Limnophilaspiroketone, a Highly Oxygenated Phenolic Derivative from *Limnophila geoffrayi*

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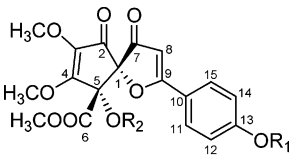
A highly oxygenated phenolic spiroketone, limnophilaspiroketone (**1**), was isolated from the aerial parts of *Limnophila geoffrayi* collected in Thailand. The structure of **1** was determined based on spectroscopic data interpretation. This novel isolate, obtained as a major secondary metabolite constituent, was verified as a racemate using the Mosher ester method.

Limnophila geoffrayi Bon. (Scrophulariaceae) is used as an antidote for the detoxification of poisons and is considered as a vegetable in northeastern Thailand.[1] This species is also used as a traditional medicine for its antipyretic, expectorant, and galactogogue properties.[1] Flavonoids[2,3] and triterpenoids[4,5] are the major constituents of plants in the genus *Limnophila*. In the course of an investigation on potential cancer chemopreventive agents from plants,[6−8] an EtOAc-soluble partition part of a MeOH extract of the aerial parts of the edible species *L. geoffrayi* was selected for study. Repeated chromatography of this extract led to the isolation of a novel phenolic derivative, limnophilaspiroketone (**1**), and nine known compounds, including a prenylated benzoic acid, three flavones, and five triterpene acids. The new compound, limnophilaspiroketone (**1**), represents an unprecedented highly oxygenated spiroketone-possessing phenolic metabolite, and it was further established as a natural racemate. Nevadensin and isothymusin, two flavones isolated in the present study, were reported from *L. geoffrayi* recently.[1] The isolation and structure elucidation of limnophilaspiroketone (**1**) and the evaluation of the quinone reductase induction activity of all isolates obtained are reported herein.



1 $R_1 = R_2 = H$
1a $R_1 = Ac$ $R_2 = H$
1b $R_1 = R_2 = Ac$

Compound **1** was isolated as a major component by repeated chromatography. Initially, the NMR data (Table 1) of **1** were acquired using $CDCl_3$, in which, in addition to three methoxy singlets and two deuterium-exchangeable broad singlets, only one olefinic singlet at δ_H 5.89 and two aromatic doublets at δ_H 7.48 and 6.83 were observed. However, the ^{13}C NMR spectrum of compound **1** exhibited 16 signals, assigned as 10 quaternary, 3 methine, and 3 methoxy carbons, based on the DEPT135 data. One-bond proton and carbon connectivity was established by correlations observed in the HMQC spectrum of **1**. The two relatively high-intensity methine carbon signals at δ_C 129.7 (C-11 and C-15, d) and 116.2 (C-12 and C-14, d) were correlated with the two-proton aromatic signals at δ_H 7.48 (2H, d, J = 8.7 Hz, H-11 and H-15) and 6.83 (2H, d, J = 8.7 Hz, H-12 and H-14), respectively. The chemical shifts and coupling constants of these signals, in combination with the observed $^1H-^1H$ COSY (H-11/H-15 with H-12/H-14) and HMBC correlations, suggested the presence of a para-substituted aromatic ring. Therefore, 18 carbon atoms were present in **1**, which was confirmed by the molecular formula of $C_{18}H_{16}O_9$ determined from its EIMS (*m/z* 376 $[M]^+$) and HRESIMS (*m/z* 775.1472 $[2M + Na]^+$, calcd for $C_{36}H_{32}O_{18}Na$, 775.1486) data. Except for the para-substituted aromatic ring and three methoxy groups, the remaining nine carbons would contribute seven unsaturation values on the basis of the determined molecular formula of compound **1**.

Generally, two- and three-bond HMBC correlations are very useful for the structure elucidation of natural products. However, in the case of compound **1**, eight of the nine remaining signals were quaternary carbons, and therefore, comparatively few correlations could be observed in the HMBC spectrum. The signals of two deuterium-exchangeable protons were observed at δ_H 8.41 and 4.32 as very broad singlets in the ^1H NMR spectrum of **1** recorded in $CDCl_3$. Therefore, no HMBC correlations were obtained from these two signals to any carbons, and the observed HMBC data were not adequate to elucidate the structure of this unusual isolate. In an attempt to obtain a better resolution for certain carbon signals and a sharper pattern for the deuterium-exchangeable signals, the NMR data of **1** were then acquired in both acetone-d_6 and methyl sulfoxide-d_6 (Supporting Information). As expected, the ^1H NMR resonances of OH-5 and OH-13 were much sharper in these solvents. In the HMBC spectra, valuable correlations were obtained from OH-5 to C-1, C-4, C-5, and C-6. Careful analysis of the observed HMBC correlations (Fig-

