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An Isoflavone from *Leiophyllum buxifolium* and Its Antiproliferative Effect

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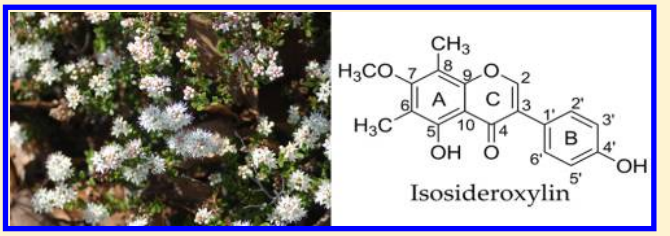
Supporting Information

ABSTRACT: A new *C*-methylisoflavone, isosideroxylin (**1**), and a known *C*-methylflavone, sideroxylin (**2**), were isolated from the EtOAc extract of the leaves of *Leiophyllum buxifolium*. The two compounds were evaluated with the sulforhodamine B assay for their antiproliferative effects against ER$^-$ MDA-MB-231 and ER$^+$ MCF-7 breast cancer cell lines and the NIH3T3 mouse fibroblast cell line. Isosideroxylin (**1**) displayed a selective antiproliferative effect against MDA-MB-231 cells.



Terrestrial plants have been a significant source of novel cytotoxic compounds.[1] The New Jersey Pine Barrens (NJ pinelands) is a vast wild ecosystem from which little chemical or bioactivity exploration of the plants has been done. In our continuing search for naturally occurring compounds with interesting biological activities, bioassay-guided fractionation of *Leiophyllum buxifolium* (Berg.) Elliott (=*Kalmia buxifolia* (Berg.) Gift & Kron, Ericaceae), commonly known as sand myrtle, collected from the NJ pinelands, led to the isolation of a new *C*-methylisoflavone, isosideroxylin **1**, and a known *C*-methylflavone, sideroxylin **2**. The antiproliferative effects of **1** and **2** on the MDA-MB-231 and MCF-7 breast cancer and the NIH3T3 mouse fibroblast cell lines were evaluated.

Ericaceae, commonly known as the heath or heather family, comprises many edible, medicinal, and poisonous plants. Phytochemical studies of *Vaccinium macrocarpon* (cranberry) uncovered three proanthocyanidin trimers that prevent the attachment of *Escherichia coli*, the principal bacteria involved in urinary tract infections.[2] Cytotoxic grayanane-type diterpenoids, grayanotoxins, have been found mainly in the genera *Kalmia*,[3] *Rhododendron*,[4] *Pieris*,[5] and *Leucothoe*.[6] Triterpenoids have been reported in the genera *Vaccinium*,[7] *Rhododendron*,[8] and *Craibiodendron*.[9] A number of common antioxidant anthocyanins and flavonoids (quercetin, kaempferol, and others), ubiquitous flavonoid aglycones, and some new glycosides of these have been reported in species in the Ericaceae.[10] Cytotoxic grayanotoxin I and dihydrochalcones have been reported from *K. latifolia*.[11] The phytochemistry of *L. buxifolium* has not been reported previously. None of the reported toxins that could be expected are present in the crude active extract of *L. buxifolium* based on our HRMS dereplication results. A bioassay-guided fractionation that led to the compounds responsible for the activity was necessary.

C-Methylflavones have been found in several plant families,[12] although they have been most extensively studied in the Myrtaceae.[12a,b] *C*-Methylflavones show a variety of different activities. The 6,8-dimethylkaempferol 3-*O*-methyl ether and oleanolic acid were found to be cytotoxic to a leukemia cell line.[13] The 4′,5-dihydroxy-6,8-dimethyl-7-methoxyflavanone from *Callistemon lanceolatus* DC. (Myrtaceae) showed a promising neuroprotective effect.[14] Sideroxylin (**2**), isolated from *Hydrastis canadensis* L. (Ranunculaceae), has antibacterial properties.[12a] There is also a report of **2** in *K. latifolia* (Ericaceae), although no bioactivity was reported.[12h] Our report is the first of the *C*-methylisoflavone **1** and antiproliferative activity comparison of **1** and **2** in mammalian cell lines. The isolation and structure elucidation of **1** and **2**, as well as their antiproliferative activities, are described.

