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ORIGINAL ARTICLE

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Plant growth regulation effects of triterpenoid saponins

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Abstract To investigate structure–activity relations between the sugar chain structures of triterpenoid saponins and their plant growth regulation effects, several monodesmosidic saponins with betulin as an aglycon were synthesized by chemical and enzymic reactions. Three triterpenoids (betulin, betulinic acid, oleanolic acid) and synthesized betulin glycosides were submitted to germination and growth regulation tests on alfalfa seeds. We concluded the following. Betulin had a slight growth inhibitory effect on alfalfa radicles. Betulin glycosides exhibited stronger effects than betulin, and betulin glycosides with two to four glucose residues as a sugar moiety had the greatest inhibitory activity. These characteristics of growth inhibitory effects were considerably different from those of phenolic compounds so far reported. Some betulin glycosides also showed a significant growth regulation effect on alfalfa hypocotyls. However, hypocotyl growth was less affected than radicle growth for all betulin glycosides. Among the triterpenoids, betulinic acid had stronger growth inhibitory effects on alfalfa radicles than betulin, suggesting the importance of the carboxyl group at the C-28 position for the inhibitory effects of lupane-type triterpenoids. On the other hand, no germination regulation effects on alfalfa seeds were observed for any of the betulin glycosides or triterpenoids examined.

Key words Betulin · Betulin glycosides · Triterpenoids · Triterpenoid saponins · Plant growth regulation effects

Introduction

The saponins are a group of plant glycosides in which hydrophilic sugars are attached to a lipophilic steroid or triterpenoid moiety.[1] They are widely distributed in higher plants, and it is well known that they show various biological activities such as antimicrobial, antitermitic, molluscicidal, and anti-human immunodeficiency virus-1 (HIV-1) protease.[2–5] Recently, the relations between the chemical structures of saponins and their biological activities have been examined using isolated or synthesized saponins. Matsuda et al.[6] investigated the effects of escins Ia, Ib, IIa, and IIb isolated from the horse chestnut on acute inflammation in animals and indicated the importance of the 21-angeloyl group or 2′-*O*-xylopyranosyl moiety. Furthermore, the effects of sugar compositions of the sugar moiety on the hemolytic activity and stimulating activity for fruiting of *Pleurotus ostreatus* have also been reported.[7,8]

Saponins are usually found with some concentration in roots, foliage, and seeds.[1,9–11] It has been reported that allelopathic potentials, which show stimulating or inhibiting activity for growth of other plant species, are exuded from seeds of various plant species into the environment.[12] These facts prompted us to study the plant growth regulation effects of saponins. There have been many investigations on the plant growth regulation effects of phenolic compounds[13,14] but none on the saponins.

We synthesized several monodesmosidic saponins with betulin as an aglycon, and these triterpenoid saponins were submitted to the germination and plant growth regulation assays using alfalfa seeds. The relations between the sugar chain structures of these saponins and their plant growth regulation activities are discussed. The activities of other triterpenoids were also examined.

Materials and methods

Spectrometry

Proton nuclear magnetic resonance (^{1}H-NMR) and carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra were recorded on a JEOL Alpha-500 NMR spectrometer. Pyridine-d_5 was used as the solvent. Fast atom bombard-

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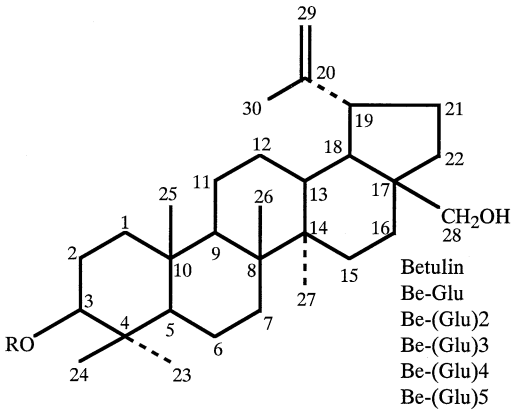
Fig. 1. Structures of betulin and synthesized betulin glycosides. G*n* (n = 1, 2, 3, 4, 5, 6) indicates a glucosyl unit. G1 is an innermost unit binding to betulin; G2 is a second one from G1; and G3, G4, G5, and G6 are the third, fourth, fifth and sixth units, respectively, from G1. G6 is the outermost unit



