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A NEW REARRANGED CHOLESTANE GLYCOSIDE FROM *ORNITHOGALUM SAUNDERSIAE* BULBS EXHIBITING POTENT CYTOSTATIC ACTIVITIES ON LEUKEMIA HL-60 AND MOLT-4 CELLS

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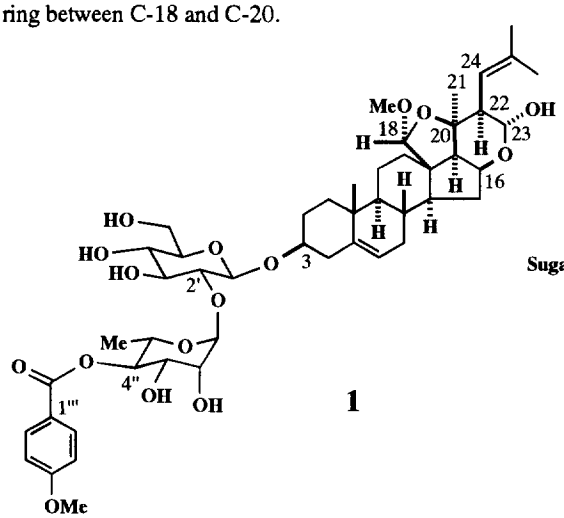
Abstract: A new rearranged cholestane glycoside (**1**) was isolated from *Ornithogalum saundersiae* bulbs by monitoring the cytostatic activity on leukemia HL-60 cells. Compound **1** was exceptionally cytostatic against HL-60 and MOLT-4 cells, which was revealed to be mediated partially through induction of apoptosis.

Ornithogalum saundersiae (Liliaceae) is a perennial plant which is native to Natal, Swaziland and the eastern Transvaal, and widely cultivated as an excellent garden plant. We have found that the MeOH extract of *O. 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}). Further fractionation of the *n*-BuOH phase was established by passing it through column chromatography on Diaion HP-20 eluted with H_2O gradually enriched with MeOH. The MeOH eluate fraction was highly cytostatic to HL-60 cells (0.8 ng ml^{-1}). A series of chromatographic separations of the MeOH fraction gave **1** (0.00008%, fresh weight) as an active constituent responsible for HL-60 cells cytostasis.

Compound **1** of $[\alpha]_D$ -62.0° (MeOH) was analyzed as being $C_{48}H_{68}O_{16}$ by negative-ion FAB-MS (*m/z* 899 $[M - H]^-$), ^{13}C NMR spectrum (48 carbons) and elemental analysis (Found: C, 59.38; H, 8.11%. Calcd: C, 59.25; H, 7.87%). The presence of a *p*-methoxybenzoyl ester group in **1** was shown by the IR (ν_{max} 1705 cm^{-1}), UV [λ_{max} 257 nm (log ε = 4.02)], ^1H NMR [δ 8.28 and 7.03 (each d, *J* = 8.9 Hz), and 3.76 (3H, s)] and ^{13}C NMR [δ 123.8 (C), 132.5 (CH x 2), 114.3 (CH x 2), 164.0 (C), 166.6 (C=O), and 55.6 (OMe)] spectra,[3] and by alkaline methanolysis of **1** with 3% NaOMe in MeOH, giving methyl *p*-methoxybenzoate. Acid hydrolysis of **1** with 1 M HCl in dioxane - H_2O (1 : 1) at 100° for 1h yielded D-glucose and L-rhamnose

in a ratio of 1 : 1.[4]

The ^{1}H NMR spectrum of **1** showed signals for four methyls at δ 1.85, 1.82, 1.47 and 0.99 (each s), an acetal proton at δ 4.83 (s), a hemiacetal proton at δ 5.52 (d, J = 8.6 Hz), and two olefinic protons at δ 5.72 (d, J = 10.1 Hz) and 5.53 (br d, J = 5.0 Hz) arising from the aglycone moiety. The above functional group data and ^{13}C NMR spectrum[5] allowed to assign the aglycone structure of **1** to be identical to that of a cholestane triglycoside previously isolated by us from the same plant source as a cAMP phosphodiesterase inhibitor.[6] The aglycone is unique in structure having a rearranged carbon skeleton, 24(23→22)*abeo*-cholestane, with formation of a six-membered hemiacetal ring between C-16 and C-23 and a five-membered acetal ring between C-18 and C-20.



