Inhibition of Human 20S Proteasome by Compounds from Seeds of *Psoralea corylifolia*

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Psoraleae Semen is the dried seed of *Psoralea corylifolia* L. (Fabaceae) which is commonly used in traditional Chinese medicine to alleviate asthma and diarrhea and to treat vitiligo and alopecia areata.[1] Also, this crude drug has been used for the treatment of enuresis, pollakiuria, painful feeling of cold in the waist or knees and weak kidney.[1] Modern pharmacological and clinical studies have shown that the extracts of *P. corylifolia* possess different biological activities, such as antihyperglycemic, antidepressant, antimicrobial, antitumor and antidermatophytic effects.[1] The chemical constituents of *P. corylifolia* include coumarins such as psoralen and isopsoralen, *etc*. and flavone components such as psoralidin and bavachalcone, *etc*.[2]

The ubiquitin-proteasome proteolytic system plays an important role in selective protein degradation and regulates cellular events, including apoptosis, and inflammation.[3] The 26S proteasome is composed of 20S core and 19S regulator. Since proteasomes interact primarily with endogenous proteins, inhibition of the proteolytic action of the proteasome may block the signaling action of the transcription factor NF-κB and, thus, inhibit the completion of the cell cycle and hence the mitotic proliferation of cancerous cells, leading to cell death by apoptosis and inhibition of angiogenesis and metastasis.[4] Cancer cells have been shown to be more sensitive to the proapoptotic effects of proteasome inhibition than normal cells in preclinical evaluation.[5] Thus, proteasome inhibitors can be potential anticancer agents.

As part of a project to discover proteasome inhibitory compounds from natural products, several plants were screened using an assay for human 20S proteasome inhibition. A MeOH extract from Psoraleae Semen with potent 20S proteasome inhibitory activity was chosen for further chemical investigation. The MeOH extract of Psoraleae Semen was fractionated depending on the polarity into four fractions, of which each fraction was evaluated on the 20S proteasome inhibitory activity. The inhibitory activities of the fractions against 20S proteasome were described in a previous report.[6] Since the $CHCl_3$ fr. (fraction) showed the most potent 20S proteasome inhibitory activity,[6] the $CHCl_3$ fr. was subjected to chemical investigation for further study. This study reports on the isolation of the compounds from the psoraleae semen and their inhibitory activities on human 20S proteasome.

There has been a report that the extract of *Psoralea corylifolia* inhibited mitochondrial complex I and proteasome activities in SH-SY5Y, a human neuroblastoma cell line, with respect to neurodegenerative diseases such as Parkinson's and Alzheimer's diseases.[7] However, the compounds that are responsible for its inhibitory activity have not been identified so far. To identify the active compounds from the extract of Psoraleae Semen, bioassay-guided fractionation was performed using silica gel column chromatography, followed by reversed-phase HPLC, resulting in the isolation of compounds **1**-**8**. The structures of the isolated compounds were identified as psoralen (**1**), isopsoralen (**2**), bakuchiol (**3**), isobavachromene (**4**), 6-prenylnaringenin (**5**), corylin (**6**), bavachinin (**7**), and Δ^3,2-hydroxybakuchiol (**8**). The structures of the isolated compounds were identified analyzing their NMR data (Fig. 1) along with comparing their spectral data with those in the literature.[2,8] In case of compound **8**, initial ^{1}H-NMR data was exactly identical to that of Δ^3,2-hydroxybakuchiol.[9] However, **8** easily underwent dehydration to furnish $\Delta^{1,3}$-bakuchiol (**9**) of which spectral characteristic was consistent with the structure **9**.[9]

To investigate the 20S proteasome inhibitory activity of theisolated compounds from Psoraleae Semen, the commercially available purified human erythrocyte 20S proteasome was used. Suc-Leu-Leu-Val-Tyr linked to fluorogenic aminomethylcou-

