New Neolignan and Dihydrostilbene Derivatives from Pouzolzia sanguinea Inhibit NO Production in LPS-Activated BV2 Cells

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Abstract

Three dihydrostilbene derivatives (1-3) and 5 neolignans (4-8) were isolated from the ethyl acetate-soluble fraction of Pouzolzia sanguinea. Two new compounds (1 and 4) were obtained. Their structures, as well as their absolute configuration, were elucidated by means of high-resolution electrospray ionization mass spectrometry, nuclear magnetic resonance, and circular dichroism spectral data. Compounds 1-8 inhibited NO production in lipopolysaccharide-activated BV2 cells with half-maximal inhibitory concentration (IC50) values ranging from 22.7 ± 1.5 to 61.2 ± 3.1 µM. Of these, compounds 1 and 2 exhibited the most NO inhibitory activity with IC50 values of 22.7 ± 1.5 and 25.1 ± 2.1 µM, respectively, in comparison with the positive control, NG-monomethyl-L-arginine, IC50 value 22.1 ± 1.2 µM.

Keywords
pouzobistilbene A, pouzosanoside A, Pouzolzia sanguinea (Blume) Merr., NO inhibitor, anti-inflammation

Results and Discussion

Compound 1 was obtained as a yellow amorphous powder. The negative-mode high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) of 1 showed a pseudo-molecular ion peak at m/z 567.1438 [M + Cl]− indicating a molecular formula of C30H28O9 (calculated for [C30H28O9Cl]−, 567.1427) (Supplemental Figures S1, S1a). The 1H nuclear magnetic resonance (NMR) spectrum of compound 1...
contained signals of a 1,3,4-trisubstituted benzene ring ($\delta_H$ 7.00 (1H, d, $J = 1.5$ Hz), 6.92 (1H, dd, $J = 8.0, 1.5$ Hz), 6.79 (1H, d, $J = 8.0$ Hz)], a 1,3,5-trisubstituted benzene ring ($\delta_H$ 6.07 (overlapped 3H, s)], and a methoxy group ($\delta_H$ 3.77 (3H, s)] (Supplemental Figure S2). The 13C NMR and heteronuclear single-quantum correlation (HSQC) spectra of 1 showed 15 carbon signals, including 6 nonprotonated aromatic carbons ($\delta_C$ 159.0 (2C), 148.8, 147.0, 142.2, and 135.0), 6 aromatic methines ($\delta_C$ 120.0, 116.0, 111.4, 109.0 (2C), and 101.9), 2 oxygenated aliphatic methines ($\delta_C$ 85.9 and 58.8), and 1 methoxy ($\delta_C$ 56.3) carbon signal (Supplemental Figures S3, S4). The above 1H and 13C NMR spectral data and expected molecular formula of 1, C30H28O9, suggested compound 1 to be a dihydrostilbene-dimer derivative. Moreover, the above 1H and 13C NMR spectral data of 1 were shown to be similar to those of pouzoliagnan K (2), except slightly different in carbon chemical shift values for aliphatic methines (Table 1). The heteronuclear multiple bond correlations (HMBCs) between H-2′ ($\delta_H$ 7.00) and H-6′ ($\delta_H$ 6.92) and C-4′ ($\delta_C$ 147.0), and between H-5′ ($\delta_H$ 6.79) and methoxy proton ($\delta_H$ 3.77) and C-3′ ($\delta_C$ 148.8) indicated the presence of a 4-hydroxy-3-methoxyphenyl group (Figure 1). Assignment of an oxygenated methine (C-2) was then confirmed by its carbon chemical shift ($\delta_C$ 85.9) and the HMBC correlations between H-2′ ($\delta_H$ 7.00) and H-6′ ($\delta_H$ 6.92) and C-2. The observed correlation spectroscopy (COSY) cross peak between H-2 ($\delta_H$ 5.45) and H-3 ($\delta_H$ 3.60) revealed the direct linkage between C-2 and C-3 (Supplemental Figure S6). The HMBC correlation between H-3 ($\delta_H$ 3.60) and C-1″ ($\delta_C$ 142.2) demonstrated the location of a 1,3,5-trisubstituted benzene ring at C-3 (Supplemental Figure S5). The deshielded carbon signals of C-3″ and C-5″ ($\delta_C$ 159.0) indicated the presence of 2 hydroxy groups at C-3″. The observed correlation spectroscopy (COSY) cross peak between H-2 ($\delta_H$ 5.45) and H-3 ($\delta_H$ 3.60) revealed the direct linkage between C-2 and C-3 (Supplemental Figure S6). The HMBC correlation between H-3 ($\delta_H$ 3.60) and C-1″ ($\delta_C$ 142.2) demonstrated the location of a 1,3,5-trisubstituted benzene ring at C-3 (Supplemental Figure S5). The deshielded carbon signals of C-3″ and C-5″ ($\delta_C$ 159.0) indicated the presence of 2 hydroxy groups at C-3″. 

