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A Bioactive Resveratrol Trimer from the Stem Bark of the Sri Lankan Endemic Plant *Vateria copallifera*

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Abstract

A new resveratrol trimer, vateriferol (**1**), having four *cis*-oriented methine protons and constituting four contiguous stereocenters, was isolated from the bark extract of *Vateria copallifera* by bioassay-guided fractionation using a combination of normal, reversed phase, and size exclusion column chromatography. The structure was established based on its spectroscopic data. Vateriferol (**1**) was evaluated in vitro for its antioxidant capacity, enzyme inhibitory activity, growth inhibitory activity on a number of cancer cell lines, neuroprotective activity, and anti-inflammatory activity. Vateriferol (**1**) exhibited AChE inhibitory activity (IC₅₀ 8.4 ± 0.2 μM), ORAC activity (2079 ± 0.20 TE/g), and neuroprotective activity at 1.5 μM using PC12 cells deprived of oxygen and glucose and lowered NO levels in lipopolysaccharide-stimulated SIM-A9 microglial cells at 14.7 and 73.6 μM. Vateriferol (**1**) exhibited weak cytotoxic potency (<50% growth inhibition) against the tested cell lines at 147.2 μM.

Graphical Abstract

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ASSOCIATED CONTENT

Supporting Information

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¹H, ¹³C, COSY, NOESY, HMQC, and HMBC spectra of vateriferol (**1**); comparison of ¹H and ¹³C chemical shifts of vateriferol (**1**) and the two known isomers melapinol B and rockiiol (PDF)

Notes

The authors declare no competing financial interest.

The genus *Vateria* belongs to the Dipterocarpaceae family and consists of three species, namely, *V. indica*, *V. macrocarpa*, and *V. copallifera*.¹ *Vateria* species are found in the evergreen forests of Western Ghats from North Karnataka to Kerala and in Sri Lanka.² *V. copallifera* (Retz.) Alston is a plant endemic to Sri Lanka, locally known as “Hal”. In the traditional system of medicine, a decoction of the *V. copallifera* bark has been used in the treatment of diarrhea, ulcers, rheumatic pains, and diabetes mellitus.³ It has also been used in arresting fermentation of palm tree sap in the local jaggery industry, and the fruit flour is used in the preparation of food items.^{4,5} Two resveratrol trimers, copalliferol A and copalliferol B, together with stemonoporol, which is isomeric with copalliferol A, have been reported from the acetone extract of the bark of *V. copallifera*.^{4,6} In addition, chemical studies on the bark of *V. copallifera* extracted with boiling petroleum ether (60–80 °C), benzene, and MeOH afforded sitosterol, β amyrin acetate, β -amyrin, and dipterocarpol.⁷

Previous research on the acetone seed extract of *V. copallifera* reported mosquito larvicidal activity against third instar larvae of *Culex quinquefasciatus* and *Aedes aegypti* and antibacterial activity against methicilin-resistant *Staphylococcus aureus*.⁸ As for the isolated compounds, copalliferol A and copalliferol B exhibited antibacterial activity against *Oxford staphylococcus* and *Escherichia coli*.^{4,6}

Previously, melapinol B (2), a stereoisomer with *cis*-8a,7a, *trans*-7a,8b, and *cis*-8b,7b methine protons, was isolated from the EtOH extract of *Vitis amurensis* (Vitaceae) roots and the MeOH extract of *Shorea* sp. “melapi” (Dipterocarpaceae) heartwood (Figure 1).^{9,10} A second stereoisomer, rockiiol C (3), with *trans*-8a,7a, *cis*-7a,8b, and *cis*-8b,7b methine protons, was isolated from the acetone extract of the seed cake of *Paeonia rockii* (Paeoniaceae)¹¹ (Figure 1). The all-*cis* and all-*trans* isomers have not been reported to date. The all-*cis* isomer in particular could be interesting, as the three *cis*-oriented aromatic rings in close proximity can be expected to be arranged parallel in space compared to other stereoisomers (Figure 2), which may affect its interaction with cellular macromolecules and, therefore, the biological properties.

Herein we report the isolation, characterization, and biological activity of vateriferol (1), the first resveratrol trimer with all-*cis* configuration.

RESULTS AND DISCUSSION

Following preliminary bioactivity screening of the EtOH extract of coarsely powdered bark of *V. copallifera*, fresh plant material was extracted successively with hexanes, EtOAc, and

EtOH. The EtOAc and EtOH extracts were combined based on the similarities shown in the bioactivity studies (Table 1) and the TLC profiles. Bioactivity-guided fractionation of the combined extracts by dry column flash chromatography, followed by repeated normal and reversed phase chromatography, and finally by Sephadex LH 20–100 size exclusion chromatography led to the isolation of compound **1** from fraction FA5.