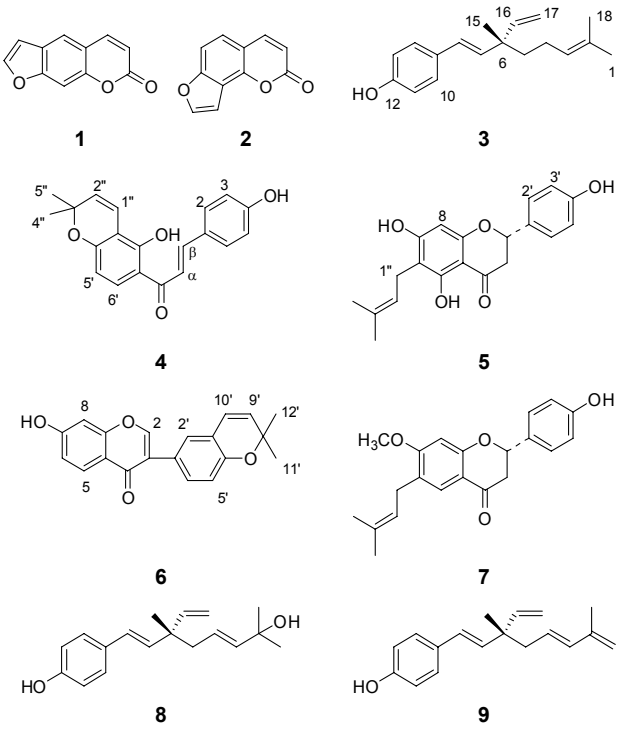

Figure 1. Chemical structures of the isolated compounds from Psoraleae Semen.

Table 1. The inhibitory activities of the 20S proteasome by compounds isolated from Psoraleae Semen

Compounds	Psoralen (**1**)	Isopsoralen (**2**)	Bakuchiol (**3**)	Isobava Chromene (**4**)	6-Prenyl naringenin (**5**)	Corylin (**6**)	Bavachinin (**7**)
IC_{50} (μM)[a]	> 100	> 100	30.5 ± 1.5	24.3 ± 1.2	46 ± 1.2	> 100	49 ± 1.5

[a]IC_{50} (50% inhibition concentrations) were calculated from a log dose inhibition curve and expressed as the mean ± SD of triplicate experiments. Epoximicin was used as the positive control (IC_{50} = 65 nM).

marin (Suc-LLVY-AMC) was used as a substrate to determine the chymotrypsin-like activity of 20S proteasome. The inhibitory activity was determined by measuring the generation of free AMC using a fluorescence plate reader after adding Suc-LLVY-AMC to incubated mixtures of the isolated compounds and 20S proteasome. The inhibitory activities of the isolated compounds against 20S proteasome are shown in Table 1. Compounds **3**, **4**, **5**, and **7** inhibited the activity of 20S proteasome in a dose-dependent manner with IC_{50} values of 30.5 ± 1.5, 24.3 ± 1.2, 46 ± 1.2, and 49 ± 1.5 μM, respectively. Compound **8** was not evaluated using this assay since **8** was easily dehydrated to **9**. A known proteasome inhibitor, epoximicin (IC_{50} = 65 nM), was employed as a positive control in the assay.

Since the first proteasome inhibitor, Bortezomib (Velcade™, Millenium) was approved by the US FDA for the treatment of multiple myeloma in 2003,[10] natural products as well as synthetics have been vigorously investigated for working as proteasome inhibitors and antitumor agents. Several proteasome inhibitors such as NPI-0052,[11] lactacystin,[12] withaferin A,[13] celastrol,[14] gliotoxin,[15] ginsenoside Rd,[16] *etc* were already identified from natural sources including microsomes. Yang *et al*. reported the relationship between proteasomal chymotrypsin-like activity and tumor growth, demonstrating that treatment of withaferin A resulted in inhibition of tumor growth in association with inhibition of the tumor tissue proteasomal chymotrypsin-like activity.[13]

Several biological studies of Psoraleae Semen have been performed.[17] As mentioned in the above, there has been a report that the extract of Psoraleae Semen inhibited mitochondrial complex I and proteasome activities in SH-SY5Y, assuming that the interplay of oxidative damage due to reactive species generation, mitochondrial complex I inhibition, and proteasomal dysfunction may be responsible for the reduced cell viability in SH-SY5Y neuroblastoma cells treated with Psoraleae Semen, possibly leading to increase in the risk of development of neurodegenerative diseases such as Parkinson's and Alzheimer's diseases.[7] However, even though the inhibitory activity of *P. corylifolia* on proteasome has been reported before,[7] the compounds from the extract that may be responsible for the activity were identified in this study for the first time. Moreover, 20S proteasome purified from human erythrocyte was used to determine the chymotrypsin-like activity of proteasome in this study whereas proteasome-containing SH-SY5Y cells which were lysed with cell lysis buffer were used without purification in the above mentioned report.