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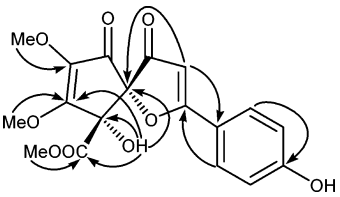

Figure 1. HMBC correlations of **1**.

ure 1) in acetone-d_6, chloroform-d, and methyl sulfoxide-d_6, in combination with a consideration of the determined molecular formula, suggested the structure of compound **1** to be as shown, which was assigned the trivial name, limnophilaspiroketone. Compound **1** was acetylated to afford mono- (**1a**) and diacetate (**1b**) derivatives.

Thus, limnophilaspiroketone (**1**) represents an unprecedented highly oxygenated spiroketone-possessing phenolic derivative. To obtain long-range correlations to support the structure of this novel isolate, a series of HMBC spectra were acquired with different relaxation delay values of 150, 250, and 400 ms, instead of 70 ms as more generally used. Additional correlations from OMe-6 at δ_H 3.89 to C-5 at δ_C 80.3, and from OMe-4 at δ_H 4.22 to OMe-3 at δ_C 59.9, were observed in the HMBC spectrum (500 MHz, in acetone-d_6), with a relaxation delay of 250 ms (Supporting Information). These correlations were also supportive of the proposed structure of limnophilaspiroketone (**1**). The relative configuration of **1** was determined as 1*S* and 5*S* based on the observed NOESY correlation from OH-5 to H-12 and H-14.

The optical rotation values of limnophilaspiroketone (**1**) and its acetylation products (**1a** and **1b**) were found to be zero using both Na (589 nm) and Hg (578, 546, 435, and 365 nm) source lamps. To determine if compound **1** is a racemate, it was treated with (*S*)-MTPA-Cl using a convenient Mosher ester method.[9,10] Two sets of the signals were observed in the ^{1}H NMR spectrum (Supporting Information) of the resulting (*R*)-MTPA ester of **1**, which suggested that limnophilaspiroketone (**1**) is a racemate. This was confirmed by the lack of Cotton effects in the circular dichroism (CD) spectrum of **1**. The occurrence of racemic natural products is rare, although this is a well-known phenomenon among the plant lignans.[11]

In addition to the novel phenolic derivative, limnophilaspiroketone (**1**), nine known compounds, betulinic acid,[12] 4-*epi*-hederagenin,[13] 3-farnesyl-4-hydroxybenzoic acid,[14] gardenin B,[15] 6β-hydroxyoleanolic acid,[16] isothymusin,[1] nevadensin,[1] rotungenic acid,[17] and uncaric acid,[18] were also isolated in this study. The structures of these known compounds were identified by physical (mp, $[\alpha]_D$) and spectroscopic (^{1}H NMR, ^{13}C NMR, 2D NMR, and MS) data measurement and by comparison with published values.

Induction of Phase II drug-metabolizing enzymes, such as quinone reductase, is considered a relevant mechanism for achieving protection against the toxic and neoplastic effects of many carcinogens.[19,20] All isolates obtained in the present study were evaluated for their potential cancer chemopreventive activity utilizing an in vitro assay to determine quinone reductase induction.[19,20] While the initial EtOAc extract of *L. geoffrayi* was weakly active in this assay (concentration to double induction, CD, 9.4 μg/mL), all compounds isolated, including **1**, were found to be inactive (CD, >20 μg/mL).

