Arch Pharm Res Vol 27, No 3, 291-294, 2004

Archives of Pharmacal Research
http://apr.psk.or.kr

# Di- and Sesqui-Terpenoids Isolated from the Pods of *Sindora sumatrana* and Their Potential to Inhibit Lipopolysaccharide-Induced Nitric Oxide Production

**Dae Sik Jang, Hye-Young Min[1], Yeon-Hee Jeong[1], Sang Kook Lee[1], and Eun-Kyoung Seo[1]**

*Division of Molecular Life Sciences and [1]College of Pharmacy, Ewha Womans University, Seoul 120-750, Korea*

*(Received February 23, 2004)*

Activity-guided fractionation of the *n*-hexane and $CHCl_3$-soluble fractions of *Sindora sumatrana* using a bioassay based on the inhibition of lipopolysaccharide (LPS)-induced nitric oxide (NO) production by inducible nitric oxide synthase (iNOS) in murine macrophage RAW 264.7 cells led to the isolation of the known compound, (+)-7β-acetoxy-15,16-epoxy-3,13(16),14-clerodatriene-18-oic acid (**2**) as an active constituent. In addition, a new *trans*-clerodane diterpenoid, (+)-2-oxokolavenic acid (**1**), together with six known compounds, (+)-3,13-clerodadiene-16,15-olide-18-oic acid (**3**), (+)-7β-acetoxy-3,13-clerodadiene-16,15-olide-18-oic acid (**4**), (+)-7β-acetoxy-16-hydroxy-3,13-clerodadiene-16,15-olide-18-oic acid (**5**), β-caryophyllene oxide (**6**), clovane-2β,9β-diol (**7**), and caryolane-1,9β-diol (**8**) were isolated and found to be inactive. The structure of compound **1** was determined using physical and spectroscopic methods such as 1D and 2D-NMR experiments. The known compounds **2**-**8** were identified by the spectroscopic data and by comparison with the published values. Of eight isolates (**1**-**8**), only compound **2** exhibited an iNOS inhibitory activity with $IC_{50}$ value of 51.6 μM.

**Key words:** *Sindora sumatrana*, Leguminosae, Diterpenoids, Sesquiterpenoids, (+)-2-Oxokolavenic acid, Nitric oxide synthase

## INTRODUCTION

In our ongoing project directed toward the discovery of novel naturally occurring inducible nitric oxide synthase (iNOS) inhibitory agents (Je *et al.*, 2004; Lee *et al.*, 2002), the pods of *Sindora sumatrana* Miq. (Leguminosae) were chosen for more detailed investigation, since the *n*-hexane and $CHCl_3$-soluble fractions of the MeOH extract significantly inhibited lipopolysaccharide (LPS)-induced nitric oxide production in murine macrophage RAW 264.7 cells. Nitric oxide (NO) is a short-lived molecule exerting many physiological functions, and produced from L-arginine by NO synthase (NOS) (Moncada, 1999). NO can stimulate tumor growth and metastasis by promoting migratory, invasive, and angiogenic abilities of tumor cells (Lala and Chakraborty, 2001). Thus, an inhibitor of iNOS can be considered as a therapeutic agent for certain cancers.

Correspondence to: Eun-Kyoung Seo, Ph.D., Natural Products Chemistry Laboratory, College of Pharmacy, Ewha Womans University, Seoul 120-750, Korea
Tel: 82-2-3277-3047, Fax: 82-2-3277-3051
E-mail: Yuny@ewha.ac.kr

*S. sumatrana* is a large tree of lowland forests, often by rivers, distributed throughout South East Asia. The wood when wounded produced copious amounts of viscous blood-red exudates, and is taken as a treatment for infections of the urinary tract when mixed with *Scoparia dulcis* leaves and alum (Elliott and Brimacombe, 1987). Previous phytochemical investigations on this plant have isolated several sesquiterpenoids and diterpenoids (Heymann *et al.*, 1994a; Heymann *et al.*, 1994b; Heymann *et al.*, 1994c).

