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A DNA Strand-Nicking Principle of a Higher Plant, *Caesalpinia sappan*

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To find anticancer agents from higher plants, DNA strand-scission assay method was employed for bioassay-guided fractionation as well as for screening the crude extracts. During the screening, an ethyl acetate extracts of the heartwood of *Caesalpinia sappan* L. (Leguminosae) exhibited potent DNA strand-scission activity. Therefore, the ethyl acetate extracts of the dried heartwood of *C. sappan* was subjected to the bioassay-guided fractionation, which led to the isolation of a known compound, brazilin (**1**) as the active constituent. In addition, caesalpine J (**2**) was also isolated as an inactive constituent.

Key words: DNA strand-scission, *Caesalpinia sappan*, Brazilin

INTRODUCTION

The dried heartwood of *Caesalpinia sappan* L. (Legumir osae), Sappan Lignum, has been used in traditional medicine to increase blood circulation, and to treat various illnesses such as pain, tetanus, and blood clotting of ladies (Kim *et al.*, 1997). Its various biological activities include a vasorelaxing activity (Xie *et al.*, 2000), anticonvulsant activity (Baek *et al.*, 2000), and acetylcholinesterase inhibitory activity (Lee *et al.*, 1997). Phytochemical studies on this plant, have resulted in reports upon various compounds including, homoisoflavonoids (Namikoshi *et al.*, 1987a) and various phenolic compounds (Nagai *et al.*, 1986; Namikoshi *et al.*, 1987b; Nagai and Nagumo., 1987; Fuke *et al.*, 1985).

To discover anticancer agents in higher plants, a DNA strand-scission assay method has been employed for bioassay-guided fractionation and for the screening the crude extracts. Several natural products such as biphenyl compounds (Seo *et al.*, 1999) and benzophenones (Seo *et al.*, 2000) have been isolated from Guttiferae plants, and have been reported to possess DNA strand-nicking ability. During our screening efforts, the MeOH extracts of Sappan Lignum was found to exhibit a potent ability to cleave DNA strand, therefore, bioassay-guided fractionation was used to isolate the active principle in the extract.

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MATERIALS AND METHODS

General experimental procedure

Optical rotations were measured on a P-1010 digital polarimeter (JASCO, Japan) at 25°C. The UV spectrum was obtained using a U-3000 spectrophotometer (Hitachi, Japan) and the IR spectrum was recorded on a FTS-135 FT-IR spectrometer (Bio-Rad, USA). NMR experiments were run on a Unity INOVA 400 MHz FT-NMR (Varian, CA), and TMS was used as an internal standard. Mass data were obtained using a JMS-700 MStation HRMS spectrometer (JEOL, Japan). Flash column chromatography was carried out on Si gel 60 (70-230 mesh, Merck, Darmstadt, Germany). Column chromatography was monitored by TLC (Silica gel 60 F_{254} plates, 0.25 mm thickness) with visualization under UV light (254 and 365 nm) and 1% sulfuric acid in EtOH.

Chemicals

Chemicals and reagents were of the highest purity. Bleomycin sulfate, cacodylic acid, cupric chloride, ferrous sulfate, ethylenediaminetetraacetic acid (EDTA), bromophenol blue, xylene cyanole FF, ficoll, boric acid, lauryl sulfate, glycerol, and Trizma base were all purchased from Sigma-

Aldrich (St. Louis, MO, USA). Electrophoresis grade agarose and pBR322 plasmid DNA were obtained from Gibco BRL (Life Technologies, Grand Island, NY, USA). SYBR Green I Nucleic Acid Gel Stain was obtained from Roche (Indianapolis, IN, USA).