| No. | $\delta_C^{ab}$ | $\delta_H^{ae}$ (mult., $J$ in Hz) | $\delta_C^{ab}$ | $\delta_H^{ae}$ (mult., $J$ in Hz) |
|-----|----------------|----------------------------------|----------------|----------------------------------|
| 2   | 85.9           | 5.45 (brd, 5.0)                  | 89.1           |                                  |
| 3   | 58.8           | 3.60 (dd, 1.5, 5.0)              | 63.8           |                                  |
| 4   | 58.8           | 3.60 (dd, 1.5, 5.0)              | 63.8           |                                  |
| 5   | 85.9           | 5.45 (brd, 5.0)                  | 89.1           |                                  |
| 1′  | 135.0          |                                  | 134.4          |                                  |
| 2′  | 111.4          | 7.00 (d, 1.5)                    | 111.0          |                                  |
| 3′  | 148.8          |                                  | 148.8          |                                  |
| 4′  | 147.0          |                                  | 147.2          |                                  |
| 5′  | 116.0          | 6.79 (d, 8.0)                    | 116.0          |                                  |
| 6′  | 120.0          | 6.92 (dd, 1.5, 8.0)              | 119.9          |                                  |
| 1″  | 142.2          |                                  | 141.7          |                                  |
| 2″  | 109.0          | 6.07 (s)                        | 107.8          | 2′                                  |
| 3″  | 159.0          |                                  | 159.4          | 3′                                  |
| 4″  | 101.9          | 6.07 (s)                        | 102.3          | 4′                                  |
| 5″  | 159.0          |                                  | 159.4          | 5′                                  |
| 6″  | 109.0          | 6.07 (s)                        | 107.8          | 6′                                  |
| 1‴  | 135.0          |                                  | 134.4          | 7‴                                  |
| 2‴  | 111.4          | 7.00 (d, 1.5)                    | 111.0          | 8‴                                  |
| 3‴  | 148.8          |                                  | 148.8          | 9‴                                  |
| 4‴  | 147.0          |                                  | 147.2          | 3-OCH3                              |
| 5‴  | 116.0          | 6.79 (d, 8.0)                    | 116.0          | 5-OCH3                              |
| 6‴  | 120.0          | 6.92 (dd, 1.5, 8.0)              | 119.9          | 3″-OCH3                             |
| 1‴‴ | 142.2          |                                  | 141.7          | 5″-OCH3                             |
| 2‴‴ | 109.0          | 6.07 (s)                        | 107.8          | Glc-1                              |
| 3‴‴ | 159.0          |                                  | 159.4          | Glc-2                              |
| 4‴‴ | 101.9          | 6.07 (s)                        | 102.3          | Glc-3                              |
| 5‴‴ | 159.0          |                                  | 159.4          | Glc-4                              |
| 6‴‴ | 109.0          | 6.07 (s)                        | 107.8          | Glc-5                              |
| 3″-OCH3 | 56.3         | 3.77 (s)                       | 56.4           | Glc-6                              |
| 3″″-OCH3 | 56.3         | 3.77 (s)                       | 56.4           |                                  |

