ micro-melting point apparatus (uncorrected). Optical rotations were measured on a JASCO DIP-1000 digital polarimeter. IR spectrum (KBr) was taken on a JASCO A-102 spectrometer. 1D and 2D NMR spectra were measured on a JNM ALPHA-500 spectrometer ($^1$H 500 MHz, $^{13}$C 125 MHz). FABMS and HRMS were obtained using a JEOL JMS-DX302 spectrometer. GC was recorded on a HP-5890 SERIEIS II spectrometer. Preparative HPLC was carried out on a liquid chromatography LC-10 (Japan Analytical Industry Co., Ltd.) equipped with a RI detector by using an

ODS column (Waters). Analytical TLC and preparative TLC were performed on Merck precoated Si gel 60 $F_{254}$S plates and RP-18 $F_{254}$S plates, respectively. Compounds were visualized by spraying with 10% $H_2SO_4$ followed by heating.

**Plant Material.** The rhizomes of *Dioscorea collettii* var. *hypoglauca* (Dioscoreaceae) were collected in April 1994, from Zhejiang Province (People's Republic of China) and identified by Prof. Zerong Jiang (Division of Pharmacognosy, Shenyang Pharmaceutical University, China). A voucher specimen is deposited at the Division of Pharmacognosy of Shenyang Pharmaceutical University (no. 104).

**Extraction and Isolation.** The air-dried powdered rhizomes (1500 g) of *D. collettii* var. *hypoglauca* were extracted with 75% EtOH (10 l × 3). The EtOH solutions were concentrated in vacuo to give 133.5 g of residue. A suspension of the resulting extract in $H_2O$ was partitioned successively with $CHCl_3$, EtOAc, and *n*-BuOH to afford four corresponding fractions: fractions DC (4.5 g), DE (4.2 g), DB (13.5 g), and DH (102.1 g), respectively. Fractions DC (4.5 g) and DE (4.2 g) were subjected to VLC (vacuum liquid chromatography)[14] on Si gel to give three bioactive spirostanol saponins.[8] Fraction DB (13.5 g) was subjected to column chromatography on Si gel and further separated by preparative HPLC (ODS column, 5 $\mu$M, 250 mm × 20 mm i.d.; solvent, MeOH−$H_2O$ 50:50; flow rate, 3.0 mL/min) to give eight active furostanol saponins[9] along with a bioactive fraction enriched with **1**. This fraction was further purified by preparative HPLC with MeOH−$H_2O$ (ODS column, 5 $\mu$M, 250 mm × 20 mm i.d.; solvent, MeOH−$H_2O$ 48:52; flow rate, 3.0 mL/min) and preparative TLC (Si gel PR-18) developed by $CH_3CN$−$H_2O$ (33:67) to yield compounds **1** (60.7 mg) and **2** (4.5 mg).

**Hypoglaucin G (1):** white amorphous powder; mp 154−156 °C; $[\alpha]^{24}_D$ − 48.6° (*c* 0.205, MeOH); IR (KBr) $\nu_{max}$ 3400 (OH), 2950, 1705 (C═O), 1040 (C−O) $cm^{-1}$; HRMS *m/z* $[M + Na]^+$ 1085.5186 (calcd for $C_{51}H_{82}O_{23}Na$, 1085.5145); $^1H$ NMR (500 MHz, $C_5D_5N$) and $^{13}C$ NMR (125 MHz, $C_5D_5N$), Table 1.

**Compound 2:** white amorphous powder; mp 270−273 °C; IR (KBr) $\nu_{max}$ 3420 (OH), 2933, 1646 (C═O), 1046 (glycosyl C−O) $cm^{-1}$; $^1H$ NMR (500 MHz, $C_5D_5N$) and $^{13}C$ NMR (125 MHz, $C_5D_5N$), Table 1.

**Acid Hydrolysis of 1 and 2.** Each compound (a few milligrams) was refluxed with 1N $H_2SO_4$ in 50% $Me_2CO$ for 5 h. The aglycon was identified as pregna-5,16-dien-3$\beta$-ol-20-one by co-TLC with an authentic sample. The reaction mixture was dried by blowing $N_2$ gas over it at room temp. For GC analysis, the residue was trimethylsilyated with hexamethyldisilazane and trimethylchlorosilane in 2:1 ratio[15] at room temperature, GC, SE30 capillary column (12 m × 0.22 mm i.d.); detector, FID (270 °C); column temperature 170−210°, rate 5° $min^{-1}$; carrier gas, $N_2$ (30 mL/min); retention time (min), rhamnose (3.72) and glucose (7.12).

**Cell Lines and Culture Medium.** The solid tumor cell lines of HT-29, HCT-15, LNCaP, and PC-3 were obtained from the American Tissue Culture Collection (ATCC, Rocksville, MD) and cultured with RPMI 1640 (Gibco-BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco) and 100 IU/mL penicillin and 100 $\mu$g/mL streptomycin. T47D and MDA-MB-231 cells were purchased from ATCC and cultured in the same medium, except with 5 $\mu$g/mL insulin.

**Cytotoxicity Assay.** Cellular viability in the presence and absence of tested agents was determined using the standard MTT (3-[4,5,-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma) assay as described by Mosmann.[16] Briefly, a 0.2-mL cell suspension containing 1000 cells was seeded to each well of a 96-well microtiter plate (Becton Dickinson Labware, NJ), and new medium containing different tested samples was added 24 h after seeding. After culturing for 5 days at 37 °C, 50 $\mu$L of MTT solution (2 mg/mL) was added, and the culture was continued for an additional 4 h. The medium was removed by aspiration, and cells were dissolved in DMSO (200 $\mu$L/well) for 10 min. The plate was read immediately on a microtiter plate reader (Dynatech MR5000) at the wavelength of 540 nm. Absorbance taken from cells grown in the absence of test agents was taken as 100% cell survival (control). For each compound, the viability was plotted against concentration, and the $IC_{50}$ (concentration required to reduce viability by 50%) value against the cancer cell lines was calculated. The anticancer drug VP16 (etoposide) (Sigma) was used as the positive control.

***Pyricularia oryzae* Bioassay.** See the previous report of Kobayashi et al.[5] The positive controls employed two antifungal agents, griesolfuvin and fusarelin A, which exhibited activity with MMDC values of 50 and 15 $\mu$M, respectively.

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