Antimutagenic Constituents from the Thorns of *Gleditsia sinensis*

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Antimutagenic activity-guided fractionation of an extract prepared from the thorns of *Gleditsia sinensis* LAM. led to the isolation of one triterpenoid and four steroids, which were identified as *D:C*-friedours-7-en-3-one (1), stigmast-4-ene-3,6-dione (2), stigmastane-3,6-dione (3), stigmasterol (4), and β-sitosterol (5). Triterpenoid 1 was found for the first time in a natural source and the steroids 2—5 were first isolated from this plant. Stigmasterol was the most active antimutagen, showing 51.2% and 64.2% reduction of the induction factor against the mutagens MNNG and NQO, respectively, in the SOS chromotest. Some NMR data of the steroids 2 and 3 obtained have to be revised.

Key words *Gleditsia sinensis*; antimutagenic activity; stigmasterol; *D:C*-friedours-7-en-3-one

Gleditsia sinensis LAM. (Leguminosae), a perennial shrub, is widely distributed throughout China and in the Gyeongju city area in Korea. Its thorns, called "Jo Gak Ja" in Korea and "Zao Jiao Ci" in China, have been used in traditional medicine for the treatment of swelling, suppuration, carbuncle and skin diseases.[1)] Korean "Jo Gak Ja" is much longer and thicker than Chinese "Zao Jiao Ci". The anomalous fruits of this plant have been reported to contain 15 triterpenoid saponins, called gledithiosides A—K and N—Q,[2)] and its leaves are known to contain 6 flavonoids.[3)] However, until now there has been no report on the constituents of the thorns and their biological activities, except the anti-inflammatory[4)] and antihistaminic effects[5)] of the water extract.

As a part of our continuing search[6,7)] for antimutagenic agents from natural sources, we have investigated the antimutagenic activity of the crude methanolic extracts from "Jo Gak Ja". Activity-guided fractionation of the extract indicated that the methylene chloride fraction exhibited most potently the antimutagenicity. In this study, we isolated one triterpenoid for the first time from a natural source and four steroidal components first from *Gleditsia sinensis*. The structure identification and their antimutagenic activities are discussed.

Results and Discussion

In order to search for any active constituents by activity-guided fractionation, the antimutagenic and antigenotoxic properties of the total extracts and fractions of the thorns of this plant were investigated by the Ames test and the SOS chromotest. For antimutagenic activity, the inhibitory effect of the test samples was examined in a plate incorporation assay against mutagens NPD and NaN_3 using *Salmonella typhimurium* test strains TA98 and TA100, respectively, without S9 activation. Among the tested samples, the methylene chloride fraction showed the strongest effect at a dose of 1 mg/plate in the Ames test revealing 42.6% and 40.7% inhibition ratios of revertant CFU (colony forming unit) per plate against NPD and NaN_3, respectively (Table 1). No mutagenic activities of the methylene chloride fraction or the solvent DMSO were detected (data not shown), indicating that these are not mutagenic. Although DMSO is reported to block the mutagenic and carcinogenic activity of benzene,[8)] this solvent exhibited no antimutagenicity in our assay system. In the SOS chromotest using the test strain *Escherichia coli* PQ37, the methylene chloride fraction exhibited the lowest induction factor at a concentration of 100 μg/reaction tube against the mutagens, MNNG and NQO (Fig. 1). The above results indicate that the methylene chloride fraction has the strongest antimutagenic and antigenotoxic properties of the fractions tested. The active methylene chloride fraction was found to contain a series of triterpenoid or steroid compounds (Liebermann–Burchard test) which were separated by repeated column chromatography or by preparative TLC to furnish five components (**1**—**5** in order of elution, Fig. 2).

Compound **1** was isolated as colorless needles and had a molecular formula of $C_{30}H_{48}O$ by HR-EI-MS (Found *m/z* 424.3703, Calcd 424.3705 for $M^{+\cdot}$). Its ^{1}H- and ^{13}C-NMR data (Table 2) including COSY spectrum indicated that the structure of **1** must be *D:C*-friedours-7-en-3-one, a particular

Table 1. Inhibitory Effect of Methanol Extract and Fractions from *Gleditsia sinensis* on the Mutagenicity Induced by NPD in *S. typhimurium* TA98 and Sodium Azide in *S. typhimurium* TA100