Compound **1** was obtained as a brown, amorphous solid (3.8 mg, 0.0038%), $[\alpha]_D^{25} +145$ (c 0.2, MeOH). The HRESIMS data (m/z 679.1990, $[M + H]^+$, calcd for $C_{42}H_{30}O_9$, 679.1968) together with the complete assignment of 1H and ^{13}C NMR data determined through 1H – 1H COSY, APT, HMQC, and HMBC experiments established the molecular formula as $C_{42}H_{30}O_9$ (Table 2). The UV absorbance maximum at $\lambda = 319$ nm indicated the presence of a strong nonconjugated phenyl ring system. The IR absorption bands at 1451 and 1605 cm^{-1} indicated the presence of aryl rings, and the band at 3303 cm^{-1} indicated the presence of hydroxy groups.

The analysis of ^{13}C NMR and APT spectra established the presence of 42 carbon resonances, including 18 aromatic methine, four sp^3 methine, 10 quaternary, and 10 oxygenated tertiary carbons (Table 2). The 1H NMR spectrum indicated the presence of 22 protons (Table 2). The 1H NMR resonances at δ 6.05 (2H, d, $J = 8.7$ Hz, H-3a, 5a), 6.26 (2H, d, $J = 8.7$ Hz, H-2a, 6a), 6.33 (2H, d, $J = 8.6$ Hz, H-3b, 5b), 6.75 (2H, d, $J = 8.6$ Hz, H-2b, 6b), 6.84 (2H, d, $J = 8.7$ Hz, H-3c, 5c), and 7.44 (2H, d, $J = 8.7$ Hz, H-2c, 6c), together with the ^{13}C NMR data, delineated the presence of a characteristic six sets of AA'BB'-type hydrogens (Table 2; Figures S1 and S2, Supporting Information). Further, two sets of meta-coupled aromatic hydrogens at δ 6.65 (1H, d, $J = 2.5$ Hz, H-14c), 6.34 (1H, d, $J = 2.5$ Hz, H-12c), and 6.10 (2H, d, $J = 2.2$ Hz, H-10a, 14a), and 6.13 (1H, t, $J = 2.2$ Hz, H-12a), an aromatic singlet at δ 6.89 (1H, s, H-12b), and four aliphatic methine hydrogens at δ 5.36 (1H, d, $J = 3.4$ Hz, H-7b), 4.40 (1H, dd, $J = 3.4, 9.3$ Hz, H-8b), 3.85 (1H, dd, $J = 2.3, 9.3$ Hz, H-7a), and 4.68 (1H, d, $J = 2.3$ Hz, H-8a) were present (Table 2; Figure S1, Supporting Information). Comparison of the reported 1H and ^{13}C NMR chemical shifts of the AA'BB'-type hydrogens, meta-coupled aromatic hydrogens, aromatic hydrogen singlets, and the aliphatic hydrogens of melapinol B (**2**) isolated from melapi (*Shorea* sp.) and rockiiol C (**3**) isolated from *Paonia rockii*^{9,11} with those observed for compound **1** indicated a close similarity (Figure 1). A comparison of the 1H and ^{13}C NMR chemical shifts of the three isomers are given in Table S1, Supporting Information. The characteristic chemical shift of the ortho-coupled hydrogens of melapinol B (**2**) was also observed at δ_H 6.26 (H-2a, 6a) in compound **1**.⁹

1H – 1H COSY data displayed couplings between signals δ_H 7.44 (H-2c, 6c) and 6.84 (H-3c, 5c); δ_H 6.75 (H-2b, 6b) and 6.33 (H-3b, 5b); δ_H 6.26 (H-2a, 6a) and 6.05 (H-3a, 5a); δ_H 5.36 (H-7b) and 4.40 (H-8b); δ_H 4.40 (H-8b) and 3.85 (H-7a); and δ_H 3.85 (H-7a) and 4.68 (H-8a) (Figure 3; Figure S3, Supporting Information).

The HMBC correlations between carbon resonance δ_C 51.6 (C-8b) and δ_H 4.68 (8a); δ_C 141.2 (C-9b) and δ_H 3.85 (H-7a), 4.68 (H-8a), 5.36 (H-7b); δ_C 120 (C-10b) and δ_H 6.89 (H-12b); δ_C 116.4 (C-8c) and δ_H 6.65 (H-14c); and δ_C 134.5 (C-9c) and δ_H 6.33 (H-12c) and 4.40 (H-8b), together with correlations between carbon resonances at δ_C 130.7 (C-2b)

and 155.2 (C-11c) and δ_{H} 5.36 (H-7b) and δ_{C} 135.5 (C-1a) with δ_{H} 4.40 (H-8b), further indicated that the six benzene rings (A1, B1, C1 and A2, B2, C2), the four methine carbons, and the olefinic C-7c and C-8c originated from three resveratrol units (A–C) (Figure 3; Figure S5, Supporting Information).

Long-range HMBC correlations were observed between aliphatic protons at δ_{H} 5.36 (H-7b) and 4.40 (H-8b) with quaternary carbons δ_{C} 141.1 (C-9b) and 134.5 (C-9c), respectively. The 2D structure of compound **1** was concluded to be as shown in Figure 3.

