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# Isolation of Dineolignans, Saucernetin-7 and -8, with Nitric Oxide Inhibitory Activity and NMR assignment from *Saururus chinensis*

**Kyung-Tae Lee, Hyun-Ju Jung[1] and Hee-Juhn Park[1]***
College of Pharmacy, Kyung-Hee University, Seoul 131-760, Korea
[1]Department of Botanical Resources, Sangji University, Wonju 220-702, Korea

**Abstract** - Two dineolignans (**1**, **2**) with nitric oxide inhibitory activities were isolated from *Saururus chinensis* (Saururaceae) using silica gel column chromatography. Although the structures, saucernetin-7 (**1**) and –8 (**2**), have been already reported, NMR assignment of the two compounds was completed aided by 2D-NMR spectroscopy including $^{1}H$-$^{1}H$ COSY, $^{1}H$-$^{13}C$ COSY, HMBC and NOESY NMR spectra. Compounds **1** and **2** exhibited significant nitride oxide inhibitory activity in LPS-induced RAW 264.7 cells with $IC_{50}$ values of 11.3μM and 7.1μM, respectively.

**Key words** - *Saururus chinensis*, Saururaceae, Dineolignan, Lignan, Saucernetin, NMR assignment

## Introduction

NO has diverse physiological roles and also contributes to the immune defense against viruses, bacteria, and other parasites. However, excess production of NO is associated with various diseases such as arthritis, diabetes, stroke, septic shock, autoimmune diseases, chronic inflammatory diseases, and atherosclerosis (Bredt and Snyder 1994).

*Saururus chinensis* (Lour.) Baill (Saururaceae) has been used to treat edema, jaundice, and gonorrhea (Chung *et al.*, 1900) in the folk medicine in Korea. Several constituents such as lignans, neolignans (Rao *et al.*, 1990), acyclic diterpenes (Rajbhandari *et al.*, 2001), aristolactams (Rao and Reddy *et al.*, 1990) and particularly manassantin A and –B (Rao and Alvarez *et al.*, 1990) classified into dineolignans have been isolated from the genus *Saururus*. In addition, the constituents of sauchinone and its stereoisomers, a phenylpropanoid (sarisan), lignans (galbacin and saucernetin) (Sung *et al*, 2000), diarylbutane lignans (Ahn *et al.*, 2001), and tetrahydrofuran-type sesquilignans (Sung and Huh *et al.*, 2001), furanoditerpenes (Hwang *et al.*, 2002) were isolated from *S. chinensis*. It has been reported that sauchinone, a lignan from *S. chinensis*, attenuates $CCl_4$-induced toxicity in primary cultures of rat hepatocytes (Sung *et al.*, 2000).

We have isolated dineolignans, saucernetins 7 (**1**) and 9 (**2**) with nitric oxide inhibitory activity from *S. chinensis* in the course of isolation of anti-inflammatory constituents. Since the two compounds were established as the isomers of manassantin A and –B, respectively, which has been isolated from *S. chinensis*, the structural difference between the former compounds and the latter ones is presented based on the 2D-NMR spectral data including HMBC- and NOESY NMR. Although the structures have been already known (Min *et al.*, 2001), full NMR assignment is herein reported.

*Corresponding author. E-mail : hjpark@sangji.ac.kr

## Materials and Methods

### General Experimental Procedures

Optical rotations were measured using a JASCO DIP-370 digital polarimeter. The IR spectra were measured with a JASCO IR report-100 infrared spectrometer. The $^{1}H$- and $^{13}C$-NMR spectra were recorded on a Bruker AMX 500 using TMS as an internal reference. The EI-MS was recorded on a VG high resolution GC/MS (model, Autospexc-Ultima) spectrometer. Silica gel 60 (Merck No. 5715) was used for normal phase column chromatography.