Treatment	Revertants per plate	
	TA98	TA100
Spontaneous	23±4	148±3
NPD	589±18[a)]	—
NaN_3	—	280±8[a)]
MeOH extract	429±15[d)] (17.1)	257±6[d)] (8.2)
Methylene chloride fraction	338±6[c)] (42.6)	166±5[c)] (40.7)
Ethyl acetate fraction	388±12[b)] (34.1)	193±12[b)] (31.1)
n-BuOH fraction	412±8[b)] (30.1)	238±10 (15.0)

Values represent mean±S.E. of three independent experiments. The values in parentheses are the inhibition rates (%). 10 μl of mutagens (100 μg/ml) and 10 μl of fractions (10 mg/ml) were used for the test. *a*) Significantly different from the spontaneous group, $p<0.001$. *b*) Significantly different from the NPD- or NaN_3-treated group, $p<0.01$. *c*) Significantly different from the NPD- or NaN_3-treated group, $p<0.01$. *d*) Significantly different from the NPD- or NaN_3-treated group, $p<0.05$.

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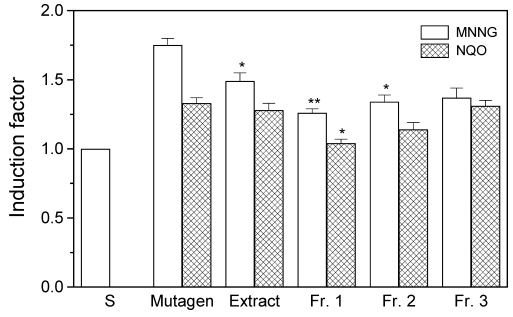

Fig. 1. Comparison of the Antigenotoxic Activity of the Extract and Fractions against the Mutagens, MNNG and NQO (Negative Controls) Using *E. coli* PQ37 as a Test Strain

10 μl of mutagens (100 μg/ml) and 10 μl of fractions (10 mg/ml) were used for the test. S: spontaneous, Fr. 1: methylene chloride fraction, Fr. 2: ethyl acetate fraction, Fr. 3: butanol fraction. Level of statistical significance: $**p<0.05$ and $*p<0.01$ with respect to mutagen values from three experiments.

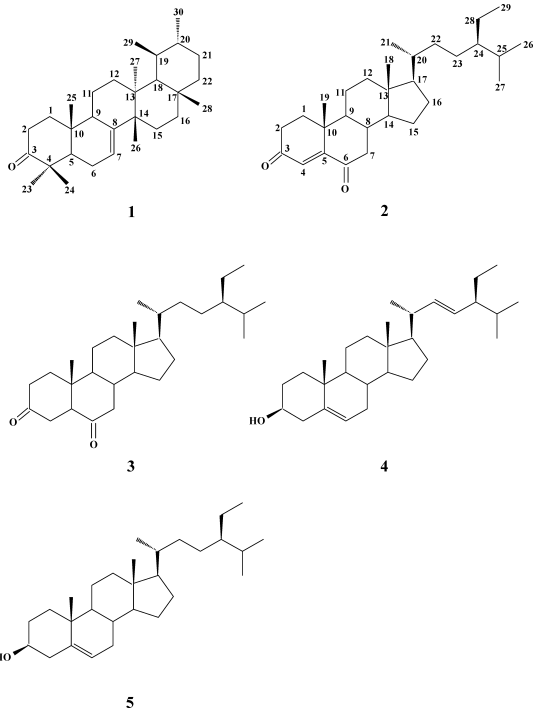

Fig. 2. Chemical Structures of the Isolated Components from *Gleditsia sinensis*

group of triterpenes. To the best of our knowledge, this is the first report of its occurrence as a natural product, although a few *D:C*-friedours-7-enes have been isolated from some plants.[9] Compound **1** has been reported by synthesis from *D:C*-friedours-7-en-3β-ol by CrO_3 oxidation[9] and our spectroscopic data for **1** are in a good agreement with literature data for the synthetic compound. The unusual chemical shift of C-28 (*ca.* 38 ppm) could be explained by a complete lack of interactions between C-28 and *syn*-diaxial hydrogens on γ-carbon atoms and the distinction between C-13 and C-14 was achieved by single-frequency irradiation of H-7.[9] The relative stereochemistry in our isolated compound **1** could be confirmed by direct comparison of its carbon chemical shifts with those of a synthetic compound. Compound **2** was isolated as coloress amorphous crystals. It was identified by mass spectrometry and by comparison of ^{1}H- and ^{13}C-NMR