Experimental Section

General Experimental Procedures. Melting points were determined on a Fisher-Johns melting-point apparatus and are uncorrected. Optical rotations were obtained using a Perkin-Elmer 241 polarimeter. UV spectra were recorded with a Beckman DU-7 spectrometer. CD spectra were measured with a JASCO J-810 spectropolarimeter. IR spectra were run on an ATI Mattson Genesis Series FT-IR spectrometer. NMR experiments were conducted on Bruker DPX-300 and DRX-500 MHz spectrometers with tetramethylsilane (TMS) as internal standard. FABMS and HRFABMS were obtained on a VG 7070E-HF sector-field mass spectrometer, and EIMS and HREIMS on a Finnigan MAT 95 sector-field mass spectrometer operating at 70 eV. Thin-layer chromatographic (TLC) analysis was performed on Kieselgel 60 F_{254} (Merck) plates (silica gel, 0.25 mm layer thickness), with compounds visualized by dipping plates into 10% (v/v) H_2SO_4 reagent (Aldrich, Milwaukee, WI) followed by charring at 110 °C for 5−10 min. Silica gel (Merck 60A, 70−230 or 200−400 mesh ASTM) and Sorbisil C_{18} reversed-phase silica gel (Sigma, St. Louis, MO) were used for column chromatography. Preparative TLC was performed on Kieselgel 60 F_{254} (Merck) plates (silica gel, 0.25 mm layer thickness). All solvents used for chromatographic separations were purchased from Fisher Scientific (Fair Lawn, NJ) and distilled before use.

Plant Material. The aerial parts of *Limnophila geoffrayi* Bonati were collected in Ayuthaya Province, Thailand, in July 2002, and the plant was identified by N. Bunyapraphatsara. A voucher specimen has been deposited at the Herbarium of the Department of Pharmaceutical Biology (PBM02729), Faculty of Pharmacy, Mahidol University, Bangkok 10400, Thailand.

Quinone Reductase Assay. For the evaluation of plant extracts, fractions, and pure isolates as inducers of quinone reductase (QR), cultured mouse Hepa 1c1c7 cells were used as described previously.[19,20]

Extraction and Isolation. The dried and milled plant material (5 kg) was extracted with MeOH (3 × 20 L) by maceration. The extracts were combined and concentrated in vacuo at 40 °C. The concentrated extract was suspended in 90% MeOH and then partitioned with petroleum ether (3 × 4 L) to afford a petroleum ether-soluble syrup on drying. Next, the aq. MeOH extract was concentrated and suspended in H_2O (4 L) and partitioned with EtOAc (3 × 4 L) to give an EtOAc-soluble extract and an aqueous residue. The EtOAc-soluble extract showed moderate activity in the QR induction assay (CD value 9.4 μg/mL).

The EtOAc-soluble extract (240 g) was chromatographed over silica gel as stationary phase using a $CHCl_3$−MeOH gradient (from 1:0 to 0:1 v/v) as mobile phase to afford 16 pooled fractions (fractions 4−19). Of these, fractions F004, F005, F006, F007, and F008 showed the most potent QR-inducing activity (CD values 5.1, 11.4, 9.3, 7.2, and 8.5 μg/mL, respectively). Fractions F004 and F005 [eluted with $CHCl_3$; 5.3 g] were combined and then chromatographed over Sephadex LH-20 ($CHCl_3$−MeOH 1:1 v/v) followed by recrystallization from MeOH to give gardenin B (40 mg, 0.0008%). Nevadensin (38 g, 0.760%) was obtained as a major component as a yellow powder by recrystallization (from MeOH) from fraction F006 [eluted with $CHCl_3$−MeOH 99:1 v/v; 89 g]. The rest of fraction F006 was purified further over a silica gel column, with *n*-hexanes−EtOAc−MeOH (70:26:4 to 60:30:10 v/v) as the solvent system, yielding betulinic acid (13 mg, 0.00026%) and 3-farnesyl-4-hydroxybenzoic acid (64 mg, 0.0013%). Fractions F004 and F005 [eluted with $CHCl_3$−MeOH 49:1 v/v; 26 g] were combined and then chromatographed over a silica gel column, with *n*-hexanes−EtOAc−MeOH (60:38:2 to 50:40:10 v/v) as the solvent system, resulting in eight subfractions (fractions F036−043). Fraction F036 was further purified by reversed-phase low-pressure liquid chromatography over C_{18} silica gel, with MeOH−H_2O (9:1 v/v), to give 6β-hydroxyoleanolic acid (134 mg, 0.00268%) and uncaric acid (12 mg, 0.00024%). Isothymusin (420 mg, 0.0084%) was isolated from fraction F037 by recrystallization (from petroleum ether−EtOAc 1:1 v/v). Fraction F038 was further fractionated over C_{18} silica gel, with MeOH−H_2O (9:1 v/v), yielding 4-*epi*-hederagenin (14 mg, 0.00028%) and rotungenic acid (5.0

mg, 0.00010%). The new compound, limnophilaspiroketone (**1**, 460 mg, 0.0092%), was isolated by recrystallization (from MeOH) from fraction F041.