Isosideroxylin (**1**) was isolated as yellow granular crystals. The positive HR-ASAP-MS[15] of **1** showed an $[M + H]^+$ ion at *m/z* 313.1058 (Supporting Information, Figure S1), which, taken together with the ^{13}C NMR data analysis, indicated the molecular formula $C_{18}H_{16}O_5$, indicating 11 indices of hydrogen deficiency. The FT-IR spectrum showed the presence of hydroxy (3300 cm^{-1}) and carbonyl (1750 cm^{-1}) groups (Figure S2). The strong absorbance band at 240−280 nm and the weak absorbance band at 320−380 nm in the UV spectrum were consistent with an isoflavone nucleus (Figure S3).[16] The ^1H NMR spectrum (Table 1, Figure S4) displayed the resonances of two methyl groups (δ_H 2.11, 3H s; δ_H 2.24, 3H s), one methoxy group (δ_H 3.75, 3H s), one deshielded methine proton (δ_H 8.49, 1H s), and four aromatic protons (δ_H 7.38, 2H d; δ_H 6.84, 2H d) that share the same 8.0 Hz coupling constant, suggesting the presence of a *para*-disubstituted aromatic moiety. The ^{13}C NMR (Table 1, Figure S5), DEPT135, and HSQC data (Figures S5 and S6) revealed the presence of two methyls (δ_C 8.64, δ_C 8.55); one methoxy (δ_C 60.8); five methines, of which one was an oxygenated olefinic

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Table 1. NMR Spectroscopic Data (400 MHz for ^{1}H, 100 MHz for ^{13}C, DMSO-d_6) for 1

position	δ_C, type	δ_H (J in Hz)
2	155.1, CH	8.49, s
3	122.8, C	
4	181.6, C	
5	157.4, C	
6	113.6, C	
7	162.7, C	
8	108.8, C	
9	153.2, C	
10	107.9, C	
1′	121.2, C	
2′, 6′	130.5, CH	7.38, d (8.0)
3′, 5′	115.6, CH	6.84, d (8.0)
4′	158.3, C	
CH_3-6	8.64, CH_3	2.11, s
CH_3-8	8.55, CH_3	2.24, s
OCH_3-7	60.8, CH_3	3.75, s
OH-5		13.00, s
OH-4′		5.05, s

methine (δ_C 155.1) and the other four were symmetrical aromatic methines (δ_C 130.5, δ_C 115.6); nine quaternary sp^2 carbons including four oxygenated aromatic carbons (δ_C 162.7, 158.3, 153.2, and 157.4); and a carbonyl carbon (δ_C 181.6), collectively corresponding to the molecular formula, $C_{18}H_{14}$. The remaining two hydrogens were assigned to the two remaining O atoms as OH groups. The HMBC correlations from CH_3-6 (δ_H 2.11) to C-5 (δ_C 157.4), C-6 (δ_C 113.6), and C-7 (δ_C 162.7), from OCH_3-7 (δ_H 3.75) to C-7 (δ_C 162.7), and from CH_3-8 (δ_H 2.24) to C-7 (δ_C 162.7), C-8 (δ_C 108.8), and C-9 (δ_C 153.2) suggested the presence of a pentasubstituted aromatic moiety (Figure 1). The deshielded C-4′ (δ_C 158.3) resonance indicated the presence of a OH and, hence, a *para*-disubstituted aromatic B-ring.

The NMR data of **1** were similar to those of sideroxylin **2**, a flavone of the same molecular formula isolated as another active compound in this study. The aforementioned A-, B-, and C-ring moieties in compound **1** were also observed in **2**, as supported by the ^{1}H−^{1}H COSY and HMBC experiments (Figures S7 and S8). The obvious differences between **1** and **2** were a C-2 methine (δ_C 155.1, δ_H 8.49) in **1** and an oxygenated tertiary sp^2 C-2 (δ_C 164.6) in **2**, as well as a quaternary sp^2 C-3 (δ_C 122.8) in **1** and a C-3 methine (δ_C 103.4, δ_H 6.90) in **2**, suggesting an isoflavone skeleton in **1**. This conclusion was supported by the chemical shifts of the C-2 resonances (δ_C 155.1 in **1** and δ_C 164.6 in **2**), the shifts of the C-3 resonances (δ_C 122.8 in **1** and δ_C 103.4 in **2**), and the HMBC correlations between C-3 (δ_C 122.8) and H-2′ and H-6′ (δ_H 7.38) in **1** and C-2 (δ_C 164.6) and H-2′ and H-6′ (δ_H 7.98) in **2**. The chemical shifts of the C-2 methine (δ_C 155.1, δ_H 8.49) suggested a direct oxygen linkage. Three additional HMBC correlations from H-2 (δ_H 8.49) to C-9 (δ_C 153.2), C-3 (δ_C 122.8), and C-4 (δ_C 181.6) connected the B-ring to the A-ring via the oxygen-C2 chain. The two aromatic rings accounted for eight out of 11 indices of hydrogen deficiency required by the molecular formula. The olefinic C-2−C-3 bond and the C-4 carbonyl group contributed two indices of hydrogen deficiency; the remaining index connected the C-4 carbonyl group to C-10, hence completing the C-ring of an isoflavonoid skeleton.