In conclusion, our data show that Psoraleae Semen may exhibit the antitumor activity by inhibiting human 20S proteasome and bakuchiol (**3**), isobavachromene (**4**), 6-prenylnaringenin (**5**), and bavachinin (**7**) from this plant can contribute to the inhibitory activity on human 20S proteasome.

Experimental Section

Plant material. The seeds of *P. corylifolia* were purchased from the Daegu pharmacopoeia market in South Korea. A voucher specimen has been deposited in Natural Products Chemistry Laboratory of School of Biotechnology, Yeungnam University.

Reagents and instruments. NMR spectra were recorded with Varian's standard pulse program of Varian VNS spectrometer at 250 MHz, 300 MHz, and 600 MHz. EI-MS spectra were recorded with Micromass spectrum (AUTOSPEC, UK). TLC was done using Kieselgel 60F254 (Merck) and RP-18 (Whatman). Column chromatography was done using silica gel (70-230 mesh, Merck). Agilent 1200 series HPLC system equipped with a quaternary pump, a degasser, an injector, a column thermostat, and diode array detector (DAD) was used for purification of compounds. All HPLC separations were carried out using an Eclipse XDB-C_{18} semi-preparative column (9.4 × 250 mm, 5 μm, Agilent Technologies, USA) at a flow rate of 2 mL/min. A commercially available 20S Proteasome Assay Kit (AK-740, BIOMOL International, LP.) was used, which provided the purified, human erythrocyte 20S proteasome, assay buffer, and substrate Suc-LLVY-AMC. Proteasome inhibition was determined using a fluorescence plate reader (FluorOptima, BMG Labtech Ltd., UK) to measure fluorescence of free AMC from Suc-LLVY-AMC.

Extraction, fractionation and isolation. The dried Psoraleae Semen (1 kg) was extracted with MeOH and the extracts were successively partitioned with *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH. The $CHCl_3$ fraction (96.5 g) with potent 20S proteasome inhibition activity[6] was subjected to silica gel column chromatography eluting with *n*-hexane and EtOAc gradient system (10:1 → 10:4), affording twenty-seven fractions (Fr. 1 ~ Fr. 27). Fraction 3 gave a colorless oil, compound **3**. Fraction 13 and 15 were crystallized from *n*-hexane, EtOAc, and MeOH to yield compounds **1** and **2**, respectively. Fraction 16 was subjected to repeated column chromatography with a gradient elution of *n*-hexane/EtOAc (10:1 → 10:1.5), resulting in 19 subfractions (Fr. 16-1 ~ Fr. 16-19). Compound **4** was isolated through repeated column chromatography eluted with Hexane and CH_2Cl_2 gradient solvents followed by reversed-phase HPLC (30 to 100% CH_3CN in H_2O over 40 min). Fraction 24 of the $CHCl_3$ fraction was chromatographed on a silica gel column with a gradient elution of *n*-hexane and EtOAc (10:1 → 1:1) to afford fifty subfractions (Fr. 24-1 ~ Fr. 24-50). Fr. 24-7 and Fr. 24-20 were subjected to semi-preparative HPLC (20 to 100% CH_3CN in H_2O over 40 min) to afford compounds **5** and **6**, respectively. Compounds **7** and **8** were purified from Fr. 24-26 and Fr. 24-30, respectively through semi-preparative HPLC (10 to 100% CH_3CN in H_2O over 45 min).

