Arch Pharm Res Vol 24, No 1, 44-50, 2001 Medicinal Chemistry & Natural Products

Constituents and the Antitumor Principle of *Allium victorialis* var. *platyphyllum*

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(Received September 9, 2000)

To search for cytotoxic components from *Allium victorialis*, MTT assays on each extract and an isolated component, gitogenin 3-O-lycotetroside, were performed against cancer cell lines. Cytotoxicities of most extract were shown to be comparatively weak, though IC_{50} values of $CHCl_3$ fraction was found to be <31.3-368.4 μg/ml. From the incubated methanol extract at 36°C, eleven kinds of organosulfuric flavours were predictable by GC-MS performance. The most abundant peak was revealed to be 2-vinyl-4*H*-1,3-dithiin (**1**) by its mass spectrum. Further, this extract showed significant cytotoxicities toward cancer cell lies. Silica gel column chromatography of the n-butanol fraction led to the isolation of gitogenin 3-O-lycotetroside (**3**) along with astragalin (**4**) and kaempferol 3, 4'-di-O-β-D-glucoside (**5**). This steroidal saponin exhibited significant cytotoxic activities (IC_{50}, 6.51-36.5 μg/ml) over several cancer cell lines. When compound **3** was incubated for 24 h with human intestinal bacteria, a major metabolite was produced and then isolated by silica gel column chromatography. By examining parent- and prominent ion peak in FAB-MS spectrum of the metabolite, the structure was speculated not to be any of prosapogenins of **3**, suggesting that spiroketal ring were labile to the bacterial reaction. These suggest that disulfides produced secondarily are the antitumor principles.

Key words: *Allium victorialis*, Liliaceae, Organosulfuric, Steroidal saponin, Cytotoxicity

INTRODUCTION

Allium victorialis var. *platyphyllum* (Liliaceae) is a perrenial herb wildly distributed in Mt. Chiri, Mt. Odae and Ullung Island. The leaves and bulbs have been used not only as mountain-edible herbs but also as functional foods for improving gastritis and heart failures (Moon, 1984). *A. victorialis* is closely related to *A. sativum* (garlic) though external morphology of these two plants distinctly differentiated with each other (Lee, 1985). It was reported that the leaves contains 2-3% of carbohydrate and ascorbic acid whereas the bulbs possesses organosulfuric compounds. These organosulfuric compounds exist in the form of S-alkenyl- or S-alky-L-cysteine sulfoxide but these hydrophilic compounds are converted to disulfides with garlic smell when alliinase is activated by tissue injury (Lawson *et al.*, 1991). Therefore, the aging indicates the conversion of the particular amino acids in garlic related-plants to flavoring disulfides. In addition, steroidal saponin is found in *Allium* species (Matsuura *et al.*, 1989).

Wijaya (1996) reported anti-platelet aggregation of Allium species have positive correlation with the content of organosulfuric substances (Moon, 1984). Allicin, one of the disulfide substances is known to have anti-platelet aggregation (Liakopoulou-Kyriakides *et al.*, 1985). Wu *et al.* (1982) searched the changes of the organosulfurics contents in shallots by processing. Garlic, one of *Allium* species, has the activities of anti-platelet aggregation (Liakopoulou-Kyriakides *et al.*, 1985), antihepatotoxicity (Hikino *et al.*, 1986) and antitumor activity (Singh *et al.*, 1998; Siegers *et al.*, 1999). In the present study, we found the antitumorically active components by extensive study on *A. victorialis* var. *platyphyllum*. We focused on the compounds responsible for the antitumor activities of this plant. We proposed that the structure of steroidal saponin might be changeable by human intestinal bacteria.

MATERIALS AND METHODS

Apparatus

Melting points were determined on a Yanagimoto micro-

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melting point apparatus and are uncorrected. Optical rotations were measured on a JASCO DIP-360 digital polarimeter at 25°C. IR spectra were recorded on a Hitachi 260-01 spectrometer in KBr disks. EIMS (ionization voltage 70 eV) and FABMS were measured with a JEOL JMS DX-300 spectrometer. ^{1}H- and ^{13}C-NMR spectra were taken on a JEOL JNM-GX 400 spectrometer with TMS as an internal standard.

