Convallasaponin A, a New 5β-Spirostanol Triglycoside from the Rhizomes of *Convallaria majalis*

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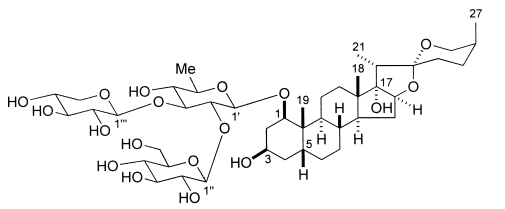
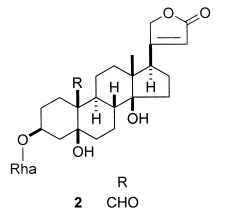
The rhizomes of *Convallaria majalis* have been analyzed for their steroidal glycoside constituents, resulting in the isolation of a new 5β-spirostanol triglycoside, named convallasaponin A, along with two known cardenolide glycosides and a known cholestane glycoside. The structure of convallasaponin A was determined on the basis of extensive spectroscopic analysis, including 2D NMR data, and the results of hydrolytic cleavage. The cardenolide glycosides showed tumor specific cytotoxic activity.

Key words *Convallaria majalis* L.; Liliaceae; 5β-spirostanol triglycoside; convallasaponin A; cardenolide glycoside; cytotoxic activity

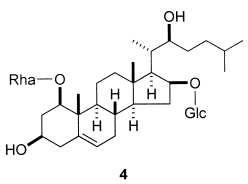
Convallaria majalis L. is a perennial plant belonging to the family Liliaceae and is distributed in Europe, Asia, and North America. Its rhizomes are known to contain several cardenolide glycosides[1] and were used as a cardiotonic agent. However, this plant is cultivated only for ornamental purposes because an extract prepared from the rhizomes showed potent toxicity and produced harmful side effects on the heart. A literature survey concerning the secondary metabolites of *C. majalis* showed that it has been suggested to contain steroidal saponins,[2,3] as well as cardenolide glycosides, but no systematic phytochemical examinations have been carried out on this plant. The present investigation on the steroidal glycoside constituents of the rhizomes of *C. majalis* has resulted in the isolation of a new 5β-spirostanol triglycoside, named convallasaponin A, (**1**), along with two known cardenolide glycosides (**2**, **3**) and a known cholestane glycoside (**4**). The paper deals with the structure elucidation of convallasaponin A (**1**) on the basis of extensive spectroscopic analysis, including 2D NMR data, and the results of hydrolytic cleavage. The cytotoxic activities of **1**—**4** against human submandibular gland carcinoma (HSG) cells and human periodontal ligament fibroblasts (HPLF) are also described.



1



R
2 CHO
3 CH_2OH



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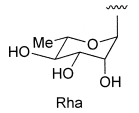
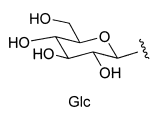


Rha



Glc

Results and Discussion

The fresh rhizomes of *C. majalis* L. were extracted with hot MeOH, and the MeOH extract was passed through a porous-polymer polystyrene resin (Diaion HP-20) column. A series of chromatographic separations of the 80% MeOH eluate fraction, in which steroidal glycosides were enriched, resulted in the isolation of compounds **1** (14.1 mg), **2** (173 mg), **3** (63.1 mg), and **4** (39.1 mg). Compounds **2**—**4** were identified as 5β,14β-dihydroxy-19-oxo-3β-[(α-L-rhamnopyranosyl)oxy]card-20,22-enolide (convallatoxin),[4] and 5β,14β,19-trihydroxy-3β-[(α-L-rhamnopyranosyl)oxy]card-20,22-enolide (convallatoxol),[4] and (22*S*)-16β-[(β-D-glucopyranosyl)oxy]-3β,22-dihydroxycholest-5-en-1β-yl α-L-rhamnopyranoside,[5] respectively. This is the first isolation of glycoside of a cholestane derivative from *C. majalis*.