Betulin	H
Be-Glu	1β G1
Be-(Glu)2	1β G1 (4,1) β G2
Be-(Glu)3	1β G1 (4,1) β G2 (4,1) α G3
Be-(Glu)4	1β G1 (4,1) β G2 (4,1) α G3 (4,1) α G4
Be-(Glu)5	1β G1 (4,1) β G2 (4,1) α G3 (4,1) α G4 (4,1) α G5
Be-(Glu)6	1β G1 (4,1) β G2 (4,1) α G3 (4,1) α G 4 (4,1) α G 5 (4,1) α G6

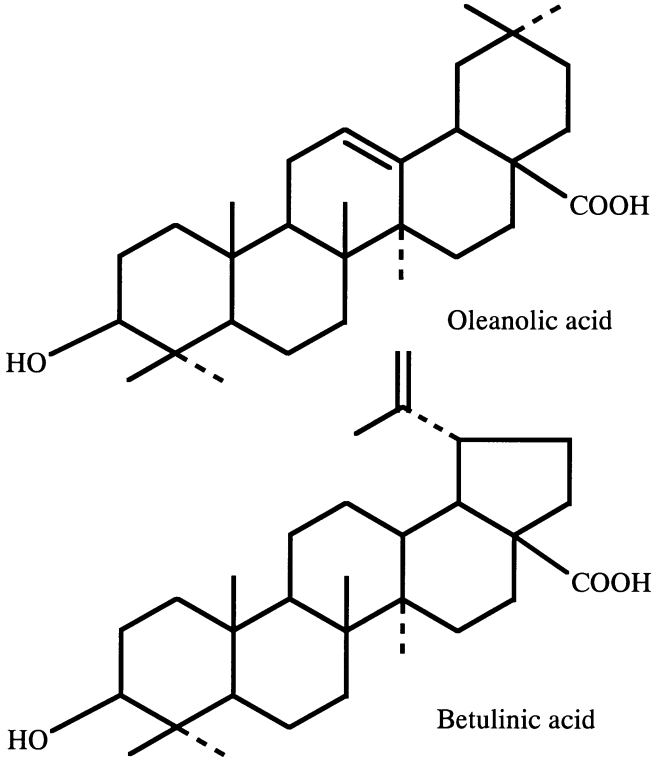


Fig. 2. Structures of the triterpenoids used in this study

ment (FAB) mass spectra were obtained using a JEOL HX-110A spectrometer. Optical rotations were analyzed by a JASCO DIP-140 digital polarimeter. Chloroform was used as the solvent.

Isolation of triterpenoids

Betulin (Fig. 1) and oleanolic acid (Fig. 2) were isolated from dichloromethane extracts of the outer bark of shirakamba (*Betula platyphylla* Sukatchev var. *japonica* Hara) and were identified by their analytical data from ^{13}C-NMR and specific rotations and references in the literature.[15–17] Betulin: colorless needles (EtOH), $[\alpha]_D$ + 18.1° (c 0.16, $CHCl_3$); oleanolic acid: colorless needles (EtOH), $[\alpha]_D$ + 78.5° (c 1.66, $CHCl_3$). Betulinic acid (Fig. 2) was purchased from Extrasynthese (France). These triterpenoids were submitted to the subsequent plant growth assay.

Synthesis of betulin glycosides

28-*O*-Acetylbetulin was synthesized by the method described in our previous paper.[18] Acetobromoglucose 2.31 g (Sigma Chemical Company) and 0.91 g of 28-*O*-acetylbetulin (molar ratio 3:1) was suspended in 82 ml of dried nitromethane, and 1.42 g of mercury cyanide was added. The suspension was stirred at room temperature for 24 h. At the end of the reaction, the reaction products were diluted with 20 ml of nitromethane and washed with water until the pH of the aqueous layer became neutral. The nitromethane layer was dried with anhydrous sodium sulfate, the sodium sulfate was filtered off, and the filtrate was evaporated to dryness under reduced pressure. The residue was chromatographed on a silica gel column using *n*-hexane–acetone (2:1) as eluent, and 765 mg of betulin-3-yl β-D-glucoside pentaacetate was obtained. The pentaacetate was treated at room temperature with 80 ml of methanol (MeOH) containing 1.25 ml of MeONa. After neutralizing the solution with AG 50W-X4 resin (H^+ form, Bio-Rad) and separating the resin, the filtrate was evaporated to give 487 mg of betulin-3-yl β-D-glucoside (Be-Glu) (Fig. 1).