Plant material

Allium victorialis var. *platyphyllum* was collected in August 1998 on Is. Ullung, Kyungbuk Province, Korea, and the plant was divided into two parts, the aerial parts and the bulbs. Each part was dried avoiding direct sunlight and pulverized. For the extraction of disulfide substances, fresh leaves were preserved in refrigerator at -10°C and treated within two days.

Extraction and fractionation

The bulbs (1.5 kg) were extracted three times with MeOH under reflux. The MeOH extract was filtered and evaporated on a rotary evaporator under reduced pressure to give a viscous mass (510 g) of MeOH extract. This material was suspended in H_2O and partitioned with $CHCl_3$, EtOAc, and n-BuOH to give a $CHCl_3$-soluble fraction (70 g), EtOAc-soluble fraction (90 g) and *n*-BuOH-soluble fraction (135 g) being dried *in vacuo*.

Isolation of compounds 1 and 2

A part of EtOAc-soluble fraction (10 g) was subjected to column chromatography over silica gel (Merck, Art. No. 7734, Germany). The column was eluted over silica gel (280 g) with $CHCl_3$-MeOH-H_2O (7:3:1, lower phase) and two subfraction 1 and 2 were taken. These were dried *in vacuo* and recrystalized from MeOH to afford compound **1** (120 mg) and **2** (210 mg).

Compound 1: Pale yellowish needles from MeOH, mp 230-233°C; $FeCl_3$, Mg-HCl test: positive; IR ν_{max} (KBr) cm^{-1}: 3500-3100 (broad, OH), 1650 (α, β-unsaturated ketone), 1605, 1505 (aromatic C=C), 1100-1000 (glycoside); ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 8.03 (2H, d, J=8.8 Hz, H-2', 6'), 6.87 (2H, d, *J*=8.8 Hz, H-3', 5'), 6.43 (1H, d, *J*=1.9 Hz, H-8), 6.20 (1H, d, *J*=1.9 Hz, H-6), 5.44 (1H, d, *J*=7.0 Hz, anomeric proton); ^{13}C-NMR (75.5 MHz, DMSO-d_6): literature (Park *et al*, 1991).

Compound 2: Pale yellowish needles from MeOH, mp 207-210°C; $FeCl_3$ test, Mg-HCl test: positive; IR ν_{max} (KBr) cm^{-1}: 3500-3100 (broad, OH), 1655 (α, β unsaturated ketone), 1594, 1590, 1482 (aromatic C=C), 1100-1000 (glycoside); ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 8.11 (2H, d, J=8.8 Hz, H-2', 6'), 7.14 (2H, d, J=8.8 Hz, H-3', 5'), 6.45 (1H, d, J=1.9 Hz, H-8), 6.21 (1H, d, J=1.9 Hz, H-6), 5.46 (1H, d, J=7.1 Hz, anomeric proton), 5.36 (1H, d, J=7.1 Hz, anomeric proton); ^{13}C-NMR (75.5 MHz, DMSO-d_6): Kaempferol moiety-155.8 (C-2), 133.9 (C-3), 177.3 (C-4), 161.4 (C-5), 100.1 (C-6), 164.6 (C-7), 94.0 (C-8), 156.7 (C-9), 104.3 (C-10), 123.9 (C-1'), 130.8 (C-2', 6'), 116.0 (C-3', 5'), 159.4 (C-4'); 3-Glc-101.0 (C-1), 74.4 (C-2), 76.7 (C-3), 70.1 (C-4), 77.8 (C-5), 60.8 (C-6); 4'-Glc-99.9 (C-1), 73.4 (C-2), 76.7 (C-3), 69.8 (C-4), 77.3 (C-5), 60.8 (C-6).

Isolation of compound 3

A part of *n*-BuOH-soluble fraction (15 g) was subjected to column chromatography over silica gel and eluted with $CHCl_3$-MeOH-H_2O (65:35:10, lower phase) to yielded subfraction-3. This one was dried *in vacuo* and purified through sephadex LH-20 column chromatography and followed by being recrystallized from water-saturated *n*-BuOH to give compound 3 (1.6 g).