Convallasaponin A (**1**) was isolated as an amorphous solid and showed an accurate $[M+H]^+$ ion at *m*/*z* 889.4843 in the high-resolution electronspray-ionization mass spectrum (HR-ESI-MS), corresponding to the empirical molecular formula $C_{44}H_{72}O_{18}$. The glycosidic nature of **1** was shown by strong IR absorptions at 3388 cm^{-1} and 1076 cm^{-1}. The ^{1}H-NMR spectrum of **1** showed signals for four steroid methyls at δ 1.39 (s), 1.23 (d, J=7.2 Hz), 1.06 (d, J=7.0 Hz), and 0.99 (s), and three anomeric protons at δ 5.13 (d, J=7.9 Hz), 4.85 (d, J=7.8 Hz), and 4.55 (d, J=7.6 Hz). Acid hydrolysis of **1** with 0.5 M HCl yielded D-glucose, D-quinovose, and D-xylose. Identification of these sugars, including their absolute configuration, was carried out by direct HPLC analysis of the hydrolysate. The above data and an acetalic ^{13}C-NMR signal at δ 110.3[6] suggested that **1** is a spirostanol triglycoside. The ^{13}C-NMR spectrum of **1** showed a total of 44 resonance lines, 17 of which were attributed to the sugar units. This implied a $C_{27}H_{44}O_5$ molecular formula for the aglycone moiety, indicating that three oxygen atoms were incorporated into the spirostane skeleton. Analysis of the ^{1}H–^{1}H shift correlation spectroscopy (COSY), totally correlated spectroscopy (TOCSY), ^{1}H-detected heteronuclear multiple quantum coherence (HMQC), and ^{1}H-detected heteronuclear multiple-bond connectivities (HMBC) spectra allowed all the ^{1}H- and

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^{13}C-NMR signals arising from the aglycone moiety to be assigned. A broad singlet signal at δ 4.04 ($W_{1/2}$=8.9 Hz) was shown to be coupled with two pairs of methylene groups at δ 2.04 and 1.47 (H_2-2), and δ 1.89 and 1.60 (H_2-4). The former exhibited proton spin-coupling correlations with an oxymethine proton at δ 4.12 (H-1), whereas the latter showed correlations with a methine proton at δ 2.39 (H-5). The methine protons at δ 4.12 and 2.39 were associated with the one-bond coupled carbons at δ 78.9 (C-1) and 31.9 (C-5), respectively, by the HMQC spectrum. The angular Me-19 (δ 1.39) showed long-range correlations not only with its attached carbon at δ 39.3 (C-10) but also with the 78.9 (C-1) and 31.9 (C-5) resonances. On the other hand, the three-proton doublet signal at δ 1.23 assignable to Me-21 showed a coupling correlation with a methine proton at δ 2.22 (H-20) with a J value of 7.2 Hz. Although the methine proton at δ 2.22 did not show further spin-coupling correlation, it exhibited an HMBC correlation with an oxygenated quaternary carbon at δ 90.0 (C-17). Long-range correlations were also observed from H-16 (δ 4.45), Me-18 (δ 0.99), and Me-21 to C-17. Thus, the aglycone of **1** was revealed to be a spirostanol derivative having oxygen atoms at C-1, C-3, and C-17. NOE correlations between H-2axial(ax) and H-3, H-5 and Me-19, H-8 (δ 1.73) and Me-19, H-9 (δ 1.26) and H-2ax/H-4ax/H-14 (δ 2.18), H-14 and H-16, H-16 and H-26ax (δ 3.26), H-20 and Me-18/H-23ax (δ 1.93), and between H-23ax and Me-27 observed in the phase-sensitive NOE correlation spectroscopy (NOESY) spectrum of **1**, as well as the small proton coupling constants of H-1 (br s, $W_{1/2}$=6.8 Hz), H-3 (br s, $W_{1/2}$=8.9 Hz), and H-26ax ($^3J_{\text{H-25,H-26ax}}$=0.5 Hz>), provided evidence for the steroid ring fusions of A/B *cis* (5β), B/C *trans*, C/D *trans*, D/E *cis*, and the 1β, 3β, 17α, 20α, 22α, and 25*S* configurations (Fig. 1). Although the genuine aglycone of **1** could not be obtained by acid hydrolysis, all of these data indicated that the aglycone of **1** is (25*S*)-5β-spirostane-1β,3β,17α-triol, which is a new steroidal sapogenin.