Measured at $^1$CD$_3$OD, $^{125}$MHz, 500 MHz.
and C-5″. The dimer structure of a dihydrostilbene fragment was formed by a C-C linkage at C-3 and an ether bridge at C-2, which were confirmed by HMBC correlations of H-3 (δ_H 3.60)/C-4 (δ_C 58.8) and H-2 (δ_H 5.45)/C-5 (δ_C 85.9) (Supplemental Figure S5). Thus, the planar structure of 1 was established and recognized to be similar to that of pouzolignan K (2, Figure 2), a dihydrostilbene derivative previously isolated from aerial parts of *P. zeylanica* var *microphylla*. It is interesting that the key nuclear Overhauser effect spectroscopy correlations of 1 (Supplemental Figure S7) were found to be similar to those of 2 including interactions between H-2 (δ_H 5.45) and H-2″/H-6″ (δ_H 6.07), H-3 (δ_H 3.60) and H-2′ (δ_H 7.00)/H-6′ (δ_H 6.92). However, the difference in the 13C NMR data between 1 and 2 (Table 1) indicated that compound 1 is a diastereomer of 2, having trans-configuration between the 2 benzene rings A and B. The meso-structure of 1 was agreed by its lack of optical activity shown by polarimetry and the absence of a Cotton effect in its circular dichroism spectrum (Supplemental Figure S8). Therefore, structure of 1 was proposed as shown in Figure 2, a new compound, named as pouzobistilbene A.

Compound 4 was also obtained as a yellow amorphous powder. A pseudo-molecular ion peak at *m/z* 635.2119 [M + Cl]− (Supplemental Figures S12, S12a) suggested a molecular formula for 4 as C_{28}H_{40}O_{14} (calculated for [C_{28}H_{40}O_{14}Cl]−, 635.2112), indicating 9 degrees of unsaturation. The 1H NMR spectrum of 4 showed 4 aromatic proton signals [δ_H 6.83 and 6.60 (each 2 H, s)], an anomeric proton [δ_H 4.60 (1H, d, J = 7.5 Hz)], and 4 methoxy groups [δ_H 3.88 and 3.87 (each, 6 H, s)] (Supplemental Figure S13). The 13C NMR and HSQC spectra of 4 (Supplemental Figures S14, S15) revealed signals for 28 carbons. Twelve aromatic carbons at δ_C 154.3 (2C), 148.9 (2C), 140.2, 136.2, 134.6, 131.0, 106.9 (2C), and 106.2 (2C) were assigned for 2 symmetric 1,3,4,5-tetrasubstituted benzene rings. The presence of a sugar unit was indicated by a signal for an anomeric proton (δ_H 4.60; δ_C 105.2). Two sets of COSY cross peaks (Supplemental Figure S17) including H-7 (δ_H

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**Figure 1.** Key HMBC and COSY correlations of compounds 1 and 4. COSY, correlation spectroscopy; HMBC, heteronuclear multiple bond correlation.