Psoralen (1). Colorless crystals. ^{1}H-NMR (250 MHz, $CDCl_3$)

δ 7.78 (1H, d, J = 9.6 Hz, H-4), 7.68 (1H, d, J = 2.4 Hz, H-2'), 7.67 (1H, s, H-5), 7.46 (1H, br s, H-8), 6.82 (1H, dd, J = 0.9, 2.4 Hz, H-3'), 6.36 (1H, d, J = 9.6 Hz, H-3); ^{13}C-NMR (62.5 MHz, $CDCl_3$) δ 161.10 (C-2), 156.45 (C-7), 152.09 (C-9), 146.93 (C-2'), 144.06 (C-4), 124.89 (C-6), 119.85 (C-5), 114.70 (C-3), 106.39 (C-3'), 99.91 (C-8); EIMS *m/z* (rel. int.) 186 $[M]^+$ (98), 158 $[M - CO]^+$ (100).

Isopsoralen (2). Colorless crystals. ^{1}H-NMR (250 MHz, $CDCl_3$) δ 7.78 (1H, d, J = 9.6 Hz, H-4), 7.66 (1H, br d, J = 2.1 Hz, H-2'), 7.40 (1H, d, J = 8.5 Hz, H-6), 7.34 (1H, d, J = 8.5 Hz, H-5), 7.09 (1H, m, H-3'), 6.36 (1H, d, J = 9.6 Hz, H-3); ^{13}C-NMR (62.5 MHz, $CDCl_3$) δ 160.81 (C-2), 157.35 (C-7), 148.50 (C-9), 145.88 (C-2'), 144.50 (C-4), 123.83 (C-5), 116.92 (C-8), 114.12 (C-3), 113.52 (C-10), 108.80 (C-6), 104.09 (C-3'); EIMS *m/z* (rel. int.) 186 $[M]^+$ (100), 158 $[M - CO]^+$ (85).

Bakuchiol (3). Colorless oil. ^{1}H-NMR (250 MHz, $CDCl_3$) δ 7.14 (2H, d, J = 8.4 Hz, H-10, 14), 6.68 (2H, d, J = 8.4 Hz, H-11, 13), 6.18 (1H, d, J = 16 Hz, H-8), 5.98 (1H, d, J = 16 Hz, H-7), 5.79 (1H, dd, J = 10, 17 Hz, H-16), 5.04 (1H, br t, H-3), 4.93 (2H, m, H-17), 1.89 (2H, m, H-5), 1.59 (3H, s, H-18), 1.50 (3H, s, H-1), 1.41 (2H, s, H-4), 1.11 (3H, s, H-15); ^{13}C-NMR (62.5 MHz, $CDCl_3$) δ 131.1 (C-9), 127.3 (C-10), 115.4 (C-11), 154.2 (C-12), 115.4 (C-13), 127.3 (C-14), 135.8 (C-8), 126.4 (C-7), 42.4 (C-6), 41.2 (C-5), 23.2 (C-4), 124.8 (C-3), 131.2 (C-2), 17.6 (C-1), 25.6 (C-18), 23.2 (C-15), 145.8 (C-16), 111.9 (C-17).

Isobavachromene (4). Yellow amorphous powder. ^{1}H-NMR (600 MHz, $CDCl_3$) δ 7.82 (1H, d, J = 16 Hz, H-β), 7.69 (1H, d, J = 9.0 Hz, H-6'), 7.55 (2H, d, J = 8.4 Hz, H-2,6), 7.42 (1H, d, J = 16 Hz, H-α), 6.86 (2H, d, J = 8.4 Hz, H-3,5), 6.74 (1H, d, J = 10 Hz, H-1''), 6.36 (1H, d, J = 9.0 Hz, H-5'), 5.57 (1H, d, J = 10 Hz, H-2''), 1.45 (6H, s, H_3-4'',5'').

6-Prenylnaringenin (5). Yellow amorphous powder. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 12.41 (1H, s, 5-OH), 7.30 (2H, br d, J = 8.4 Hz, H-2',6'), 6.79 (2H, br d, J = 8.4 Hz, H-3',5'), 5.96 (1H, s, H-8), 5.40 (1H, dd, J = 3, 13 Hz, H-2), 5.12 (1H, br t, J = 6.9 Hz, H-2''), 3.25 (2H, br d, J = 6.9 Hz, $H_α$-1'', $H_β$-1''), 3.17 (1H, dd, J = 13, 17 Hz, $H_β$-3), 2.67 (1H, dd, J = 3, 17 Hz, $H_α$-3), 1.69 (3H, s, H_3-5''), 1.61 (3H, s, H_3-4'').