### Plant Material

The underground parts of *S. chinensis* (2kg) were purchased from Keochang herb farm (Keochang, Korea) and identified by B. T. Ahn, one of the authors. Voucher specimen has been deposited under No. CNUP 3053 in College of Pharmacy, Chungbuk National University, Korea.

### Extraction and Isolation

The dried plant material (2kg) was cut into small pieces and extracted repeatedly with 80% MeOH (3×6L). The combined methanolic extract was concentrated *in vacuo* and the aqueous suspension of this dried extract was successively partitioned with hexane, $CHCl_3$,

EtOAc and BuOH. $CHCl_3$ fraction (20g) was chromatographed on silica gel (70-230 mesh, 600g) using hexane with increasing amounts of EtOAc to give 8g of the fraction with anti-inflammatory action *in vitro* (data not shown) when eluted with 60% EtOAc/hexane. This material was further separated using silica gel (70-230 mesh, 40g) column chromatography with the eluent of benzene/acetone to give **1** (2.21g) and **2** (1.92g).

Saucernetin-7 (**1**, $C_{42}H_{52}O_{11}$): pale brown solid, $[\alpha]_D^{25}$ –9.3° (MeOH, c=0.30); IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3500, 1610, 1590; $^1$H-NMR ($CDCl_3$, 500 MHz) δ: Table 1; $^{13}$C-NMR ($CDCl_3$, 125 MHz) δ: Table 2; EIMS (rel. int.): m/z 732 ($[M]^+$, 43), 538 (96, $[M-C_{11}H_{14}O_3]^+$), 520 (45), 370 (10), 357 (8), 192 (94), 180 (38), 165 (100).

Saucernetin-8 (**2**, $C_{41}H_{48}O_{11}$): pale brown solid, $[\alpha]_D^{25}$ –16.2° (MeOH, c=0.40); IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3500, 1610, 1590; $^1$H-NMR ($CDCl_3$, 500 MHz) δ: Table 1; $^{13}$C-NMR ($CDCl_3$, 125 MHz) δ: Table 2; EIMS (rel. int.): m/z 716 (32, $[M]^+$), 698 (7, $[M-H_2O]^+$), 538 (41, $[M-C_{10}H_{10}O_3]^+$), 520 (13), 370 (69), 339 (50), 192 (100), 165 (94), 151 (85), 121 (68).

**Nitrite assay**

Nitrite accumulation, an indicator of NO synthesis, was measured in the culture medium by Griess reaction (Kim *et al.*, 2000). Briefly, 100μl of cell culture medium were mixed with 100μl of Griess reagent [equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phos-

Table 1. $^1$H NMR data of **1** and **2** isolated from *S. chinensis* (500 MHz, $CDCl_3$)

| Hyd. No. | Saucernetin 7 (**1**) | Saucernetin 8 (**2**) |
|---|---|---|
| 1, 1′ | - | - |
| 2, 2′ | 6.92[1)] (2H, d, 1.8 Hz[2)]) | 6.92-6.86 (2H) |
| 3, 3′ | - | - |
| 4, 4′ | - | - |
| 5, 5′ | 6.84 (2H, d, 8.2 Hz) | 6.84 (1H, d, 8.2 Hz)<br>6.78 (1H, d, 7.9 Hz) |
| 6, 6′ | 6.83 (2H, dd, 1.8 & 8.3 Hz) | 6.82-6.85 (2H, dd-like) |
| 7, 7′, | 5.47 (2H, d, 6.0 Hz) | 5.46 (2H, d, 5.9 Hz) |
| 8, 8′ | 2.30 (2H, m) | 2.30 (2H, m) |
| 9, 9′ | 0.73 (2×3H, d, H=6.6 Hz) | 0.72 (2×3H, d, 6.5 Hz) |
| $OCH_3$ | | 3.92 (s), 3.93 (s) |
| 1″, 1‴ | - | - |
| 2″, 2‴ | 6.94 (2H, d, 1.8 Hz) | 6.84 (1H, d, 1.8 Hz)<br>6.94 (1H, d, 1.8 Hz) |
| 3″, 3‴ | - | - |
| 4″, 4‴ | - | - |
| 5″, 5‴ | 6.99 (2H, d, 8.2 Hz) | 6.99 (1H, d, 8.1 Hz)<br>6.98 (1H, d, 7.2 Hz) |
| 6″, 6‴ | 6.94 (2H, dd, 1.8 & 8.2 Hz) | 6.94 (1H, dd, 1.8 & 8.2 Hz)<br>6.86-6.92 (1H, dd-like) |
| 7″, 7‴ | 4.66 (2H, d, 8.3 Hz) | 4.65 (2H, d, 8.3), 4.62 (2H, d, 8.3 Hz) |
| 8″, 8‴ | 4.13 (2H, dd, 6.3 & 8.3 Hz) | 4.13 (1H, dq, 8.3 & 6.5 Hz)<br>4.12 (1H, dq, 8.3 & 6.4 Hz) |
| 9″, 9‴ | 1.17 (2×3H, d, 6.3 Hz) | 1.17 (3H, d, 6.5 Hz), 1.18 (3H, d, 6.4 Hz) |
| $-OCH_2O-$ | - | 5.95 (2H, s) |
| $OCH_3$ | 3.88 (3H, s), 3.89 (2×3H, s) | 3.88 (s), 3.89 (s) |