Table 2. ^{1}H- and ^{13}C-NMR Data for Components **2** and **3**

H/C	δ ^{1}H		δ ^{13}C	
	2	**3**	**2**	**3**
1	2.15; 1.63	2.09; 1.62	35.53	38.13
2	2.54; 2.46	2.42; 2.35	33.81	37.39
3	—	—	202.37	211.23
4	6.17	2.59; 2.32	125.45	37.00
5	—	2.59	161.08	57.55
6	—	—	199.22	209.09
7	2.68; 2.04	2.39; 2.01	46.82	46.64
8	1.87	1.85	34.20	38.05
9	1.33	1.34	50.97	53.52
10	—	—	39.81	41.26
11	1.67; 1.47	1.65; 1.44	20.86	21.69
12	2.11; 1.27	2.07; 1.25	39.12	39.40
13	—	—	42.72	43.02
14	1.26	1.26	56.54	56.64
15	1.55; 1.10	1.54; 1.09	23.96	24.02
16	1.90; 1.27	1.88; 1.28	28.01	28.05
17	1.15	1.16	55.84	56.04
18	0.72	0.69	11.88	12.02
19	1.17	0.96	17.50	12.56
20	1.39	1.37	36.02	36.06
21	0.94	0.93	18.74	18.70
22	1.33; 1.03	1.34; 1.02	33.96	33.85
23	1.16 (2H)	1.16 (2H)	26.01	26.09
24	0.93	0.93	45.78	45.83
25	1.68	1.67	29.11	29.16
26	0.82	0.81	19.82	19.02
27	0.84	0.84	19.01	19.81
28	1.28; 1.23	1.28; 1.22	23.05	23.08
29	0.85	0.85	11.96	11.97

data to the previously reported compound[10] as stigmast-4-ene-3,6-dione. However, the ^{13}C-NMR-APT spectrum clearly proved that the carbon assignments[10] at C-8, C-10 and C-12 should be revised (*cf.* Table 2). Compound **3** was isolated as coloress amorphous crystals. Its structure, stigmastane-3,6-dione was derived from the detailed NMR analysis and by comparison of its data to the known compound.[11] However, the assignment of some carbon signals (C-2, 3, 6, 8, 12, 14, 17, 18, 19)[11] have been revised on the basis of our detailed NMR analysis (COSY, HMQC and HMBC). Compounds **4** and **5** were separated from the mixture of both components by preparative TLC using hexane/EtOAc 4 : 1, affording colorless amorphous solids. Both compounds could be detected on TLC (bluish color) by spraying phosphomolybdic acid reagent and were identified as stigmasterol (**4**) and β-sitosterol (**5**) by comparison of their spectra with those of authentic samples.

Compounds **1**—**5** were evaluated for their antigenotoxic activities by the SOS chromotest. When a final concentration of 10 μg per reaction tube of the compounds was applied to *E. coli* PQ37, stigmasterol (**4**), one of the phytosterols, showed more or much more effective antigenotoxic activity against both mutagens used (MNNG and NQO) in comparison with that of other components (Fig. 3). Its induction factors were reduced by 51.2% and 64.2% against the mutagens MNNG and NQO, respectively. Another phytosterol, β-sitosterol (**5**) revealed slightly weaker activity than stigmasterol against both mutagens. The effect of the test samples was not compared with that of a positive control because no standard compound is yet known, and the above experiments showed

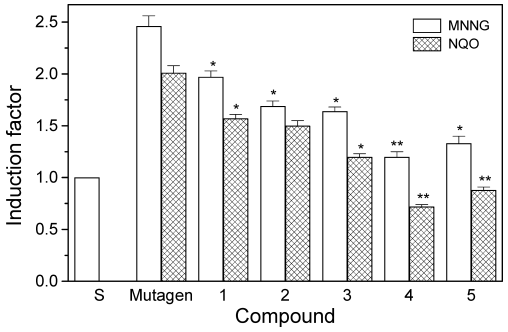

Fig. 3. Antigenotoxic Activity of the Isolated Components against the Mutagens, MNNG and NQO (Negative Controls) Using *E. coli* PQ37 as a Test Strain

10 μl of mutagens (100 μg/ml) and 10 μl of compounds (1 mg/ml) were used for the test. Level of statistical significance: $^{**}p<0.05$ and $^{*}p<0.01$ with respect to mutagen values from three experiments.

only that each sample inhibits the induction of genotoxicity by the mutagens (negative control).