In the present study, activity-guided fractionation of the *n*-hexane and $CHCl_3$-soluble residues of *S. sumatrana* using the iNOS assay led to the isolation and identification of an active *trans*-clerodane diterpenoid, (+)-7β-acetoxy-15,16-epoxy-3,13(16),14-clerodatriene-18-oic acid (**2**), along with a new *trans*-clerodane diterpenoid, (+)-2-oxokolavenic acid (**1**) and six di- and sesquiterpenoids of known structures (**3**-**8**) as inactive constituents.

## MATERIALS AND METHODS

### Plant material

The pods of *Sindora sumatrana* Miq. were collected in

Surabaya, Indonesia, in 2001 and were identified by professor Tri Windono (University of Surabaya, Indonesia). A voucher specimen (No. 07/DT/XI/2001) has been deposited at University of Surabaya, JL. Raya Kalirungkut, Surabaya 60293, Indonesia.

### General experimental procedures

Optical rotations were obtained using a DIP-1000 digital polarimeter (Jasco, Japan) at 25°C. UV and IR spectra were recorded with a U-3000 spectrophotometer (Hitachi, Japan) and a FTS 135 FT-IR spectrometer (Bio-Rad, CA), respectively. LR and HREIMS were recorded on an Autospec M393 mass spectrometer (Micromass, U.K.) operated at 70 eV. NMR experiments were conducted on a Unity INOVA 400 MHz FT-NMR (Varian, CA), and TMS was used as an internal standard.

TLC analysis was performed on Kieselgel 60 $F_{254}$ (Merck) plates (silica gel, 0.25 mm layer thickness), with compounds visualized by dipping plates into 10% (v/v) $H_2SO_4$ reagent (Aldrich) followed by charring at 110°C for 5-10 min. Silica gel (Merck 60A, 200-400 mesh ASTM) and Sephadex LH-20 (Amersham Pharmacia Biotech) were used for column chromatography. All solvents used for chromatographic separations were distilled before use.

### Extraction and isolation

The dried and milled plant material (500 g) was extracted with MeOH (3×3 L) by maceration. The extracts were combined and concentrated *in vacuo* at 40°C. The concentrated extract (43.4 g) was suspended in distilled water (1 L) and then partitioned with *n*-hexane (3×1 L) to afford *n*-hexane-soluble syrup on drying (4.0 g). Next, the aqueous extract was partitioned again with $CHCl_3$ (3×1 L) to give a $CHCl_3$-soluble extract and an aqueous residue. The $CHCl_3$-soluble extract (20 g) was chromatographed over silica gel as stationary phase using a $CH_2Cl_2$-MeOH gradient (from 1:0 to 0:1 v/v) as mobile phase to afford 10 pooled fractions (F01-F010). Fraction F04 [eluted with $CH_2Cl_2$-MeOH (99:1 v/v); 3.9 g] was chromatographed over silica gel as stationary phase using *n*-hexane-EtOAc gradient (from 9:1 to 1:1 v/v) as mobile phase to afford nine subfractions (F0401-F0409) and compound **2** (218 mg). Fractions F0406 [eluted with *n*-hexane-EtOAc (7:3 v/v); 190 mg] and F0408 [eluted with *n*-hexane-EtOAc (3:2 v/v); 180 mg] were purified over a further Sephadex LH-20 column, with 100% MeOH, to give compounds **3** (43 mg) and **4** (132 mg), respectively.

Fractions, F05 and F06 [eluted with $CH_2Cl_2$-MeOH (197:3 v/v), 5.1 g], were combined and then chromatographed on silica gel with gradient mixtures of *n*-hexane-acetone (from 4:1 to 1:1), yielding, in turn, the new compound **1** (9.4 mg) and the known compounds **7** (140 mg), **8** (64 mg), and **5** (16 mg). A known sesquiterpenoid, compound **6** (145 mg) was isolated from the *n*-hexane-soluble extract (4.0 g) by chromatography over silica gel using *n*-hexane-EtOAc gradient (from 19:1 to 0:1 v/v) as mobile phase.