Be-Glu: FAB-MS *m/z*: 605 [(M + H)$^+$] , 425 [(M + H − glucose)$^+$], 697 [(M + H + glycerol)$^+$]; ^{1}H-NMR (500 MHz, C_5D_5N): δ0.79 (3H, s, CH_3-25), 0.98 (3H, s, CH_3-26), 1.02 (3H, s, CH_3-24), 1.09 (3H, s, CH_3-27), 1.33 (3H, s, CH_3-23), 1.78 (3H, s, CH_3-30), 3.44 (1H, dd, J = 11.6 and 4.2 Hz, H-3), 3.68 (1H, d, J = 10.5 Hz, H-28), 4.10 (1H, d, J = 10.0 Hz, H-28), 4.44 (1H, dd, J = 11.6 and 5.4 Hz, H-G1-6), 4.62 (1H, dd, J = 11.6 and 2.0 Hz, H-G1-6), 4.74 (1H, brs, H-29), 4.90 (1H, brs, H-29), 4.98 (1H, d, J = 7.6 Hz, H-G1-1); ^{13}C-NMR (125 MHz, C_5D_5N): δ15.0 (C-27), 16.2 (C-26), 16.4 (C-25), 16.9 (C-24), 18.5 (C-6), 19.3 (C-30), 21.1 (C-11), 25.7 (C-12), 26.8 (C-15), 27.6 (C-2), 28.2 (C-23), 30.0 (C-16), 30.4 (C-21), 34.6 (C-22), 34.9 (C-7), 37.1 (C-10), 37.6 (C-13), 39.1

(C-1), 39.6 (C-4), 41.2 (C-8), 43.0 (C-14), 48.3 (C-19), 48.5 (C-17), 49.1 (C-18), 50.7 (C-9), 55.9 (C-5), 59.4 (C-28), 63.0 (C-G1-6), 71.9 (C-G1-4), 75.8 (C-G1-2), 78.3 (C-G1-5), 78.8 (C-G1-3), 88.9 (C-3), 106.9 (C-G1-1), 110.0 (C-29), 151.2 (C-20).

Betulin-3-yl β-D-cellobioside [Be-$(Glu)_2$], betulin-3-yl β-D-(4-*O*-β-D-maltosyl)-glucoside [Be-$(Glu)_3$] and betulin-3-yl β-D-(4-*O*-β-D-maltotriosyl)-glucoside [Be-$(Glu)_4$] (Fig. 1) were prepared by the method described in our previous paper.[18]

Betulin-3-yl β-D-(4-*O*-β-D-maltotetraosyl)-glucoside [Be-$(Glu)_5$] and betulin-3-yl β-D-(4-*O*-β-D-maltopentaosyl)-glucoside [Be-$(Glu)_6$] were synthesized from α-cyclodextrin as a donor and Be-$(Glu)_2$ as an acceptor by the transglycosylation of cyclodextrin glycosyltransferase (CGTase) from *Bacillus macerans* by the same method as was used for Be-$(Glu)_3$ and Be-$(Glu)_4$.[18] The enzymic reaction products were extracted with *n*-butanol (*n*-BuOH), and the *n*-BuOH extract was evaporated to dryness and dissolved in MeOH. The solution was chromatographed by preparative high-performance liquid chromatography (HPLC) on a column of Shim-pack CLC-ODS (Shimadzu Corporation, Japan) to afford Be-$(Glu)_5$ and Be-$(Glu)_6$ (Fig. 1).