Compound 3: Colorless needles(from *n*-BuOH saturated with H_2O), mp 255-260 (dec.), $[\alpha]_D$ -75.8° (c=0.38, pyridine), ^{1}H-NMR(500 MHz, pyridine-d_5): 0.68 (3H, d, *J*= 5.2 Hz, H-27), 0.78 (3H, s, H-18), 0.80 (3H,s, H-19) 1.10 (3H, d, *J*=6.8 Hz, H-21), 4.90 (1H, d, *J*=7.8 Hz), 5.20 (1H, d, *J*=7.9 Hz), 5.24 (1H, d, *J*=7.7 Hz), 5.71 (1H, d, *J*=7.7 Hz); ^{13}C-NMR (125 MHz, pyridine-d_5): Table I.

Acid hydolysis of 3: Compound **3** was hydrolyzed in 5%-H_2SO_4 in MeOH-H_2O (2:8) under reflux for 3 h. After neutralization with NH_4OH followed by extraction with $CHCl_3$, the aqueous layer was evaporated *in vacuo* to give a residue. The resulting residue was applied to a TLC plate and developed with EtOAc-MeOH-H_2O-AcOH (13:6:3:3). The R_f values of the product were identical to those of L-galactose, D-glucose and D-xylose.

Partial hydrolysis of 3

For partial hydrolysis of **3**, this compound (500 mg) was refluxed in 50 ml of 0.25 M HCl/50% MeOH for 20 min. The reactant composed of gitogenin and prosapogenins was subjected to column chromatography over silica gel (60 g). The column was eluted with $CHCl_3$-MeOH-H_2O (4:1:0.1) to yield **3a** (60 mg), **3b** (31mg) and **3c** (20 mg).

3a: colourless needles, mp 220-227°C, $[\alpha]_D$ + 1.0 (pyridine: *c*=1.0). FAB-MS m/z 941 $[M+Na]^+$; **3b**: colourless needles, mp 220-223, $[\alpha]_D$ -30.0°C (pyridine, *c*=1.0), FAB-MS m/z 779 $[M+Na]^+$. **3c**: colourless needles, mp 220-222°, FAB-MS m/z 617 $[M+Na]^+$.

Metabolism of gitogenin 3-O-lycotetroside (3) by human intestinal bacteria

To find out the degraded metabolites of compound **3**

Table I. ^{13}C-NMR spectral data of gitogenin 3-O-β-lycotetroside (75.5 MHz, *pyridine-d_5*)

Carbon	3	A[a]	Carbon	3	B[b]
1	45.5	45.5	3-O-Gal		
2	70.7	70.7	1	103.3	102.8
3	84.2	84.2	2	72.5	73.1
4	34.1	33.9	3	75.1	75.1
5	44.6	44.6	4	79.4	79.9
6	28.1	28.1	5	76.1	76.2
7	32.2	32.1	6	60.5	60.6
8	34.5	34.6	4'-O-Glc		
9	54.3	54.4	1	104.7	104.8
10	36.8	36.8	2	81.3	81.3
11	21.4	21.4	3	86.9	86.9
12	40.0	40.2	4	70.7	70.8
13	40.7	40.8	5	78.7	78.7
14	56.3	56.4	6	62.7	62.5
15	32.1	32.4	2''-O-Glc		
16	82.1	81.1	1	104.8	105.1
17	63.0	63.0	2	75.1	75.3
18	16.6	16.6	3	78.1	78.2
19	13.4	12.3	4	71.3	71.1
20	42.0	42.0	5	77.6	77.8
21	15.0	15.0	6	63.0	63.0
22	109.2	109.1	3''-O-Xyl		
23	31.8	31.8	1	104.9	105.1
24	29.2	29.2	2	75.7	75.6
25	30.6	30.6	3	78.5	77.6
26	66.8	66.8	4	70.4	70.5
27	17.3	17.3	5	67.3	67.3

A:3-O-β-(3'''-O-β-D-xylopyranosyl)lycotetrosylgitogenin,
B:Yamogenin 3-O-β-lyco-tetroside

