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CHOLESTANE GLYCOSIDES WITH POTENT CYTOSTATIC ACTIVITIES ON VARIOUS TUMOR CELLS FROM *ORNITHOGALUM SAUNDERSIAE* BULBS

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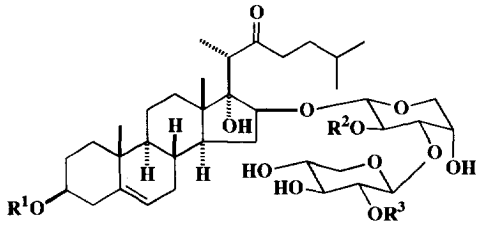
Abstract: Five cholestane glycosides (**1** - **5**) including three new ones (**3** - **5**) with potent cytostatic activity on leukemia HL-60 cells were isolated from *Ornithogalum saundersiae* bulbs. Compound **1**, a main constituent in the bulbs, was revealed to be exceptionally cytostatic against various malignant tumor cells and effective to mouse P388 leukemia in *in vivo* evaluation.

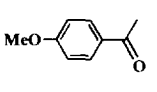
During the course of our systematic survey of the bioactive constituents with medicinal potential from higher plants, we have found that the MeOH extract of *Ornithogalum saundersiae* bulbs[1] exhibited extremely potent cytostatic activity on human promyelocytic leukemia HL-60 cells with an IC_{50} value of 31 ng ml^{-1}.[2] On fractionation of the MeOH extract into *n*-BuOH-soluble phase and H_2O phase, the cytostatic activity appeared only in the *n*-BuOH phase (17 ng ml^{-1}). The *n*-BuOH phase was passed through a Diaion HP-20 column eluting with H_2O gradually enriched with MeOH. The MeOH eluate fraction was highly cytostatic to HL-60 cells (0.8 ng ml^{-1}), and subjected to silica-gel and ODS silica-gel column chromatographies, and to reversed-phase HPLC to furnish compounds **1** (437 mg), **2** (24.3 mg), **3** (19.0 mg), **4** (50.1 mg) and **5** (28.7 mg) as the active constituents responsible for HL-60 cells cytostasis.

Compounds **1** - **5** were obtained as amorphous solids and their molecular formulas were determined by negative-ion FABMS, ^{13}C NMR data with the help of various DEPT spectra and elemental analysis to be $C_{47}H_{68}O_{15}$, $C_{48}H_{70}O_{16}$, $C_{48}H_{68}O_{14}$, $C_{53}H_{78}O_{20}$ and $C_{54}H_{78}O_{19}$, respectively.

Compounds **1** and **2** were identified by their IR, ^{1}H NMR and ^{13}C NMR spectra as 3β,16β,17α-trihydroxycholest-5-en-22-one 16-*O*-{*O*-(2-*O*-4-methoxybenzoyl-β-D-xylopyranosyl)-(1→3)-2-*O*-acetyl-α-L-arabinopyranoside} and 3β,16β,17α-trihydroxycholest-5-en-22-one 16-*O*-{*O*-(2-*O*-3,4-dimethoxybenzoyl-β-D-xylopyranosyl)-(1→3)-2-*O*-acetyl-α-L-arabinopyranoside}, which were previously isolated from the same plant source.[3]



	R^1	R^2	R^3
1	H	Ac	*p*-methoxybenzoyl
1a	H	H	H
2	H	Ac	3,4-dimethoxybenzoyl
3	H	Ac	(*E*)-cinnamoyl
4	Glc	Ac	*p*-methoxybenzoyl
5	Glc	Ac	(*E*)-cinnamoyl



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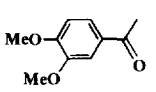

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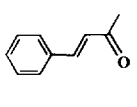

(*E*)-cinnamoyl

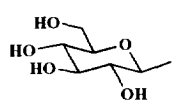

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Table 1. Cytostatic Activity of 1, 1a and 2 - 5 and Controls on HL-60 Cells

Compounds	IC_{50} (μM)
1	0.00025
1a	0.19
2	0.00020
3	0.00029
4	0.00024
5	0.00012
etoposide	0.025
ADM	0.0072
MTX	0.012