[1)]Unit represents δ (ppm).
[2)]Value represents coupling constants.

phoric acid and 0.1% (w/v) naphtylethylenediamine-HCl] and incubated at room temperature for 10 min, and then the absorbance at 550nm was measured in a microplate reader. Fresh culture medium was used as the blank in all experiments. The amount of nitrite in the samples was calculated from a sodium nitrite standard curve freshly prepared in culture medium.

## Results and Discussion

Column chromatography of $CHCl_3$ fraction produced the two compounds, **1** (saucernetin 7) and **2** (saucernetin 8). In the $^1$H-NMR spectrum of **1**, the peaks due to the inner two phenylpropanoid moieties were observed indicating that it belongs to a type of lignans. However, it was presumed that **1** is a tetrameric phenylpropanoid from the molecular ion (m/z 732, $[C_{42}H_{52}O_{11}]^+$) in the mass spectrum. This spectrum also showed a prominent ion peak at m/z 538 due to the fragment ion that a phenylpropanoid moiety has been eliminated. The NMR data of **1** was compared with literature data (Rao and Alvarez, 1983) on manassantin A which has been isolated from *S. cernuus*. The spectral data both measured in $CDCl_3$ were similar between **1** (saucernetin 7) and manassantin A but different, indicating that **1** belongs to dineolignan distinguishable from manassantin A. Six aromatic hydrogen peaks were shown as coupled with *o*-, *m*-, and both *o*- and *m*-type from the coupling constants as shown in Table 1. The data shown in Table **1** exhibited the exact symmetricity which is different from other dineolignans including **2** described below. Since the optical rotation of manassantin A, $[\alpha]_D$ –100° (Rao and Alvarez, 1983), is significantly different from **1**, $[\alpha]_D^{25}$ –9.3°, the latter compound could be assigned as a stereoisomer of the former. Dimethyltetrahydrofuran, a central ring system of this diarylbutane-type lignan, showed three kinds of proton peaks (each 2H in methine and 6H in methyl) due to the chains of phenylpropanoid moieties at δ 0.73 (2×3H, H-9,9′), 2.30 (2H, H-8, 8′), δ 5.47 (2H, H-7, 7′) whereas other two chains were observed by being precisely overlapped at δ 1.17 (2×3H, H-9″, 9‴), 4.13 (2H, H-8″, 8‴), 4.66 (2H, H-7″, 7‴), respectively, as shown in Table 1. The $^{13}$C-NMR data shown