(100 mg) by human intestinal bacteria, compound **3** was anaerobically incubated for 24 h with human fecal suspension. Then the metabolites were extracted with EtOAc and analyzed by TLC. Main metabolite **3d** was obtained using silica gel column (2 × 20 cm) chromatography {eluent, $CHCl_3$-MeOH (10:1)} from the metabolite mixture and further elution with the solvent ($CHCl_3$-MeOH-H_2O, lower phase) yielded **3e**. FABMS of these two isolates were taken. (**3d**: FAB-MS m/z (%) 433 (20), 415 (100); **3e**: FAB-MS 637 (25) $[M+Na]^+$, 329 (63), 176 (100))

The time-course of the metabolism of compound **3** by human intestinal bacteria was measured as follows. A reaction mixture containing 2 mg compound 3 and 0.1 g fecal human feces in a final volume of general anaerobic media (GAM, Nissui Pharm. Co., Japan). The mixture was incubated at 37°C for 24 h and an aliquot (1 ml) of the reaction mixture was periodically extracted twice with EtOAc. The chromatograms of these compounds were quantitatively assayed with a TLC scanner after apraying with 5% H_2SO_4 solution followed by heating in a dry oven at 110 °C for 15 min.

Preparation of disulfide extract

Both fresh leaves (50 g) of *A. victorialis* and fresh bulbs of *A. sativum* were homogenized with 50 ml MeOH, respectively, and incubated at 37°C. The incubated ones were filtered and reduced to a half volume, respectively and followed by being extracted with diethyl ether. Each extract was washed with small volume of water and dehydrated with anhydrous sodium sulfate. The final extracts were evaporated under reduced pressure and the dried substances were dissolved in MeOH. The solutions were preserved at -20°C for the application of gas chromatography-mass spectroscopy.

GC-MS analysis

The GC (Varian 3400) column for detecting organosulfuric substances was SE-54 (30 m × 0.3 mm, 0.25 μm film thickness). The used detector was MS (TSQ-700, Finnigan Mat). The initial temperature was 40°C and increased by 2°C per min and injection temperature was 250°C.

MTT assay

The *in vitro* tests cytotoxicity against HepG-2 (human hepatoblastoma cell), Vero-P128 (monkey kidney cell), P-388 (mouse lymphoma leukemia cell), L-1210 (mouse lymphomatic leukemia cell), L-1210 (human promyelocytic leukemia cell) and SNU-5 (human stomach cancer cells) were performed essentially according to the method described previously (Denizot *et al.*, 1996). Cells were seeded into 96 microtiter plates and incubated overnight. The test samples were dissolved in dimethylsulfoxide (DMSO) and were added in serial dilution (the final DMSO concentrations in all assay did not exceeded 0.01%). Twenty four hour after seeding, 100 μl new media or test compounds were added and the plates were incubated for 48 h. Cells were washed once before adding 50 μl FBS-free medium containing 5 μg/ml (MTT) concentration. After 4 h incubation at 37°C, the medium was discarded and formazan blue formed into the cells was extracted by adding 50 μl DMSO. Optical density was measured at 540 nm.

RESULTS AND DISCUSSION

Chemical structure

Physicochemical and spectroscopic data of compound **1** were shown on the experimental section. According to the data, compound **1** was identified as astragalin (kaempferol 3-O-β-D-glucoside). Two anomeric protons due to sugar moieties of compound **2** were shown at δ 5.36 and 5.46 on ^{1}H-NMR spectrum. Overall signals in ^{13}C-NMR spectrum

of compound 2 resembled those of astragalin (**1**) (Park *et al.*, 1991), though the former had signals due to one more sugar than the latter. When ^{13}C-NMR data of this compound was compared with those of astragalin and kaempferol 4′-O-β-D-glucopyranoside (Agrawal, 1989), **2** was obviously found that the two glucoses linked to C_3-OH and $C_{4'}$-OH of kaempferol. The assignment of ^{13}C-NMR data of compound **1** was done for the first time in the present study, though Yoshida *et al* (1987). have reported the first isolation of this compound from a natural source.

