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Short communication

Lignan, sesquilignans and dilignans, novel HIV-1 protease and cytopathic effect inhibitors purified from the rhizomes of *Saururus chinensis*

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ABSTRACT

Five lignans were isolated from the ethyl acetate extracts of *Saururus chinensis* rhizomes and evaluated for anti-HIV-1 activity. Their structures were elucidated as two dilignans, manassantin A (**1**), manassantin B (**2**), two sesquilignans, saucerneol B (**3**) and saucerneol C (**4**), and a new lignan, saururin B (**5**) by spectroscopic analysis. Of these components, manassantin A (**1**) and saururin B (**5**) showed dose-dependent inhibitory activities on HIV-1 protease with IC_{50} values of 38.9 and 5.6 μM. In addition, manassantins A (**1**), B (**2**) and saucerneol B (**3**) inhibited HIV-1-induced cytopathic effects in a human T lymphoblastoid cell line with IC_{100} values of 1.0, 1.0 and 0.2 μM, respectively. Of these active constituents, saucerneol B (**3**) showed the most potent and selective anti-HIV-1 activity (IC_{100} of 0.2 μM, CC_0 of >125.0 μM, and SI of >520.8).

The etiological agent of the acquired immune deficiency syndrome (AIDS), considered one of the greatest challenges in medicine in the 20th century, is human immunodeficiency virus (HIV). Although the anti-HIV drugs now available have significantly extended the life span and improved the quality of life of HIV/AIDS patients, the appearance of drug-resistant virus strains added urgency to the search for new anti-HIV agents and targets. Highly active antiretroviral therapy (HAART), i.e. combination therapies with different classes of antiviral drugs was introduced for the effective therapy of HIV/AIDS, and has transformed this condition from a rapid lethal infection to a chronic condition that can be controlled for many years (Cos et al., 2008). In addition, many kinds of natural products are still considered to be a useful strategy for the development of anti-HIV-1 agents. They include alkaloids, sulfated polysaccharides, polyphenolics, flavonoids, coumarins, phenolics, tannins, triterpenes, etc. (Cos et al., 2008). Herbal medicine with *in vitro* bioassays for inhibition of the HIV-1-induced cytopathic effect (CPE), and the HIV essential enzymes such as reverse transcriptase, protease, and integrase would seem to be an effective research approach (Lee et al., 2008a,b, 2009).

In this study, we investigated anti-HIV compounds from the rhizomes of *Saururus chinensis* Baill (Saururaceae), a Korean special medicinal plant. The underground part of this plant (Saururi Rhizoma, 三白草根) has been used in the treatment of edema, beriberi, gonorrhea, leucorrhea, jaundice and leproma in Korean folk medicine (Jung and Shin, 1990). Various lignans have been reported from the underground parts of this plant (Sung et al., 2001; Sung, 2006; Hwang et al., 2003), and their anti-inflammatory and anti-cancer activities have also been studied (Hwang et al., 2003; Kwon et al., 2005). However, anti-HIV-1 components have not been isolated from this plant. The present study describes the isolation of anti-HIV-1 substances from the rhizomes of *S. chinensis* and their anti-HIV-1 activities.

S. chinensis Bail (Saururaceae) was cultivated in the Medicinal Plant Garden of the College of Pharmacy, Seoul National University in September of 1998, and a voucher specimen (SNUPH-0058) has been deposited in the Herbarium of our University.

The underground part of *S. chinensis* (Saururi Rhizoma) was extracted and lignans were isolated as previously described (Sung et al., 2001). The air-dried, powdered underground parts of *S.*