Table 2. $^{13}$C NMR data of **1** and **2** isolated from plants. (125 MHz, $CDC_{13}$)

| Carbon No. | Saucernetin 7 (**1**) | Saucernetin 8 (**2**) |
|---|---|---|
| 1, 1′ | 136.51 | 136.54, 136.47 |
| 2, 2′ | 110.91 | 110.92 |
| 3, 3′ | 146.49 | 146.45, 146.33 |
| 4, 4′ | 148.88 | 148.88, 150.6 |
| 5, 5′ | 118.75 | 118.75, 118.64 |
| 6, 6′, | 110.12 | 110.12, 110.92 |
| 7, 7′, | 83.36 | 83.38 |
| 8, 8′ | 44.24 | 44.20 |
| 9, 9′ | 14.89 | 14.87 |
| $OCH_3$ | 55.88 | 55.88 |
| 1″, 1‴ | 132.63 | 132.62 |
| 2″, 2‴ | 120.00 | 120.00, 121.08 |
| 3″, 3‴ | 149.04 | 149.03, 147.75 |
| 4″, 4‴ | 150.60 | 150.58, 147.38 |
| 5″, 5‴ | 118.73 | 118.71, 108.10 |
| 6″, 6‴ | 110.15 | 110.15, 107.55 |
| 7″, 7‴ | 78.41 | 78.40 |
| 8″, 8‴ | 84.08 | 83.90, 83.97 |
| 9″, 9‴ | 17.04 | 17.01, 16.89 |
| $-OCH_2O-$ | - | 101.02 |
| $OCH_3$ | 55.91 | 55.86, 55.90 |

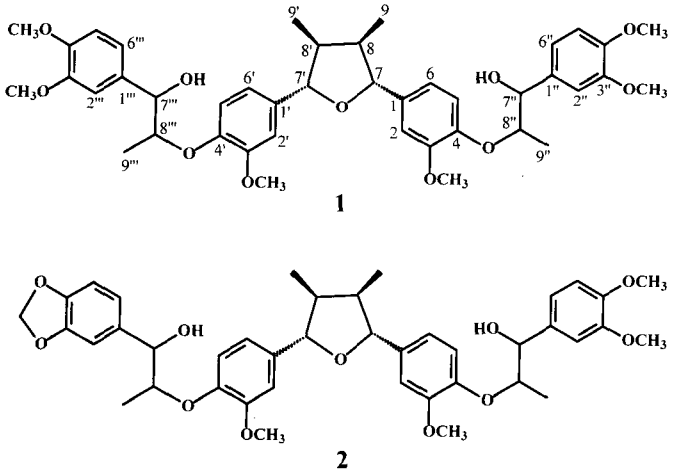

Fig. 1. Structure of **1** and **2** isolated from *S. chinensis*.

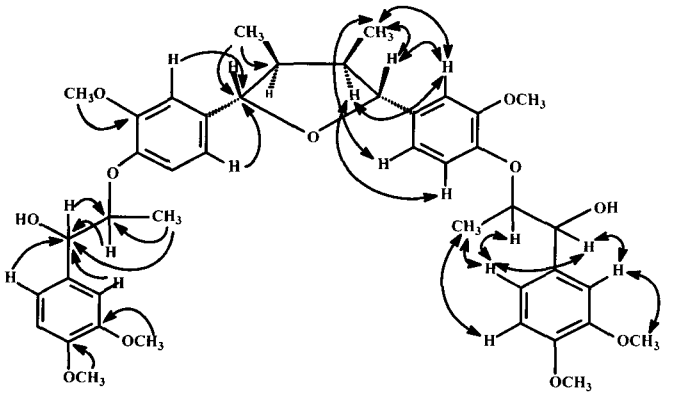

Fig. 2. HMBC- and NOESY correlations on **1**. (HMBC correlation (→) on the left half of the symmetrical structure and NOESY correlation (↔) on the right half).