The deshielded OH-5 (δ_H 13.00) resonance indicated its hydrogen bonding to the C-4 carbonyl group. This left the remaining OH group (OH-4′, δ_H 5.15) for placement as the *para*-substituent of the B-ring. Accordingly, the structure of compound **1** was defined as 4′,5-dihydroxy-7-methoxy-6,8-dimethylisoflavone and named isosideroxylin, the isoflavonoid isomer of the flavone sideroxylin (**2**).

Sideroxylin (**2**) was isolated as dark green crystalline needles. The positive HR-ASAP-MS[15] of **2** showed the $[M + H]^+$ ion at m/z 313.1031, which, taken together with the ^{13}C NMR data analysis, indicated a molecular formula of $C_{18}H_{16}O_5$. The NMR data were in agreement with the data reported for **2**.[14]

Both compounds passed the filter for removal of pan assay interference compounds (PAINS) created by Baell and Holloway[17] and were deemed worthy of antiproliferative activity evaluation.

Compounds **1** and **2** were found to be active in the brine shrimp assay[18] in a dose-dependent manner. The LD_{50} values of **1** and **2** were 20.2 and 80.1 μM, respectively. *C*-Methylated flavones, together with *C*-prenylated flavones and isoflavones, show a wide variety of bioactivities, including anti-influenza,[19] antioxidant,[20] and cytotoxicity.[21] Considering the isoflavone skeleton of **1** and the reported estrogenic activity of isoflavones from plant sources,[22] we chose an ER-positive human breast cancer cell line (MCF-7) and an ER-negative human metastatic breast adenocarcinoma cell line (MDA-MB-231) to investigate the antiproliferative potential of **1** and **2**. The NIH3T3 mouse fibroblast cells were used as a noncancerous control cell line.

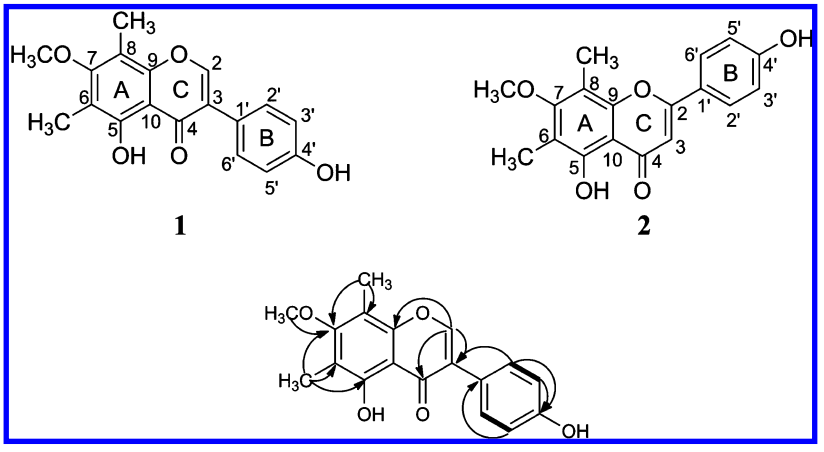

Figure 1. Key COSY (bold bonds) and HMBC correlations (^{1}H → ^{13}C) for compound **1**.

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Table 2. Effects of Compound 1 and 2 on Cell Proliferation[a]

compound	MDA-MB-231	MCF-7	NIH3T3
1	7.0 ± 4.2	>40	>40
2	36.9 ± 0.6	14.7 ± 6.7	>40
podophyllotoxin	0.079 ± 0.009	0.017 ± 0.004	0.084 ± 0.016

[a]The results are expressed as the mean values of IC_{50} ± SD in μM (n = 3).