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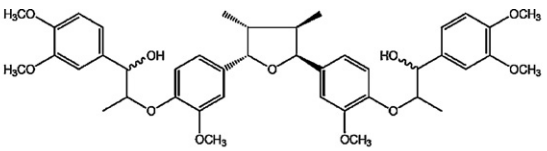
chinensis (5.5 kg) were defatted with *n*-hexane and were then extracted with MeOH in an ultrasonic apparatus which, upon removal of the solvent in vacuo, yielded a methanolic extract (900 g). The MeOH extract was then suspended in distilled water and was partitioned successively with EtOAc and *n*-BuOH. The EtOAc fraction (100 g) was fractionated by column chromatography over silica gel to obtain six fractions 1–6, sequentially. From active fraction 3 (32.5 g, 85.3%), compounds **1** (38.2 mg), **2** (50.3 mg) and **3** (30.5 mg) were obtained by silica gel and semipreparative RP-HPLC. In addition, compounds **4** (25.3 mg) and **5** (40.3 mg, retention time, 7.16 min) were purified from the active fraction 4 (93.2%) by semipreparative RP-HPLC [mobile phase, H_2O–MeOH–AcCN (10:20:20)]. Manassantin A (**1**); white amorphous powder; $[\alpha]_D^{25}$-111° (c 1.0, $CHCl_3$): manassantin B (**2**); white amorphous powder; $[\alpha]_D^{25}$-144° (c 0.9, $CHCl_3$): saucerneol B (**3**); white powder; $[\alpha]_D^{25}$-58° (c 0.6, $CHCl_3$): saucerneol C (**4**); brown oil; $[\alpha]_D^{25}$-66° (c 1.3, $CHCl_3$): saururin B (**5**); brown oil; $[\alpha]_D^{25}$-44° (c 1.1, $CHCl_3$), IR (KBr) V_{max}; 3444 (OH), 2960, 1634, 1280, ^{1}H NMR (300 MHz, CD_3OD) δ 0.83 (6H, d, J = 6.8 Hz, H-9, 9′), 1.77 (2H, *m*, H-8, 8′), 2.31 (2H, *dd*, J = 8.4, 13.9 Hz, H-7a, 7a′), 2.56 (2H, *dd*, J = 5.6, 13.9 Hz, H-7b, 7b′), 5.85 (4H, *s*, –OCH_2O–), 6.34 (2H, *s*, H-3, 3″), 6.49 (2H, *s*, H-6, 6′), ^{13}C NMR (75 MHz, CD_3OD) δ 14.3 (C-9, 9′), 35.4 (C-7, 7′), 37.2 (C-8, 8′), 98.2 (C-3, 3′), 100.8 (–OCH_2O–), 110.1 (C-6, 6′), 119.6 (C-1, 1′), 141.1 (C-5, 5′), 145.8 (C-4, 4′), 147.8 (C-2, 2′); EIMS (70 eV) (*m*/*z*) (rel. int.); 358 (48) [M^+], 151 (100).

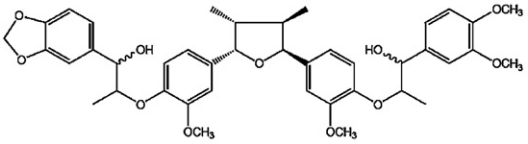
In order to determine the anti-HIV-1 protease (PR) activity of the compounds, a HIV-1 PR assay was performed as described previously (Lee et al., 2008a). The fused recombinant HIV-1 PR and synthetic undecapeptide [His-Lys-Ala-Arg-Val-Leu-(pNO_2-Phe)-Glu-Ala-Ile-Ser-NH_2] as a substrate were purchased from BACHEM Feinchemikalien AG (Bubendorf, Switzerland). The proteolytic activity against HIV-1 PR of the test samples was determined using HPLC with the synthetic undecapeptide. To a reaction mixture (25 μL) containing 50 mM of acetate buffer (pH 5.0), 2.5 μg of the substrate, 2.5 μL of a DMSO solution of the test compound, and 2.5 μL of recombinant HIV-1 PR (0.175 μg protein) were added and incubated at 37 °C for 15 min. The reaction was stopped by addition of 2.5 μL of 10% trifluoroacetic acid. The hydrolysate and remaining substrate were quantitatively analyzed by HPLC. The HPLC system was composed of LC9A liquid chromatography, a SPD-6A UV spectrophotometric detector, a SLC-6B autoinjector and an integrator C-R6A Chromatopac (Shimadzu Corporation, Kyoto, Japan). Five microliters of the reaction mixture were injected into a RP-18 column (4.6 mm × 150 mm) (YMC Inc., Kyoto, Japan) and were eluted with a gradient of acetonitrile (15–40%) in 0.1% trifluoroacetic acid at a flow rate of 1.0 mL/min. The elution profile was monitored at 280 nm. The substrate and p-NO_2-Phe-bearing hydrolysate were eluted at 11.6 and 6.6 min, respectively. Acetyl-pepstatin, which is widely used as a positive control, showed an IC_{50} of 0.09 μM under these conditions.