**Figure 2.** Chemical structure of compounds 1-8 isolated from *Pouzolzia sanguinea*.
5.16)/H-8 (δ_H 4.29)/H-9 (δ_H 3.63 and 3.25) and H-2′/C-6′ (δ_C 2.68)/H-8′ (δ_H 1.85)/H-9′ (δ_H 3.59) indicated 2 individual carbon chains C-7/C-8/C-9 and C-7′/C-8′/C-9′, respectively (Figure 1). Carbon chemical shift values of C-9 (δ_C 61.2) and C-9′ (δ_C 62.1) suggested 2 hydroxymethylene groups, and carbon chemical shift values for C-7 (δ_C 82.4) and C-8 (δ_C 86.9) were assigned for 2 oxygenated methines. The HMBC correlations between H-7 (δ_H 5.16) and C-1 (δ_C 131.0)/C-2/C-6 (δ_C 106.2), H-7′ (δ_H 2.68) and C-1′ (δ_C 134.6)/C-2′/C-6′ (δ_C 106.9) (Supplemental Figure S16) indicate 1,3,4,5-tetrasubstituted benzene rings located at C-7 and C-7′, respectively. Four methoxy groups at C-3, C-5, C-3′, and C-5′ formed 2 sets of symmetric 1,3,4,5-tetrasubstituted benzene rings. This deduction was also supported by HMBC correlations between aromatic proton δ_H 6.83 (H-2, H-6)/methoxy proton δ_H 148.9 (C-3, C-5), aromatic proton δ_H 6.60 (H-2′, H-6′)/methoxy proton δ_H 3.88 and carbon δ_C 154.3 (C-3′, C-5′). Additionally, chemical shifts of C-8 (δ_C 86.9) and C-4′ (δ_C 140.2), and the HMBC correlation between H-8 (δ_H 4.29) and C-4′ indicated the connection between C-8 and C-4′ via an ether linkage. Also, the HMBC correlation between aromatic proton Glc δ_H 7.1 (δ_C 113.0, 112.6, 101.6, 101.4, 100.4, 100.2)/C-2/C-6 (δ_C 86.9) were assigned for 2 oxygeneated methines. The presence of D-glucose in compound 4 was further confirmed by acid hydrolysis, converting the sugar residue to its thiocarboxymethylazidolidine derivative, and comparison of its high-performance liquid chromatography (HPLC) retention time with those of authentic D-glucose derivatives prepared in a similar manner (Supplemental Material). According to the J coupling constant of the anomeric proton (J = 7.5 Hz) and 5 remaining carbons carbons (δ_C 78.1, 77.8, 75.6, 71.4, and 62.6) suggested a β-glycopyranosyl group. The presence of D-glucose in compound 4 was further confirmed by acid hydrolysis, converting the sugar residue to its thiocarboxymethylazidolidine derivative, and comparison of its high-performance liquid chromatography (HPLC) retention time with those of authentic D-glucose derivatives prepared in a similar manner (Supplemental Material). According to the J coupling constant of the anomeric proton (J = 7.5 Hz) and 5 remaining carbons carbons (δ_C 78.1, 77.8, 75.6, 71.4, and 62.6) suggested a β-glycopyranosyl group.

Finally, the negative Cotton effect at 238 nm (−1.48 mdeg) indicated an absolute configuration (7R,8S).9 Consequently, the structure of compound 4 was established as a new compound named as pouzosanoside A.

The other isolated compounds were determined to be pouzolignan K (2) (Supplemental Figures S9–S11),3 pouzolignan D (3),3 thro-guaiaicylglycerol-β-O-4′-coniferyl alcohol (5),10 thro-guaiaicylglycerol-β-O-4′-dihydroconiferyl alcohol (6),11 erythro-guaiaicylglycerol-β-O-4′-coniferyl alcohol (7),2 and erythro-guaiaicylglycerol-β-O-4′-dihydroconiferyl alcohol (8)12 by comparison of their NMR data with the data reported in the literature.

Neolignans and stilbenes derivatives are naturally occurring polyphenols found in the plant kingdom. Neolignans are formed by dimerization of 2 phenylpropanoid (C6–C3) units. However, stilbene derivatives also occur in the form of monomeric stilbenes (1,2-diphenylethylene core) and oligomeric stilbenes. Their chemical structures are characterized by the presence of terminal phenyl groups, which are functionalized with various numbers of hydroxyl and/or methoxy groups. Neolignans and stilbenes have a wide range of biological properties such as antioxidant, anti-inflammatory, anti-neurodegenerative, antitumor, antiviral, and antimicrobial activities.13,14 In this work, the anti-inflammatory activity of compounds 1-8 was evaluated by their inhibiting effect on NO production in LPS-stimulated BV2 cells.15 At a high concentration up to 80 µM, compounds 1-8 did not show any cytotoxic effect on the BV2 cells (cell viability in range from 96.2% ± 2.0% to 113.2% ± 4.3%, Table 2). Thus, the NO inhibitory activity of the compounds was not affected by cytotoxic activity. As shown in Table 2, dihydrostilbene-dimer derivatives 1 and 2 exhibited the most NO inhibitory activity with half-maximal inhibitory concentration (IC50) values of 22.7 ± 1.5 and 25.1 ± 2.1 µM, respectively. Their NO inhibitory effects were better than those of neolignans 4-8, which showed IC50 values ranging from 35.8 ± 1.9 to 61.2 ± 3.1 µM.