Podophyllotoxin was used as the positive control. The cells were incubated with the compounds for 72 h. Cell proliferation was assessed by the sulforhodamine B assay.[23] Both compounds exhibited modest antiproliferative activity against the cancer cell lines (Table 2). Compound **1** showed inhibition of the MDA-MB-231 cell line with an IC_{50} value of 7.0 μM and showed weak inhibition against the MCF-7 cell line (IC_{50} > 40 μM). Compound **2** showed inhibition of the MDA-MB-231 and MCF-7 cell lines with IC_{50} values of 36.9 and 14.7 μM, respectively (dose−response curves are in the Supporting Information, Figure S9). Neither compound was inhibitory to the NIH3T3 mouse fibroblasts at the concentrations examined (IC_{50} > 40 μM). The IC_{50} values of the positive control are within the range of previously reported values.[24]

The similarity between the isoflavone skeleton of **1** and isoflavone phytoestrogens suggests a possible estrogenic effect on the ER^+ MCF-7 cell line. The lower potency of **1** against the MCF-7 cell line and the greater potency against the MDA-MB-231 cell line somewhat suggest a phytoestrogenic effect, but the lack of growth stimulation in the MCF-7 cells, when treated with low concentrations of **1**, does not support the hypothesis of a phytoestrogenic effect of **1**. Among a collection of 6,8-dimethyl-substituted flavones, 7-OCH_3 and 5-OH substitution showed higher cytotoxicities than other substitution patterns; C-4′ methoxylation exhibited even higher cytoxicity.[25] Since **1** and **2** share the A-ring substitution pattern, we concluded the 5-hydroxy-7-methoxy-6,8-dimethyl substitution pattern on the A-ring is essential for the observed antiproliferative activity. In addition, 4′-*O*- methylation may similarly increase potency of compounds **1** and **2**. This is the first report of the presence of an isoflavone in the Ericaceae. The presence of a *C*-methylisoflavone in the Ericaceae supports the presence of an unreported isoflavone synthesis pathway in this family.

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were measured on a DigiMelt MPA160 melting point apparatus (SRS, USA), and the uncorrected values are reported. NMR spectra were acquired on a Bruker Avance (400 MHz for ^1H and 100 MHz for ^{13}C) spectrometer (Billerica, MA, USA) equipped with a Bruker 5 mm BBO probe with DMSO-d_6 as solvent and internal standard (^1H, 2.50 ppm). HRASAPMS[15] data were acquired with an atmospheric solids analysis probe (ASAP) interfaced with an Orbitrap Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Attenuated total reflectance Fourier transform-infrared spectroscopy (ATR-FT-IR) was recorded as a thin film on a ZnSe plate on a Nicolet iS10 Smart Performer spectrometer (Thermo Scientific, USA). Low-pressure column chromatographic separations were accomplished with an open column (30 × 500 mm) on silica gel (grade 60, 230−400 mesh) (Thermo Scientific, USA). HPLC isolations and analyses were performed on an Agilent 1100 dual-pump system equipped with a model G1315B DAD detector (200−600 nm) and a model G1313A autosampler (Santa Clara, CA, USA). HPLC-grade solvents (Sigma-Aldrich, St. Louis, MO, USA) were used throughout.

Plant Material. The above-ground parts of *L. buxifolium* were collected from the NJ pinelands (N39.702426 W74.386216) in June of 2012 and 2013 and authenticated by one of us (J.R.P.). Voucher specimens are deposited at the herbarium of the University of the Sciences in Philadelphia. Fresh leaves were separated and freeze-dried.

Extraction and Isolation. Dried leaves of *L. buxifolium* (166 g) were ground and extracted four times with 1.5 L of CH_2Cl_2/MeOH (1:1, v/v) at room temperature on a rotary shaker (150 rpm, 24 h). The extracts were pooled, and CH_2Cl_2 was removed *in vacuo*. The remaining extract (1.8 L) was combined with 100 mL of HPLC H_2O and partitioned with petroleum ether (1.8 L). The MeOH fraction (1.8 L) was applied to a 40 g polyamide (Sigma-Aldrich) open column and eluted with five column volumes of CH_2Cl_2/MeOH (1:1) to remove tannins. The 2.8 L polyamide elution was coated onto 90 g of diol resin (Grace, Columbia, MD, USA), loaded onto 90 g of uncoated diol resin, and eluted by flash chromatography with *n*-hexane, CH_2Cl_2, EtOAc, MeOH, and H_2O (200 mL each) in two batches. The fractions were assessed in the brine shrimp lethality assay,[18] and the active EtOAc fraction (117LE23, 3.1240 g), which gave greater than 50% lethality, was further separated by silica gel flash chromatography with *n*-hexane/EtOAc mixtures of increasing polarities (1:3 to 1:5), affording fractions A−H. A portion of the active fraction A (100% lethality in the brine shrimp lethality assay) was further fractionated by preparative TLC developed with *n*-hexane/EtOAc (7:3), affording fractions 1−12. Profiling chromatography of the active fractions 6−10 was done by isocratic HPLC (MeOH/H_2O, 70:30, v/v, with 0.2% HOAc, 0.6 mL/min) on a Beckman C_{18} column (4.6 × 250 mm, 5 μm), 28 °C. Fraction 6 was further purified by preparative TLC and developed with *n*-hexane/EtOAc (1:1), affording subfractions 1−6. The most active subfraction, 3, was further purified on a YMC C_{18} column (10 × 250 mm, 4 μm) under isocratic conditions (MeOH/H_2O, 80:20, v/v, 3.0 mL/min), yielding compound **1** (6.5 mg) at t_R 15 min. Compound **2** (0.6 mg) was crystallized from the fraction 7 solution at 4 °C.

