Cytotoxic Activities and Structure–Cytotoxic Relationships of Steroidal Saponins

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We have systematically examined the cytotoxic activities of the steroidal saponins mainly isolated from the Liliaceae plants against HL-60 human promyelocytic leukemia cells and found several structure–activity relationships. Some steroidal saponins evaluated in the assay system showed considerable cytotoxic activities, which were almost as potent as that of etoposide used as a positive control. The activities were found to be sensitive to the monosaccharides constituting the sugar moieties and their sequences, as well as to the structures of the aglycons.

Key words steroidal saponin; cytotoxic activity; HL-60 cell; structure–activity relationship

Steroidal saponins are naturally occurring glycosides that possess properties such as producing foam, hemolytic activity, toxicity to fish, and complex formation with cholesterol. Some of the steroidal saponins isolated recently have been shown to be antidiabetic,[1] antitumor in association with the modification of the immune system,[2] antitussive,[3] and platelet aggregation inhibitors.[4] We systematically examined the cytotoxic activities of the steroidal saponins mainly isolated from the Liliaceae plants against HL-60 human promyelocytic leukemia cells and found several structure–activity relationships. In this paper, we report the structure–activity relationships of the steroidal saponins *versus* HL-60 cell growth, and also the results of the National Cancer Institute (NCI) 60 cell line assay of a representative saponin.

MATERIALS AND METHODS

Materials NMR spectra were recorded on a Bruker DRX-500 (500 MHz for ^{1}H-NMR) spectrometer using standard Bruker pulse programs. Silica gel (Fuji-Silysia Chemical, Aichi, Japan) and ODS silica gel (Nacalai Tesque, Kyoto, Japan) were used for column chromatography. Computer calculations were performed using the molecular-modeling software Macro-model 6.0 on a Silicon Graphics work station, COMTEC 4D/O2 10000SC. The following materials and reagents were used for cell culture and assay of cytotoxic activity: microplate reader, Inter Med Immuno-Mini NJ-2300 (Tokyo, Japan); 96-well flat-bottom plate, Iwaki Glass (Chiba, Japan); HL-60 cells, ICN Biomedicals (Costa Mesa, CA, U.S.A.); RPMI 1640 medium, GIBCO BRL (Gland Island, NY, U.S.A.); MTT, Sigma (St. Louis, MO, U.S.A.). All other chemicals used were of biochemical reagent grade. Compounds **1**—**3**, **6**, **8**—**10**, **12**, **29** and **30** were isolated from *Triteleia lactea*,[5] **4** and **17** from *Allium narcissiflorum*,[6] **18** from *Smilax china*,[7] **5** from *Tacca esquirolii*,[8] **7** and **11** from *Paris polyphylla* var. *chinensis*,[9] **13**, **14**, **19** and **20** from *Lilium brownii* var. *colchesteri*,[10] **22** and **26** from *A. jesdianum*,[11] **23** and **32** from *Reineckea carnea*,[12] **24** and **28** from *A. karataviense*,[13] **25**, **27**, **33** and **34** from *A. ampeloprasum*,[14] **35** and **36** from *Nolina recurvata*,[15] **37**—**39**, **45** and **49** from *Ruscus aculeatus*,[16] **40**—**44** and **46** from *Dracaena draco*,[17] **47** and **48** from *Sansevieria cylindrica*,[18] and **50** from *D. surculosa*.[19] Compounds **15**, **16**, **21**, and **31** were prepared from **5**, **6**, **23**, and **23**, respectively.

Preparation of 15, 16, and 31 A solution of **5** (27 mg) in 0.2 M HCl (dioxane–H_2O, 1 : 1, 2 ml) was heated at 95 °C for 30 min under an Ar atmosphere. After cooling, the reaction mixture was neutralized by passage through an Amberlite IRA-93ZU (Organo, Tokyo, Japan) column, and chromatographed on silica gel using a gradient mixture of $CHCl_3$–MeOH–H_2O (60 : 10 : 1; 40 : 10 : 1) and ODS silica gel using MeOH–H_2O (4 : 1) to give **15** (2.0 mg).[20] By the same procedure, **16** (1.8 mg)[21] and **31** (12 mg)[22] were prepared from **6** (23 mg) and **23** (50 mg), respectively. The structures of **15**, **16**, and **31** were ascertained by analysis of their IR, ^{1}H-, and ^{13}C-NMR spectra.

