Eucophylline, a Tetracyclic Vinylquinoline Alkaloid from *Leuconotis eugenifolius*

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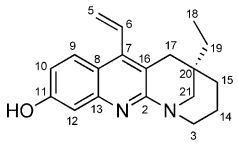
Eucophylline (**1**), a new tetracyclic vinylquinoline alkaloid, was isolated from the bark of *Leuconotis eugenifolius* together with leucophyllidine (**2**). The structure and absolute configuration of **1** were elucidated on the basis of 2D NMR correlations and simulated CD analysis. Leucophyllidine (**2**) showed iNOS inhibitory activity and decreased the iNOS protein expression dose-dependently.

Leuconotis eugenifolius DC. (Apocynaceae) is found in Sumatra, Borneo, and peninsular Malaysia. It is restricted to the northwest region. Medicinally, the latex of this plant was once used for the treatment of worm infections and yaws by applying it on infected skin.[1] Recently, a new type of bisindole alkaloid, leucophyllidine,[2] and a tetracyclic ring-opened oxindole alkaloid, leucolusine,[3] were isolated from *Leuconotis griffithii*. We also recently reported new bisindole alkaloids, bisleuconothine A[4] and bisleucocurine A,[5] from *L. griffithii*, which showed cell growth inhibitory activity against various human cancer cell lines. Our efforts to identify biogenetically interesting alkaloids with biological activity from *L. eugenifolius* led to the isolation of a new tetracyclic vinylquinoline alkaloid, eucophylline (**1**). This paper describes the structure elucidation of **1** on the basis of spectroscopic data and simulated CD analysis by Turbomole 6.1 and iNOS inhibitory activity of leucophyllidine (**2**).



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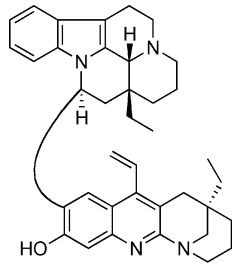

2

The alkaloidal fraction prepared from a MeOH extract of the bark of *L. eugenifolius* was subjected to Sephadex LH-20, silica gel, and amino silica gel columns and ODS HPLC to give eucophylline (**1**, 1.9 mg, 0.00039%) together with leucophyllidine (**2**),[2] eburnamine,[6] leuconolam,[7] and rhazinilam.[8] Eucophylline (**1**), brown powder, $[\alpha]^{27}_{D}$ +27 (*c* 0.75, MeOH), showed the molecular formula $C_{19}H_{22}N_2O$, which was determined by HRESITOFMS [*m*/*z* 295.1816 $(M + H)^+$, Δ +0.6 mmu]. The UV spectrum (227 and 347 nm) indicated a vinylquinoline system.[8] IR absorption was characteristic of an amino or hydroxyl group (3680 cm^{-1}). ^1H and ^{13}C NMR data (see Experimental Section) suggested the presence of six sp^3 methylene, one sp^3 quaternary, one sp^2 methylene, four sp^2 methine, and six sp^2 quaternary carbons and one methyl group. Among them, two sp^3 methylenes (δ_C 56.8; δ_H 3.16 and 3.65, and

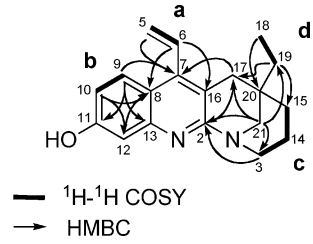

Figure 1. Selected 2D NMR correlations for eucophylline (**1**).

δ_C 58.5; δ_H 2.90 and 3.10) and three sp^2 quaternary carbons (δ_C 148.9, 160.0, and 162.3) were ascribed to those bearing a nitrogen or an oxygen atom. The gross structure of **1** was deduced from detailed analyses of two-dimensional NMR data, including $^1H-^1H$ COSY, HSQC, and HMBC spectra in CD_3OD (Figure 1). The $^1H-^1H$ COSY and HSQC spectra revealed connectivities of four partial structures, **a** (C-5−C-6), **b** (C-9−C-10), **c** (C-3, C-14−C-15), and **d** (C-18−C-19), as shown in Figure 1. The connectivity of partial structures **a** and **b** was revealed by the HMBC correlations of H-5 and H-9 to C-7 (δ_C 146.5) and of H-6 to C-8 (δ_C 120.0). HMBC correlations of H-18 to C-20 (δ_C 31.7), H-19 to C-15 (δ_C 37.0) and C-17 (δ_C 37.6), and H-21 to C-3 (δ_C 56.8), C-17, and C-19 (δ_C 36.1) established the connections between partial structures **c** and **d**. The correlations from H-10 to C-12 (δ_C 109.1) and from H-9 to C-11 (δ_C 160.0) established an OH at C-11, and chemical shifts of C-13 (δ_C 148.9) and C-2 (δ_C 162.3) indicated the presence of an imine function. Furthermore, HMBC correlations of H-17 to C-2 and C-7, and H-6 to C-16 (δ_C 122.6), established the connections among C-2, C-7, and C-17 through C-16. The presence of a 4-vinylquinolin-7-ol core was indicated from the above observed correlations. HMBC cross-peaks of H-3 and H-21 to C-2 suggested the connection among C-2, C-3, and C-21 through a nitrogen atom. Thus, the gross structure of **1** was assigned as shown in Figure 1. On the basis of the proposed structure, **1** could be implied to be the half unit of a known bisindole alkaloid, leucophyllidine (**2**).[2] Alkaloid **1** showed almost the same ^1H and ^{13}C NMR chemical shifts as those of **2** except for the existence of a proton at C-10 and coupling constants of H-9, -10, and -12.