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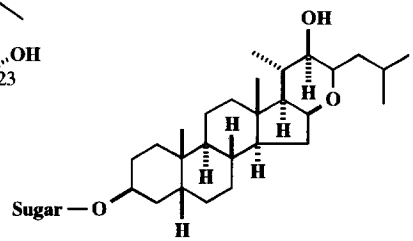

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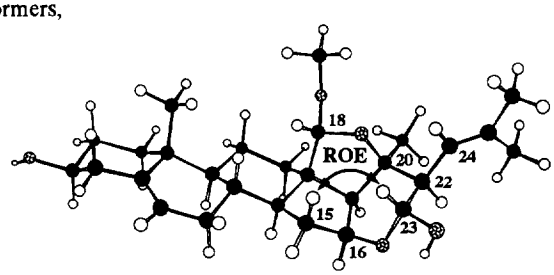
The conformation of the six-membered hemiacetal ring was examined through molecular mechanics calculations using the MM2 force field as implemented in Macro-model 4.0. The starting geometries were generated by a systematic Monte Carlo conformational search. The most stable conformer thus found was taken as starting structures for molecular dynamics calculations *in vacuo* at 296 K with a path length of 100 ps and followed by minimizing random structures sampled after multiple 1 ps interval. In this run, two stable conformers (A: 139.0 kJ mol^{-1}; B: 139.7 kJ mol^{-1}) were obtained at 296 K, whose boltzmann populations were 77% and 23%, respectively. In the two conformers, the torsion angle of H_{22}-C_{22}-C_{23}-H_{23} was almost identical to each other; -179.5° for the conformer A and -178.0° for B. The difference was recognized at the torsion angle of H_{22}-C_{22}-C_{24}-H_{24} between the two conformers; -163.8° for A and +157.1° for B. The experimental coupling constants, $^3J_{22\text{-H},23\text{-H}}$ = 8.6 Hz and $^3J_{22\text{-H},24\text{-H}}$ = 10.1 Hz, corresponded to the theoretical values within 1 Hz difference (9.5 Hz and 10.7 Hz, respectively), which were calculated through the application of the given dihedral angles, taking into account



Calculated conformer A

the boltzmann populations, to the advanced Karplus-type equation proposed by Altona *et al.*[7] The above findings proved the six-membered hemiacetal ring to be a half-chair form, which was well supported by an ROE correlation between 15β-H and 23-H. Previously, the conformation of the six-membered hemiacetal ring of the related cholestane glycoside with a five-ring system aglycone (**2**) has been shown to be almost a boat-form through molecular mechanics and molecular dynamics calculations.[8] The five-membered acetal ring formed between C-18 and C-20 is considered to leave the six-membered hemiacetal ring for a half-chair form in the aglycone of **1**.

Next, our attention was turned to the structure of the saccharide portion, which must bear a *p*-methoxybenzoyl group. The presence of a β-D-glucopyranosyl [$^4C_1$: δ_H 5.10 (d, J = 7.6 Hz); δ_C 100.5 (CH)] and an α-L-rhamnopyranosyl [$^1C_4$: δ_H 6.43 (br s); δ_C 101.8 (CH)] units in **1** was readily revealed by the result of acid hydrolysis and spin coupling information provided from the ^1H-^1H COSY and HOHAHA spectra. In the HMBC spectrum ($^nJ_{C,H}$ = 8 Hz), the anomeric proton of the rhamnose showed a $^3J_{C,H}$ correlation with C-2' (δ 77.6) of glucose, and that of glucose with C-3 (δ 78.0) of the aglycone, leading to rhamnosyl-(1→2)-glucosyl structure attached to C-3 of the aglycone. The ester linkage in the rhamnose C-4 hydroxy position of **1** was formed from *p*-methoxybenzoic acid, as was evident in the ^1H NMR paramagnetic chemical shift due to acylation; the 4"-H proton of the rhamnose was deshielded to appear at δ 6.13 (dd, J = 9.5, 9.5 Hz).[9]

From the data mentioned above, the full structure of **1** was established.