### Assay methods

Measurements of NO formation by iNOS activity were performed in cultured LPS-induced RAW 264.7 macrophage cells. The cells were maintained in DMEM-supplemented with penicillin-streptomycin and 10% fetal bovine serum (FBS) at 37°C, in 5% $CO_2$ of humidified air. To evaluate the inhibitory activity of test materials on iNOS, the cells in 10% FBS-DMEM without phenol red were plated in 24-well plates ($5\times10^4$ cells/mL), and then incubated for 24 h. The cells were replaced with new media, and then incubated with 1 μg/mL of LPS and test samples. After additional 20 h incubation, the media were removed and analyzed for nitrite accumulation as an indicator of NO production by the Griess reagent. Briefly, 150 μL of Griess reagent (0.1% naphthylethylenedianine and 1% sulfanilamide in 5% $H_3PO_4$ solution) were added to 100 μL of each supernatant from LPS, or sample-treated cells in triplicate. The plates were read at 570 nm against a standard curve of sodium nitrite. The percentage inhibition was expressed as [1-(NO level of sample/NO level of vehicle treated-control)] × 100.

**(+)-(13*E*)-2-Oxo-3,13-clerodien-15-oic acid [(+)-2-oxokolavenic acid] (1)**

Colorless solid: $[\alpha]_D^{25}$ +2.2° (*c* 0.42, $CHCl_3$); IR $\nu_{max}$ NaCl $cm^{-1}$: 2928, 2875, 1689, 1669, 1643, 1459, 1438, 1250, 1152, 755; UV $\lambda_{max}$ MeOH nm (log ε): 223 (3.98); $^1$H-NMR ($CDCl_3$, 400 MHz) δ: 5.73 (1H, s, H-3), 5.68 (1H, s, H-14), 2.40 (1H, dd, *J* = 17.6, 13.2 Hz, H-1ax), 2.32 (1H, dd, *J* = 17.6, 4.0 Hz, H-1eq), 2.15 (3H, d, *J* = 0.8 Hz, H-16), 2.02 (1H, td, *J* = 12.4, 4.4 Hz, H-12*trans*), 1.90 (overlapped, H-12*cis*), 1.89 (3H, d, *J* = 1.2 Hz, H-18), 1.85 (1H, dd, *J* = 13.4, 4.4 Hz, H-10ax), 1.83 (1H, dt, *J* = 12.4, 2.8 Hz, H-6eq), 1.51 (1H, m, H-7), 1.47 (1H, m, H-8), 1.41-1.44 (2H, m, H-11), 1.38 (overlapped, H-6ax), 1.30 (1H, m, H-7), 1.12 (3H, s, H-19), 0.85 (3H, d, *J* = 6.0 Hz, H-17), 0.83 (3H, s, H-20); $^{13}$C-NMR ($CDCl_3$, 100 MHz) δ: 200.1 (C-2), 172.5 (C-4), 170.8 (C-15), 162.9 (C-13), 125.5 (C-3), 115.0 (C-14), 45.7 (C-10), 39.9 (C-5), 38.8 (C-9), 36.1 (C-8), 35.6 (C-6), 35.5 (C-11), 34.9 (C-1), 34.3 (C-12), 26.9 (C-7), 19.5 (C-16), 19.0 (C-18), 18.4 (C-19), 17.9 (C-20), 15.7 (C-17); LREIMS *m/z* (rel. int.): 318 ([M]$^+$, 12), 303 ([M-Me]$^+$, 10), 300 ([M-$H_2O$]$^+$, 23), 285 ((M-Me-$H_2O$]$^+$, 21), 259 ([M-Me-$CO_2$]$^+$, 27), 205 ([$C_{14}H_{21}O$]$^+$, 82), 135 ([$C_9H_{10}O$]$^+$, 68), 121 (70), 109 (100), 95 (83); HREIMS *m/z*: 318.2190 ([M]$^+$, calcd for $C_{20}H_{30}O_3$, 318.2195).

