1-1-2014

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Abstract

A new 1,6-benzoxazocine-5-one alkaloid has been isolated as its butyl acetal derivative (1) along with peristrophine from the n-BuOH and EtOAc fractions of the crude MeOH extract of the aerial parts of Peristrophe lanceolaria growing in Thailand. The structures of these compounds were elucidated on the basis of their spectroscopic data. These compounds were isolated for the first time from P. lanceolaria. The EtOAc and n-BuOH fractions also possessed significant antioxidant activity with IC50 values of 57 and 50 µg/mL, respectively (DPPH method), whereas 1 had an IC50 value of 23 µg/mL.

Disciplines

Medicine and Health Sciences | Social and Behavioral Sciences

Publication Details

Prapalert, W., Santiarworn, D., Liawruangrath, S., Liawruangrath, B. & Pyne, S. G. (2014). A new 1,6-benzoxazocine-5-one alkaloid isolated from the aerial parts of Peristrophe lanceolaria. Natural Product Communications: an international journal for communications and reviews, 9 (10), 1433-1435.

A New 1,6-Benzoxazocine-5-one Alkaloid isolated from the Aerial Parts of *Peristrophe lanceolaria*

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A new 1,6-benzoxazocine-5-one alkaloid has been isolated as its butyl acetal derivative (**1**) along with peristrophine from the *n*-BuOH and EtOAc fractions of the crude MeOH extract of the aerial parts of *Peristrophe lanceolaria* growing in Thailand. The structures of these compounds were elucidated on the basis of their spectroscopic data. These compounds were isolated for the first time from *P. lanceolaria*. The EtOAc and *n*-BuOH fractions also possessed significant antioxidant activity with IC_{50} values of 57 and 50 µg/mL, respectively (DPPH method), whereas **1** had an IC_{50} value of 23 µg/mL.

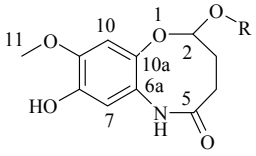
Keywords: 1,6-Benzoxazocine-5-one alkaloid, Antibacterial activity, Antioxidant activity, *Peristrophe lanceolaria*.

The plant *Peristrophe lanceolaria* (Acanthaceae) is widely distributed in warm temperate and tropical regions of Africa and Asia, especially in the north of Thailand. It has been used as a traditional medicine for treating glossitis, smallpox, infected wounds, rashes, and fever [1]. A previous report on the bioactivity of the CH_2Cl_2 extract of *P. lanceolaria* showed cytotoxic activity against a T-lymphoblast cell line and the ability to scavenge TPA-induced superoxide anion radical formation in differentiated HL-60 cells, whereas the MeOH extract was able to inhibit the superoxide anion radical. Both the CH_2Cl_2 and MeOH extracts showed antiplasmodial and antioxidant activities in an oxygen radical antioxidant capacity assay [2]. Previous phytochemical investigations of this plant revealed the presence of pelargonidin-3-β-glucoside [3-4]. In the current study we determined the antioxidant and antibacterial activities and chemical composition of the extract from the aerial parts of *P. lanceolaria.*

Our preliminary phytochemical screening of the crude methanol extract of *P. lanceolaria* revealed the presence of saponins, steroids and flavonoids.

The antibacterial activity of the methanol extract using the agar disc diffusion method exhibited moderate activity against *Bacillus subtilis* at pH 6 and pH 7.2 with inhibition zones of 8.0 and 10.0 mm, respectively. This extract was inactive against *Micrococcus luteus*, *Bacillus cereus* and *Escherichia coli*.