The spectral data of **3** were essentially analogous with those of **1** and **2** and suggestive of a cholestane glycoside of the same type. The presence of an *E*-cinnamoyl group as well as an acetyl group in the molecule was indicated by the IR (ν_{max} 1695 cm^{-1}, an α,β-unsaturated ester carbonyl), UV (λ_{max} 277 nm, log ε 4.36), ^{1}H NMR [δ 7.95 and 6.83 (each 1H, d, J = 16.0 Hz, *trans*-olefinic group)] and ^{13}C NMR [δ 135.1 (C-1), 128.6 (C-2, -6), 129.5 (C-3, -5), 130.8 (C-4), 145.2 (C-7), 119.3 (C-8) and 166.3 (C-9)] spectra. Alkaline hydrolysis of **3** with 4% potassium hydroxide in EtOH gave *E*-cinnamic acid and a cholestane glycoside, the latter was identical to the product produced by alkaline treatment of **1**. The ester linkages at the arabinose C-2 and the xylose C-2 hydroxy positions of **3** were formed from an acetic acid and an *E*-cinnamic acid, respectively, as was evident from the three-bond coupled C,H-correlations between the ester carbonyl carbons and the carbinol protons in the HMBC spectrum optimized for an $^nJ_{C,H}$ of 8 Hz; the downfield-shifted proton signal at δ 5.61 (dd, J = 8.8, 6.8 Hz) assignable 2-H of the arabinose was correlated to the ester carbonyl carbon due to the acetyl group at δ 169.5, and the other downfield-shifted ptoton at δ 5.51 (dd, J = 8.9, 7.7 Hz) assignable to 2-H of the xylose was correlated to the carbonyl carbon of the *E*-cinnamoyl group at δ 166.3.[4] The structure of **3** was thus characterized as 3β,16β,17α-trihydroxycholest-5-en-22-one 16-*O*-{*O*-(2-*E*-cinnamoyl-β-D-xylopyranosyl)-(1→3)-2-*O*-acetyl-α-L-arabinopyranoside}.

The ^{1}H NMR spectrum of **4** showed three anomeric proton signals at δ 5.09 (d, J = 7.6 Hz), 4.96 (d, J = 7.7 Hz) and 4.53 (d, J = 6.2 Hz). Acid hydrolysis of **4** with 1 M HCl in dioxane - H_2O (1 : 1) gave D-glucose, D-xylose and L-arabinose in a ratio of 1 : 1 : 1 as the carbohydrate compounds.[5] On comparison of the whole ^{13}C NMR spectrum of **4** with that of **1**, a set of additional six signals corresponding to a terminal β-D-glucopyranosyl appeared at δ 102.5 (C-1), 75.2 (C-2), 78.4 (C-3), 71.6 (C-4), 78.4 (C-5) and 62.7 (C-6), and

the signal due to C-3 of the aglycone was displaced downfield by 7.1 ppm, whereas the signal due to C-2 and C-4 were shifed upfield by 2.5 and 4.3 ppm, respectively, suggesting that the C-3 hydroxyl group of the aglycone was the glycosylated position to which the additional D-glucose was linked. This was confirmed by a long-range C,H correlation from the anomeric proton of the glucose at δ 4.96 to the C-3 oxymethine carbon at δ 78.3. The structure of **4** was formulated as 3β,16β,17α-trihydroxycholest-5-en-22-one 3-*O*-β-D-glucopyranoside 16-*O*-{*O*-(2-*O*-4-methoxybenzoyl-β-D-xylopyranosyl)-(1→3)-2-*O*-acetyl-α-L-arabinopyranoside}.

The NMR data of **5** showed that it was identical to **3** in terms of the structures of the aglycone and the acylated diglycoside attached to C-16 of the aglycone, but differed from it in the presence of a terminal β-D-glucopyranosyl moiety. On comparison of the ^{13}C NMR spectrum of **5** with that of **3**, the glycosylation-induced downfield shift was recognized at the aglycone C-3, accompanied by the upfield shifts at C-2 and C-4. The structure of **5** was formulated as 3β,16β,17α-trihydroxycholest-5-en-22-one 3-*O*-β-D-glucopyranoside 16-*O*-{*O*-(2-*E*-cinnamoyl-β-D-xylopyranosyl)-(1→3)-2-*O*-acetyl-α-L-arabinopyranoside}.

Compounds **3** - **5** are new naturally occurring constituents.