*Isosideroxylin (**1**):* yellow granular crystals; mp 159.1−160.9 °C; UV (MeOH) λ_{max} (log ε) 270 (3.28), 350 (3.13) nm; FT-IR (film) ν_{max} 3300 (−OH), 2905, 1650 (C═O), 1600, 1525, 1450, 1210, 1090, 1010, 805 cm^{-1}; ^1H (400 MHz) and ^{13}C (100 MHz) NMR (DMSO-d_6), see Table 1; HR-ASAP-MS m/z 313.1058 $[M + H]^+$ (calcd for $C_{18}H_{17}O_5$, 313.1078).

*Sideroxylin (**2**):* dark green crystalline needles; mp 243.0−245.9 °C; UV (MeOH) λ_{max} (log ε) 280 (3.46), 340 (3.45) nm; HR-ASAP-MS m/z 313.1031 $[M + H]^+$ (calcd for $C_{18}H_{17}O_5$, 313.1078). The MS and 1D and 2D NMR data matched the literature values.

Cytotoxicity and Antiproliferative Assays. The toxicities of the crude extract and fractions were evaluated against brine shrimp (*Artemia salina*).[18a,c] Samples were dissolved in DMSO and added into 48-well plates in artificial seawater at a final concentration of 50 μg/mL; each well contained 10 newly hatched (24 h) brine shrimp. Brine shrimp lethality was determined after 24 h by counting the number of dead shrimp in each well.[18b]

The sulforhodamine B (SRB) (Sigma-Aldrich) assay[26] was used to test the effects of **1** and **2** on the growth of estrogen receptor positive (ER^+) MCF-7 and estrogen receptor negative (ER^-) MDA-MB-231 human breast cancer and the NIH3T3 mouse fibroblast cell lines. All of the cultures showed morphologies that were consistent with these cell lines. Podophyllotoxin in DMSO and DMSO (0.5%) were used as the positive and negative controls, respectively. The compounds were dissolved in DMSO before dilution with sterile distilled H_2O to known concentrations and plated into each well.[26] The cell lines were seeded into the 96-well plates in growth medium (EMEM + 10% NuSerum for the MDA-MB-231 line, EMEM + 10% FBS for the MCF-7 line, and DMEM + 5% NuSerum +1% antibiotics for the NIH3T3 line) at 1 × 10^4 cells/well. The final concentration of the test compounds was

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1.25, 2.5, 5, 10, 20, and 40 μM in each well. The cells were incubated for 72 h (37 °C, 5% CO_2, 95% RH). A plate containing only the cell suspension in medium was plated 3 h before the 72 h end point as a nongrowth control (day 0). The cells in each well were fixed by addition of 50 μL of 50% (w/v) trichloroacetic acid (TCA) (4 °C) to a final TCA concentration of 10% followed by incubation at 4 °C for 1 h. The plates were washed six times with demineralized H_2O and air-dried. The dried plates were stained with 50 μL of 0.4% (w/v) SRB in 1% HOAc for 1 h at room temperature. The plates were rinsed quickly six times with 1% HOAc to remove unbound SRB and again air-dried. The bound SRB was dissolved in 100 μL of 10 mM unbuffered Tris base. Dissolved SRB was determined at A_{510} with a microplate spectrophotometer (Spectra Max Plus 384, Molecular Devices, Sunnyvale, CA, USA).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Copies of spectra for compound **1** and the dose response curves of **1** and **2**. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.5b00100.

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Notes

The authors declare no competing financial interest.

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