The relative structure of **1** was assigned by ROESY correlations as shown in a computer-generated 3D drawing (Figure 2). The ROESY correlations of H-14/H-17a and H-15a/H-3a and H-21b suggested that the piperidine ring (N−C(3)−C(14)−C(15)−C(20)−C(21)) of **1** took a chair conformation. The absolute configuration of C-20 was extrapolated by comparing the experimental CD spectrum with the calculated CD spectra performed by Turbomole 6.1[9] using the RI-TD-DFT-BP86/aug-cc-pVDZ[10−14] level of theory on RI-DFT-BP86/TZVP[10−13,15] optimized geometries. The conformer used for

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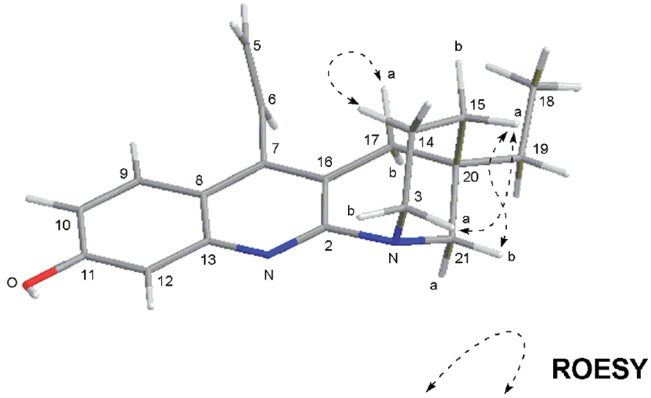


Figure 2. Selected ROESY correlations for eucophylline (**1**).

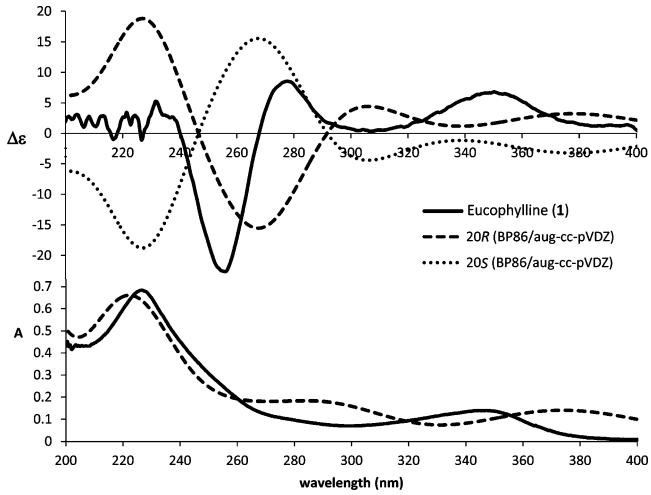


Figure 3. Experimental and calculated UV and CD spectra for (*R*)-**1** and (*S*)-**1**.[16]

CD calculations was the global minimum obtained using Monte Carlo analysis with the MMFF force field in Macromodel 9.1. Experimental and calculated spectra for the 20*R* isomer were in good agreement (Figure 3). Therefore the absolute configuration at C-20 was deduced to be *R*.

A plausible biogenetic pathway for **1** is proposed as shown in Scheme 1. The biosynthetic pathway of the vinylquinoline unit incorporating a tetrahydrobenzo[*b*][1,8]naphthyridine core was also proposed by Kam et al.[2] Biogenesis of the Aspidosperma precursor to the melodinus-type alkaloid might be transformed through a leuconolam derivative by an internal Michael addition.[1] Finally, ring closure via attack of the piperidine NH onto the carbonyl and dehydration in the precursor could lead to eucophylline (**1**).