Be-$(Glu)_5$: FAB-MS *m/z*: 1275 [$(M + Na)^+$]; ^{1}H-NMR (500 MHz, C_5D_5N): δ0.76 (3H, s, CH_3-25), 0.95 (3H, s, CH_3-26), 0.97 (3H, s, CH_3-24), 1.05 (3H, s, CH_3-27), 1.28 (3H, s, CH_3-23), 1.74 (3H, s, CH_3-30), 3.33 (1H, dd, J = 11.5 and 4.5 Hz, H-3), 3.64 (1H, d, J = 11.0 Hz, H-28), 4.71 (1H, brs, H-29), 4.85 (1H, d, J = 7.0 Hz, H-G1-1), 4.86 (1H, brs, H-29), 5.20 (1H, d. J = 7.0 Hz, H-G2-1), 5.77 (1H, d, J = 4.5 Hz, H-G3-1), 5.78 (1H, d, J = 4.5 Hz, H-G4-1), 5.92 (1H, d, J = 4.5 Hz, H-G5-1); ^{13}C-NMR (125 MHz, C_5D_5N): δ14.9 (C-27), 16.1 (C-26), 16.3 (C-25), 16.8 (C-24), 18.4 (C-6), 19.2 (C-30), 21.0 (C-11), 25.7 (C-12), 26.6 (C-15), 27.5 (C-2), 28.1 (C-23), 30.0 (C-16), 30.3 (C-21), 34.5 (C-22), 34.8 (C-7), 37.0 (C-10), 37.5 (C-13), 38.9 (C-1), 39.6 (C-4), 41.1 (C-8), 42.9 (C-14), 48.3 (C-19), 48.5 (C-17), 49.1 (C-18), 50.6 (C-9), 55.8 (C-5), 59.4 (C-28), 61.7 (C-G2-6 and C-G4-6), 61.8 (C-G3-6), 62.3 (C-G1-6), 62.7 (C-G5-6), 71.9 (C-G5-4), 73.37 (C-G4-5), 73.42 (C-G3-5), 73.8 (C-G4-2), 73.9 (C-G3-2), 74.2 (C-G2-2), 74.4 (C-G5-2), 74.8 (C-G3-3), 74.9 (C-G4-3), 75.2 (C-G1-2 and C-G5-5), 75.4 (C-G5-3), 76.3 (C-G1-5), 76.8 (C-G2-5), 76.9 (C-G1-3), 77.4 (C-G2-3), 81.1 (C-G1-4), 81.4 (C-G4-4), 81.6 (C-G2-4), 81.8 (C-G3-4), 89.0 (C-3), 102.8 (C-G3-1), 102.9 (C-G4-1), 103.1 (C-G5-1), 104.7 (C-G2-1), 106.5 (C-G1-1), 109.9 (C-29), 151.2 (C-20).

Be-$(Glu)_6$: FAB-MS *m/z*: 1437 [$(M + Na)^+$]; ^{1}H-NMR (500 MHz, C_5D_5N): δ0.75 (3H, s, CH_3-25), 0.94 (3H, s, CH_3-26), 0.97 (3H, s, CH_3-24), 1.05 (3H, s, CH_3-27), 1.28 (3H, s, CH_3-23), 1.74 (3H, s, CH_3-30), 3.33 (1H, dd, J = 12.0 and 4.0 Hz, H-3), 3.64 (1H, d, J = 11.0 Hz, H-28), 4.70 (1H, brs, H-29), 4.85–4.87 (2H, m, H-G1-1 and H-29), 5.19 (1H, d, J = 8.0 Hz, H-G2-1), 5.78–5.80 (3H, m, H-G3-1, H-G4-1, and H-G5-1), 5.93 (1H, d, J = 3.5 Hz, H-G6-1); ^{13}C-NMR (125 MHz, C_5D_5N): δ14.9 (C-27), 16.1 (C-26), 16.3 (C-25), 16.9 (C-24), 18.5 (C-6), 19.3 (C-30), 21.1 (C-11), 25.7 (C-12), 26.7 (C-15), 27.6 (C-2), 28.1 (C-23), 30.1 (C-16), 30.4 (C-21), 34.6 (C-22), 34.9 (C-7), 37.1 (C-10), 37.6 (C-13), 39.0 (C-1), 39.6 (C-4), 41.2 (C-8), 43.0 (C-14), 48.4 (C-19), 48.6 (C-17), 49.2 (C-18), 50.7 (C-9), 55.9 (C-5), 59.4 (C-28), 61.78, 61.85, and 61.94 (C-G2-6, C-G3-6, C-G4-6, and C-G5-6), 62.3 (C-G1-6), 62.8 (C-G6-6), 72.0 (C-G6-4), 73.45 and 73.48 (C-G3-5, C-G4-5 and C-G5-5), 73.8 (C-G5-2), 73.9 (C-G3-2 and C-G4-2), 74.3 (C-G2-2), 74.5 (C-G6-2), 74.9 (C-G3-3 and C-G4-3), 75.0 (C-G5-3), 75.3 (C-G1-2 and C-G6-5), 75.4 (C-G6-3), 76.3 (C-G1-5), 76.8 (C-G2-5), 77.0 (C-G1-3), 77.5 (C-G2-3), 81.3 (C-G1-4), 81.5 (C-G2-4 and C-G5-4), 82.0 (C-G3-4 and C-G4-4), 89.0 (C-3), 102.87 (C-G3-1), 102.97 and 103.03 (C-G4-1 and C-G5-1), 103.1 (C-G6-1), 104.8 (C-G2-1), 106.5 (C-G1-1), 110.0 (C-29), 151.2 (C-20).