Acid hydrolysis of compound **3** produced an aglycone and D-glucose, L-galactose and D-xylose shown on TLC. On FAB mass spectrum of compound **3**, *m/z* 1073 $[M+Na]^+$ was shown indicating four sugars in this saponin. Partial hydrolysis of compound **3** and further isolation of the prosapogenins yielded the three compounds, **3a**, **3b** and **3c**. From FAB-MS data of the isolates, the ions $[M+Na]^+$ were shown on *m/z* 941 (**3a**), 779 (**3b**) and 617 (**3c**), respectively. The structures shown on Fig. 1 were presumed from only FAB-MS. Compound **3a** has a D-xylopyranosyl-(1→4)-D-glucopyranosyl-(1→4)-L-arabinopyranosyl moiety. But, compound **3b** bears inner two sugars and **3c** contains the most inner sugar (L-arabinose). Lycotetrose of spirostane steroids is very commonly occurred in the saponin of Allium species. As shown in Table I, the ^{13}C-NMR data of compound **3** were compared with those of 3-O-β-(3‴-O-β-D-xylopyranosyl) lycotetrosylgitogenin (Nakano *et al.*, 1989) and yamogenin 3-O-β-lycotetroside (Son *et al.*, 1990). The assignment was done for the first time in the present study on the basis of ^{1}H-^{1}H COSY, ^{1}H-^{13}C COSY NMR spectra. Therefore, this compound was identified as gitogenin 3-O-β-lycotetroside (Matsuura *et al.*, 1989).

For confirming the existence of disulfide substances in the incubated extracts, several of disulfides were shown on GC chromatogram. These are volatile disulfides secondarily metabolized from S-alkenyl or S-alkycystein derivatives by alliinase. The structures of relatively abundant cyclic disulfides are shown on Fig. 1.

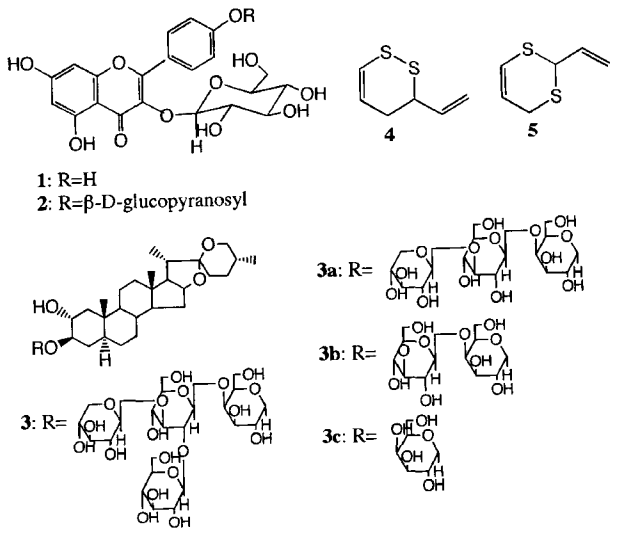

Fig. 1. Structures of the components of *Allium victorialis* var. *platyphyllum* (**1-5**) and **3a**, **3b** and **3c**

Cytotoxicity

It is well known that cysteine derivatives contained in native forms show no cytotoxicities while the transformed disulfides the activity (Hikino *et al.*, 1986; Siegers *et al.*, 1999; Munday *et al.*, 1999; Chalier *et al.*, 1998). For the cytotoxicity of allicin, allylic structure contributes to the cytotoxicity maybe in the apoptotic mechanism. MTT assays of MeOH extract, its fractions and the incubated extract were conducted against HepG-2, Vero-P128, P-388, L-1210 cell lines. As shown in Table II, the incubated extract and gitogenin 3-O-lycotetroside showed significant activities but others negligible activities. Although **3** showed a potent activity, *n*-BuOH fraction containing this saponin showed a very weak cytotoxicity. It seems that other substances in that fraction disturbed the activity. However, the incubated extract showed the most significant activity among the tested samples. That extract was confirmed to have diallysulfides by GC-MS analysis as already been reported. The most abundant component was shown to be 2-vinyl-

Table II. Cytotoxic activities of MeOH extract, its fractions and gitogenin 3-O-lycotetroside from the bulbs of *Allium victorialis* var. *platyphyllum*

Treatment	IC_{50}[a] (μg/ml)			
	HepG-2	Vero-P128	P-388	L-1210
MeOH extract	>1000	>1000	695.0	405.3
$CHCl_3$ fraction	368.4	373.6	<31.3	<31.3
EtOAc fraction	325.2	368.4	62.9	34.6
n-BuOH fraction	>1000	>1000	>1000	438.7
Incubated extract	93.5	96.8	24.5	88.9
Incubated extract of garlic	88.9	33.2	15.2	54.3
Gitogenin 3-*O*-lycotetroside	17.9	14.6	36.5	6.5

[a]IC_{50} is defined as the concentration which resulted in a 50% decrease in cell number. The values reprsent the mean of three independent experiments.