Plant materials

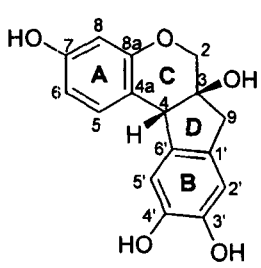
The heartwood of *C. sappan* was purchased at the Gyungdong Market in Seoul, Korea in January 2001. A voucher specimen (No. EAB089) was deposited at the Herbarium of College of Pharmacy, Ewha Womans University.

Extraction and isolation

The dried heartwood of *C. sappan* (3 kg) was extracted with MeOH (5 L×5) at room temperature for 24 h by percolation. The MeOH extracts were concentrated *in vacuo* and suspended in water. It was then partitioned with *n*-hexane (1 L×5) followed by ethyl acetate (1 L×5). The ethyl acetate extract (220 g) was subjected to a silica gel vacuum liquid column chromatography using CH_2Cl_2-MeOH (gradient, 100:0 → 0:100) as a solvent system, to produce 8 fractions (I~VIII). Fraction III (45 g) eluted with CH_2Cl_2-MeOH (24:1) from the first column was further separated into 9 subfractions (III-1~III-9) using another gradient mobile phase of CH_2Cl_2-MeOH (98.5:1.5 → 0:100). Fraction III-3 eluted with CH_2Cl_2-MeOH (49:1) was subjected to a C18 reversed phase column chromatography using MeOH-H_2O (1:9 → 100:0) as a solvent system to provide 8 fractions. The third of these eight fractions was purified by a revered phase (C18) preparative HPLC (YMC Jsphere ODS-H80, 2.0×25 cm, 4 mm, flow rate: 10 mL/min, UV detection: 220 nm) using a MeOH-H_2O gradient (11:39 → 1:3) to provide the pure compound **2** (30 mg, t_R 25 min). Fraction III-8 was further separated on Sephadex LH-20 using MeOH, and then subjected to revered phase preparative HPLC (YMC Jsphere ODS-H80, 2.0×25 cm, 4 mm, flow rate: 8 mL/min, UV detection: 220 nm) using MeOH-H_2O gradient (7:13 → 19:31), to provide the pure compound **1** (100 mg, t_R 11 min). The structure of **1** was identified as the known compound brazilin by analyzing spectral data such as 1D- and 2D-NMR including HSQC and HMBC experiments as well as by comparison of its spectral data with published values (Namikosh *et al.*, 1987). Compound **2** was identified as caesalpine J by comparison of spectral data with literature values (Shimokawa *et al.*, 1985; Miyahara *et al.*, 1986) and by analyzing its 1D- and 2D-NMR data.

Compound 1 (Brazilin)

$[\alpha]_D^{25}$ +90° (c 0.10, MeOH); UV, IR and ^{1}H-NMR (acetone-d_6) data were comparable to the published values (Kim *et al.*, 1997; Baek *et al.*, 2000; Kim *et al.*, 1997). ^{13}C-NMR (acetone-d_6, 100 MHz): δ = 157.6 (C-7), 155.5 (C-8a), 145.1 (C-3′), 144.8 (C-4′), 137.4 (C-6′), 132.0 (C-5), 131.5 (C-1′), 115.6 (C-4a), 112.6 (C-2′), 112.3 (C-5′), 109.6 (C-6), 104.0 (C-8), 77.8 (C-3), 70.8 (C-2), 51.2 (C-4), 43.0 (C-9). FABMS: *m/z* 309 $[M+Na]^+$.



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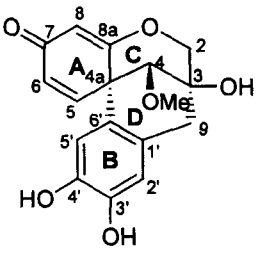

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Compound 2 (Caesalpine J)