Compound **1** exhibited almost equal or more potent cytostatic activity on HL-60 cells (IC_{50} 9.2 nM) compared to the clinically applied anticancer agents, etoposide (IC_{50} 25 nM), methotrexate (12 nM), adriamycin (7.2 nM). It is also cytostatic against human T-lymphocytic leukemia MOLT-4 cells with an IC_{50} of 3.2 nM, which is about ten times more potent than those of etoposide (38 nM), methotrexate (48 nM), and adriamycin (35 nM). The growth inhibiting activity of **1** on HL-60 cells appeared to be mediated partially through induction of apoptosis by cell morphology[10] and DNA fragmentation.[11] Flow cytomeric analysis of **1**-treated HL-60 cells also demonstrated apoptotic cells with low DNA content and showed a decrease of G_0/G_1 cells and a concomitant increase of S and/or G_2/M cells, which indicated that G_0/G_1 phase cells were preferentially undergoing apoptosis. The effect of **1** on HL-60 was prompted by Ca^{2+} and was inhibited by the presence of Zn^{2+}. This suggested involvement of Ca^{2+} dependent endonuclease activation in the **1**-induced apoptosis.[12] Previous reported cholestane triglycoside with the same aglycone structure as **1** showed little cytostatic activity, suggesting that the saccharide part bearing an aromatic acyl group was essential for the exceptional activity.

Compound **1** must be emphasized as a new class of cytostatic natural product with induction of apoptosis in malignant tumor cells and induction of apoptosis could be an efficient strategy for cancer chemotherapy, however, the yield was not much enough to explain the total cytostatic activity of the MeOH eluate fraction. The presence of other active principles in the fraction would be expected. Further survey of the cytostatic constituents is still in progress.

Table 1. Effects of **1** and anticancer agents on leukemia cell proliferation

agents	IC_{50} (nM = x 10^{-9} M)	
	HL-60	MOLT-4
1	9.2	3.2
anticancer agents		
etoposide	25.0	38.0
methotrexate	12.0	48.0
adriamycin	7.2	35.0

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. 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.
4. The monosaccharides were identified by converting them to the 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxyalditol acetate derivatives followed by HPLC analysis. Oshima, R.; Yamauchi, Y.; Kumanotani, J. *Carbohydr. Res.* **1982**, *107*, 169.
5. ^{13}C NMR of **1**: δ 37.4, 30.2, 78.0, 39.4, 141.4, 121.9, 32.6, 32.7, 50.1, 37.5, 23.0, 34.6, 57.7, 51.1, 29.8, 77.3, 53.3, 108.8, 19.8, 86.0, 27.8, 50.8, 90.9, 122.9, 134.8, 26.6 and 19.1 (C-1 - C-27), 55.5 (OMe), 100.5, 77.6, 79.7, 71.8, 78.5 and 62.7 (C-1' - C-6'), 101.8, 72.6, 70.5, 76.8, 67.2 and 18.3 (C-1" - C-6"), and 123.8, 132.5, 114.3, 164.0, 114.3, 132.5, 166.6 and 55.6 (C-1"' - C-7"', OMe).
6. Kuroda, M.; Mimaki, Y.; Sashida, Y.; Nikaido, T.; Ohmoto, T. *Tetrahedron Lett.* **1993**, *34*, 6073.
7. Haasnoot, C. A. G.; Leeuw, F. A. A. M. De; Altona C. *Tetrahedron* **1980**, *36*, 2783.
8. Kuroda, M.; Mimaki, Y.; Sashida, Y.; Hirano, T.; Oka, K.; Dobashi, A. *Chem. Pharm. Bull.* **1995**, *43*, 1257.
9. Unfortunately, no $^3J_{C,H}$ correlation between the carbonyl carbon of the *p*-methoxybenzoyl group and the 4"-H proton of the rhamnose was observed in the HMBC spectra optimized for $^nJ_{C,H}$ = 5 Hz and 8 Hz. The 4"-H proton of the rhamnose, however, was shifted downfield by 1.91 ppm in comparison of that of a steroidal saponin bearing the same saccharide sequence as **1** isolated by us from an *Allium* plant. This is believed to be a confirmative evidence for the ester linkage position.
10. Compound **1** efficiently induced morphological changes characteristic of apoptosis in HL-60 cells. Untreated cells exhibited typical non-adherent, fairly round morphology. Wright, S. C.; Kumar, P.; Tam, A. W.; Shen, N.; Varma, M.; Larrick, J. W. *J. Cellular Biochem.* **1992**, *48*, 344.
11. DNA fragmentation has been known as a characteristic feature of apoptosis. Wyllie, A. H.; Kerr, J. F.; Currie, A. R. *Int. Rev. Cytol.*, **1980**, *68*, 251.
12. DNA fragmentation in apoptotic cells has been reported to be due to Ca^{2+} dependent endonucleases, and Zn^{2+} inhibits the enzyme activities which results in blocks of apoptosis. Duke, R. C.; Chervenak, R.; Cohen, J. J. *Proc. Natl. Acad. Sci.* **1983**, *80*, 6361.

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