### Material and Methods

#### General Experimental Procedures

Optical rotation was recorded using a Jasco P-2000 digital polarimeter. HR-ESI-MS was measured on an Agilent 6530 Accurate Mass Q-TOF instrument, and 1-dimensional (1D)- and 2D-NMR spectra on a Bruker 500 MHz spectrometer; data acquisition was analyzed by MestReNova software. The circular dichroism spectrum was measured on a Chirascan spectrometer. Preparative HPLC was performed on an Agilent 1100 system using a YMC J’sphere ODS-H80 column (4 µm, 20 × 250 mm). Thin-layer chromatography was carried out on precoated silica gel 60 F254 and/or RP-18 F254S plates. Compounds were detected by spraying with H2SO4 solution (5%) followed by heating on a hot plate.

#### Plant Material

The plant samples were collected at Lam Dong province, Vietnam in March 2018. *P. sanguinea* (Blume) Merr. was identified by Dr Nguyen The Cuong, Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology (VAST). A voucher specimen (number: NCCT0318) was deposited at the Institute of Ecology and Biological Resources (VAST).

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### Table 2. Inhibitory Effect of Compounds 1-8 on NO Production in the LPS-Stimulated BV2 Cells.

| Compound | IC50 (µM) | Cell viability (%) |
|----------|-----------|--------------------|
| 1        | 22.7 ± 1.5| 98.3 ± 1.3         |
| 2        | 25.1 ± 2.1| 96.2 ± 2.0         |
| 3        | 47.6 ± 3.7| 102.7 ± 2.6        |
| 4        | 61.2 ± 3.1| 107.4 ± 4.8        |
| 5        | 38.0 ± 2.4| 111.2 ± 3.9        |
| 6        | 41.4 ± 1.8| 104.8 ± 4.1        |
| 7        | 39.7 ± 2.7| 101.6 ± 3.7        |
| 8        | 35.8 ± 1.9| 113.2 ± 4.3        |

L-NMMAa | 22.1 ± 1.2 | NT |

Abbreviations: NT, not tested; LPS, lipopolysaccharide; L-NMMA, N’-monomethyl-L-arginine.

*aCell viability was evaluated at compound concentration of 80 µM.

bL-NMMA was used as a positive control.

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Extraction and Isolation

The dried and powdered aerial parts of *P. sanguinea* (15 kg) were macerated 3 times with methanol in an ultrasonic bath (each 20 L, in 60 minutes at room temperature). The solution was evaporated under reduced pressure to give the methanol extract (730 g). This was suspended in water and extracted in turn with *n*-hexane, methylene chloride, and ethyl acetate to yield the corresponding *n*-hexane, methylene chloride, and ethyl acetate extracts, and the water layer. The ethyl acetate extract (34 g) was chromatographed on a silica gel column, eluting with methylene chloride and methanol (40/1, 20/1, 10/1, 5/1, 3/1, 1/1, v/v) to give 6 fractions E1-E6. Fraction E2 was separated on a reverse-phase C-18 column chromatography, eluting with methanol/water (1/1, v/v) to give 3 fractions E2A-E2C. Fraction E2A was purified by pre-HPLC using acetonitrile in water (22%) as mobile phase to give compounds 7 (6.3 mg, *t*ₚ 40.2 minutes) and 6 (7.5 mg, *t*ₚ 43.7 minutes). Fraction E2C was also purified by pre-HPLC using acetonitrile in water (22%) to mobile phase to give compounds 7 (9.4 mg, *t*ₚ 38.6 minutes) and 5 (8.3 mg, 40.8 minutes). Fraction E3 was chromatographed on a reverse-phase C-18 column, eluting with acetonitrile/water (1/2, v/v) to give 4 fractions E3A-E3D. Fraction E3A was first separated on a Sephadex LH-20 column, eluting with methanol/water (2/1, v/v) and then purified by pre-HPLC using acetonitrile in water (30%) to obtain compound 3 (6.8 mg, *t*ₚ 43.1 minutes). Fraction E3C was purified by pre-HPLC using acetonitrile in water (27%) to give compounds 1 (25.6 mg, *t*ₚ 44.0 minutes) and 2 (28.4 mg, *t*ₚ 45.7 minutes). Fraction E5 was chromatographed on a silica gel column, eluting with methylene chloride/methanol/water (6/1/0.1, v/v/v) to give 3 fractions E5A-E5C. Fraction E5B was first separated on a reverse-phase C-18 column, eluting with methanol/water (2/3, v/v) and then purified by pre-HPLC using acetonitrile in water (21%) to give compound 4 (25.2 mg, *t*ₚ 29.8 minutes).