Finally, our attention was directed to the structure of the sugar moiety and its linkage position to the aglycone. The ^{1}H–^{1}H COSY and TOCSY experiments with **1** allowed the sequential assignments from H-1 to CH_2-6, H-1 to Me-6, and H-1 to H_2-5 of the three monosaccharides. Their signal multiplet patterns and coupling constants enabled identification of a β-D-glucopyranosyl ($^4C_1$) unit, a β-D-quinovopyranosyl ($^4C_1$) unit, and a β-D-xylopyranosyl ($^4C_1$) unit. The relatively large J values of the anomeric protons of these sugars indicated β anomeric orientations. All the proton signals for the sugar moiety thus assigned were associated with one-bond coupled carbon signals using the HMQC spectrum. The glucosyl and xylosyl residues were considered to be the terminal units, as shown by the absence of any glycosylation shifts for their carbon signals, while the C-2 and C-3 hydroxy groups of the quinovosyl unit were suggested to be substituted by comparison of the ^{13}C-NMR shifts with those reported in the literature.[7] In the HMBC spectrum, the anomeric protons at δ 5.13 (glucosyl) and 4.85 (xylosyl) showed long-range correlations with C-2 (δ 80.6) and C-3 (δ 88.1) of the quinovosyl moiety, respectively, whose anomeric proton at δ 4.55, in turn, showed an HMBC correlation with C-1 (δ 78.9) of the aglycone. On the basis of the above data, convallasaponin A (**1**) was assumed to be a combination of a new steroidal sa-

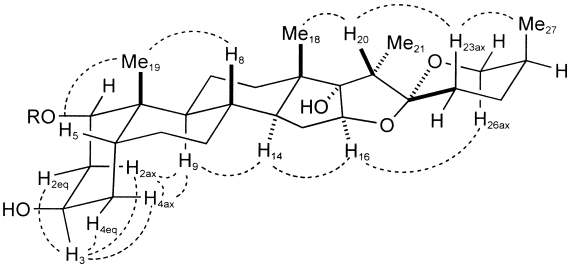

Fig. 1. NOE Correlations of **1**

Table 1. ^{1}H- and ^{13}C-NMR Spectral Data for **1** in C_5D_5N

Position	^{1}H		J (Hz)	^{13}C
1	4.12	br s	6.8 ($W_{1/2}$)	78.9
2 eq	2.04	br d	13.9	29.2
ax	1.47	br d	13.9	
3	4.04	br s	8.9 ($W_{1/2}$)	66.9
4 eq	1.89	br d	13.8	33.9
ax	1.60	ddd	13.8, 13.8, 2.5	
5	2.39	br d	13.9	31.9
6 eq	1.32			26.3
ax	1.94			
7 eq	1.10			26.2
ax	1.47			
8	1.73	dddd	11.0, 11.0, 11.0, 3.5	36.3
9	1.26			41.8
10				39.3
11	1.46 (2H)			21.2
12 eq	2.23			32.5
ax	1.55			
13				45.4
14	2.18			52.9
15	2.21			31.6
	1.50			
16	4.45			90.3
17				90.0
18	0.99	s		17.6
19	1.39	s		19.7
20	2.22	q	7.2	45.4
21	1.23	d	7.2	9.6
22				110.3
23 eq	1.45			26.5
ax	1.93	ddd	13.6, 13.6, 4.4	
24 eq	2.17			25.7
ax	1.35			
25	1.57			27.4
26 eq	4.06	br d	11.0	64.5
ax	3.26	br d	11.0	
27	1.06	d	7.0	16.3
1′	4.55	d	7.6	99.0
2′	3.74	dd	8.9, 7.6	80.6
3′	3.79	dd	8.9, 8.9	88.1
4′	3.23	dd	8.9, 8.9	75.1
5′	3.38	dq	8.9, 6.0	72.6
6′	1.37	d	6.0	18.2
1″	5.13	d	7.9	103.8
2″	3.46	dd	9.5, 7.9	75.3
3″	3.62	dd	9.5, 9.5	78.2
4″	3.84	dd	9.5, 9.5	68.0
5″	3.38	ddd	9.5, 5.3, 2.4	77.6
6‴ a	4.42	dd	11.8, 2.4	62.2
b	4.32	dd	11.8, 5.3	
1‴	4.85	d	7.8	105.0
2‴	3.49	dd	9.0, 7.8	75.3
3‴	3.57	dd	9.0, 9.0	78.5
4‴	3.73	ddd	10.0, 9.0, 5.5	70.9
5‴ a	4.02	dd	11.4, 5.5	68.0
b	3.39	dd	11.4, 10.0	

pogenin and a new triglycoside, and the full structure of **1** was formulated elucidated as (25*S*)-3β,17α-dihydroxy-5β-spiorstan-1β-yl *O*-β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-β-D-quinovopyranoside.