These synthesized betulin glycosides (Be-Glu, Be-$(Glu)_2$, Be-$(Glu)_3$, Be-$(Glu)_4$, Be-$(Glu)_5$, and Be-$(Glu)_6$) were subjected to the following bioassay.

Germination and growth regulation assays

Betulin, oleanolic acid, betulinic acid, and synthesized betulin glycosides were tested on the seeds of alfalfa (*Medicago sativa* L, Rusan 156) prepared by Sakatanotane (Tokyo, Japan). Test components were diluted 0.1% to 0.0018% with distilled water. Twenty or five seeds of alfalfa were put on the filter papers (Advantec, no. 2) impregnated with 10 ml of the above test solutions, and grown at 24°C in a light-controlled growth cabinet (24 h dark). After 4 days the number of germinated seeds and the lengths of radicles and hypocotyles were measured. Each determination was made with three replicates of 20 or 5 alfalfa seeds.

Results and discussion

Characterizations of synthesized betulin glycosides

Be-Glu was synthesized by the coupling of 28-*O*-acetylbetulin with acetobromoglucose by means of a Koenigs-Knorr-type condensation followed by deacetylation. Its structure was confirmed by FAB-MS and ^{1}H- and ^{13}C-NMR spectroscopy. In the ^{13}C-NMR spectrum of Be-Glu, the signals due to C-3 and C-G1-1 were observed at 89.0 and 106.9 ppm, respectively. Compared with the corresponding carbons of betulin and β-D-glucose, both resonances were shifted about 10 ppm to lower field, indicating the formation of a glycosidic linkage between the C-3 position of betulin and D-glucose. As the coupling constant of anomeric H-G1-1 was 7.6 Hz, this glycosidic linkage was found to be β-type. In addition, the FAB-MS spectrum of Be-Glu showed $(M + H)^+$ = 605, which was consistent with the structure shown in Fig. 1.

Be-$(Glu)_5$ and Be-$(Glu)_6$ were synthesized from α-cyclodextrin and Be-$(Glu)_2$ by transglycosylation of CGTase. Their structures were confirmed by FAB-MS and ^{1}H- and ^{13}C-NMR spectroscopy. Details of interpretation of FAB-MS and NMR results are as follows: The ^{1}H-NMR spectrum of Be-$(Glu)_5$ was extremely complicated, al-

though the assignment of each signal was accomplished by considering the total correlation spectroscopy (TOCSY) spectrum. Furthermore, the ^{1}H-^{1}H nuclear Overhauser enhancement spectroscopy (NOESY) experiment was useful for assigning the five anomeric protons. Resonance at 5.77 ppm was due to H-G3-1 by connectivity to H-G2-4 at 4.21 ppm. The coupling constant of H-G3-1 was 4.5 Hz, indicating the α configuration. The heteronuclear single-quantum coherence (HSQC) experiment then permitted assignment of the carbon signal at 81.6 ppm (C-G2-4). This signal was shifted 10.1 ppm to lower field compared with the corresponding carbon of Be-$(Glu)_2$. These NMR data indicate that Be-$(Glu)_5$ is formed by α-(1,4) transglycosylation to the nonreducing end of Be-$(Glu)_2$. In addition, the FAB-MS spectrum showed $(M + Na)^+ = 1275$, which was consistent with the structure shown in Fig. 1. The structure of Be-$(Glu)_6$ was confirmed in the same manner as above.