The methanol extract exhibited significant capacity to scavenge the DPPH radical with an IC_{50} value of 176 µg/mL. The *n*-hexane fraction and the aqueous fraction also showed the capacity of scavenging the DPPH radical with IC_{50} values of 227 and 148 µg/mL, respectively. The EtOAc and *n*-BuOH extracts exhibited the highest antioxidant activity with IC_{50} values of 57 and 50 µg/mL, respectively. These two extracts were selected for purification by column chromatography. Two alkaloids, peristrophine and compound **1** were isolated. Their structures were elucidated through



1 : R = Bu
2 : R = Me
3 : R = H

Figure 1: Chemical formulas of compounds **1**-**3**.

extensive analysis of their ^1H and ^{13}C NMR spectroscopic and MS data, especially 2D-NMR, including, ^1H-^1H COSY, HSQC, NOESY 1D, and HMBC, as well as comparison with either known or related compounds. Compound **2** was prepared from compound **1** by treatment with methanol using camphor sulfonic acid (CSA) as a catalyst. Compounds **1** and **2** showed higher activity against DPPH radicals than the EtOAc and *n*-BuOH extracts, with IC_{50} values of 23 and 8 µg/mL, respectively.

The ^1H NMR spectroscopic data of peristrophine were in full agreement with those already reported in the literature for this compound [5]. The mass spectrum of peristrophine showed a molecular-ion peak at *m/z* 272 (M^+) and in the HR-ASAP-MS at *m/z* 273.0869 $[M+H]^+$ (calcd for 273.0875), which corresponded to $C_{14}H_{12}N_2O_4$.

Compound **1** was isolated as a brick-red amorphous powder. The positive HR-ASAP-MS showed the molecular ion peak at *m/z* 296.1498 $[M+H]^+$ (calcd for $C_{15}H_{22}NO_5$ 296.1498) corresponding to $C_{15}H_{21}NO_5$ (M = 295). The ^1H and ^{13}C NMR spectra of **1** showed two aromatic proton signals at δ 6.66 (1H, *s*, H-10) and δ 6.60 (1H, *s*, H-7), which were correlated in the HSQC spectrum to ^{13}C NMR signals at δ 105.1 (C-10) and δ 109.6 (C-7), respectively. The assignments of the other protons and carbons were established by HSQC and HMBC correlations. An aromatic methoxy group showed as a singlet resonance at δ 3.80 (3H, s, H-11) which was correlated to the resonance at δ 56.5 (C-11). The methine proton, with a downfield ^1H NMR resonance at δ 5.13 (1H, d, *J*= 5.5 Hz, H-2) and an attached carbon at δ 93.0 (C-2), suggested an acetal

structure at C-2. The position of H-2 was also supported by a NOESY experiment which showed a through space interaction with protons H-1′ and H-10. The characteristic signals for two pairs of diastereotopic protons observed at δ 2.19 (1H, dd, ^2J = 13.0, J(3,4) = 9.0 Hz, H-3) and δ 2.33 (1H, m, H-3) and at δ 2.49 (1H, dd, ^2J = 13.0, J(4,3) = 9.0 Hz, H-4) and δ 2.80 (1H, dd, ^2J = 17.5, J (4,3) = 9.0 Hz, H-4), were attached to C-3 (δ 25.8) and C-4 (δ 28.7), respectively. The HMBC spectrum indicated that the carbonyl carbon at δ 175.9 (C-5) was adjacent to C-4 (δ 28.7). The chain of acyclic aliphatic proton resonances at δ 3.47 (2H, m, H-1′), δ 1.57 (2H, m, H-2′), δ 1.35 (2H, m, H-3′), and δ 0.90 (3H, t, J = 7.5 Hz, H-4′) were associated with their attached carbons, C-1′ (δ 67.6), C-2′ (δ 31.5), C-3′ (δ 19.1) and C-4′ (δ 13.7), respectively.The optical rotation of **1** ($[\alpha]_D^{25}$ +29.2, *c* 0.00525 g/mL, $CHCl_3$) confirmed the presence of a chiral carbon; however, the absolute configuration of **1** was not determined.

We initially suspected that **1** was not a natural product but an artifact produced from the reaction of the natural product, the hemi-acetal compound **3**, with the *n*-butanol used in the extraction processes. Compound **3**, however, could not be isolated from the crude extracts nor could its methyl acetal analogue **2**, which could have formed from **1** or **3** by an acetal exchange reaction with the MeOH used extensively in the separation and chromatography processes. However, the significant optical rotation of **1**, $[\alpha]_D^{25}$ +29.2 (*c* 0.53, $CHCl_3$), would indicate that it was formed enzymatically and was a natural product.