The relative configuration of compound **1** was determined by NOESY experiments and taking into consideration the coupling constants of the four methine protons H-7a, H-8a, H-7b, and H-8b in relation to the coupling constants of the aliphatic protons of the two known resveratrol trimers melapinol B (**2**) and rockiiol C (**3**).^{9,11}

NOESY cross-peaks between H-7a/H-8a, H-7b/H-8b, and H-7a-H-8b were observed for the four aliphatic protons (Figure S6, Supporting Information). The coupling constants of H-7b (3.4 Hz) and H-8b (3.4, 9.3 Hz) of **1** and the coupling constants of H-7b (3.3 Hz) and H-8b (3.3, 9.1 Hz) of rockiiol C (**3**) were similar and indicated a *cis* orientation of 7b and 8b, which is supported by the similar NOE interactions observed for H-7b/H-8b of compound **1** compared to rockiiol C (**3**). The coupling constants of 9.3 Hz of H-7a and 9.3 Hz of H-8b of compound **1** were in agreement with the coupling constants of 9.3 and 9.1 Hz, respectively, observed for the same protons of rockiiol C (**3**), indicating their *cis* orientation, which was confirmed by their NOE interactions. However, the coupling constant of 2.3 Hz of H-8a and H-7a of compound **1** were not in agreement with the coupling constant of 1.5 Hz of the *trans*-oriented H-8a and H-7a of rockiiol C (**3**), but were more consistent with the coupling constants of 2.2 and 2.4 Hz, respectively, of the *cis*-oriented H-8a and H-7a of melapinol B (**2**). This, along with the NOE interaction observed between H-7a and H-8a of compound **1** as in melapinol B (**2**) confirms the *cis* orientation of the two protons (Figure 4). Therefore, the *cis* orientation of the four aliphatic protons was evidenced by their vicinal coupling constants and reinforced by NOESY interactions. A weaker, but clear NOE interaction between H-7b and H-7a of compound **1** confirmed their *cis* orientation.

The stereochemical difference between melapinol B (**2**), rockiiol C (**3**), and vateriferol (**1**) lies in the orientation of the four aliphatic protons (Figures 1 and 4). All four aliphatic protons of compound **1** are *cis*-oriented. On the basis of these results, compound **1** is identified as a new compound and named vateriferol (**1**) with relative configuration as depicted in Figure 4. To determine the absolute configuration, the electronic circular dichroism (ECD) spectrum of vateriferol (**1**) was measured in MeOH and compared with the computed ECD spectra of the two enantiomers generated by density functional theory (DFT) and time-dependent density functional (TDDFT) theory calculations using the Gaussian16 program package. The experimental ECD spectrum of vateriferol (**1**) showed a strong positive Cotton effect near 280 nm for the L_b transition of the aromatic moieties and correlated with the calculated ECD spectrum of the enantiomer with the (7a β ,8a β , 7b β ,8b β) hydrogen orientations shown in Figure 1. The isolation and characterization of vateriferol (**1**) augments the resveratrol oligomer diversity in the Dipterocarpaceae plant family.

Resveratrols have received considerable attention due to their cytotoxic and neuroprotective activities. In particular, a previous study has discussed the 5 α -reductase enzyme inhibitory activity of melapinol B (2).⁹ Thus, vateriferol (1) was evaluated for its antioxidant, enzyme inhibitory, cytotoxic, neuroprotective, and anti-inflammatory activities. In the DPPH free radical scavenging assay, vateriferol (1) exhibited weak DPPH free radical scavenging ability and ORAC activity compared to the positive standard Trolox (Table 1). The enzyme inhibitory activity was evaluated against two cholinesterase (ChE) enzymes: acetylcholine esterase (AChE) and butyrylcholinesterase (BChE), protease enzymes α -chymotrypsin and elastase, and the glutathione *S*-transferase (GST) enzyme. The results are summarized in Table 1. The inhibition of cholinesterase enzymes is considered a potential treatment for the cognitive impairments associated with Alzheimer's disease, Parkinson's disease, senile dementia, and myasthenia gravis.¹² Vateriferol (1) exhibited strong AChE inhibitory activity in a dose-dependent manner with an IC₅₀ value of $8.4 \pm 0.2 \mu\text{M}$ when compared to the standard inhibitor galantamine ($13.9 \pm 0.3 \mu\text{M}$) (Table 1). Resveratrol trimer α -viniferin isolated from *Caragana chamlague* (Leguminosae) exhibited marked AChE inhibitory activity as opposed to the weak AChE inhibitory activity exhibited by the monomer, resveratrol.¹³ Therefore, the AChE inhibition exhibited by vateriferol (1) further confirms that the trimeric arrangement of resveratrol monomers is an important feature for the AChE inhibitory activity of resveratrol oligomers. In contrast, a weak BChE inhibition was observed at the tested concentration of $14.7 \mu\text{M}$, indicating that vateriferol (1) may not be the factor responsible for the observed BChE inhibition of the extracts (Table 1).