The inhibitory activity on the HIV-1-induced cytopathic effect (CPE) was described previously (Ma et al., 2002; Zhong et al., 2005). The human T lymphoblastoid cell line (MT-4) and human leukemia T-cell line (MOLT-4) infected with HTLV-1 were maintained at 37 °C under 5% CO_2 in a RPMI-1640 medium (Flow Laboratories, Irvine, Scotland) supplemented with 10% fetal calf serum (FCS, Flow Laboratories, North Ryde, Australia), 100 μg/mL of streptomycin (Meiji Seika, Tokyo, Japan) and 100 U/mL of penicillin G (Banyu Pharmaceutical, Tokyo, Japan). HIV-1 (strain HTLV-III_B) was obtained from the supernatant of MOLT-4/HTLV-III_B cells. Initially, MT-4 cells were infected for 1 h with HIV-1 (strain HTLV-III_B) at 0.001 $CCID_{50}$ (50% cell culture infective dose)/cell. For $CCID_{50}$ determinations, supernatants were collected on the tenth day after the beginning of treatment and were stored at −80 °C. Prior to testing, they were thawed and clarified by centrifugation and filtration through a 0.45 μm filter. The $CCID_{50}$ was determined according to the literature (Johnson and Byington, 1990). In brief, an assay of confluent cell monolayers in a 96-well plates with a 0.1 mL suspension containing 100 median $CCID_{50}$ of virus and serial twofold dilutions of the test samples were added simultaneously. To act as a virus control and a cell control, the virus suspension and a maintenance medium, respectively, without samples were added. The plates were incubated at 37 °C under a humidified CO_2 atmosphere for 3–6 days. The virus-induced CPE was scored under light microscopy in comparison with the virus control and cell control. The cells were then resuspended at 1×10^5 cells/mL in RPMI 1640 medium and 200 μL/well of the cell suspension was cultured for 5 days in a 96-well plates containing various concentrations (9 doses, maximum 125–100 μM and minimum 0.06–0.03 μM) of the test compounds. After 5 days, the inhibitory concentration (IC_{100}) of the test sample required to completely prevent the HIV-1-induced CPE was examined through an optical microscope, and the cell growth was visualized to determine the cytotoxic concentration (CC_0) that reduced the viability of MT-4 cells. SI, the selectivity index, was determined to assess whether the observed anti-HIV-1 activity was specific. Azidothymidine (AZT, TCI, Tokyo, Japan) and dextran sulfate 8000 (DS 8000, Sigma, St. Louis, MO) were used as reference compounds. The IC_{100} and CC_0 values of AZT were 0.0039 and >1.0 μg/mL, while those of DS 8000 were 1.95 and >1000 μg/mL, respectively.