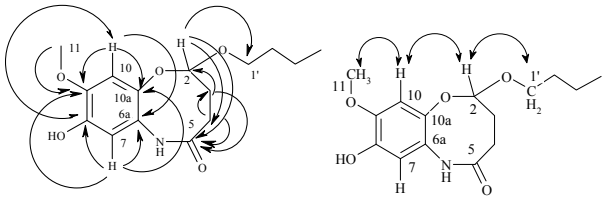


Figure 2: HMBC and NOESY 1D correlations of compound **1**.

Further support for the acetal feature of **1** was its conversion to **2** upon stirring a solution of **1** in methanol containing a catalytic amount of camphor sulfonic acid. This produced the corresponding methyl acetal derivative **2**, as a racemate, in 87% yield. Compound **1** was notably stable in methanol solution at neutral pH and thus the possibility of **1** arising from the reaction of hypothetical **3** with *n*-butanol in the extraction process under neutral conditions seems unlikely.

Compound **2** was a brick-red amorphous powder. The ^{1}H and ^{13}C NMR spectra of **2** were quite similar to those of **1**, the only difference being the absence of the *O*-butoxy group, but a sharp singlet resonance for a methoxy group was represented at δ 3.40 (3H, s, H-2′). The positive HR-ASAP-MS showed the molecular ion peak at *m/z* 254.1040 $[M+H]^+$ (calcd for $C_{12}H_{16}NO_5$ 254.1028) corresponding to $C_{12}H_{16}NO_5$ (M = 253).

Experimental

General procedures: ^{1}H, ^{13}C and 2D NMR spectra were recorded relative to $CDCl_3$ (δ= 7.26 and 77.0 ppm, respectively) on a Varian Unity (VNMRS PS54 500 MHz) NMR spectrometer with tetramethylsilane (TMS) as the internal standard. Low resolution electrospray ionization mass spectra (LR-ESI-MS) were measured on a Waters Platform LCZ (Single Quadrupole) mass spectrometer. High resolution atmospheric solids analysis probe mass spectra (HR-ASAP-MS) were obtained with a Waters Xevo (Quadrupole Time-of-flight) mass spectrometer using leucine enkephalin as an internal standard. Optical rotations were measured using a Jasco P-2000 polarimeter (USA) in a 10 cm path length cell. TLC analysis were performed on precoated silica gel aluminum-backed plates (60 GF_{254} silica gel, Merck Germany), and the compounds were first viewed under UV light at λ 254 nm and 365 nm, then stained either with Dragendorff's reagent (5 mL of solution A (1.7 g $BiO(NO_3)$ in 100 mL 20% AcOH) + 5 mL of solution B (40 g KI in 100 mL water) + 20 mL AcOH + 70 mL water) or stained with cerium-ammonium-molybdate, CAM (400 mL 10% H_2SO_4 (aq.), 10 g ammonium molybdate, 4 g cerric ammonium sulfate) followed by heating. Column chromatography was carried out using silica gel (40-630 μm, Merck). *N*-hexane or light petroleum, ethyl acetate and methanol were used as mobile phases with gradient elution.

Plant material: The aerial parts of *P. lanceolaria* were collected from the Medicinal Plant Garden, Faculty of Pharmacy, Chiang Mai University (CMU), Thailand. A voucher specimen (voucher number Prapalert W.2) was deposited at the herbarium of the Department of Biology, CMU. Plant material was identified by Mr James F. Maxwell from the Department of Biology, CMU.

Extraction: The aerial parts of *P. lanceolaria* (21 kg) were air dried for 2 days and then dried at 50ºC in a hot-air oven for 24 h and ground into powder. The dried plant powder (4.074 kg) was extracted with 10 L methanol for 7 days and then filtered. The residue was extracted again with methanol (3 x 5 L) for 21 days (7 days for each extraction). The filtrates were evaporated under vacuum to give the crude MeOH extract as a dark green residue (497.4 g).