Protease inhibitors synthesized by plants as defense molecules against pathogens and adverse abiotic conditions were investigated as antiviral, anti-inflammatory, antibacterial, and anticarcinogenic agents.¹⁴ Protease inhibitory activity was evaluated using the two serine proteases α -chymotrypsin and elastase enzymes. Vateriferol (1) exhibited 55.70% inhibition of α -chymotrypsin at a single-dose concentration of $147.2 \mu\text{M}$. However, due to the limited availability of compound 1 and the overall moderate activity exhibited compared to the standard inhibitor, the dose-dependent activity of vateriferol (1) against α -chymotrypsin was not evaluated. With respect to elastase inhibitory activity, weak activity was exhibited by vateriferol (1) at the tested concentration of $44.2 \mu\text{M}$ (Table 1).

Glutathione-*S*-transferase inhibitors are important adjuvants during chemotherapy to increase the effectiveness of cancer chemotherapeutic agents.¹⁵ A weak percent inhibitory activity (34.70%) of GST was observed with vateriferol (1) at the tested concentration of $1.5 \mu\text{M}$ (Table 1).

Five cancer cell lines, namely, human colon cancer HCT116 cells, human breast cancer MCF7 cells, human hepatocellular carcinoma HepG2 cells, human cervical cancer HeLa cells, and human skin melanoma SK-Mel 5 cells, were used to evaluate the cytotoxic activity of vateriferol (1) using methylene blue staining. Vateriferol (1) exhibited weak cytotoxic potency (<50% growth inhibition) at the tested concentration of $147.2 \mu\text{M}$ against all five cell lines. The weak cytotoxicity exhibited by vateriferol (1) is in agreement with literature reports of weak growth inhibition of various cancer cells such as human breast, colon, and liver cancer cells by resveratrols.^{16,17} The cytotoxic potential of a compound could depend on the sensitivity of the cell line against the tested compound or the test concentration.¹⁶

The neuroprotective activity was investigated by assessing the ability of vateriferol (**1**) to protect rat pheochromocytoma (PC12) cells (commonly used as a model for neuroprotection) against insult caused by oxygen and glucose deprivation (OGD).^{18,19} As shown in Figure 6, cells subjected to OGD stress showed a highly significant reduction in viability (about 65% cell viability; $p < 0.001$) compared to control cells without OGD (100% cell viability). Interestingly, vateriferol (**1**) significantly increased cell viability (88.29% cell viability) at 1.5 μM compared to the OGD group ($p < 0.05$). However, vateriferol (**1**) did not significantly increase survival in response to OGD at other concentrations, either higher or lower than 1.5 μM (Figure 6). This observation suggests that vateriferol (**1**) exhibits a bell-shaped dose–response curve with respect to its promising neuroprotective effects. There are a number of potential explanations for this type of response curve. For example, high concentrations of vateriferol (**1**) itself may be toxic, thereby masking a neuroprotective effect. Further investigations are required to elucidate this hypothesis.

Next, vateriferol (**1**) was screened for anti-inflammatory activity against LPS-stimulated SIM-A9 microglial cells using NO release assay.^{19,20} As shown in Figure 7, NO release from microglia was significantly increased by lipopolysaccharide (LPS) challenge ($p < 0.0001$). Treatment of microglia with vateriferol (**1**) at 14.7 and 73.6 μM significantly inhibited LPS-induced NO release ($p < 0.01$ and $p < 0.0001$, respectively), suggesting a concentration-dependent anti-inflammatory activity. However, to prove this hypothesis, further studies are required to validate the anti-inflammatory activity of vateriferol (**1**), using specific anti-inflammatory assays at higher concentrations.

In conclusion, the phytochemical study of the stem bark of *V. copallifera* resulted in the isolation and the characterization of a new resveratrol trimer, vateriferol (**1**), with all-*cis* configuration at the four contiguous methine-type stereo-centers. Vateriferol (**1**) exhibited marked AChE inhibitory activity with an IC_{50} of $8.4 \pm 0.2 \mu\text{M}$, ORAC activity with $2079 \pm 0.20 \text{ mg TE/g}$ of compound, and 88.29% neuro-protective activity at 1.5 μM treatment and significantly inhibited LPS-induced NO release at 14.7 and 73.6 μM . Collectively, the results add value to the chemotaxonomic identification of species and indicate that vateriferol (**1**) could be a potential therapeutic candidate for management of AD and related pathological conditions.

EXPERIMENTAL SECTION

General Experimental Procedures.