Several cardenolides and bufadienolides have been reported to show cytotoxic activity against cultured tumor cells.[8,9] The cardenolide glycosides (**1**, **2**) also exhibited potent cytotoxic activity against HSG cells with IC_{50} values at 0.012 and 0.028 μg/ml, respectively, whereas **1** and **2** did not cytotoxic to normal HPLF at a sample concentration of 0.10 μg/ml.

Experimental

Optical rotations were measured using a JASCO DIP-360 (Tokyo, Japan) automatic digital polarimeter. IR spectra were recorded on a JASCO FT-IR 620 spectrophotometer. NMR spectra were recorded on a Bruker DRX-500 spectrometer (500 MHz for ^{1}H-NMR, Karlsruhe, Germany) using standard Bruker pulse programs. Chemical shifts are given as the δ-value with reference to tetramethylsilane (TMS) as an internal standard. ESI-MS data were obtained on a Micromass LCT mass spectrometer (Manchester, U.K.). Diaion HP-20 (Mitsubishi-Chemical, Tokyo, Japan), Sephadex LH-20 (Pharmacia, Uppsala, Sweden), silica gel (Fuji-Silysia Chemical, Aichi, Japan), and octadecylsilanized (ODS) silica gel (Nacalai Tesque, Kyoto, Japan) were used for column chromatography. TLC was carried out on precoated Kieselgel 60 F_{254} (0.25 mm, Merck, Darmstadt, Germany) and RP-18 F_{254} S (0.25 mm thick, Merck) plates, and spots were visualized by spraying with 10% H_2SO_4 followed by heating. HPLC was performed by using a system comprised of a CCPM pump (Tosoh, Tokyo, Japan), a CCP PX-8010 controller (Tosoh), an RI-8010 detector (Tosoh) or a Shodex OR-2 detector (Showa-Denko, Tokyo, Japan), and a Rheodyne injection port. A Capcell Pak C18 column (10 mm i.d.×250 mm, 5 μm, Shiseido, Tokyo, Japan) was used for preparative HPLC. The following reagents were obtained from the indicated companies: Dulbecco's modified Eagle medium (DMEM) (Gibco, Grand Island, NY, U.S.A.); fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, U.S.A.); penicillin, streptomycin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) (Sigma, St. Louis, MO, U.S.A.). All other chemicals used were of biochemical reagent grade.

Plant Material *Convallaria majalis* was purchased from a garden center in Itoi-noen, Japan, in April 1998 and identified by Dr. Yutaka Sashida, emeritus professor of the Tokyo University of Pharmacy and Life Sciences. A voucher specimen has been deposited in our laboratory (voucher No. 98-4-27-CM, Laboratory of Medicinal Pharmacognosy).