It has been reported that a mixture of stigmasterol and β-sitosterol[12] or stigmasterol only[13] reduced the number of micronucleated polychromatic erythrocytes induced by mitomycin C in the micronucleus test. β-Sitosterol (**5**) is known to inhibit the mutagenicity of *N*-methyl-*N*-nitrosourea[14] or tetracycline.[15] The antimutagenic activity of stigmasterol (**4**), however, has not yet been reported using SOS chromotest which is regarded as a more general antimutagenicity assay with accuracy and rapidity.

When stigmasterol was added to LB broth to give a final concentration up to 20 μg/ml, the cell growth of *E. coli* PQ37 after 24 h was not inhibited, which suggests that its antimutagenic effects are not due to a bactericidal effect of the compound in the presence of a mutagen. However, because the present results were obtained from an *in vitro* assay using a bacterial strain, compounds **4** and **5** would not directly be applied to mammals as an anticancer agent, but should further be estimated in an *in vivo* test using animal models to prove their clinical values.

Experimental

General Procedures *E. coli* PQ37 (*sfi*A::Mud(Ap *lac*)*cts lac*ΔU169 *mal*+, *uvr*A, *gal*E *gal*Y, PhoC, *rfa*) and *S. typhimurium* TA98 and TA100 were used as the test strains for antigenotoxicity (SOS chromotest) and for antimutagenicity (Ames test), respectively. The mutagens MNNG (1-methyl-3-nitro-1-nitrosoguanidine) and NQO (4-nitroquinoline-1-oxide) were purchased from Sigma Co. (St Louis, MO, U.S.A.). Melting points were measured on an Electrothermal IA9100 apparatus (Reallabware Co., Watford Herts, U.K.) and are uncorrected. NMR spectra were recorded on a Varian UNITY-500 or Varian GEMINI-200 (Varian Inc., Palo Alto, CA, U.S.A.) spectrometer using $CDCl_3$ as a solvent. Mass spectra were obtained on a Finnigan MAT95 or Finnigan LCQ (Thermo Electron Corporation, West Palm Beach, FL, U.S.A.). Specific rotations were measured on a Jasco DIP-370 polarimeter (Jasco Inc., Commerce Dr. Easton, MD, U.S.A.). HPLC analysis was carried out with a Shimadzu LC-10AD system (Kyoto, Japan) equipped with an Eclipse XDB-C18 column (4.6 mm×25 cm) (Agilent, Palo Alto, CA, U.S.A.) using a linear gradient of CH_3CN (40%→60%, flow rate: 1 ml/min)) at 254 nm at room temp.

Plant Material Dried thorns of *Gleditsia sinensis* were purchased from one of the oriental drugstores in Gyeongju, Korea and identified by Prof. Byung-Soo Kang, College of Oriental Medicine, Dongguk University, Gyeongju, Korea. A voucher specimen of this plant material is deposited at the Herbarium (DG-GS001) of this college.

Extraction and Isolation The powdered thorns (1.2 kg) of commercially available *G. sinensis* were extracted with 80% methanol (2×3 l) under reflux. The extract were concentrated to dryness *in vacuo* at 40 °C to give a brown oily extract (50 g, yield 4.2%), which was successively fractionated with 200 ml of each of CH_2Cl_2 (5 g), EtOAc (9 g) and *n*-BuOH (6.3 g) in sequence to yield the corresponding dried extracts. The CH_2Cl_2 fraction (3 g) was chromatographed on a silica gel column (5—40 μm, 5×70 cm) (Merck & Co., Inc., Whitehouse Station, NJ, U.S.A.). Elution was initiated with CH_2Cl_2 providing 30 fractions (10 ml of each after the first spot on UV). Fractions 4—11, 18—20 and 25—28 afforded compounds **1** (25 mg), **2** (2 mg) and **3** (2 mg), respectively. Further isolation using the above column with hexane/EtOAc 4 : 1 gave 15 fractions (20 ml each), of which the fractions 5—9 and 11—13 furnished compounds **4** (35 mg) and **5** (15 mg), respectively. Compounds **2** and **3** were purified by preparative TLC (CH_2Cl_2/MeOH 50 : 1, Kieselgel 60F_{254}, 0.5 mm, 20×20 cm, Merck) and the other compounds were recrystallized with MeOH. The purity of the isolated compounds **1**—**5** was analyzed by reverse-phase HPLC to be 95.0% (t_R=104.4 min), 98.5% (t_R=98.9 min), 97.2% (t_R=123.4 min), 96.8% (t_R=84.2 min) and 93.7% (t_R=75.6 min), respectively. All compounds **1**—**5** showed a positive Liebermann–Burchard test (bluish green). TLC (silica gel), CH_2Cl_2/MeOH 50 : 1, *Rf* **1**: 0.86, **2**: 0.62, **3**: 0.53, **4**: 0.36, **5**: 0.33.