Preparation of 21 A mixture of **23** (98 mg) and PtO_2 (6.9 mg) in $CHCl_3$–MeOH (1 : 1, 10 ml) was stirred under an H_2 atmosphere at room temperature for 12 h. The reaction mixture, after removal of the catalyst by filtration, was subjected to an ODS silica gel column eluting with MeOH–H_2O (2 : 1) to give **21** (19 mg).[23] The structure of **21** was ascertained by analysis of its IR, ^{1}H-, and ^{13}C-NMR spectra.

Cell Culture Assay HL-60 cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum supplemented with L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. The leukemia cells were washed and resuspended in the above medium to 3×10^4 cells/ml, and 196 μl of this cell suspension was placed in each well of a 96-well flat-bottom plate. The cells were incubated in 5% CO_2/air for 24 h at 37 °C. After incubation, 4 μl of EtOH–H_2O (1 : 1) solution containing the sample was added to give the final concentrations of 0.1—20 μg/ml and 4 μl of EtOH–H_2O (1 : 1) was added into control wells. The cells were further incubated for 72 h in the presence of each agent, and then cell growth was evaluated using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) reduction assay.[24] Briefly, after termination of the cell culture, 10 μl of 5 mg/ml MTT in phosphate buffered saline was added to each well and the plate was further reincubated in 5% CO_2/air for 4 h at 37 °C. The plate was then centrifuged at 1500 ***g*** for 5 min to precipitate cells and MTT formazan. An aliquot of 150 μl of the supernatant was removed from each well, and 175 μl of dimethyl sulfoxide (DMSO) was added to dissolve the MTT formazan crystals. The plate was mixed on a microshaker for 10 min, and then read on a mi-

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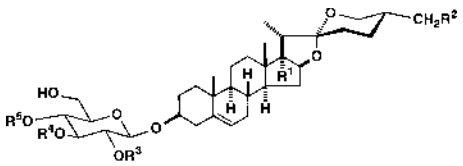
croplate reader at 550 nm. A dose response curve was plotted for each compound, and the concentration giving 50% inhibition (IC_{50}) was calculated. Data are mean values of three experiments performed in triplicate.

Conformational Analysis 1000-Step systematic Monte Carlo conformation searches were carried out with the AMBER* force field as implemented in Macro-model 6.0 to predict the fully optimized lowest energy structure.[25] Energies were minimized with the Polak–Ribiere (PR) conjugate gradient minimizer, and convergence was obtained when the gradient root mean square was less than 0.001 kJ Å^{-1} M. Throughout this article, all molecular mechanics (MM) calculations assumed a dielectric constant of 1.0. The molecular dynamics (MD) simulations were carried out with Macromodel beginning with the lowest energy structures obtained by the Monte Carlo conformation search. The following options were used in the MD calculations; time step: 1 fs, equilibration time period: 10 ps, and production run time period: 1000 ps. Initial kinetic energy was added to all atoms as random velocities. Translational and rotational momentum was reset to zero every 0.1 ps. To maintain a constant temperature, the system was coupled to an external temperature bath set at 300 K. Coupling between bath and molecule was updated every 0.2 ps. In the production run time at 300 K the conformers were sampled every 1 ps, followed by energy minimizations using the AMBER* force field. The final MM calculations provided the fully optimized lowest energy structures as shown in this article.