4H-1,3-dithiin (**5**) and another vinylthiin-type component was 3,4-dihydro-3-vinyl-1,2-dithiin (**4**) (Nishimura *et al.*, 1988). These two compounds and other disulfides with allyl groups may show electrophilic attack to the eucaryotic cells as in the reports of cytotoxicities of costunolide (Kim *et al.*, 1999) and others. A sulfur-containing amino acid and its glucoside has been isolated from garlic (Mutsch-Eckner *et al.*, 1993). There have been various reports that the aged metabolites of disulfides are responsible for various biological activities. A hydrophilic substance, S-allylmercapto-cysteine, was reported not to have a cytotoxicity but a hepatoprotective activity (Hikino *et al.*, 1986).

As shown in Table II, compound 3 showed sighificant cytotoxicities in the range of 6.51-36.5 μg/ml though *n*-BuOH fraction has shown no significant cytotoxicities. Many steroidal saponins with the genin spirostane-type are well known to have cytotoxicity and, particularly, cytostatic action of a steroidal saponin isolated from *Balanites aegyptica* has been also reported (Petit *et al.*, 1991). On the examination of cytotoxicities of the isolated saponin, **3**, and *n*-BuOH fraction containing **3**, it seems likely that a cytotoxic steroidal saponin may be attenuated. Only pure substance (**3**) showed cytotoxicities toward tumor cell lines. Nevertheless, many biological activities such as anti-platelet aggregation activity (Peng *et al.*, 1995), anti-tumour-promoter activity (Mimaki *et al.*, 1996) and cholesterol absorption disturbance activity (Harris *et al.*, 1997) have been reported.

Based on our previous studies on the degradation of saponins by human intestinal bacteria (Kim *et al.*, 1998), we expected that the structures and biological activities of steroidal saponins could be changed by the action of human intestinal bacteria. One of the aims to the present study is to elucidate the structural change of steroidal saponin by human intestinal bacteria. From the incubation with human intestinal bacteria for 24 h, the metabolite **3e** was obtained by column chromatography. As shown in Fig. 3, the molecular weight obtained from the FAB-MS spectrum of **3e** did not belong to any of **3c** (gitogenin 3-O-galactoside) and **3b** (gitogenin 3-O-β-D-glucosyl (1→6)-α-L-galactoside). This suggests that the structural changes in sugar- and genin parts were induced. There has been reports that some bacteria opened the spiroketal structure

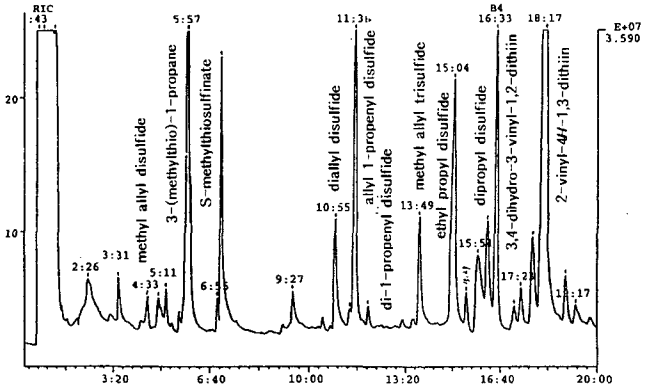

Fig. 2. Gas chromatogram of the volatile organosulfuric fraction obtained from the aerial part of *Allium victorialis* var. *platyphyllum*.