Limnophilaspiroketone (1): white amorphous powder; mp 200−202 °C; $[\alpha]^{22}_{D}$ 0° (*c* 0.5, MeOH; measured at 589, 578, 546, 435, and 365 nm); UV (MeOH) λ_{max} (log ϵ) 236 (2.44), 270 (2.54), 332 (2.84) nm; IR ν_{max} (film) 3384, 1756, 1701, 1633, 1569, 1503, 1439, 1013 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, TMS) δ 8.41 (1H, br s, OH-13), 7.48 (2H, d, J = 8.7 Hz, H-11 and H-15), 6.83 (2H, d, J = 8.7 Hz, H-12 and H-14), 5.89 (1H, s, H-8), 4.32 (1H, br s, OH-5), 4.25 (3H, s, OMe-4), 3.931 (3H, s, COOMe), 3.925 (3H, s, OMe-3); ^{13}C NMR (75 MHz, $CDCl_3$, TMS) δ 194.5 (C-7, s), 187.5 (C-9, s), 185.4 (C-2, s), 169.1 (C-6, s), 164.3 (C-4, s), 162.1 (C-13, s), 138.2 (C-3, s), 129.7 (C-11 and C-15, d), 118.9 (C-10, s), 116.2 (C-12 and C-14, d), 98.4 (C-8, d), 94.6 (C-1, s), 78.9 (C-5, s), 60.7 (OMe-4, q), 59.7 (OMe-3, q), 54.5 (COOMe, q); ^1H NMR and ^{13}C NMR data obtained in CD_3COCD_3 and DMSO-d_6, see Supporting Information; EIMS *m/z* 376 $[M]^+$ (21), 317 (77), 289 (10), 261 (6), 231 (6), 203 (6), 145 (6), 142 (100), 127 (27), 121 (16), 118 (13), 115 (6), 44 (9); HRESIMS *m/z* 775.1472 $[2M + Na]^+$ (calcd for $C_{36}H_{32}O_{18}$-Na, 775.1486).

Limnophilaspiroketone 13-monoacetate (1a): $[\alpha]^{22}_{D}$ 0° (*c* 0.4, MeOH; measured at 589, 578, 546, 435, and 365 nm); ^1H NMR (300 MHz, $CDCl_3$, TMS) δ 7.79 (2H, d, J = 8.8 Hz, H-11 and H-15), 7.24 (2H, d, J = 8.8 Hz, H-12 and H-14), 6.03 (1H, s, H-8), 4.27, 3.94, 3.93 (each 3H, s, −OMe), 3.99 (1H, s, OH-5), 2.34 (3H, s, OAc-13); EIMS *m/z* 418 $[M]^+$ (30), 359 (82), 331 (11), 317 (100), 289 (9), 231 (8), 145 (11), 142 (54), 127 (16), 118 (19), 89 (9), 43 (19).

Limnophilaspiroketone 5,13-diacetate (1b): $[\alpha]^{22}_{D}$ 0° (*c* 0.2, MeOH; measured at 589, 578, 546, 435, and 365 nm); ^1H NMR (300 MHz, $CDCl_3$, TMS) δ 7.84 (2H, d, J = 8.8 Hz, H-11 and H-15), 7.23 (2H, d, J = 8.8 Hz, H-12 and H-14), 5.94 (1H, s, H-8), 4.30, 3.93, 3.90 (each 3H, s, −OMe), 2.33 (3H, s, OAc-13), 2.14 (3H, s, OAc-5); EIMS *m/z* 460 $[M]^+$ (27), 390 (9), 373 (11), 359 (67), 344 (10), 331 (20), 317 (72), 289 (9), 273 (13), 247 (20), 231 (6), 214 (100), 205 (35), 182 (33), 163 (18), 142 (35), 121 (35), 43 (55).

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Supporting Information Available: The ^1H, ^{13}C, and HMBC (relaxation delay 250 ms) NMR spectra of **1**, ^1H NMR spectra of the 13-monoacetate, 5,13-diacetate, and (*R*)-MTPA esters of **1**, and ^1H and ^{13}C NMR data of **1** in acetone-d_6 and DMSO-d_6 (total 11 pages). This material is available free of charge via the Internet at http://pubs.acs.org.

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