RESULTS AND DISCUSSION

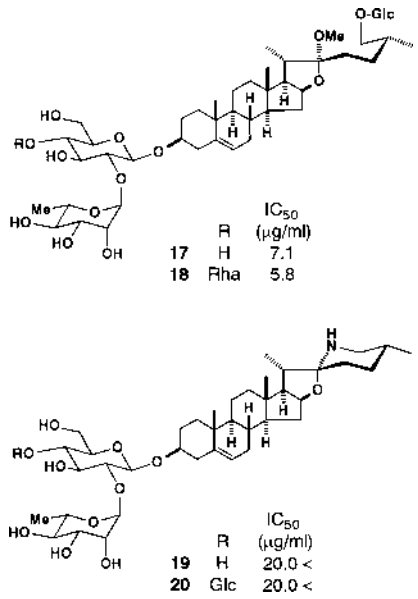
A. Structure–Activity Relationships of (25*R*)-Spirost-5-en-3β-ol (Diosgenin) Glycoside Derivatives Diosgenin β-D-glucoside (**1**) showed no cytotoxic activity against HL-60 cells (IC_{50} >20 μg/ml), and the attachment of an α-L-rhamnosyl group at C-2 of the glucosyl moiety led to the appearance of considerable activity (**2**: IC_{50} 1.8 μg/ml). Further addition of an α-L-rhamnosyl, an α-L-arabinofuranosyl or a β-D-glucosyl, with the exception of a β-D-galactosyl, to C-3 or C-4 of the inner glucosyl moiety either gave no influence on the activity or slightly increased the activity (**3**—**7**: IC_{50} 0.5—3.3 μg/ml); the attachment of a β-D-galactosyl at C-3 of the glucosyl residue led to a decrease in the activity (**8**: IC_{50} 9.2 μg/ml). Although the introduction of a C-17α hydroxyl group to the aglycon of the active saponins slightly reduced their cytotoxicities (**9**—**12**: IC_{50} 1.5—13.2 μg/ml), that of a C-27 hydroxyl group led to a considerable decrease in the activity (**13**, **14**: IC_{50} >20.0 μg/ml). Among α-L-rhamnosyl-(1→2)-β-D-glucoside (**2**), α-L-rhamnosyl-(1→3)-β-D-glucoside (**15**), and α-L-rhamnosyl-(1→4)-β-D-glucoside (**16**) of diosgenin, only diosgenin α-L-rhamnosyl-(1→2)-β-D-glucoside (**2**) exhibited cytotoxic activity. Rotating frame nuclear Overhauser and exchange effects (ROE) correlations were observed between the protons of the anomeric center of each monosaccharide and its linkage site in the ROE correlation spectroscopy (ROESY) spectra of **2**, **15**, and **16**. Comparison of the molecular models of **2**, **15**, and **16**, which were constructed on the basis of the ROE information, and MM and MD calculation studies, suggested that the three-dimensional structure of the diglycoside moiety contributed to the activity. In the cytotoxic saponin (**2**), the diglycoside existed in a conformation having a vertical orientation against the steroid plane of the aglycon, while in **15** and **16**, the diglycoside and the steroid skeleton were on the same plane. Furostanol saponins usually exhibit no specific properties of saponins, but the furostanol glycosides bearing an α-L-rhamnosyl-(1→2)-β-D-glucosyl or an α-L-rhamnosyl-(1→2)-[α-L-rhamnosyl-(1→4)]-β-D-glucosyl at C-3 of the aglycon (**17**, **18**) were found to be cytotoxic to HL-60 cells. The corresponding spirosolan glycoside (**19**, **20**) of **2** and **4** showed no cytotoxicity at 20.0 μg/ml.



	R^1	R^2	R^3	R^4	R^5	IC_{50} (μg/ml)
1	H	H	H	H	H	20.0 <
2	H	H	Rha	H	H	1.8
3	H	H	Rha	Glc	H	2.1
4	H	H	Rha	H	Glc	1.8
5	H	H	Rha	Rha	H	2.0
6	H	H	Rha	H	Rha	3.3
7	H	H	Rha	H	Araf	0.5
8	H	H	Rha	Gal	H	9.2
9	OH	H	Rha	H	H	5.1
10	OH	H	Rha	Glc	H	5.0
11	OH	H	Rha	H	Araf	1.5
12	OH	H	Rha	Gal	H	13.2
13	H	OH	Rha	H	H	20.0 <
14	H	OH	Rha	H	Glc	20.0 <
15	H	H	H	Rha	H	20.0 <
16	H	H	H	H	Rha	20.0 <

Glc: β-D-glucopyranosyl
Gal: β-D-galactopyranosyl
Rha: α-L-rhamnopyranosyl
Araf: α-L-arabinofuranosyl

Fig. 1



	R	IC_{50} (μg/ml)
17	H	7.1
18	Rha	5.8
19	H	20.0 <
20	Glc	20.0 <

Fig. 2

B. Structure–Activity Relationships of Saponins with a Lycotetraose Group Lycotetraose, β-D-glucosyl-(1→2)-[β-D-xylosyl-(1→3)]-β-D-glucosyl-(1→4)-β-D-galactose, often occurs as a glycoside group of steroidal saponins. The activity of the A/B *trans* (H-5α) steroids with lycotetraose