**(+)-7β-Acetoxy-15,16-epoxy-3,13(16),14-clerodatriene-18-oic acid (2)**

Colorless amorphous solid: $[\alpha]_D^{25}$ +85.6° (*c* 0.4, $CHCl_3$) {lit.

$[\alpha]_D$ +88° (*c* 2.2, $CHCl_3$) (Heymann *et al.*, 1994c)}. $^1$H- and $^{13}$C-NMR and MS data were in agreement with the reported values (Heymann *et al.*, 1994c).

## RESULTS AND DISCUSSION

A known diterpenoid, (+)-7β-acetoxy-15,16-epoxy-3,13 (16),14-clerodatriene-18-oic acid (**2**) (Heymann *et al.*, 1994c) was isolated from the *n*-hexane and $CHCl_3$-soluble fractions of the pods of *Sindora sumatrana* by bioassay-guided fractionation using iNOS inhibition assay as an active constituent. In addition, a new *trans*-clerodane diterpenoid, (+)-2-oxokolavenic acid (**1**) along with six known diterpenoids, (+)-3,13-clerodadiene-16,15-olide-18-oic acid (**3**) (Heymann *et al.*, 1994c), (+)-7β-acetoxy-3,13-clerodadiene-16,15-olide-18-oic acid (**4**) (Heymann *et al.*, 1994c), (+)-7β-acetoxy-16-hydroxy-3,13-clerodadiene-16,15-olide-18-oic acid (**5**) (Heymann *et al.*, 1994c), β-caryophyllene oxide (**6**) (Heymann *et al.*, 1994a), clovane-2β,9β-diol (**7**) (Heymann *et al.*, 1994a), and caryolane-1,9β-diol (**8**) (Heymann *et al.*, 1994a) were isolated and found to be inactive. The structures of the known compounds (**2-8**) were identified by physical and spectroscopic data (mp, $[\alpha]_D$, MS, $^1$H- and $^{13}$C-NMR) and by comparison with the published values of the compounds isolated from the same plant.

Compound **1** was obtained as colorless solid and gave a protonated molecular ion at *m/z* 318.2190 $[M]^+$ by HREIMS, consistent with an elemental formula of $C_{20}H_{30}O_3$. Assignments for the resonances of all of the hydrogen and carbon atoms in the molecule were made by application of one- and two-dimensional NMR experiments ($^1$H-NMR, $^{13}$C-NMR, DEPT, COSY, HSQC, HMBC, and NOESY). The $^1$H-NMR spectrum of **1** showed resonances for five methyl signals (3H) at $\delta_H$ 0.83 (s), 1.12 (s), and 0.85 (d, *J* = 6.0 Hz), indicating two tertiary and one secondary methyl on saturated carbons, and at $\delta_H$ 1.89 (d, *J* = 1.2 Hz) and 2.15 (d, *J* = 0.8 Hz) on unsaturated carbons. The latter both exhibited long-range allylic coupling (*ca* 1 Hz) to olefinic protons at $\delta_H$ 5.73 and 5.68 (Hasan *et al.*, 1982). The signal at $\delta_H$ 2.15 is characteristic of a β-methyl group in a clerodan-15-oic type α,β-unsaturated acid with the *E*-configuration (Heymann *et al.*, 1994c). The $^{13}$C-NMR and DEPT experiments with **1** showed six quaternary carbons including a carbonyl carbons (δ 200.1), as well as four tertiary carbons, comprising two $sp^2$ carbon (δ 125.5 and 115.0), five secondary carbons, and six methyl groups.