$[\alpha]_D^{25}$ +461.0° (c 0.15, MeOH); UV (MeOH) λ_{max} (log e) 285 (4.0), 241 (4.4), 213 (4.7) nm; IR data were comparable to the published values (Shimokawa *et al.*, 1985). ^{1}H-NMR (acetone-d_6, 400 MHz): δ = 6.96 (1H, d, *J* = 10 Hz, H-5), 6.65 (1H, s, H-2′), 6.43 (1H, s, H-5′), 6.40 (1H, dd, *J* = 10, 1.6 Hz, H-6), 5.47 (1H, d, *J* = 1.2 Hz, H-8), 4.12 (1H, dd, *J* = 10.4, 1.6 Hz, H-2b), 4.06 (1H, d, *J* = 10.4 Hz, H-2a), 3.85 (1H, s, H-4), 3.55 (3H, s, OCH_3), 3.20 (2H, d, *J* = 8.8 Hz, H-9a and 9b). ^{13}C-NMR (acetone-d_6, 100 MHz): δ = 189.0 (C-7), 175.7 (C-8a), 148.3 (C-5), 146.5 (C-3′), 144.7 (C-4′), 131.3 (C-6), 127.4 (C-1′), 125.8 (C-6′), 116.1 (C-2′), 113.8 (C-5′), 110.3 (C-8), 85.5 (C-4), 76.2 (C-2), 69.2 (C-3), 62.1 (OCH_3), 53.4 (C-4a), 43.2 (C-9). EIMS: *m/z* 316 $[M]^+$(5), 230 (4), 149 (4), 87 (10), 62 (100).

DNA strand-scission assay

The DNA strand-scission assay was conducted using a modification of the procedure described by Sugiyama *et al.* and Chaudhuri *et al.* (Sugiyama *et al.*, 1985; Chaudhuri *et al.* 1995). In brief, the assay reaction mixtures (40 μL total volume) contained 25 mM of cacodylate buffer pH 7.0, 0.3 mM $CuCl_2$, and 500 ng of supercoiled DNA pBR322 as a substrate, and various concentrations of the test compounds (initially dissolved in 0.5 μL of 100% DMSO, to produce a final concentration of 1.25% DMSO). The reaction mixture was incubated for 30 min at 25°C, protected from light, then stopped by adding 5 μL of stop solution (7 mM EDTA, 0.15% bromophenol blue, 75% glycerol). The reaction mixture was analyzed by electrophoresis at 80 volts for 7 h on a 1% agarose gel in 0.5 TBE buffer (45 mM Tris-borate, 1 mM EDTA), and then stained with SYBR Green I fluorescence, and photographed using luminescence image analyzer, LAS-1000plus (Fuji film, Japan). The band densities of pBR322 were measured using Image Gauge software (Fuji film, Japan). Each experiment included DMSO and bleomycin sulfate as negative and positive controls, respec-