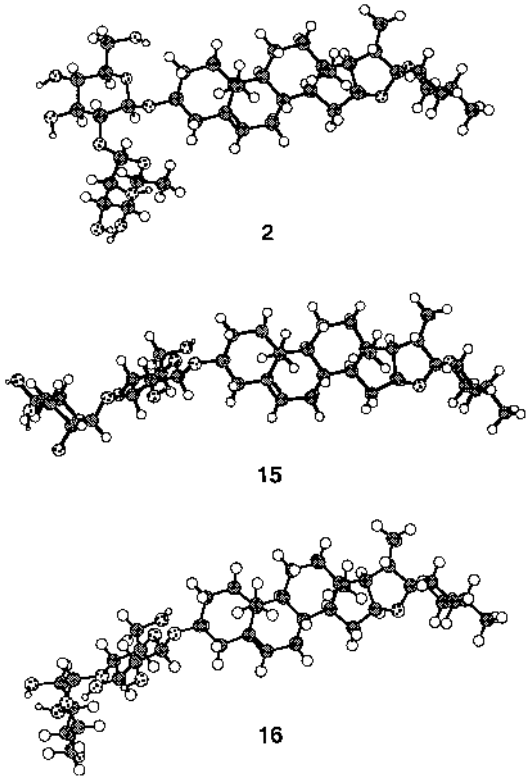
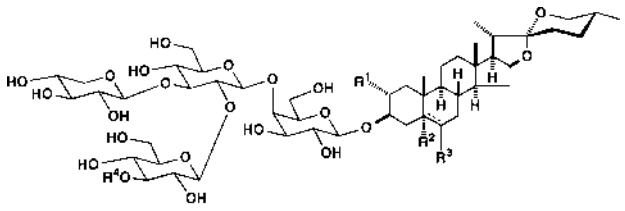


Fig. 3. Calculated Preferred Conformations of **2**, **15**, and **16**

(**21**, **22**) was as potent as that of the corresponding C-5(6) unsaturated saponins (**23**, **24**). Introduction of one hydroxyl group to the A or B ring of the aglycon slightly enhanced the activity (**22**, **24**, **25**). However, further hydroxylation significantly reduced the activity (**26**—**28**: IC_{50} >20.0 μg/ml). The furostanol saponins with lycoteraose (**32**—**34**) were inactive in contrast with the furostanols (**17**, **18**) mentioned in the section **A**. The terminal xylosyl moiety was revealed to be essential for the activity as was evident from the fact that the partial hydrolysate (**31**) of **23** exhibited no activity. Further glycosyl formation at C-3 of the terminal glucosyl moiety with a β-D-glucosyl or an α-L-rhamnosyl group did not affect the activity (**29**, **30**).

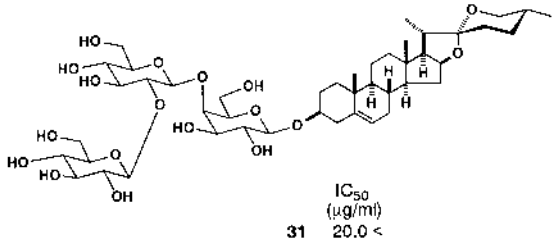
C. Cytotoxicities of Saponins with a Glycosyl Group at C-1 of the Aglycon These saponins showed no activity (**35**—**44**, **47**) with the exceptions of those possessing some acyl groups at the glycosyl moiety (**45**, **46**, **48**, **49**). A (25*S*)-spirost-5-ene-1β,3β-diol derivative (**50**), whose glycoside moiety is composed of two deoxypyranoses, that is, D-fucose and L-rhamnose, was also cytotoxic. These data led us to assume that the presence of a certain degree of lipophilicity in the sugar moiety is essential for exhibiting the cytotoxic activity in this type of saponin.

D. NCI 60 Cell Line Screening of 2 Compound **2** was subjected to the NCI 60 cell line assay.[26] The mean concentrations required to achieve GI_{50}, total growth inhibition (TGI), and LC_{50} levels against the panel of cells tested were 2.4 μM, 6.5 μM, and 13.5 μM, respectively.[27] The differential cellular sensitivity of **2** was moderate for the cell lines, and it did not exhibit a specific activity towards any particular subpanel of cells. However, some cell lines were relatively sensitive to it. These cell lines included the leukemia RPMI-8226 (GI_{50}: 0.69 μM), non-small cell lung cancer A549/ATCC

