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


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Received April 19, 2005

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 Flavonoids2,3 and triterpenoids4,5 are the major constituents of plants in the genus *Limnophila*. In the course of an investigation on potential cancer chemopreventive agents from plants,6 a MeOH extract of the aerial parts of the edible species *Limnophila 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 triterpenes. The new compound, limnophilaspiroketone (1), represents an unprecedented highly oxygenated spiroketone-containing phenolic metabolite, and it was further established as a natural racemate. Nevadensin and isothymsin, 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.

![Diagram of Limnophilaspiroketone](https://example.com/diagram)

Compound 1 was isolated as a major component by repeated chromatography. Initially, the NMR data (Table 1) of 1 were acquired using CDCl₃, in which, in addition to three methoxy singlets and two deuterium-exchangeable broad singlets, only one olefinic singlet at δH 5.89 and two aromatic doublets at δH 7.48 and 6.83 were observed. However, the ¹³C 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 δC 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 δH 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 C₁₈H₁₆O₉ determined from its EIMS (m/z 376 [M⁺]) and HRESIMS (m/z 775.1472 [2M⁺ + Na⁺]⁺, calcd for C₃₆H₃₂O₁₈Na, 775.1468). 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 δH 8.41 and 4.32 as very broad singlets in the ¹H NMR spectrum of 1 recorded in CDCl₃. 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-d₆ and methyl sulfoxide-d₆ (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-
Figure 1. HMBC correlations of 1.

ure 1) in acetone-d₆, chloroform-d₆, and methyl sulfoxide-
-d₆ 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 unpre-
cedented 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 δH 3.89 to C-5 at δC
80.3, and from OMe-4 at δH 4.22 to OMe-3 at δC 59.9, were
observed in the HMBC spectrum (500 MHz, in acetone-
d₄), with a relaxation delay of 250 ms (Supporting Informa-
tion). These correlations were also supportive of the
proposed structure of limnophilaspiroketone (1). The rela-
tive configuration of 1 was determined as 1S and 5S 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 con-
venient Mosher ester method. 5,10 Two sets of the signals
were observed in the 1H NMR spectrum (Supporting Infor-
mation) 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, limnophila-
spiroketone (1), nine known compounds, betulinic acid, 12
4-epi-hederagenin, 13 3-farnesyl-4-hydroxybenzoic acid, 14
gardenin B, 15 6β-hydroxyxoleanolic acid, 16 isothyminusin, 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, [α]D) and
spectroscopic (1H NMR, 13C 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. 13,14 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. 13,14 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 spectrophotometer. 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 spectrom-
eter operating at 70 eV. Thin-layer chromatographic (TLC)
analysis was performed on Kieselgel 60 F₂₅₄ (Merck) plates
(silica gel, 0.25 mm layer thickness), with compounds visual-
ized by dipping plates into 10% (v/v) H₂SO₄ 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 C₁₀ reversed-phase silica gel (Sigma, St. Louis, MO)
were used for column chromatography. Preparative TLC was
performed on Kieselgel 60 F₂₅₄ (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), Fac-
ulty of Pharmacy, Mahidol University, Bangkok 10400, Thai-
land.

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. 15,16

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 H₂O
(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 CHCl₃–MeOH
gradient (from 1:0 to 0:1 v/v) as mobile phase to afford 16
poled 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
CHCl₃–5.3 g] were combined and then chromatographed over
Sephadex LH-20 (CHCl₃–MeOH 1:1 v/v) followed by recrys-
tallization 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–EtOAc–
MeOH (70:26:4 to 60:30:10 v/v) as the solvent system, yielding
betulinic acid (13 mg, 0.00002%) and 3-farnesyl-4-hydroxybenzoic
acid (64 mg, 0.0013%). Fractions F004 and F005 [eluted with
CHCl₃–MeOH 49:1 v/v; 26 g] were combined and then chromato-
graphed over a silica gel column, with n-hexanes–ETOAc–
MeOH (70:26:4 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 chro-
matography over C₁₁₈ silica gel, with MeOH–H₂O (9:1 v/v), to
give 6β-hydroxyxoleanolic acid (134 mg, 0.00268%) and uncaric
acid (12 mg, 0.00024%). Isothyminusin (420 mg, 0.0084%) was
isolated from fraction F037 by recrystallization (from petroleum
ether–ETOAc 1:1 v/v). Fraction F038 was further frac-
tionated over C₁₁₈ silica gel, with MeOH–H₂O (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; [α]D20 0° (c 0.5, MeOH; measured at 589, 578, 546, 435, and 365 nm); UV (MeOH) 430 (log ε 4.24), 270 (2.54), 332 (2.84) nm; IR νmax (film) 3384, 1756, 1701, 1633, 1569, 1503, 1439, 1013 cm⁻¹; 1H NMR (300 MHz, CDCl3, 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, OMe-3); 13C NMR (75 MHz, CDCl3, TMS) δ 194.5 (C-7, s), 187.5 (C-9, s), 185.4 (C-2, s), 169.1 (C-4, s), 164.3 (C-6, s), 164.3 (C-4, s), 159.7 (C-5, s), 60.7 (OMe-4, q), 59.7 (OMe-3, q), 54.5 (COOMe, q); 1H NMR and 13C NMR data obtained in CD3COCD3 and DMSO-d6 (total 11 pages). This material is available free of charge via the Internet at http://pubs.acs.org.

**Supporting Information Available:** The 1H, 13C, 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 13C NMR data of 1 in acetone-d6 and DMSO-d6 (total 11 pages). This material is available free of charge via the Internet at http://pubs.acs.org.

**References and Notes**


NP050132B