The isolated compounds strongly suppressed the growth of leukemia HL-60 cells, showing the IC_{50} values ranging between 0.1 and 0.3 nM, which are much more potent in comparison to the clinically applied anticancer agents, etoposide, adriamycin (ADM) and methotrexate (MTX) used as positive controls (Table 1). Further detailed examination of **1**, a main constituent in *O. saundersiae* bulbs, discovered that it exhibited exceptionally potent cytostatic activities on various malignant tumor cells such as mouse mastrocarcinoma, human pulmonary adenocarcinoma, human pulmonary large cell carcinoma and human pulmonary squamous cell carcinoma including ADM-resistant P388 leukemia and camptothecin (CPT)-resistant P388; the activities are around 10 - 100 times more potent than those of the clinically applied anticancer agents, mitomycin C (MMC), ADM, cisplatin (CDDP), CPT and taxol (TAX) (Table 2). It should be emphasized that **1** showed little toxicity

Table 2. Cytostatic Activities of 1 and Clinically Applied Anticancer Agents on Various Malignant Tumor Cells

Malignant cells	IC_{50} (μg/ml)					
	1	MMC	ADM	CDDP	CPT	TAX
CCD-19Lu	1.5	2	2	10	2	2
P388	0.00013	0.01	0.003	0.05	0.005	0.01
P388/ADM	0.00077					
P388/CPT	0.00010					
FM3A	0.00016					
A-549	0.00068					
Lu-65	0.00020					
Lu-99	0.00020	0.01	0.002	0.001	0.001	0.002
RERF-LC-AI	0.00026					
CCRF-CEM	0.00016	0.02	0.01	0.005	0.005	0.001

CCD-19Lu (human normal pulmonary cell)
P388 (mouse leukemia)
P388/ADM (adriamycin-resistant P388)
P388/CPT (camptothecin-resistant P388)
FM3A (mouse mastrocarcinoma)
A-549 (human pulmonary adenocarcinoma)
Lu-65 (human pulmonary large cell carcinoma)
Lu-99 (human pulmonary large cell carcinoma)
RERF-LC-AI (human pulmonary squamous cell carcinoma)
CCRF-CEM (human leukemia)

to normal human pulmonary cells. The deacyl derivative (**1a**) of **1** was significantly less potent, indicating that a structural requirement for the potent activity is the acyl groups attached to the diglycoside moiety.

Compound **1** was also found to be cytostatic in the U.S. National Cancer Institute 60-cell *in vitro* screen,[6] with a mean IC_{50} of 0.78 nM and a mean IC_{100} of 58 nM. Melanoma cell lines were particularly sensitive to **1**. The pattern of differential response to **1** was used to probe the testing data of pure compounds in the NCI screen.[7] Striking correlations (Pearson correlation coefficients of 0.60 - 0.83) were found at all three levels of response between **1** and the cephalostatins.[8] Structurally, **1** could be considered analogous to one half of the cephalostatin molecule, with oxidation of the cholestane skeleton at C-16 and C-17. Compound **1** was not hemolytic in human erythrocytes at 100 $\mu g\ ml^{-1}$.

In *in vivo* evaluation made up to the present, **1** was remarkably effective versus mouse P388 with an increased life span of 59 % by only one time adiministration of 0.01 $mg\ kg^{-1}$.

Thus the potentiality of **1** as a new anticancer agent from a higher plant is evident.

References and Notes

1. Fresh bulbs of *O. saundersiae* (16.2 kg) were purchased from Heiwaen, Japan. The plant specimen is on file in our laboratory.
2. The assay was carried out according to a modified method of Sargent and Tayler. Sargent, J. M.; Tayler, C. G. *Br. J. Cancer* **1989**, *60*, 206.
3. Kubo, S.; Mimaki, Y.; Terao, M.; Sashida, Y.; Nikaido, T.; Ohmoto T. *Phytochemistry* **1992**, *31*, 3969.
4. 2D NMR spectra were measured in a mixed solvent of pyridine-d_5 and methanol-d_4 (11 : 1) to remove exchangeable protons and minimize signal overlap.
5. The monosaccharides were identified by converting them to the 1-[(*S*)-*N*-acetyl-α-methylbenzyl-amino]-1-deoxyalditol acetate derivatives followed by HPLC analysis. Oshima, R.; Yamauchi, Y.; Kumanotani, J. *Carbohydr. Res.* **1982**, *107*, 169.
6. Boyd, M. R.; Paull, K. D.; Rubinstein, L. R. in *Cytotoxic Anticancer Drugs: Models and Concepts for Drug Discovery and Development* **1992**, pp. 11 - 34, Kluwer Academic Publishers, Amsterdam.
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8. Pettit, G. R.; Inoue, M.; Kamano, Y.; Herald, D. L.; Arm, C.; Dufresne, C.; Christie, N. D.; Schmidt, J. M.; Doubek, D. L.; Krupa, T. S. *J. Am. Chem. Soc.* **1988**, *110*, 2006.

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