Optical rotation was measured on a Rudolph Research Analytical Autopol IV automatic polarimeter (MeOH, c in g/100 mL). The ECD spectrum was recorded in MeOH using an Applied Photophysics Chirascan ECD spectrometer. UV spectra were recorded using a Thermo Scientific Evolution 260 Bio spectrophotometer (Shanghai, China, designed in the USA). IR spectra were obtained on a Thermo Scientific Nicolet iS5 FT-IR spectrophotometer (Madison, WI, USA). NMR spectra were recorded on a Bruker Avance III NMR spectrometer with a CryoProbe (Germany). The proton (^1H) and carbon (^{13}C) frequencies are 600.2 and 150.9 MHz, respectively. Chemical shifts (δ) in ppm were referenced using the corresponding solvent signals: δ_{H} at 2.05 and δ_{C} at 29.92 for acetone-

d_6 . Off-line FID processing was conducted with the Bruker TopSpin 3.2 software. HRESIMS data were obtained using a Waters SYNAPT (Milford, MA, USA) mass spectrometer. Silica gel 60, particle size 0.035–0.070 mm, supplied by Fluka, Switzerland (220–440 mesh), and silica gel, 230–400 mesh (Sorbent Technologies, GA, USA) were used for column chromatography. Analytical TLC analysis was carried out on silica gel plates (250 μ m, 250 μ M with UV254, Sorbent Technologies). Sephadex LH-20–100 was purchased from Sigma Chemical Company, USA. All procedures were carried out at room temperature using organic solvents of analytical grade (Sigma, USA).

Collection and Identification.

A bark sample of *V. copallifera* was collected from Gampaha district, Sri Lanka. A voucher specimen (HTS-VCB-001) was deposited at the Herbal Technology Section at the Industrial Technology Institute, Sri Lanka. Samples were identified by using DNA barcoding of gene regions matK (GenBank accession no. KR338463) and trnH-psbA (GenBank accession no. KU687000).

Extraction and Isolation.

Air-dried and powdered bark of *V. copallifera* (50 g) was initially extracted with EtOH (250 mL $\times$ 3) at room temperature with each extraction lasting 2 h using a mechanical stirrer. The removal of solvent was done under reduced pressure using a rotary evaporator to afford an extract (12.43 g). Approximately, 100 g of air-dried and powdered bark of *V. copallifera* was sequentially extracted with hexanes, EtOAc, and EtOH (500 mL $\times$ 3 each) at room temperature. The filtrates were combined and concentrated in vacuo to obtain the hexanes extract as an oily solid (6 g) and dry solid masses weighing 20 and 9 g for the EtOAc and EtOH extracts, respectively.

The EtOAc and EtOH extracts showed similarities in the TLC profiles. Therefore, the EtOAc (3 g) and EtOH (3 g) extracts were combined and subjected to silica gel dry column flash chromatography (DCFC). The DCFC was performed on a silica gel column (6 cm $\times$ 12 cm, 220–440 mesh), eluting initially with a gradient solvent system of hexanes/EtOAc (7:3, 1:1, and 3:7 v/v) and 100% EtOAc, followed by a gradient solvent system of EtOAc/MeOH (10:1, 49:2, 13:1, and 9:1 v/v) 100 mL each to obtain 16 fractions (FA1–FA16). Fractions were screened for selected bioactivities, and FA5 showed more than 50% AChE, α -chymotrypsin enzyme inhibitory, and DPPH free radical scavenging activity at 50, 200, and 150 μ g/mL, respectively (Table 1). FA5 (140 mg) was subjected to silica gel column chromatography (5 cm $\times$ 30 cm, 230–400 mesh), eluting with a solvent system of EtOAc/hexanes (7:3, v/v), to obtain three fractions (FA5–1–FA5–3). Fraction A5–2 (32 mg) was further purified by Sephadex LH-20 (93.15 g of Sephadex swelled in MeOH) column chromatography (1 cm $\times$ 45 cm) eluted with CH₂Cl₂/MeOH (1:1, v/v) to obtain eight fractions (FA5–2-1–FA5–2-8), and compound **1** (3.8 mg) was obtained from F.A5–2-8.

Vateriferol (1): amorphous, brown solid; $[\alpha]_D^{25} +145$ (c 0.2, MeOH); UV (DMSO) $\lambda_{\max}$ (log ϵ) 319; IR (KBr) $\nu_{\max}$ 3303 (OH), 1605, 1451 (aromatic ring) cm^{-1} ; for ¹H (acetone- d_6 , 600 MHz) and ¹³C (methanol- d_4 , 150 MHz) NMR spectroscopic data, see Table 2; HRESIMS (m/z 679.1990 ($[M + H]^+$, calcd for C₄₂H₃₀O₉, 679.1968).

Antioxidant Assays.

2,2-Diphenyl-1-picrylhydrazyl (DPPH[•]) Free Radical Scavenging Assay.—The radical scavenging activity of the extracts, fractions, and compound **1** was determined using the stable DPPH[•] free radical as previously described.¹⁹ Dose–response activity was calculated for extracts, and the activity of the fractions and compound **1** was screened at a single dose of 150 $\mu\text{g mL}^{-1}$ and 36.8 μM , respectively. A methanolic solution of DPPH[•] (100 μM) was added to the test sample and allowed to incubate in the dark for 10 min. The absorption was measured at 517 nm using a Spectra Max Plus 384 (Molecular Devices, CA, USA). The percent radical scavenging ability was calculated.¹⁹ Trolox and MeOH were used as the positive standard and the blank, respectively.