Extraction and isolation of the bioactive compounds: The crude MeOH extract (497.4 g) was suspended in 400 mL of water and partitioned with *n*-hexane (3 x 800 mL) to yield the crude *n*-hexane extract (dark green sticky syrup, 89.6 g). The aqueous layer was then extracted with ethyl acetate (3 x 600 mL) and *n*-butanol (3 x 500 mL) to yield the crude ethyl acetate extract (dark brown solid, 7.8 g) and the crude *n*-butanol extract (red-brown solid, 39.0 g), respectively. A portion of the ethyl acetate extract (3.20 g) was separated by CC on flash silica gel (40-630 mesh, 150 g) using gradient elution from 1:1-0:100 *n*-hexane/EtOAc followed by 98:2-70:30 EtOAc/MeOH to give 4 fractions. Fraction 3 (2.55 g) was then subjected to CC over silica gel (40-630 mesh, 130 g) using gradient elution from 80:20 - 0:100 *n*-hexane/EtOAc to afford 5 fractions. Fraction 2 (256 mg) was then subjected to CC over silica gel (40-630 mesh, 40 g) using gradient elution from 95:5-70:30 *n*-hexane/EtOAc to provide 4 fractions. Fraction 4 (20 mg) was purified by preparative TLC (30:70 *n*-hexane/EtOAc) over Kieselgel 60 F_{254} (Merck) to yield pure peristrophine (7 mg). A portion of the *n*-butanol extract (5.54 g) was subjected to flash CC over silica gel (40-630 mesh, 250 g) using gradient elution from 100:0-70:30 EtOAc/MeOH; 148 fractions were collected (50 mL each) and their composition was monitored by TLC; those fractions showing similar TLC profiles were grouped into 8 major fractions. Fraction 2 (800 mg) was then subjected to flash CC over silica gel (40-630 mesh, 50 g) using gradient elution from 95:5-0:100 *n*-hexane/EtOAc, followed by 10:90-1:1 EtOAc/MeOH; 130 fractions were collected (30 mL each) and monitored by TLC, then grouped into 11 major fractions. Fraction 5 (46 mg) was further purified by CC over silica gel (40-630 mesh, 10 g) using gradient elution from 100:0-98:2 EtOAc/MeOH to yield pure compound **1** (10.5 mg).

Preparation of compound 2: Compound **1** was converted to its methyl acetal derivative by treatment with camphor sulfonic acid (CSA) in MeOH to give **2** in 87% yield. In brief, 4.5 mg of **1** was dissolved in 0.5 mL of MeOH, then 10 μL of 1% CSA was added,

and stirred at room temperature for 18 h. After evaporation of the solvent, the residue was dissolved in 1 mL CH_2Cl_2 and washed with saturated $NaHCO_3$ solution. Then, the product was further purified by CC over silica gel using ethyl acetate as eluent to yield **2** (3.4 mg).

Phytochemical screening of the methanol extract: The crude MeOH extract was screened for phytochemical constituents using the methods described by Chhetri *et al*. [6] and Egwailhide and Gimba [7].

Antibacterial activity: The MeOH extract was tested for antimicrobial activity against *Bacillus subtilis* BGE, *Micrococcus luteus* ATCC 9341, *B. cereus* ATCC 11778, and *Escherichia coli* ATCC 11303 using the agar disc diffusion method. Penicillin, sulfadimidine, streptomycin, erythromycin, oxytetracyclin and ciprofloxacin were used as the positive controls. In brief, working organism suspensions were added to test agar, pH 6, 7.2 and 8 (working trimethoprim suspension was added to the test agar at pH 7.2). A paper disc (6 mm in diameter) was placed on the surface of the agar and soaked with 10 µL of 500 mg/mL of the extract solutions. After incubating at 30-37°C for 24 h, the antibacterial activities were evaluated by measuring the diameter of the inhibition zones.

Antioxidant activity: The antioxidant activities of the extracts were studied by measuring the scavenging activity on DPPH, *2,2′*-diphenyl-*1*-picrylhydrazyl, free radicals [8].