Corylin (6). Yellow cristals. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.33 (1H, s, H-2), 7.96 (1H, d, J = 8.7 Hz, H-5), 7.30 (1H, dd, J = 2.4, 8.1 Hz, H-6'), 7.29 (1H, br s, H-2'), 6.90 (1H, dd, J = 2.1, 8.7 Hz, H-6), 6.85 (1H, d, J = 2.1 Hz, H-8), 6.78 (1H, d, J = 8.1 Hz, H-5'), 6.43 (1H, d, J = 9.9 Hz, H-10'), 5.78 (1H, d, J = 9.9 Hz, H-9'), 1.40 (6H, s, 2 × CH_3); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 175.0 (C-4), 163.2 (C-7), 157.9 (C-9), 153.6, 152.6 (C-4', C-2), 131.7, 130.1 (C-2', C-6'), 127.7 (C-5), 127.4, 124.9 (C-9', C-10'), 123.6 (C-1'), 122.2 (C-5'), 121.0 (C-3), 116.9 (C-3'), 116.0 (C-10), 115.7 (C-6), 102.6 (C-8), 76.7 (C-8''), 49.0, 28.2 (C-11' or C-12').

Bavachinin (7). Yellow amorphous powder. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.65 (1H, s, H-5), 7.33 (2H, d, J = 8.4 Hz, H-2', 6'), 6.86 (2H, d, J = 8.4 Hz, H-3', 5'), 6.42 (1H, s, H-8), 5.36 (1H, dd, J = 3.0, 13 Hz, H-2), 5.25 (1H, t, J = 6.6 Hz, H-2''), 3.82 (3H, s, 7-OMe), 3.22 (2H, d, J = 6.6 Hz, H_2-1''), 3.00 (1H, dd, J = 13, 17 Hz, $H_β$-3), 2.74 (1H, dd, J = 3.0, 17 Hz, $H_α$-3), 1.71 (3H, s, H_3-4''), 1.67 (3H, s, H_3-5'').

Δ^3,2-Hydroxy bakuchiol (8). Oil. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.21 (2H, d, J = 8.4 Hz, H-10, 14), 6.74 (2H, d, J = 8.4 Hz, H-11, 13), 6.23 (1H, d, J = 17 Hz, H-8), 6.03 (1H, d, J = 17 Hz, H-7), 5.86 (1H, dd, J = 11, 17 Hz, H-16), 5.60 (2H, m, H-3, 4), 5.02 (1H, d, J = 11 Hz, H-17b), 4.99 (1H, d, J = 17 Hz, H-17a), 2.20 (2H, d, J = 6 Hz, H-5), 1.28 (6H, s, H-1, 18), 1.15 (1H, s, H-15).

Proteasome inhibition assay. The inhibition assay was done as follows.[6] 0.03% sodium dodecyl sulfate was added to assay buffer to activate the 20S proteasome's chymotrypsin-like activity. The assay buffer was added to the blank and sample plate, and a diluted solution of positive control was added to the inhibitor wells. The enriched proteasome fraction was diluted to a final assay concentration of 50 μg/mL using assay buffer. This diluted fraction was then added to each well, and then, the plate was preincubated for 10 min at 37 °C to allow the inhibitor and enzyme to interact. The enzymatic reaction was started by adding Suc-LLVY-AMC substrate to a final concentration of 10 μM. The chymotrypsin-like enzymatic activity of the proteasome was determined by measuring the generation of free AMC using a fluorescent plate reader (FluorOptima, BMG LabTech Ltd., UK) capable of excitation at a wavelength of 355 nm and detection of emitted light at 460 nm. Epoximicin was used as the positive control (IC_{50} 65 nM) which is a rapid, potent and irreversible inhibitor of the 20S proteasome chymotrypsin-like activity. It can also inhibits the proteasome's trypsin-like and peptidyl-glutamyl peptide hydrolase activities, but at 100- and 1,000-fold slower rate, respectively.

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