Oxygen Radical Absorbance Capacity (ORAC) Assay.—Oxygen radical absorbance capacity of the extracts, fractions, and compound **1** was determined by measuring the decay of fluorescence of fluorescein as previously described.¹⁹ A 100 μL amount of 4.8 μM fluorescein solution, 40 μL of 75 mM phosphate-buffered saline (PBS) (pH 7.0), and test sample (1–3 $\mu\text{g mL}^{-1}$) were mixed and incubated at 37 °C for 5 min. Upon completion of incubation, 50 μL of 40 mg mL^{-1} 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) was added. The decaying of fluorescence was measured for 35 min at an excitation wavelength of 494 nm and an emission wavelength of 535 nm on a Spectra Max-Gemini EM (Molecular Devices). ORAC value was calculated based on net area under the curve and is expressed as mg Trolox equivalent per gram (mg TE/g) of extract.¹⁹ Trolox and PBS were used as the reference standard and the blank, respectively.

Enzyme Inhibitory Assays.

Acetylcholinesterase and Butyrylcholinesterase Inhibition Assay.—AChE and BChE inhibition assays were performed according to literature protocols.¹⁹ Dose–response activity was calculated for the extracts. The activities of the fractions were measured at 10 and 50 $\mu\text{g mL}^{-1}$, and the activity of compound **1** was measured at 14.7 μM . A 200 μL reaction volume contained 10 μL of AChE (0.002 U/mL) or 4 μL of BChE (0.5 U/mL) and 0.1 M sodium phosphate buffer (pH 8.0) and test sample. Following 15 min of incubation of the mixture at 25 °C, the reaction was initiated by the addition of 10 μL of the substrates acetylthiocholine iodide or butyrylthiocholine iodide (0.71 and 8 mM solutions, respectively) and 20 μL of 0.5 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DNTB) in 0.1 M Na_3PO_4 buffer (pH 7). The absorbance was measured at 412 nm. Galantamine was used as the standard inhibitor. The incubation mixture without enzymes and the test sample were used as the blank.

α -Chymotrypsin Inhibition Assay.—The α -chymotrypsin inhibition assay was performed by mixing 103 μL of 50 mM Tris-HCl buffer containing 10 mM CaCl_2 (pH 7.6), 15 μL of α -chymotrypsin (40 U/mL), and different concentrations of the EtOH extract (for measuring the dose–response activity) or the fractions (200 $\mu\text{g mL}^{-1}$) or compound **1** (147.2 μM). The mixture was incubated at 30 °C for 25 min. The reaction was initiated by the addition of 80 μL of *N*-succinyl-L-phenylalanine-*p*-nitroanilide (4 mM, final), and the absorption was measured at 410 nm. Chymostatin was used as the standard inhibitor. A blank was performed using the buffer.

Elastase Inhibition Assay.—The elastase inhibition assay was performed according to the method described.¹⁹ The total reaction volume of 250 μL contained different concentrations of the EtOH extract (for measuring the dose–response activity) or the fractions (100 $\mu\text{g mL}^{-1}$) or compound **1** (44.1 μM) and 10 μL of elastase dissolved in sterile water (1 U/mL) mixed in 0.2 M Tris-HCl (pH 8.0). The reaction mixture was preincubated at 37 °C for 25 min, and 10 μL of the substrate, *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide (dissolved in buffer at 5 mM), was added to the mixture. The absorption was measured at 410 nm. Quercetin was used as a positive control. Tris-HCl (0.2 M, pH 8.0) was used as the blank.

Glutathione S-Transferase Inhibition Assay.—GST inhibition was measured through the conjugation rate of the substrate 1-chloro-2,4-dinitrobenzene (CDNB) to reduced glutathione (GSH) facilitated by GST as previously described.²¹ A 250 μL reaction volume contained 10 μL of 30 mM CDNB, 10 μL of 30 mM GSH, 1.9 μL of GST enzymes (6.5 U/mL), and 0.1 M K_3PO_4 buffer pH 6.5. The EtOH extract and compound **1** were dissolved in distilled H_2O and were tested separately for dose–response activity at a concentration of 1.5 μM . The absorbance was monitored at 340 nm. Tannic acid was used as a positive control. Potassium phosphate buffer was used as the blank. For all the enzyme inhibitory assays, absorbance was measured using a Spectra Max Plus 384 (Molecular Devices). Percent enzyme inhibitions were calculated using the kinetic parameter V_{max} .^{19,21}

Cytotoxicity Assay.

The cells were seeded into 24-well plates at plating densities ranging from 15 000 to 50 000 cells/mL based on cell growth characteristics and were grown in Dulbecco's modified Eagle's medium (DMEM) (Cellgro, Manassas, VA, USA) containing 10% cosmic calf serum (Hyclone) and 1% penicillin–streptomycin (Lonza) under the following conditions; 37 °C, 10% CO_2 , 95% air, and 100% relative humidity for 24 h. Compound **1** (final concentration 147.2 μM) was added for the single-dose assay. Cells without test sample served as a control. After 48 h of treatment, growth inhibition was determined using methylene blue staining. Absorbance was read at 530 nm with a Spectra Max M5 multiwell plate reader (Molecular Devices). The optical density of untreated cells (control) was considered to represent 100% viability.²¹

Oxygen Glucose Deprivation and MTT Assay.