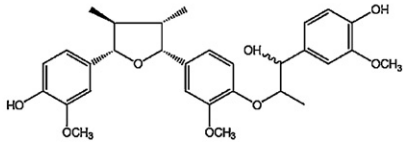
In the results, five components (**1**–**5**) were isolated by bioactivity-guided fractionation and isolation technique from the rhizomes of *S. chinensis* as HIV-1 protease inhibitors (Fig. 1). Their structures were elucidated as two dilignans, manassantin A (**1**) and manassantin B (**2**); and two sesquilignans, saucerneol B (**3**) and saucerneol C (**4**), via spectroscopic analysis in comparison with these values in the literature (Sung et al., 2001; Sung, 2006; Hwang et al., 2003). The structure of compound **5** was determined as described below. Compound **5** was obtained as brown oil, its IR spectrum showed a strong hydroxyl absorption band at 3444 cm^{-1}, and showed a brown color on treatment with an anisaldehyde sulfuric acid solution. The base molecular ion peak, *m*/*z* 358 of EIMS spectrum indicated the molecular formula $C_{20}H_{22}O_6$. The ^{1}H NMR spectra showed signals to indicate two methyl protons [δ 0.83, 6H, *d*, J = 6.8 Hz, H-9, 9′], two methylenedioxy protons [δ 5.85, 4H, *s*], four aromatic protons [δ 6.34, 2H, *s*, H-3, 3′], [δ 6.49 (2H, *s*, H-6, 6′)], two methine protons [δ 1.77, 2H, *m*, H-8, 8′], and two methylene protons [δ 2.31, 2H, *dd*, J = 8.4, 13.9 Hz, H-7a, 7a′], [δ 2.56, 2H, *dd*, J = 5.6, 13.9 Hz, H-7b, 7b′]. These data were characteristic of symmetrical 2,3-dimethyl-1,4-diarylbutane-type lignan. ^{13}C NMR spectra supported the above assumption [δ 14.3 (C-9, 9′), 35.4 (C-7, 7′), 37.2 (C-8, 8′), 98.2 (C-3, 3′), 100.8 (–OCH_2O–), 110.1 (C-6, 6′), 119.6 (C-1, 1′), 141.1 (C-5, 5′), 145.8 (C-4, 4′), 147.8 (C-2, 2′)]. In addition, the correlation the proton signals at [7a, 7′a, 7b, 7′b] and the carbon signals of [8, 8′, 9, 9′, 6, 6′, 1, 1′ 2, 2′] of the HMBC (heteronuclear multiple bonding correlation) spectrum also confirmed 2,3-dimethyl-1,4-diarylbutane-type lignan. Specially, signals of two protons [δ 5.85, 4H, *s*], two carbons [100.8 (–OCH_2O–)], and EIMS [*m*/*z* 151] showed presence of methylendioxy groups in the aromatic regions. In addition, the correlations of the protons of [methylenedioxy] and the carbons of [4, 4′, 5, 5′], the correlations of the protons of [3, 3′] and the carbons of [1, 1′ 2, 2′ 4, 4′, 5, 5′], and the correlations of the protons of [6, 6′] and carbons of [2, 2′, 4, 4′, 5, 5′] of the HMBC spectrum indicated an hydroxyl group at the 2 position and a methylenedioxy group at the 4, 5 positions of the phenyl groups; thus, the same symmetrical structure such as for saururin A (Ahn et al., 2001). But this compound has a negative optical activity −44°, different from that of saururin A (*meso* form, 8S,8′R configuration, 0°, Ahn et al., 2001), the same relative stereochemistry as sauriols A, B, and



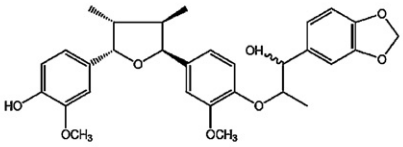
manassantin A (**1**)



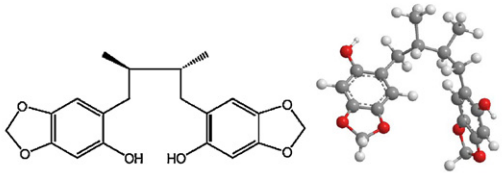
manassantin B (**2**)



saucerneol B (**3**)



saucerneol C (**4**)



saururin B (**5**)

rel-(8R,8′R)-2,2′-dihydroxy-4,5:4′,5′-bis-(methylenedioxy)-8,8′-lignans

Fig. 1. Structures of dilignans **1**, **2**, sesquilignans, **3**, **4**. and a lignan (**5**) from *S. chinensis*.

dihydroguaiaretic acid (non-*meso* form, 8R,8′R configuration, negative optical activity, Kubanek et al., 2000). Therefore, the structure of **5** was concluded to be *rel*-(8R,8′R)-2,2′-dihydroxy-4,5:4′,5′-bis-(methylenedioxy)-8,8′-lignan, and was labeled as saururin B for the first time from nature (Fig. 1).

In previous papers, manassantins A and B inhibited NF-κB activation, PMA-induced ICAM-1 expression and hypoxia-activated HIF-1 (Hwang et al., 2003; Hodges et al., 2004), and prevented the action of monocyte adhesion to HUVEC (Kwon et al., 2005). On the other hand, Hwang et al. (2003) reported that saucerneols D and E inhibited NF-κB-dependent reporter gene expression. Saururin A was reported as possessing low density lipoprotein-antioxidant activity (Ahn et al., 2001).