D:C-Friedours-7-en-3-one (**1**): Colorless needles, mp 212—214 °C (239—240 °C),[9] $[\alpha]_D^{25}$ −4.1° (c=0.4, $CHCl_3$), EI-MS (70 eV) *m/z* (rel. int.): 424 ($M^{+\cdot}$, 20), 409 (9), 257 (15), 246 (18), 245 (100), 218 (11), 205 (24), HR-EI-MS *m/z*: 424.3703 (Calcd for $C_{30}H_{48}O$: 424.3705).

Stigmast-4-ene-3,6-dione (**2**): Colorless amorphous crystals, mp 168—169 °C (170—172 °C,[16] 160—164 °C),[10] $[\alpha]_D^{25}$ −21.4° (c=0.15, $CHCl_3$) (−60.5°),[17] ^{1}H- and ^{13}C-NMR: Table 1, CI-MS *m/z* (rel. int.): 427.22 (M+H, 100).

Stigmastane-3,6-dione (**3**): Coloress amorphous crystals, mp 192—194 °C (195—198 °C),[10] $[\alpha]_D^{25}$ +26.7° (c=0.19, $CHCl_3$) (+9.5°, above 400 μm, ORD),[18] ^{1}H- and ^{13}C-NMR: Table 1, EI-MS (70 eV) *m/z* (rel. int.): 428 (M^+, 65), 413 (7), 287 (29), 245 (14), 231 (26), 149 (55), 137 (58), 98 (100).

Stigmasterol (**4**): Colorless amorphous solids, mp 163—164 °C, $[\alpha]_D^{25}$ −48.5° (c=0.26, $CHCl_3$) (−50°),[19] EI-MS (70 eV) *m/z* (rel. int.): 412 (M^+, 100), 397 (8), 369 (21), 351 (24), 314 (9), 300 (39), 271 (42), 255 (52), 213 (14).

β-Sitosterol (**5**): Colorless amorphous solids, mp 137—140 °C, $[\alpha]_D^{25}$ −29.2° (c=0.2, $CHCl_3$) (−34.6°),[18] EI-MS (70 eV) *m/z* (rel. int.): 414 (M^+, 60), 396 (90), 381 (66), 354 (17), 329 (29), 303 (53), 288 (17), 275 (39), 255 (69), 231 (37), 213 (100), 199 (45).

Compounds **1**, **4** and **5** were identified by direct comparison with authentic samples (mp, ^{1}H- and ^{13}C-NMR).

Antimutagenic Activity Assay The antigenotoxicity test was performed by the method of Quillardet and Hofnung[20] using 600 μl of bacterial suspension, 10 μl of mutagens (100 μg/ml of DMSO), and 10 μl of fractions (10 mg/ml of DMSO) or 10 μl of compounds (1 mg/ml of DMSO). The dosage of the samples was determined according to our previous report[6] on the antimutagenicity assay. Genotoxicity is expressed as an induction factor (SOS induction by a mutagen) and is defined as the value of Rs divided by Ro, where Rs is the enzyme activity ratio of β-galactosidase activity and alkaline phosphatase with sample and/or a mutagen, and Ro is the spontaneous Rs without sample or mutagen. The antimutagenicity test was performed essentially as described by Maron and Ames[21] using 0.1 ml of bacterial suspension, 0.1 ml of mutagens (NPD 10 μg/plate, NaN_3 1 μg/plate) and 0.1 ml of fractions (1 mg/plate).

The inhibition ratio of revertant colony forming unit (CFU) per plate was calculated by the following equation: % inhibition ratio=[1−(CFUsm−CFUp)/(CFUm−CFUp)]×100, where CFUsm is CFU with mutagen and sample, CFUm is CFU with mutagen, and CFUp is a spontaneous CFU with no mutagen or sample. The number of histidine revertants induced by the mutagen without any sample was given as 100%.

Statistics Inter-group comparisons of data were made by ANOVA, followed by *post hoc* multiple comparison analysis.

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