PC12 cells were maintained in DMEM media (Cellgro) supplemented with 5% heat-inactivated horse serum, 5% heat-inactivated fetal bovine serum, 1 mM L-glutamine, and 100 U/mL penicillin–streptomycin in a humidified 5% CO_2 incubator at 37 °C. Approximately 0.1×10^6 cells/mL were seeded onto poly-L-lysine-coated 24-well plates. Following a 24 h incubation period, DMEM was replaced with Hank's balanced salt solution (Fisher Scientific, Hanover Park, IL, USA), which contained all standard components except glucose. The cells were then treated with compound **1** at 73.6, 14.7, 1.5, 0.7, 0.1, and 7.4×10^{-2} μM and exposed to OGD conditions in an anaerobic chamber (Oxoid Anaerogen kit, England) for 3 h at 37 °C. A separate plate having cells cultured in DMEM without OGD treatment served as the control. Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Promega Corporation,

Madison, WI, USA) (Mosmann, 1983). Viable cells were quantified by measuring the absorbance at 570 nm using a Biotek Synergy H1 hybrid reader (Winooski, VT, USA). The optical density of formazan formed in untreated cells (control) was considered to represent 100% viability.²¹

Inhibitory Effects on Nitric Oxide (NO) Production in LPS-Activated Microglial Cells.

Briefly, microglia (SIM-A9) cells were seeded at a density of 0.2×10^6 cells/mL in 24-well plates in DMEM (Cellgro) supplemented with 10% fetal bovine serum, 5% horse serum, and 1% penicillin–streptomycin under a humidified 5% CO₂, 95% air atmosphere, at 37 °C. Following 24 h of incubation, cells were treated with compound **1** at 14.7 and 73.6 μ M and challenged with a concentration of 0.1 μ g mL⁻¹ LPS (Sigma, USA) for 24 h. For the measurement of the nitrite concentration in the medium, 100 μ L of cultured medium and 100 μ L of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride in 5% phosphoric acid) were mixed and incubated at room temperature for 10 min, and the absorbance was recorded at 540 nm on a Biotek Synergy H1 hybrid reader (Winooski, VT, USA). The NO inhibitory activity was defined by the ability of the test sample to decrease NO below that measured of the cells with LPS only.²¹

Computational Methods.

DFT/TDDFT calculations were performed using the Gaussian16 program package with default grids and convergence criteria.²² The initial conformation of vateriferol (**1**) was energy minimized using the B3LYP/6–31+G(d,p) method. Using the dihedral angle scans of the rings, the lowest energy conformation was found at the same B3LYP/6–31+G(d,p) level. Each ring (A1, A2, B1, and C1 in Figure 3) is symmetric around the axis along the bond connecting to the central part of the molecule, and the above scanning resulted in only one stable minimum energy conformation. The optimized conformation was further minimized at the same level in MeOH as the solvent. The enantiomer of vateriferol (**1**) was also subjected to the energy minimization under the same conditions. The optimized geometries were used to calculate ECD spectra at the B3LYP/aug-cc-pvtz level with the TDDFT method using MeOH as the solvent. The number of excited states per conformation was selected to be 50. The visualization program, Gaussview6²³ was used to extract data points of the calculated ECD spectra.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Structures of vateriferol, melapinol B, and rokciitol C.

Figure 2.

Energy-minimized ChemDraw Pro structures of vateriferol (1), melapinol B (2), rockiiol C (3), and the yet to be discovered all-*trans* isomer.

Figure 3.

Key HMBC (→) and COSY (bold lines) correlations of compound **1**.

Figure 4.
Key NOESY correlations and the relative configuration of compound **1**.

Figure 5. Experimental (black line) and calculated ECD spectra (vateriferol, red line, and the enantiomer, blue line).

Figure 6.

Neuroprotective effect of vateriferol (**1**) against OGD-induced cell death. Data are expressed as percentage of viable cells. Con = control cells without OGD and represent 100% cell viability. OGD = cells subjected to oxygen and glucose deprivation. OGD + vateriferol (**1**) = cells subjected to OGD and treated with various concentrations of compound **1**. *** $p < 0.001$, **** $p < 0.0001$ relative to control group; # $p < 0.05$ relative to OGD group.

Figure 7.

Inhibition of NO production by vateriferol (**1**) in LPS-challenged microglial cells. Data are expressed as percentage of NO released in response to LPS challenge. Con = control microglial cells treated with vehicle only. LPS = microglial cells challenged with LPS only. LPS + vateriferol (**1**) = microglial cells cotreated with LPS and vateriferol (**1**). **** $p < 0.0001$ relative to control group; ## $p < 0.01$, #### $p < 0.0001$ relative to LPS group.

Table 1.