In this study, Table 1 showed the inhibitory activities of five components from *S. chinensis* against HIV-1 protease. Only saururin B, a new lignan, inhibited the activity of this enzyme with an IC_{50} of 5.6 μM. For a reference, machilin A and *meso*-dihydroguaiaretic acid isolated from *Machilus thunbergii*, which is structurally similar to saururin B, but which does not contain hydroxyl groups at the 2 and 2′ positions, did not show any effect (data not shown). This proves that the dihydroxy groups at the 2, 2′ positions influence the inhibitory activity against HIV-1 protease considerably. In the case of sesqui- and dilignans, while **2**–**4** did not inhibit activity below 125 μM, only manassantin A showed mild inhibitory activity, with

Table 1
Inhibitory activities against HIV-1 protease by lignans **1**–**5** from *S. chinensis*.

Compounds	HIV-1 Protease IC_{50} (μM)
1	38.9 ± 0.8
2	>125
3	>125
4	>125
5	5.6 ± 0.3
Acetyl-pepstatin	0.09 ± 0.01

Three experiments were conducted with each compound and the control. All values are expressed as mean ± SEM. IC_{50}, the inhibitor concentration (μM) required to reduce by 50% the protease (PR) activity.

Table 2
Inhibitory activities of lignans **1**–**5** from *S. chinensis* on the HIV-1-induced cytopathic effects in (HTLV-III_B-infected) MT-4 cells.

Compounds	HIV-1-induced cytopathic effects		
	IC_{100} (μM)	CC (μM)	SI
1	1.0 ± 0.2	7.8 ± 0.5	8.0
2	1.0 ± 0.1	62.5 ± 0.3	63.8
3	0.2 ± 0.0	>125	>520.8
4	ND	ND	–
5	>125	>125	–
AZT	0.06–0.03	>1.0	>62.5

Three experiments were conducted with each compound and the control. All values are expressed as mean ± SEM values. IC_{100} is the minimum concentration (μM) for complete inhibition of HIV-1-induced CPE in MT-4 cells as determined by microscopic observation; CC_0, the minimum concentration for the appearance of MT-4 cell toxicity by microscopic observation; SI, the selectivity index or ratio of CC_0 to IC_{100}. ND, not determined.

an IC_{50} value of 38.9 μM (Table 1). This result shows that the *ortho*-dimethoxy group of the benzene rings may be a pharmacophore that can be used for the expression of inhibitory activity on the HIV-1 protease of dilignan.

As shown in Table 2, the isolated five lignans were also assayed in MT-4 cells for their activity against HIV-1-induced CPE. Manassantins A (**1**), B (**2**) and saucerneol B (**3**) significantly inhibited the CPE with IC_{100} values of 1.0, 1.0 and 0.2 μM, respectively, whereas the lignan (saururin B) did not show inhibitory activity below 125 μM. The virus-inhibitory activity (IC_{100}, 0.2 μM) of saucerneol B (**3**) was more potent (showing a fivefold difference) compared to those (IC_{100}, 1.0 μM) of manassantin A (**1**) and manassantin B (**2**), while the cytotoxicity of **3** (CC_0, >125 μM) was much weaker than that of **1** or **2** (CC_0, 7.8 and 62.5 μM). Therefore, the selectivity index (SI, 520.8) of **3** is higher than those of **1** and **2** (SI, 8.0 and 63.8, respectively) and also higher than that of AZT (SI, >62.5) used as a positive control.

These results show that the new lignan (saururin B), and the dilignan (manassantin A) are novel HIV-1 protease inhibitors, and that di- and sesquilignans (manassantins A, B and saucerneol B) also represent novel classes of HIV-1-induced CPE inhibitors. While the data as presented by no means prove a causal relationship between the inhibitory effects of the compounds on the HIV-1 protease (Table 1) and their potential inhibitory effects on the virus-induced CPE (Table 2), they point to the potential of compound **3** (saucerneol B) as a chemotherapeutic agent to be further pursued for the treatment of HIV-1/AIDS.

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