Germination and growth regulation effects

In this study alfalfa seeds were used for the bioassays of triterpenoid saponins and triterpenoids. Tables 1 and 2 show the germination and growth regulation effects of betulin and betulin glycosides on alfalfa. The germination rates of all compounds examined were similar to those of the control. On the other hand, the growth regulation effects on radicles varied considerably with the compound tested. Betulin significantly inhibited the radicle growth at 0.0090% and 0.0180% concentrations. Betulin glycosides [Be-Glu, Be-$(Glu)_2$, Be-$(Glu)_3$] exhibited stronger effects than betulin, and their inhibitory activity tended to increase with the increasing number of glucose residues (Table 1). Be-$(Glu)_2$ and Be-$(Glu)_3$ showed significant effects at 0.0018% concentration, whereas betulin and Be-Glu did not, indicating higher activity for Be-$(Glu)_2$ and Be-$(Glu)_3$ than for betulin and Be-Glu. From the results of statistical analyses of the radicle growth assays (Table 3), it was found that Be-Glu was more active than betulin and that there was no significant difference between Be-$(Glu)_2$ and Be-$(Glu)_3$. Therefore, it was concluded that the order of inhibitory activity is as follows; Be-$(Glu)_3$ = Be-$(Glu)_2$ > Be-Glu > betulin. These facts prompted us to examine the growth regulation effects on radicles of betulin glycosides with a sugar chain consisting of more glucose residues. Results of the radicle growth assays of Be-$(Glu)_4$, Be-$(Glu)_5$, and Be-$(Glu)_6$ are shown in Table 2 together with those of Be-$(Glu)_3$. All these compounds significantly inhibited radicle

Table 1. Germination and growth regulation effects of betulin and the betulin glycosides Be-Glu, Be-$(Glu)_2$ and Be-$(Glu)_3$ on alfalfa seeds

Concentration (%)	% Based on controls[a]		
	Germination	Radicle	Hypocotyl
Betulin			
0.0180	90	80.0 ± 3.2**	85.7 ± 2.0
0.0090	90	86.0 ± 4.0**	87.0 ± 3.0
0.0018	95	90.0 ± 3.2	88.0 ± 3.5
Be-Glu			
0.0180	100	62.8 ± 3.0**	74.1 ± 2.0*
0.0090	95	76.9 ± 4.0**	77.3 ± 3.0
0.0018	90	87.9 ± 5.0	76.7 ± 5.0
Be-$(Glu)_2$			
0.0180	95	53.8 ± 3.2**	88.9 ± 4.0
0.0090	95	72.1 ± 3.5**	88.9 ± 3.0
0.0018	90	78.2 ± 4.6**	93.8 ± 2.0
Be-$(Glu)_3$			
0.0180	100	52.9 ± 3.3**	83.3 ± 1.5
0.0090	100	67.3 ± 3.0**	92.6 ± 2.0
0.0018	95	74.8 ± 2.0**	94.7 ± 3.0

Be-Glu, betulin-3-yl β-D-glucoside; Be-$(Glu)_2$, betulin-3-yl β-D-cellobioside; Be-$(Glu)_3$, belulin-3-yl β-D-(4-*O*-β-D-maltosyl)-glucoside
For structures of Be-Glu, Be-$(Glu)_2$ and Be-$(Glu)_3$: see Fig. 1
[a] Figures are percentages based on the control and show germination or growth rates after 4 days. Each determination was made with three replicates of 20 alfalfa seeds
* $P < 0.05$; ** $P < 0.01$

Table 2. Germination and growth regulation effects of betulin glycosides Be-$(Glu)_3$, Be-$(Glu)_4$, Be-$(Glu)_5$, and Be-$(Glu)_6$ on alfalfa seeds

Concentration (%)	% Based on controls[a]		
	Germination	Radicle	Hypocotyl
Be-$(Glu)_3$			
0.040	90	51.0 ± 2.0**	83.0 ± 2.0**
0.020	100	65.0 ± 2.5**	85.0 ± 3.0**
0.004	100	81.0 ± 2.5**	96.0 ± 3.0
0.002	100	89.0 ± 2.0*	96.0 ± 2.5
Be-$(Glu)_4$			
0.040	90	49.0 ± 2.0**	79.0 ± 2.5**
0.020	90	62.0 ± 2.5**	79.0 ± 3.0**
0.004	100	81.0 ± 2.5**	94.0 ± 3.0
0.002	90	86.0 ± 2.0*	92.0 ± 2.0
Be-$(Glu)_5$			
0.040	90	64.0 ± 2.5**	93.0 ± 3.0
0.020	100	69.0 ± 2.5**	102.0 ± 3.0
0.004	90	81.0 ± 2.5**	104.0 ± 3.0
0.002	100	84.0 ± 2.0*	100.0 ± 2.0
Be-$(Glu)_6$			
0.040	100	62.0 ± 2.3**	94.0 ± 2.0
0.020	100	70.0 ± 2.5**	94.0 ± 2.5
0.004	90	81.0 ± 2.5**	98.0 ± 2.0
0.002	90	84.0 ± 2.5*	100.0 ± 25.0