LPS stimulation in J774.1 cells induces iNOS overexpression, subsequently to the NO synthesis, resulting in response to exposure to inflammatory and immunologic stimuli, and has been implicated in the pathogenesis of numerous diseases including septic shock, asthma, inflammatory bowel disease, osteoarthritis, and rheumatoid arthritis.[17] Leucophyllidine (**2**) inhibited NO production dose-dependently stimulated by LPS (IC_{50} 7.1 μM), although eucophylline (**1**) did not; both compounds showed high cell viability of J774.1 at this concentration. To evaluate the effect on expression of the iNOS protein in J774.1 cells stimulated by LPS, western blotting analysis was performed. When the cells were stimulated by LPS, iNOS protein in the cells was overexpressed, subjected to overproduction of NO. Leucophyllidine (**2**) decreased the iNOS protein expression dose-dependently.

Eucophylline (**1**) is a rare vinylquinoline alkaloid consisting of a naphthyridine core, and this is the second report of isolation of a naphthyridine derivative from a *Leuconotis* species. We also isolated the dimer alkaloid leucophyllidine (**2**), which showed inhibition of NO production, and leuconolam in this extract. The presence of these alkaloids is consistent with the proposed biogenesis of these rare vinylquinoline alkaloids.

Experimental Section

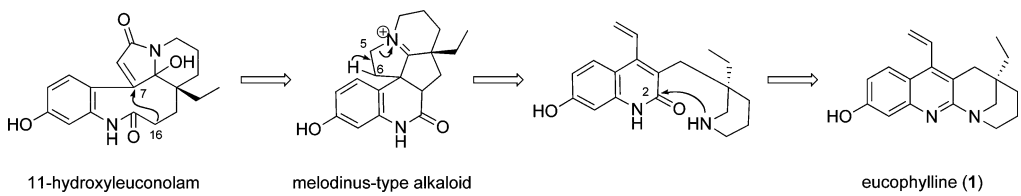
General Experimental Procedures. Optical rotations were measured on a JASCO DIP-1000 polarimeter. UV spectra were recorded on a Shimadzu UVmini-1240 spectrophotometer, and IR spectra on a JASCO FT/IR-4100 spectrophotometer. CD spectra were recorded on a JASCO J-820 polarimeter. Mass spectra were obtained using a Waters ZQ-2000 and a LTQ Orbitrap XL (Thermo Scientific) spectrometer. 1D and 2D NMR spectra were recorded on a Bruker Avance 700 spectrometer, and chemical shifts were referenced to the residual solvent peaks (δ_H 3.31 and δ_C 49.0 for methanol-d_4). Standard pulse sequences were employed for the 2D NMR experiments. HPLC was performed on a CAPCELL PAK C_{18} MG-II, 5 μm (Φ 10 × 250 mm).

Plant Material. Bark of *L. eugenifolius* DC. was collected in Mersing, Malaysia, in 2007. The botanical identification was made by Mr. Teo Leong Eng, Faculty of Science, University of Malaya. A voucher specimen (Herbarium No. KL 5476) is deposited at the Herbarium of the Department of Chemistry, University of Malaya, Kuala Lumpur, Malaysia.

Extraction and Isolation. The bark of *L. eugenifolius* (0.91 kg) was extracted with MeOH, and the extract (51 g) was treated with 3% tartaric acid (pH 2) and then partitioned with EtOAc. The aqueous layer was treated with saturated Na_2CO_3 to pH 10 and extracted with $CHCl_3$ to give an alkaloidal fraction (4.44 g). The alkaloidal fraction was subjected to a Sephadex LH-20 column, further separated using a silica gel column ($CHCl_3$ sat. with NH_4OH/MeOH, 1:0 → 0:1), an amino silica gel column ($CHCl_3$/MeOH, 1:0 → 0:1), and an ODS HPLC (MeOH/0.1% HCOOH(aq), 3:7 → 1:0, flow rate, 2 mL/min; UV detection at 254 nm, t_R 35 min) to give eucophylline (**1**, 1.9 mg, 0.00039%) together with leucophyllidine (**2**, 22.5 mg, 0.044%),[2] eburnamine (0.8 mg, 0.0016%),[6] leuconolam (7.1 mg, 0.0014%),[7] and rhazinilam (4.5 mg, 0.0089%).[8]