Peristrophine, 2-amino-7,8-dimethoxy-phenoxazine-3-one
Brick-red, amorphous powder.
^{1}H NMR (500 MHz, $CDCl_3$, δ): 7.21 (1H, s, H-9), 6.92 (1H, s, H-6), 6.50 (1H, s, H-1), 6.44 (1H, s, H-4), 5.06 (2H, br s, NH_2-11), 3.99 (3H, s, OCH_3-1), 3.97 (3H, s, OCH_3-13).
^{13}C NMR (125 MHz, $CDCl_3$, δ): 179.6 (C-3), 151.5 (C-7), 149.6 (C-10a), 147.6 (C-8), 146.3 (C-4a), 145.6 (C-2), 138.4 (C-5a), 128.6 (C-9a), 109.2 (CH-9), 103.7 (CH-4), 101.1 (CH-1), 98.7 (CH-6), 56.7 (OCH_3-12), 56.5 (OCH_3-13).
HR-ASAP-MS: *m/z* $[M+H]^+$ calcd for $C_{14}H_{13}N_2O_4$: 273.0875; found: 273.0869.

Compound 1
Brick-red, amorphous powder.
$[\alpha]_D^{25}$: +29.2 (*c* 0.53, $CHCl_3$).
^{1}H NMR (500 MHz, $CDCl_3$, δ): 6.66 (1H, s, H-10), 6.60 (1H, s, H-7), 5.13 (1H, d, *J* = 5.5 Hz, H-2), 3.80 (3H, s, H-11), 3.47 (2H, m, H-1′), 2.80 (1H, dd, ^2J = 17.5, *J*(4,3) = 9 Hz, H-4), 2.49 (1H, dd, ^2J = 13, *J*(4,3) = 9 Hz, H-4), 2.33 (1H, m, H-3), 2.19 (1H, *dd*, ^2J = 13, *J*(3,4) = 9 Hz, H-3), 1.57 (2H, m, H-2′), 1.35 (2H, m, H-3′) 0.90 (3H, t, *J* = 7.5 Hz, H-4′).
^{13}C NMR (125 MHz, $CDCl_3$, δ): 175.9 (C-5), 147.0 (C-10a),146.5 (C-8), 140.6 (C-9), 115.9 (C-6a), 109.6 (CH-10), 105.1 (CH-7), 93.0 (CH-2), 67.6 (CH_2-1′), 56.5 (CH_3-11), 31.5 (CH_2-2′), 28.7 (CH_2-4), 25.8 (CH_2-3), 19.1 (CH_2-3′),13.7 (CH_3-4′).
Positive HR-ASAP-MS: *m/z* $[M+H]^+$ calcd for $C_{15}H_{22}NO_5$: 296.1498; found: 296.1498.

Compound 2
Brick-red, amorphous powder.
^{1}H NMR (500 MHz, $CDCl_3$, δ): 6.67 (1H, s, H-10), 6.64 (1H, s, H-7), 5.12 (1H, d, *J*= 5.9 Hz, H-2), 3.83 (3H, s, H-11), 3.40 (1H, s, H-1′), 2.80 (1H, dd, ^2J = 17.6, *J*(4,3) = 9.3 Hz, H-4), 2.52 (1H, dd, ^2J = 17.6, *J*(4,3) = 9.3 Hz, H-4), 2.33 (1H, m, H-3), 2.22 (1H, dd, ^2J = 12.2, *J*(3,4) = 9.3 Hz, H-3).
^{13}C NMR (125 MHz, $CDCl_3$, δ): 175.9 (C-5), 146.8 (C-10a), 146.5 (C-8), 140.6 (C-9), 115.9 (C-6a), 108.9 (CH-10), 105.4 (CH-7), 94.1 (CH-2), 56.5 (CH_3-11), 54.6 (CH_3-1′), 28.8 (CH_2-4), 25.3 (CH_2-3)
Positive HR-ASAP-MS: *m/z* $[M+H]^+$ calcd for $C_{12}H_{16}NO_5$: 254.1028; found: 254.1040.

Acknowledgments - We gratefully acknowledge the Commission on Higher Education for financial support. We also thank the Graduate School, Faculty of Pharmacy, Chiang Mai University, and the School of Chemistry, University of Wollongong for laboratory facilities. We would like to thank National Research University (NRU), CHE Ministry of Education Thailand.

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