Enzyme Inhibitory and Antioxidant Activity of Extracts and Fractions^a

extract/fraction	enzyme inhibitory activity					antioxidant activity	
	AChE	BChE	α -chymotrypsin	elastase	GST	DPPH	ORAC
		PI or IC ₅₀ in (μ g mL ⁻¹ or μ M)				PI or IC ₅₀ in (μ g mL ⁻¹ or μ M)	mg TE/g of extract
EtOH	7.3 $\pm$ 0.2	1.3 $\pm$ 0.1	160.7 $\pm$ 5.2	97.0 $\pm$ 2.4	2.4 $\pm$ 0.3	114.8 $\pm$ 1.7	4700.8 $\pm$ 0.2
hexanes	12.3 $\pm$ 0.3	ND ¹	3.0% ^d	14.5% ^e	21.8% ^f	10.9% ^e	ND ³
EtOAc	5.4 $\pm$ 0.2	9.1 $\pm$ 0.1	41.1% ^d	60.3% ^e	79.3% ^f	173.8 $\pm$ 4.0	2966.9 $\pm$ 0.2
EtOH	4.7 $\pm$ 0.4	5.0 $\pm$ 0.2	76.8% ^d	92.8% ^e	66.5% ^f	114.0 $\pm$ 1.2	1575.7 $\pm$ 0.1
FA5	96.7% ^b	42.6% ^c	51.0% ^g	31.2% ^e		62.0% ^h	
vateriferol (I)	8.4 $\pm$ 0.2 ^g	19.7% ^c	55.7% ^e	10.2% ⁱ	34.7% ^f	ND ²	2079.0 $\pm$ 0.2
positive control	13.9 $\pm$ 0.3 ^g	2.0 $\pm$ 0.0 ^g	9.8 $\pm$ 0.1 ^g	733.5 $\pm$ 5.5 ^g	0.3 $\pm$ 0.1 ^g	18.4 $\pm$ 0.0 ^g	1362.8 $\pm$ 0.2

^aPI, percentage inhibition; ND¹, not detected at 300 μ g mL⁻¹; ND², not detected at 36.8 μ M; ND³, not detected at 3 μ g mL⁻¹. The positive control for AChE and BChE is galantamine. Positive controls for α -chymotrypsin, elastase, GST inhibitory activities, DPPH, and ORAC are chymostatin, quercetin, tannic acid, Trolox, and green tea, respectively. ORAC values are given in mg TE/g of extract. IC₅₀ values for extracts and fractions are given in μ g mL⁻¹. IC₅₀ values for vateriferol (I) and positive controls except for green tea are given in μ M.

^bPI at 50 μ g mL⁻¹.

^cPI at 10 μ g mL⁻¹.

^dPI at 60 μ g mL⁻¹.

^ePI at 100 μ g mL⁻¹.

^fPI at 1 μ g mL⁻¹.

^gPI at 200 μ g mL⁻¹.

^hPI at 150 μ g mL⁻¹.

ⁱPI at 44.1 μ M.

Table 2.

¹H and ¹³C NMR Spectroscopic Data^a of Compound 1

position	δ_H (J)	δ_C , APT	¹ H- ¹ H COSY	HMBC
1a		135.5, C		
2a, 6a	6.26, d (8.7)	130.9, CH	6.05	7b
3a, 5a	6.05, d (8.7)	114.5, CH	6.26	1a, 1b
4a		156.2, C		
7a	3.85, dd (2.3, 9.3)	61.2, CH	4.40, 4.68	1a, 1b, 9a, 6b, 9b, 9c
8a	4.68, d (2.3)	57.5, CH	3.85	1a, 7a, 9a, 10a, 8b, 9b, 14b
9a		149.4, C		
10a, 14a	6.10, d (2.2)	106.4, CH		10a, 12a, 11a, 8b
11a		159.5, C		
12a	6.13, t, (2.2)	101.3, CH		10a, 13a
13a		159.5, C		
1b		134.7, C		
2b, 6b	6.75, d (8.6)	130.7, CH	6.33	4a, 8a, 2b
3b, 5b	6.33, d (8.6)	114.9, CH	6.75	11c, 1b
4b		158.8, C		
7b	5.36, d (3.4)	41.7, CH	4.40	2b, 9b, 11c
8b	4.40, dd, (3.4, 9.3)	51.6, CH	5.36, 3.85	1a, 9c
9b		141.1, C		
10b		120.0, C		
11b		155.0, C		
12b	6.89, s	96.5, CH		10b, 14b, 7c
13b		152.9, C		
14b		127.6, C		
1c		124.3, C		
2c, 6c	7.44, d (8.7)	130.5, CH	6.84	
3c, 5c	6.84, d (8.7)	116.4, CH	7.44	1c, 8c
4c		156.4, C		
7c		151.9, C		
8c		116.4, C		
9c		134.5, C		
10c		125.8, C		
11c		155.2, C		
12c	6.34, d (2.5)	102.6, CH		7c, 9c, 14c
13c		155.0, C		
14c	6.65, d (2.5)	109.8, CH		14b, 8c, 12c
OH	8.69, s; 8.39, s; 8.18, s; 7.97, s; 7.98, s (2H); 7.83, s; 7.54, s			

^a¹H and ¹³C NMR data (δ) were measured in acetone-*d*₆ at 600 and 150 MHz, respectively.