in Table 2 exactly represented each two carbons due to symmetrical structure. As shown in Fig. 1, the substituents, both methyl groups and aryl moieties at C-7 and –8 or at C-7′ and –8′, established to be *trans*-geometry, respectively, by the NOESY correlation (Fig. 2) whereas the two methyls at C-7 and -7′ and the aryls at C-8 and –8′ must be configurated as *cis*-geometry, respectively, based on complete symmetricity. Manassatin A which has been isolated from *S. cernuus* is not stereochemically unsymmetric based on the different chemical shifts at each corresponding carbon of the dineolignan with just relatively symmetric structure (Rao and Alvarez, 1983)[5]. Even though the two kinds of substituents at C-7, -8 and at C-7′, -8′ could be drawn in the opposite configuration unlike in Fig. 1, both structures are exactly same. Again, all the β-configuration of substituents in tetrahydrofuran ring of **2** should be excluded since the $\delta_H$ and $\delta_C$ of the positions-7, -8 and -9 in **2** is quite different from those (in $CDCl_3$) of di-O-methyltetrahydrofuriguaiacin B (Sung and Huh *et al.*, 2001) with all the β-configurated substituents in the ring. The HMBC NMR spectrum aided the assignment of the $^{13}$C-NMR data as shown in Table 2. Therefore, saucernetin 7 (**1**) is just stereochemically different from manassantin A and the former compound has not been reported from a natural source. NMR assignment could be done on the basis of $^1$H-$^1$H COSY-, HMQC-, HMBC- and NOESY NMR spectra (Table 1 and 2).

The EIMS data of **2** exhibited the molecular ion at m/z 716 due to $C_{41}H_{48}O_{11}$ and a prominent fragment ion at m/z 538 due to $[M\text{-}C_{10}H_{10}O_3]^+$ which has been observed in the EIMS of **1**. The peaks in the $^1$H-NMR data of **2** (saururuslignan B) were shown at the similar δ values with those of **1** though they showed more complex peaks due to an unsymmetric structure. The chemical shifts at $\delta_H$ 5.95 and $\delta_C$ 101.02 due to the methylenedioxy substituted to C-3‴ and –4‴ of **2** were shown. As shown in Fig. 1, the relative structure of **2** ($[\alpha]_D^{25}$ –16.2) is the same as manassantin B ($[\alpha]_D^{25}$ –99°) but stereochemically different based on the significant discrepany of δ values in the NMR spectrum and $[\alpha]_D$ in optical rotation. All the NMR spectral peaks of **1** were also shown in those of **2** whereas the chemical shifts of the left part of the structure with methylenedioxy in Fig. 1 were shown at the significantly shifted δ values than the other part. A chemical shift $\delta_H$ 5.95 of methylenedioxy was shown as long-range coupled with $\delta_C$ 147.75 and 147.38 in the HMBC NMR spectrum suggesting that methylenedioxy is positioned between C-3″ and C-4″ . The peaks due to C-7, -8, and –9 and H-7, -8, and -9, were shown to be same with the corresponding C-7′, -8′, and –9′ and H-7′, -8′ and –9′, respectively, indicating that the stereochemistry of tetrahydrofuran ring in **2** is the same as that in **1**. Saucernetin 8 is stereochemically different from manassantin B and it has not been reported from a natural source. It is well known that lignans could be formed in plants mainly by means of the oxidative coupling of phenylpropanoids. Based on the different stereochemistry of saucernetin-7 and -8 isolated from *S. chinensis* than manassantin A and –B from *S. cernuus*, it could be suggested that the two plant species might make the coupling of phenylpropanoids in the different biogenetic progression. Compounds **1** and **2**, with $IC_{50}$ values of 11.3μM and 7.1μM, respectively, exhibited potent activities as NO inhibition in the Griess (nitrite) assay. Cytotoxic effect of **1** and **2** evaluated in the absence or presence of LPS by MTT assay did not affect the cell viability of RAW 264.7 cells even at 100μM for 24 h (data not shown). L-$N^6$-(1-iminoethyl) lysine ($IC_{50}$ 25.6μM) was used as a positive inhibitor in this assay.

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