



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 IC₅₀ 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 IC₁₀₀ 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 (IC₁₀₀ of 0.2 μM, CC₀ of >125.0 μM, and SI of >520.8).

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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 tran-

scriptase, 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, gonorrhoea, leucorrhoea, jaundice and leprosy 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 Baill (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, H₂O–MeOH–AcCN (10:20:20)]. Manassantin A (**1**); white amorphous powder; [α]_D²⁵ -111° (c 1.0, CHCl₃); manassantin B (**2**); white amorphous powder; [α]_D²⁵ -144° (c 0.9, CHCl₃); saucerneol B (**3**); white powder; [α]_D²⁵ -58° (c 0.6, CHCl₃); saucerneol C (**4**); brown oil; [α]_D²⁵ -66° (c 1.3, CHCl₃); saururin B (**5**); brown oil; [α]_D²⁵ -44° (c 1.1, CHCl₃), IR (KBr) ν_{\max} : 3444 (OH), 2960, 1634, 1280, ¹H NMR (300 MHz, CD₃OD) 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*, -OCH₂O-), 6.34 (2H, *s*, H-3, 3'), 6.49 (2H, *s*, H-6, 6'), ¹³C NMR (75 MHz, CD₃OD) δ 14.3 (C-9, 9'), 35.4 (C-7, 7'), 37.2 (C-8, 8'), 98.2 (C-3, 3'), 100.8 (-OCH₂O-), 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-(pNO₂-Phe)-Glu-Ala-Ile-Ser-NH₂] 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 μ L) containing 50 mM of acetate buffer (pH 5.0), 2.5 μ g of the substrate, 2.5 μ L of a DMSO solution of the test compound, and 2.5 μ L of recombinant HIV-1 PR (0.175 μ g protein) were added and incubated at 37 °C for 15 min. The reaction was stopped by addition of 2.5 μ L 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 \times 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-NO₂-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 IC₅₀ of 0.09 μ M 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% CO₂ in a RPMI-1640 medium (Flow Laboratories, Irvine, Scotland) supplemented with 10% fetal calf serum (FCS, Flow Laboratories, North Ryde, Australia), 100 μ g/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 CCID₅₀ (50% cell culture infective dose)/cell. For CCID₅₀ 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 μ m filter. The CCID₅₀ 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 CCID₅₀ 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 CO₂ 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 \times 10⁵ cells/mL in RPMI 1640 medium and 200 μ L/well of the cell suspension was cultured for 5 days in a 96-well plates containing various concentrations (9 doses, maximum 125–100 μ M and minimum 0.06–0.03 μ M) of the test compounds. After 5 days, the inhibitory concentration (IC₁₀₀) 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 (CC₀) 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 IC₁₀₀ and CC₀ values of AZT were 0.0039 and >1.0 μ g/mL, while those of DS 8000 were 1.95 and >1000 μ g/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 sesquidilignans, 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 C₂₀H₂₂O₆. The ¹H 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. ¹³C 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 (-OCH₂O-), 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, 7a', 7b, 7b'] 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 (-OCH₂O-)], and EIMS [*m/z* 151] showed presence of methylenedioxy 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 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

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