Pouzobistilbene A (1). Yellow amorphous powder, [α]₀ D²⁵: 0° (c 0.1, MeOH); CD (MeOH): no significant Cotton effect; HR-ESI-MS: *m/z* 567.1438 [M + Cl]⁻ (calculated for C₂₉H₃₅O₂Cl, 567.1422); ¹H NMR (CD₃OD, 500 MHz) and ¹³C NMR (CD₃OD, 125 MHz) data are given in Table 1 and Supplemental material.

Pouzosanoside A (4). Yellow amorphous powder, [α]₀ D²⁵: +27.4° (c 0.1, MeOH); CD (MeOD) mdeg₅₀: −1.48 (238); HR-ESI-MS: *m/z* 635.2119 [M + Cl]⁻ (calculated for C₃₀H₂₈O₉Cl, 635.2107); ¹H NMR (CD₃OD, 500 MHz) and ¹³C NMR (CD₃OD, 125 MHz) data are given in Table 1 and Supplemental material.

Nitric Oxide Assay

NO production was determined by nitrite concentration in the culture medium using Griess reaction. BV2 cells were maintained in Dulbecco’s modified Eagle medium supplemented with 5% fetal bovine serum and 1% penicillin-streptomycin medium. The cells were dispensed into a 96-well culture plate (4 × 10⁴ cells/well) and incubated at 37 °C in a humidified atmosphere (5% CO₂ and 95% air). After 24-hour incubation, the cells were treated with/without compounds and then stimulated with LPS (100 ng/mL) for the next 30 minutes. After an additional 24-hour incubation, the cell culture medium (50 µL) was mixed with an equal volume of Griess reagent for 10 minutes. Absorbance was read at 520 nm on an Emax reader. The amount of nitrite accumulated in the medium was obtained from a standard curve, which was built by NaNO₂ serial dilution. N⁷-monomethyl-L-arginine was used as a positive control. After medium collection for the Griess test, cell viability was estimated by adding MTT (0.2 mg/mL) and incubating for 2 hours. The supernatant was carefully aspirated and the formazan crystals were dissolved in 200 µL of dimethyl sulfoxide. Absorbance was read at 540 nm and compared with the control group. Data are expressed as mean ± SD of triplicate experiments. Statistical analysis was performed by GraphPad Prism software.

Conclusion

Two new compounds pouzobistilbene A (1) and pouzosanoside A (4), and 6 known ones pouzolignan K (2), pouzolignan D (3), *threo*-guaiacylglycerol-β-O-4'-coniferyl alcohol (5), *threo*-guaiacylglycerol-β-O-4'-diydroconiferyl alcohol (6), *erythro*-guaiacylglycerol-β-O-4'-coniferyl alcohol (7), and *erythro*-guaiacylglycerol-β-O-4'-dihydroconiferyl alcohol (8) were isolated from the methanol extract of *P. sanguinea*. Compounds 1-8 inhibited NO production in the LPS-activated BV2 cells with ICₕ₀ values ranging from 22.7 ± 1.5 to 61.2 ± 3.1 µM. Compounds 1 and 2 exhibited the most NO inhibitory activity with ICₕ₀ values of 22.7 ± 1.5 and 25.1 ± 2.1 µM, respectively.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material

Supplemental material for this article is available online.

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