On the basis of these spectral data and careful analysis of the COSY, HSQC, and HMBC NMR data (Fig. 2), it was inferred that compound **1** is a kolavane series diterpenoid acid (Aquino *et al.*, 1992; Hasan *et al.*, 1982). The relative configurations of the positions C-5, C-8, C-9, and C-10 were determined based on the key NOESY correlations (Fig. 3). All of these data were in accordance with the assignment of **1** as (13*E*)-2-oxo-3,13-clerodien-15-oic acid [2-oxokolavenic acid] and with published data of (-)-2-oxokolavenic acid (Aquino *et al.*, 1992). However, compound **1** has a positive optical rotation value ($[\alpha]_D$ +2.2°, chloroform), in contrast to its (-)-*ent*-type ($[\alpha]_D$ 56.5°, chloroform; Hasan *et al.*, 1982) ($[\alpha]_D$ 54°, chloroform; Aquino

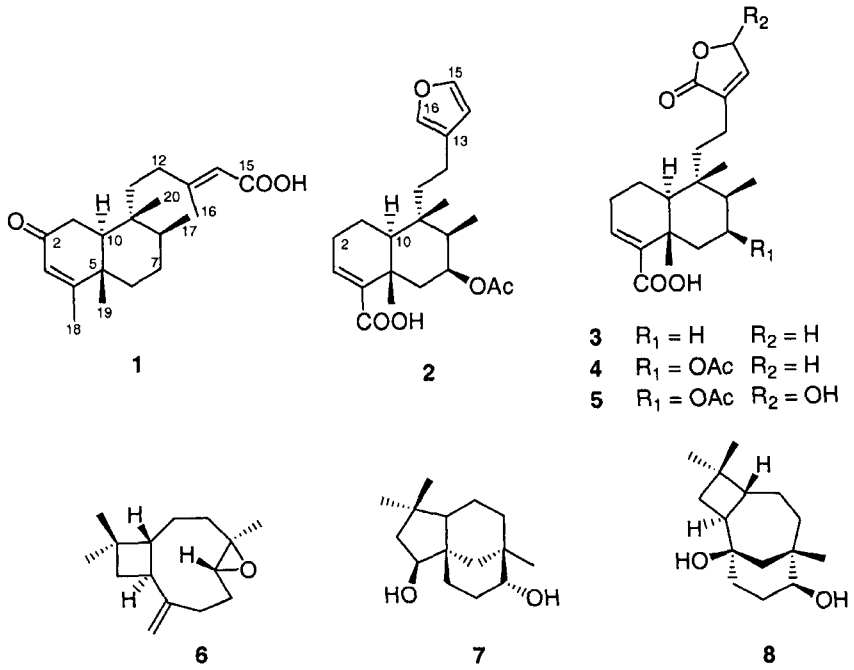


*Fig. 1. Structures of compounds* **1-8** *isolated from S. sumatrana.*

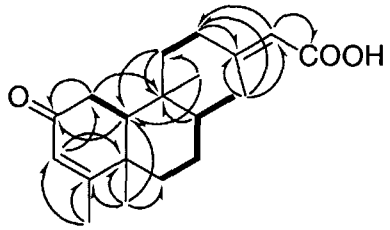


**Fig. 2.** Key correlations observed in the COSY (—) and HMBC (→) NMR spectra of **1**.

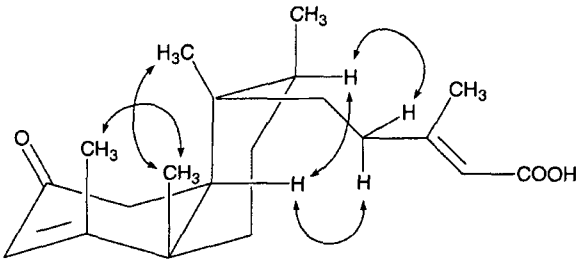


**Fig. 3.** Selected NOE correlations for **1**.

*et al.*, 1992). Furthermore, all the diterpenoids isolated from *S. sumatrana* in the present study as well as in the previous work (Heymann *et al.*, 1994c), were (+)-clerodane-type diterpenoids and it seems probable that compound **1** has the same configuration. Thus, the structure of **1** was elucidated as (+)-(13*E*)-2-oxo-3,13-clerodien-15-oic acid [(+)-2-oxokolavenic acid]. This is the first report of the normal-type 2-oxokolavenic acid, although the (-)-*ent*-type has been previously reported as a constituent of *Xylopia acethiopica* (Annonaceae) (Hasan *et al.*, 1982).

All isolates were tested for their potential to inhibit LPS-induced iNOS in murine macrophase RAW 264.7 cells. Of these compounds, only compound **2** exhibited an inhibitory activity in LPS-induced NO production with an $IC_{50}$ value of 51.6 μM [positive control: $N^{\omega}$-monomethyl-L-arginine (L-NMMA), $IC_{50}$ = 21.3 μM]. Therefore, **2** was considered as a possible potent iNOS inhibitory constituent of *S. sumatrana*. Although compound **2** was previously isolated from the same plant, this is the first report on its biological activity.