Extraction and Isolation The plant material (fresh weight, 3.4 kg) was extracted with hot MeOH twice (each 12 l). The extract was concentrated under residue pressure, and the viscous concentrate (300 g) was passed through a Diaion HP-20 column, successively eluted with 30% MeOH, 80% MeOH, MeOH, EtOH, and EtOAc (each 2 l). The 80% MeOH eluate portion (52.2 g) was chromatographed on silica gel, eluted with $CHCl_3$–MeOH–H_2O gradients (9 : 1 : 0; 4 : 1 : 0; 2 : 1 : 0; 7 : 4 : 1) and finally with MeOH alone, to give nine fractions (I—IX). Fraction II was subjected to a silica gel column eluted with $CHCl_3$–MeOH–H_2O (70 : 10 : 1), an ODS silica gel column with MeOH–H_2O (2 : 3), and a Sephadex LH-20 column with MeOH to furnish **2** (173 mg). Fraction III was separated by a silica gel column eluted with $CHCl_3$–MeOH–H_2O (60 : 10 : 1, 40 : 10 : 1) to give eight subfractions (IIIa—IIIh). Fraction IIIe was subjected to column chromatography on silica gel eluted with $CHCl_3$–MeOH–H_2O (60 : 10 : 1, 40 : 10 : 1) and ODS silica gel with MeCN–H_2O (1 : 3), and finally to preparative HPLC using MeCN–H_2O (1 : 2) to yield **3** (63.1 mg). Fraction IV was chromatographed on silica gel eluted with $CHCl_3$–MeOH–H_2O (40 : 10 : 1) to give five subfractions (IVa—IVe). Fraction IVc was purified by silica gel column chromatography eluted with $CHCl_3$–MeOH–H_2O (60 : 10 : 1) and ODS silica gel column chromatography with MeOH–H_2O (4 : 1; 3 : 1; 2 : 1) and MeCN–H_2O (1 : 2) to give **1** (14.1 mg). Fraction IVd was subjected to column chromatography on silica gel eluted with $CHCl_3$–MeOH–H_2O (60 : 10 : 1) and ODS silica gel with MeOH–H_2O (3 : 2; 8 : 5; 2 : 1) and MeCN–H_2O (1 : 2; 2 : 5) to furnish **4** (39.1 mg).

Convallasaponin A (**1**): Amorphous solid, $[\alpha]_D^{26}$ −56.0° (c=0.10, MeOH). HR-ESI-MS (positive mode) m/z: 889.4843 $[M+H]^+$ (Calcd for $C_{44}H_{73}O_{18}$: 889.4797). IR (film) ν_{max} cm^{-1}: 3388 (OH), 2925, 2871 and 2857 (CH), 1109, 1076, 1045. ^{1}H- and ^{13}C-NMR: see Table 1.

Acid Hydrolysis of 1 A solution of **1** (2.5 mg) in 0.5 M HCl (dioxane–H_2O, 1 : 1, 3 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 IA-93ZU (Organo, Tokyo, Japan) column and then chromatographed on Diaion HP-20, eluted with H_2O–MeOH (7 : 3) followed by Me_2CO–EtOH (1 : 1), to give an aglycone fraction and a sugar fraction (1.1 mg). TLC analysis of the aglycone fraction showed that it contained several unidentified artifactual compounds. The sugar fraction was dissolved in H_2O–MeOH (7 : 3) and passed through a Sep-Pak C18 cartridge (Waters, Milford, MA, U.S.A.) and Toyopak IC-SP M cartridge (Tosoh), which was then analyzed by HPLC under the following conditions: column, Capcell Pak NH2 UG80 (4.6 mm i.d.×250 mm, 5 μm, Shiseido); solvent, MeCN–H_2O (17 : 3); flow rate, 1.0 ml/min; detection, RI and OR. Identification of D-glucose, D-quinovose, and D-xylose in the sugar fraction was carried out by comparison of their retention times and optical rotations with those of authentic samples. t_R (min): 9.19 (D-quinovose, positive optical rotation), 10.82 (D-xylose, positive optical rotation), 18.14 (D-glucose, positive optical rotation).

Cell Culture Cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate in a humidified 5% CO_2 atmosphere.

Assay for Cytotoxic Activity Cells were trypsinized and inoculated at 6×10^3 to 1.2×10^4 per each 96-microwell plate (Falcon, flat bottom, treated polystyrene, Becton Dickinson, San Jose, CA, U.S.A.) and incubated for 24 h. After washing once with phosphate-buffered saline (PBS, 0.01 M phosphate buffer, 0.15 M NaCl, pH 7.4), they were treated for 24 h without or with test compounds. They were then washed once with PBS and incubated for 4 h with 0.2 mg/ml MTT in DMEM supplemented with 10% FBS. After the medium was removed, the cells were lysed with 0.1 ml dimethyl sulfoxide (DMSO) and the relative viable cell number was determined by measuring the absorbance at 540 nm of the cell lysate, using a Labsystems Multiskan (Biochromatic, Helsinki, Finland) connected to a Star/DOT Matrix printer JL-10.[10,11] The IC_{50} value, the concentration that reduces the viable cell number by 50%, was determined from the dose–response curve.

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