$Be(Glu)_3$, betulin-3-yl β-D-(4-*O*-β-D-maltosyl)-glucoside;
$Be(Glu)_4$, betulin-3-yl β-D-(4-*O*-β-D-maltotriosyl)-glucoside;
$Be(Glu)_5$, betulin-3-yl β-D-(4-*O*-β-D-maltotetraosyl)-glucoside;
$Be(Glu)_6$, betulin-3-yl β-D-(4-*O*-β-D-maltopentaosyl)-glucoside
For structures of Be-$(Glu)_3$, Be-$(Glu)_4$, Be-$(Glu)_5$, Be-$(Glu)_6$: see Fig. 1
[a] Figures are percentages based on the control and show germination or growth rates after 4 days. Each determination was made with three replicates of five alfalfa seeds
* $P < 0.05$; ** $P < 0.01$

Table 3. Growth regulation effects of betulin and betulin glycosides on alfalfa radicles

Compound	% Based on controls		
	0.0180%	0.0090%	0.040%
Betulin	80.0 ± 3.2[a]		
Be-Glu	62.8 ± 3.0[b]		
Be-$(Glu)_2$		72.1 ± 3.5[a]	
Be-$(Glu)_3$		67.3 ± 3.0[a]	51.0 ± 2.0[a]
Be-$(Glu)_4$			49.0 ± 2.0[a]
Be-$(Glu)_5$			64.0 ± 2.5[b]
Be-$(Glu)_6$			62.0 ± 2.3[b]

Values in the same column with the same superscript letter are not different at the significance level of $P < 0.05$. For further explanations, see Tables 1 and 2

growth at all concentrations examined. At high concentrations (0.020% and 0.040%), the inhibitory activity of Be-$(Glu)_3$ and Be-$(Glu)_4$ tended to be higher than that of Be-$(Glu)_5$ and Be-$(Glu)_6$. Furthermore, it was confirmed by statistical analyses that Be-$(Glu)_3$ and Be-$(Glu)_4$ were more active than Be-$(Glu)_5$ and Be-$(Glu)_6$ and that there were no significant differences between Be-$(Glu)_3$ and Be-$(Glu)_4$ and between Be-$(Glu)_5$ and Be-$(Glu)_6$ (Table 3). Consequently, it was found that Be-$(Glu)_2$, Be-$(Glu)_3$, and Be-$(Glu)_4$ had the greatest inhibitory activity among betulin and the betulin glycosides tested.

There have been many investigations on structure–activity relations of the growth regulation effects of phenolic compounds on radicles. Ohira and Yatagai studied the inhibitory effects of the groups of benzoic acid, cinnamic acid, and coumarin on the growth of alfalfa radicles and found that these effects decreased with the increasing number of phenolic hydroxyl groups.[19] In previous work, we reported the inhibitory activity of condensed tannins from the bark of *Acacia mearnsii* for the growth of alfalfa radicles.[20] The tannins, which are aqueous polyphenolic compounds, showed extremely low activity (76.4% based on the control at 0.040% concentration). As for the growth inhibitory effects of phenolic glycosides, early works demonstrated that the glycosides were less effective than their aglycons.[19,21] Furthermore, it was reported that flavonoid compounds inhibited the ATPase activity of plasma membranes isolated from oat (*Avena* spp.) roots.[22] These studies suggest that the aglycon moiety plays a substantial role in the growth inhibitory activity of phenolics and phenolic glycosides. On the other hand, the growth regulation effects of betulin and betulin glycosides showed characteristics considerably different from those of phenolic compounds. That is, betulin glycosides showed stronger effects than betulin, and betulin glycosides with two to four glucose residues had the most inhibitory activity. Therefore, the inhibitory effects of triterpenoid saponins are thought to be attributed not to the property of their aglycons but to that of the whole of their molecules. As chemical and physical properties of triterpenoid saponins vary with the sugar chain length, saponins with an adequate number of sugar residues would exhibit high activity.