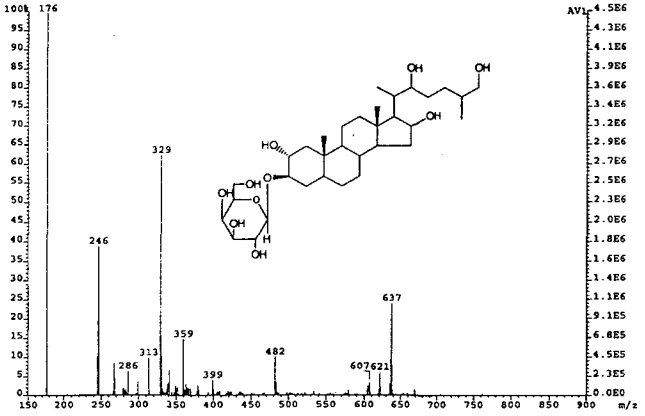

Fig. 3. FAB-MS spectrum of metabolite (**3e**) and a proposed struc-ture (3-O-galactosylcholest 2,3,16,22,26-pentaol)

Table III. Identified volatile organosulfuric components from *Allium victorialis* var. *platyphyllum*

Peak No.	compound	M^+	retention time	peak area
1	methyl allyl disulfide	120 $[C_4H_8S_2]^+$	431	8.4[a]
2	3-(methylthio)-1-propene	88 $[C_4H_8S]^+$	557	61.6
3	S-methylthiosulfinate	112 $[CH_4S_3]^+$	729	21.0
4	diallyl disulfide	146 $[C_6H_{10}S_2]^+$	1054	6.3
5	allyl 1-propenyl disulfide	146 $[C_6H_{10}S_2]^+$	1135	27.3
6	di-1-propenyl disulfide	146 $[C_6H_{10}S_2]^+$	12 5	9.1
7	methyl allyl trisulfide	120 $[C_4H_8S_3]^+$	1351	12.6
8	ethyl propyl disulfide	136 $[C_5H_{12}S_2]^+$	1458	20.3
9	dipropyl disulfide	150 $[C_6H_{14}S_2]^+$	1621	26.6
10	3,4-dihydro-3-vinyl-1,2-dithiin	144 $[C_6H_8S_2]^+$	1631	35.0
11	2-vinyl-4*H*-1,3-dithiin	144 $[C_6H_8S_2]^+$	1810	84.7

[a]Unit is mm^2

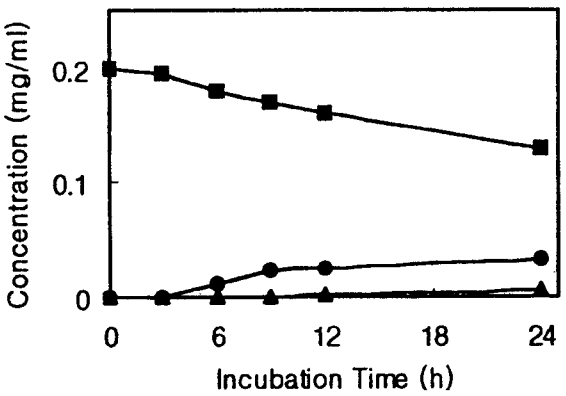

Fig. 4. Time course of the metabolism of Gitogenin-3-*O*-lycotetroside by human intestinal bacteria. ■, Gitogenin-3-O-lycotetroside; ●, metabolite **3e**; ▲, unknown metabolite.

of steroidal saponins (Mahato *et al.*, 1993). Our findings and evidence suggests the possibility that the ring E/F, probably the most labile rings, may be hydrolyzed. One of the possible structures was shown in Fig. 3. Although the full structure of the metabolites and the metabolic pathway were not established, the transforming of genin structure of steroidal saponins has no doubt. Therefore, the metabolites induced by human intestinal bacteria may be responsible for the various biological activities. As far as the antitumor activity is concerned, there is no evidence that steroidal saponins have the activity from the medicinal chemical point of view. Fig. 4 shows that the content of **3** is lowered by time-course, and that **3e** appeared in 6 h and unknown **3d** begins to appear in 24 h. In conclusion, the active substances may be attributed to the disulfides that can be produced from the aging of plant tissue.

ACKNOWLEDGEMENTS

This work was granted partly from the Korea Research Foundation.

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