## ACKNOWLEDGEMENTS

This investigation was supported by a grant of the Korea Health 21 R&D project, Ministry of Health & Welfare, Republic of Korea (01-PJ2-PG6-01NA01-0002). We are grateful to Prof. Tri Windono and Dr. Gwang-Ho Jeohn at Surabaya University, Surabaya, Indonesia for plant collection.

## REFERENCES

Aquino, R., Ciavatta, M. L., De Tommasi, N., and Gàcs-Baitz, E., Tetranorditerpenes from *Detarium microcarpum*. *Phytochemistry*, 31, 1823-1825 (1992).

Elliott, S. and Brimacombe, J., The medicinal plants of Gunung Leuser National Park, Indonesia. *J. Ethnopharmacol.*, 19, 285-317 (1987).

Hasan, C. M., Healey, T. M., and Waterman, P. G., Kolavane and kaurane diterpenes from the stem bark of *Xylopia aethiopica*. *Phytochemistry*, 21, 1365-1368 (1982).

Heymann, H., Tezuka, Y., Kikuchi, T., and Supriyatna, S., Constituents of *Sindora sumatrana* Miq. I. Isolation and NMR spectral analysis of sesquiterpenes from the dried pods. *Chem. Pharm. Bull.*, 42, 138-146 (1994a).

Heymann, H., Tezuka, Y., Kikuchi, T., and Supriyatna, S., Constituents of *Sindora sumatrana* Miq. II. Five new sesquiterpenoids from the dried pods. *Chem. Pharm. Bull.*, 42, 941-946 (1994b).

Heymann, H., Tezuka, Y., Kikuchi, T., and Supriyatna, S., Constituents of *Sindora sumatrana* Miq. III. New trans-clerodante diterpenoids from the dried pods. *Chem. Pharm. Bull.*, 42, 1202-1207 (1994c).

Je, K.-H., Han, A.-R., Lee, H.-T., Mar, W., and Seo, E.-K., The inhibitory principle of lipopolysaccaride-induced nitric oxide production from *Inula britannica* var. *chinensis*. *Arch. Pharm. Res.*, 27, 83-85 (2004).

Lala, P. K. and Chakraborty, C., Role of nitric oxide in carcinogenesis and tumour progression. *Lancet Oncol.*, 2, 149-156 (2001).

Lee, H.-T., Yang, S.-W., Kim, K. H., Seo, E.-K., and Mar, W., Pseudoguaianolides isolated from *Inula britannica* var. *chinenis* as inhibitory constituents against inducible nitric oxide synthase. *Arch. Pharm. Res.*, 25, 151-153 (2002).

Moncada, S., Nitric oxide: discovery and impact on clinical medicine. *J. R. Soc. Med.* 92., 164-169 (1999).