In regard to hypocotyl growth, only Be-Glu showed a significant growth regulation effect at 0.0180% concentration (Table 1), indicating the greater effect of Be-Glu than betulin, Be-$(Glu)_2$, and Be-$(Glu)_3$. Be-$(Glu)_3$ and Be-$(Glu)_4$ had higher inhibitory activity than Be-$(Glu)_5$ and Be-$(Glu)_6$ because the former two glycosides showed significant effects at concentrations of more than 0.020%, whereas the latter two did not (Table 2). On the whole, hypocotyl growth was less affected than radicle growth for betulin and all betulin glycosides tested. Therefore, there were no further detailed studies on the structure–activity relations. Except for a few plant species, the inhibitory effects of allelopathic compounds tested were more pronounced on radicle growth than on hypocotyl growth.[23] Ortega et al. reported that the allelopathic compound diacetyl-piquerol inhibited radicle growth and plasma membrane ATPase isolated from the plant radicle, and that the cell wall plasma membrane is one of the first targets of allelopathic compounds.[23] Therefore, the above result for the inhibitory effects on alfalfa radicle and hypocotyl growth seems reasonable. However, the germination and growth regulation assays used in this study showed that radicles always contact test compounds, whereas hypocotyls do not. The assay method used might be related to the above results.

Table 4. Germination and growth regulation effects of triterpenoids on alfalfa seeds

Concentration (%)	% Based on controls[a]		
	Germination	Radicle	Hypocotyl
Betulin			
0.1000	95	76.0 ± 2.5**	86.4 ± 4.0*
0.0250	90	79.0 ± 3.2**	84.0 ± 2.0*
0.0050	90	84.0 ± 4.0*	86.0 ± 3.0*
0.0025	95	90.0 ± 3.2	88.0 ± 3.5*
Oleanolic acid			
0.1000	90	78.0 ± 2.0**	86.0 ± 3.0*
0.0250	100	80.0 ± 2.5**	89.0 ± 3.0
0.0050	100	86.0 ± 2.5	96.0 ± 3.0
0.0025	100	93.0 ± 2.0	96.0 ± 2.5
Betulinic acid			
0.1000	90	72.0 ± 2.0**	84.0 ± 2.5*
0.0250	90	75.0 ± 2.5**	84.0 ± 3.0*
0.0050	100	80.0 ± 2.5**	85.0 ± 3.0*
0.0025	90	85.0 ± 2.0*	88.0 ± 2.0

[a] Results are percentages based on the control and show germination or growth rates after 4 days. Each determination was made with three replicates of five alfalfa seeds
* $P < 0.05$; ** $P < 0.01$

Table 4 shows the germination and growth regulation effects of betulin and other triterpenoids (oleanolic acid and betulinic acid) on alfalfa seeds. All of the triterpenoids, similar to betulin glycosides, had no significant effect on germination rates. On the other hand, they clearly inhibited radicle growth at concentrations of more than 0.0250%. Betulinic acid showed significant effects even at 0.0025% concentration, and betulin showed significant effects at 0.0050% concentration; oleanolic acid had no effect at either concentration. Therefore, it is concluded that the order of inhibitory effects on alfalfa radicle growth is as follows: betulinic acid > betulin > oleanolic acid.

That the effect of betulinic acid was higher than that of betulin suggests the importance of the carboxyl group at the C-28 position for the growth regulation effects of lupane-type triterpenoids on alfalfa radicles. All triterpenoids at the highest concentration (0.1000%) significantly inhibited hypocotyl growth. The inhibitory activity of betulin and betulinic acid was clearly higher than that of oleanolic acid because the former two showed significant effects at 0.0050% concentration, whereas the latter did not (Table 4). However, the growth regulation effects of triterpenoids on alfalfa hypocotyls were, on the whole, not pronounced, similar to betulin glycosides.

As mentioned above, betulin, betulin glycosides, and other triterpenoids exhibited no germination regulation effects on alfalfa seeds. It is reported that many low-molecular-weight phenolic compounds such as salicylic acid, *trans*-cinnamic acid, and *o*-coumaric acid inhibit the

germination of lettuce seeds, and that increased lipophilicity of phenolic compounds usually leads to increased inhibitory activity.[13] In this study even triterpenoids did not affect the germination rate of alfalfa seeds even though they are fairly lipophilic compounds.

It is assumed that the germination and growth regulation effects of triterpenoid saponins are related to the type of glycosidic linkage as well as the sugar chain length. These effects of monodesmosidic saponins with β-glycosidic linkages are under investigation to determine more detailed structure–activity relations and to prepare new functional triterpenoid saponins.

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