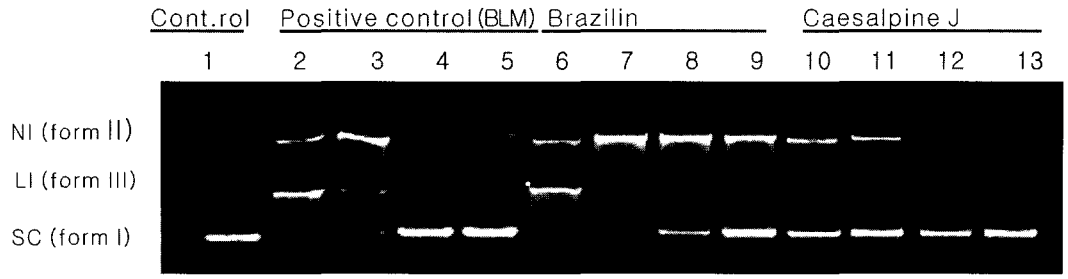

Fig. 1. The DNA strand-scission assay of supercoiled pBR322 plasmid DNA by brazilin (compound **1**) and caesalpine J (compound 2) from C. sappan. A control reaction (Cont.) was run in the absence of any Fe^{++}/bleomycin (BLM) congener (lane 1). Positive control reactions were carried out using 25 μg/mL (lane 2), 5 μg/mL (lane 3), 1 μg/mL (lane 4), and 0.2 μg/mL (lane 5) of bleomycin sulfate/Fe^{++}, respectively. The reactions contained 25 μg/mL (lane 6), 5 μg/mL (lane 7), 1 μg/mL (lane 8), and 0.2 μg/mL (lane 9) of brazilin and 25 μg/mL (lane 10), 5 μg/mL (lane 11), 1 μg/mL (lane 12), and 0.2 μg/mL (lane 13) of caesalpine J. pBR322 form I DNA denotes a supercoiled (SC) and form II DNA is produced by nicking (NI) one strand of the supercoilded DNA, while nicking both strands at proximal sites produces a linear (LI) duplex (Form III DNA).

tively. The results were calculated as the difference between the relative percents of relaxed and supercoiled pBR322.

RESULTS AND DISCUSSION

During the screening, the MeOH extracts of the dried heartwood of *C. sappan* exhibited a potent DNA strand-scission activity with an IC_{50} value of 5.9 μg/mL. Therefore, the MeOH extracts were partitioned by *n*-hexane and ethyl acetate, successively. The ethyl acetate extracts of the dried heartwood of *C. sappan* was subjected to bioassay-guided fractionation. This led to the isolation of a major active compound **1** which was identified as brazilin (**1**) by comparing its spectral data such as its ^1H and ^{13}C-NMR data with published values (Namikosh *et al.*, 1987). The inverse NMR techniques, HSQC and HMBC allowed a minor revision of previous assignments (Kim *et al.*, 1997) of the ^{13}C-NMR signals at δ_C 157.6 and 155.5 (in acetone-d_6). These signals were re-assigned to C-7 and C-8a, respectively, using the HMBC correlations of C-8a/H-2 and C-8a/H-5. In addition, caesalpine J (2) (Shimokawa *et al.*, 1985; Miyahara *et al.*, 1986) was also isolated as an inactive constituent. Compound **2** was identified as caesalpine J by comparing ^1H- and ^{13}C- NMR data with published values (Shimokawa *et al.*, 1985; Miyahara *et al.*, 1986) and by analyzing 1D and 2D NMR data. Its ^1H and ^{13}C-NMR data in acetone-d_6, which were assigned by HSQC and HMBC experiments, are reported in this paper for the first time, although the NMR data of this compound run in DMSO were previously reported (Shimokawa *et al.*, 1985; Miyahara *et al.*, 1986).

Brazilin (**1**) exhibited the DNA strand-scission activity with an IC_{50} value of 3.4 μg/ml (Fig. 1, lane 6-9), which was more potent than that of the positive control, bleomycin (IC_{50} 4.8 μg/mL, Fig. 1, lane 2-5) in the present study. On the other hand, caesalpine J (**2**) was found to be inactive in the present DNA strand-scission assay system (Fig. 1, lane 10-13). These two isolates, brazilin (**1**) and caesalpine J (**2**) each contain a catechol group, which has been thought as an essential group for the DNA strand-scission activity (Chaudhuri *et al.*, 1995; Huang *et al.*, 1996; Huang *et al.*, 1998; Seo *et al.*, 1999). The reason why brazilin has potent DNA strand-scission activity, is possibly attributed to structural differences of rings A and D in these compounds. The hydroxyl group at C-7 and 5-membered ring for the D-ring in brazilin (**1**) are probably required for its DNA strand-scission activity as well as its catechol group.

Brazilin has been previously known to have several forms of biological activity such as hypoglycemic action (Moon *et al.*, 1993; Kim *et al.*, 1998), modifying activity on altered immune function (Choi and Moon, 1997; Mok *et al.*, 1998), and anti-inflammatory activity (Hikino *et al.*, 1977). However, to the best of our knowledge, the DNA strand-scission activity of brazilin has never been previously reported.

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