Eucophylline (1): brown powder; $[\alpha]^{27}_D$ +27 (*c* 0.75, MeOH); UV (MeOH) λ_{max} 227 (ε 42 000) and 347 (ε 6900) nm; CD (MeOH) λ_{max} 231 ($\Delta\varepsilon$ 1.31), 256 ($\Delta\varepsilon$ −5.67), 278 ($\Delta\varepsilon$ 2.13), 307 ($\Delta\varepsilon$ 0), and 350 ($\Delta\varepsilon$ 1.70) nm; IR (NaCl) ν_{max} 3680 cm^{-1}; ^1H NMR (CD_3OD, 700 MHz) δ 7.95 (1H, d, J = 9.0 Hz, H-9), 7.15 (1H, J = 2.3 Hz, H-12), 7.00 (1H, J = 9.0 Hz, H-10), 6.95 (1H, dd, J = 11.7, 17.9 Hz, H-6), 5.95 (1H, dd, J = 1.7, 11.7 Hz, H-5b), 5.55 (1H, dd, J = 1.7, 17.9 Hz, H-5a), 3.65 (1H, dd, J = 5.5, 5.5 Hz, H-3b), 3.16 (1H, m, H-3a), 3.10 (1H, d, J = 13.1 Hz, H-21b), 2.90 (1H, d, J = 13.1 Hz, H-21a), 2.76 (1H, m, H-17b), 2.60 (1H, m, H-17a), 1.78 (1H, m, H-15b), 1.59 (1H, m, H-15a),1.38 (2H, q, J = 7.5 Hz, H-19), 1.32 (2H, m, H-16), 0.96 (3H, t, J = 7.5 Hz, H-18); ^{13}C NMR (CD_3OD, 175 MHz) δ 162.3 (C, C-2), 160.0 (C, C-11), 148.9 (C, C-13), 146.5 (C, C-7), 133.1 (CH, C-6), 127.6 (CH, C-9), 123.6 (CH_2, C-5a), 122.6 (C, C-16), 120.0 (C, C-8), 118.8 (CH, C-10), 109.1 (CH, C-12), 58.5 (CH_2, C-21a), 56.8 (CH_2, C-3a), 37.6 (CH_2, C-17a), 37.0 (CH_2, C-15a), 36.1 (CH_2, C-19),

Scheme 1. Plausible Biogenetic Path to Eucophylline (**1**)



31.7 (C, C-20), 20.5 (CH_2, C-14), 7.4 (CH_3, C-18); ESIMS *m/z* 295 $(M + H)^+$; HRESITOFMS *m/z* 295.1816 (M + H; calcd for $C_{19}H_{23}N_2O$, 295.1810).

Assay for NO Production by J774.1 Cell Line. The J774.1 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. Cells were seeded onto a 96-well microtiter plate at 1×10^5 cells in 100 μL per well and were preincubated for 12 h at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were cultured in the medium containing LPS (5 μg/mL) with or without the test sample of different concentrations for 24 h. The NO production was then determined by Griess assay. Then, 100 μL of the supernatant of the cultured medium was transferred to a 96-well microtiter plate, and 100 μL of Griess reagent (1% sulfanilamide, 0.1% *N*-l-naphthylethylenediamine dihydrochloride in 2.5% H_3PO_4) was added. After incubation at room temperature for 15 min, the absorbance at 540 and 620 nm was measured with a microplate reader (Benchmark Plus microplate spectrometer, Bio-Rad). L-NMMA (98%) was used as a positive control ($IC_{50} = 22$ μg/mL).

Cell Viability Assay. Cell viability was determined by MTT assay; 15 μL of MTT solution (5 mg/mL) was added into each well of the cultured medium. After further incubation for 2 h, the medium was removed, and then 50 μL of DMSO was added to resolve the formazan crystals. Optical density measurements were made using a microplate reader equipped with a two-wavelength system (550 and 700 nm). Three replicates were prepared for each sample. The ratio of the living cells was determined on the basis of the difference of the absorbance between those of samples and controls.

Western Blotting. Cells treated with test compounds were treated with lysis buffer, and the extract was passed through a 10% SDS-PAGE. The protein fraction was transferred to an Immun-Blot polyvinylidene difluoride membrane (PVDF, Bio-Rad) and blocked for 1 h with 5% skim milk. The blotted membrane was incubated with primary antibodies (diluted 1:1000) for NOS2 (C-19) antibody (Santa Cruz Biotechnology) overnight or 1:2000 for β-actin (Sigma) for 1 h, followed by goat anti-rabbit IgG (Santa Cruz Biotechnology) or mouse IgG (GE Healthcare). Protein bands were visualized with ECL solution (GE Healthcare).

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Supporting Information Available: 1D and 2D NMR spectra of **1** and iNOS protein expression levels by **2** are available free of charge via the Internet at http://pubs.acs.org.

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