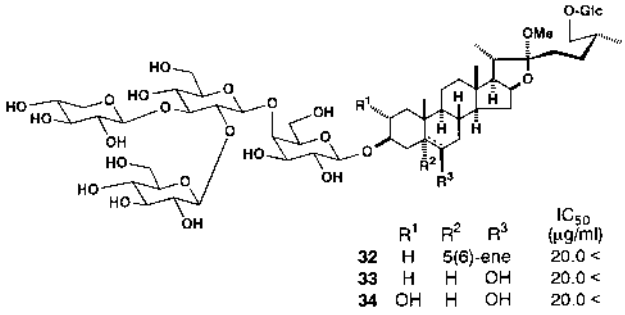


	R^1	R^2	R^3	R^4	IC_{50} (μg/ml)
21	H	H	H	H	11.6
22	OH	H	H	H	4.9
23	H	5(6)-ene		H	11.3
24	OH	5(6)-ene		H	4.1
25	H	H	β-OH	H	4.8
26	OH	H	α-OH	H	20.0 <
27	OH	H	β-OH	H	20.0 <
28	OH	OH	β-OH	H	20.0 <
29	OH	H	H	Glc	6.1
30	OH	H	H	Rha	6.0

Fig. 4



	IC_{50} (μg/ml)
31	20.0 <



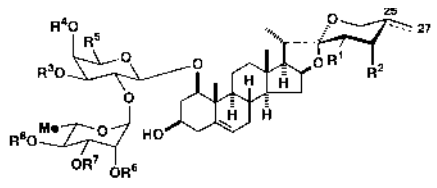
	R^1	R^2	R^3	IC_{50} (μg/ml)
32	H	5(6)-ene		20.0 <
33	H	H	OH	20.0 <
34	OH	H	OH	20.0 <

Fig. 5

(GI_{50}: 0.68 μM) and NCI-H460 (GI_{50}: 0.21 μM), colon cancer SW-620 (GI_{50}: 0.85 μM), CNS cancer U251 (GI_{50}: 0.55 μM), melanoma LOX IM VI (GI_{50}: 0.78 μM), renal cancer UO-31 (GI_{50}: 0.81 μM), and prostate cancer PC-3 (GI_{50}: 0.71 μM). The *in vivo* antitumor testing for **2** is in progress.

E. Conclusion The cytotoxic activities of some steroidal saponins may be due to their non-specific detergent effects with changing in membrane architecture, as shown by the aggregation of tumor cells at early stages after saponin treatment.[28] However, the extent of membrane damage induced by different saponins is considerably different,[28] and most recently we have found that **8** and **12** caused the death of L1210 leukemia cells through the apoptotic process.[29] These facts indicate that mechanisms other than membrane damage are also involved. Although Nohara and his co-workers have reported the cytotoxic and anti-herpes virus activities of the *Solanum* steroidal glycosides,[21,30] this communication more systematically describes the cytotoxicities and structure–activity relationships of the steroidal saponins.

Acknowledgment We wish to thank the Developmental



	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8		IC_{50} (μg/ml)
35	H	H	H	H	H	H	H	H	25(27)-ene	20.0 <
36	H	H	Xyl	H	H	H	H	H	25(27)-ene	20.0 <
37	H	H	H	H	H	H	Glc	H	25(27)-ene	20.0 <
38	H	H	H	H	CH_2OH	H	H	H	(25*R*)	20.0 <
39	H	H	H	H	CH_2OH	H	Glc	H	(25*R*)	20.0 <
40	OH	H	H	H	H	H	H	H	25(27)-ene	20.0 <
41	OH	H	H	H	H	H	H	Ac	25(27)-ene	20.0 <
42	OH	H	Xyl	H	H	H	H	H	25(27)-ene	20.0 <
43	OH	OH	H	H	H	H	H	H	25(27)-ene	20.0 <
44	OH	OH	H	H	H	H	H	Ac	25(27)-ene	20.0 <
45	H	H	Ac	HMP	H	H	H	H	25(27)-ene	3.0
46	OH	O-Fuc	H	H	H	Ac	Ac	Ac	25(27)-ene	2.6
47	OH	O-Fuc	Xyl	H	H	H	H	H	25(27)-ene	20.0 <
48	OH	O-Fuc	Xyl	H	H	Ac	Ac	Ac	25(27)-ene	1.8
49	H	H	Ac	Ac	CH_2OAc	H	H	H	(25*R*)	3.1
50	H	H	H	H	Me	H	H	H	(25*S*)	8.7

Xyl: β-D-xylopyranosyl
Fuc: β-D-fucopyranosyl

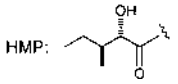


Fig. 6

Therapeutics Program, National Cancer Institute, Bethesda, MD, for performing the cytotoxic screening studies.

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