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Three Novel Rearranged Cholestane Glycosides from *Ornithogalum saundersiae* Bulbs and Their Cytostatic Activities on Leukemia HL-60 and MOLT-4 Cells

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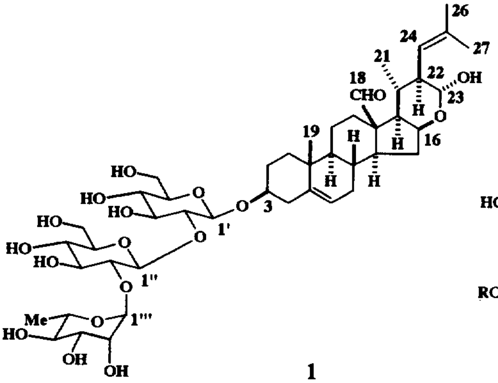
Abstract: Three novel rearranged cholestane glycosides (**1** - **3**) were isolated from *Ornithogalum saundersiae* bulbs. Their structures were determined by spectroscopic analysis. The conformation of the E-ring part of the cholestanes was studied through molecular mechanics and molecular dynamics calculation methods. Compound **3** embracing an acyl group at the saccharide part strongly suppressed the growth of leukemia HL-60 cells, which was mediated partially through induction of apoptosis.

Our random phytochemical screening of higher plants to identify compounds with medicinal potential resulted in the isolation of three novel rearranged cholestane glycosides (**1** - **3**) from the bulbs of *Ornithogalum saundersiae* belonging to the family Liliaceae. This paper briefly describes the structural elucidation of the



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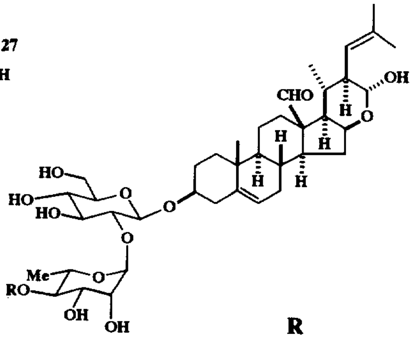

	R
2	**H**
3	***p*-hydroxybenzoyl**

novel cholestanes including the E-ring conformation based on extensive spectroscopic analysis and hydrolysis, and on molecular mechanics and molecular dynamics calculation studies. Cytostatic effects of the isolated cholestanes on human promyelocytic leukemia HL-60 and T-lymphocytic leukemia MOLT-4 cells are also discussed.

The *n*-BuOH fraction of the MeOH extract of *O. saundersiae* bulbs yielded compounds **1** (0.0017% fresh weight), **2** (0.00066%) and **3** (0.00078%) after a series of chromatographic separations.

The molecular formula of **1** was determined as $C_{45}H_{70}O_{18}$ by negative-ion FABMS showing an $[M - H]^-$ ion at *m/z* 897 and elemental analysis. The IR absorption at 1700 cm^{-1} indicated the presence of an carbonyl group in **1**. Acid hydrolysis of **1** gave D-glucose and L-rhamnose in a ratio of 2 : 1.[1] The ^{13}C NMR spectrum showed 45 resonance lines, 18 of which could be assigned to two glucoses and a rhamnose units, and three anomeric carbons were observed at δ 102.2, 102.1 and 101.2. This implied a $C_{27}H_{40}O_4$ molecular formula for the aglycone portion, which allowed us to confirm it to be a C_{27} steroid.

To clarify the full structure of **1**, detailed NMR studies, including pulsed field gradient (FG) DQFCOSY, HMQC, HMBC, phase-sensitive NOESY and ROESY spectra, were focused on it, providing useful information for the structural assignment. The FG-DQFCOSY spectrum with the help of the selective

Table 1. ^1H and ^{13}C NMR chemical shift assignments 1 in C_5D_5N - CD_3OD (11 : 1) [a]

position	^1H		J (Hz)	^{13}C	position	^1H		J (Hz)	^{13}C
1 a	1.62	br d	13.4	37.6	Glc 1'	5.05	d	7.9	101.2
b	0.94				2'	4.26	dd	8.8, 7.9	80.3
2 a	2.05	br d	11.9	30.1	3'	4.39	dd	8.8, 8.8	79.2
b	1.69				4'	4.03	dd	8.8, 8.8	71.7
3	3.88	m	20.0 [b]	78.8	5'	3.84	ddd	8.8, 6.1, 2.5	78.1
4 a	2.78	dd	13.3, 2.6	39.4	6' a	4.40	dd	11.9, 2.5	62.7
b	2.52	br dd	13.3, 13.3		b	4.22	dd	11.9, 6.1	
5	-			141.3	Glc 1"	5.76	d	7.5	102.2
6	5.32	br d	4.4	121.2	2"	4.18	dd	8.9, 7.5	79.1
7 a	1.92	br dd	13.4, 4.7	32.2	3"	4.13	dd	8.9, 8.9	79.2
b	1.48				4"	4.01	dd	8.9, 8.9	72.2
8	1.53	dddd	10.5, 10.5, 10.5, 4.7	33.6	5"	3.79	ddd	8.9, 5.5, 2.3	77.8
9	0.92			50.3	6"a	4.34	dd	11.6, 2.3	62.9
10	-			37.0	b	4.19	dd	11.6, 5.5	
11 a	1.50			22.7	Rha 1'''	6.23	br s		102.1
b	1.41	dddd	12.4, 12.4, 12.4, 3.7		2'''	4.64	br d	3.3	72.2
12 a	2.64	br d	12.4	33.6	3'''	4.57	dd	9.2, 3.3	72.4
b	1.00	ddd	12.4, 12.4, 3.7		4'''	4.21	dd	9.2, 9.2	74.2
13	-			59.5	5'''	4.89	dq	9.2, 6.2	70.1
14	1.28			52.8	6'''	1.73	d	6.2	18.8
15 a	2.34	ddd	13.2, 7.0, 7.0	33.7					
b	1.91	ddd	13.2, 13.2, 4.9						
16	4.82			69.8					
17	1.46			59.7					
18	10.10	s		207.5					
19	0.76	s		19.5					
20	1.43			31.4					
21	0.93	d	5.7	18.6					
22	2.37	ddd	11.0, 9.7, 7.7	47.6					
23	5.03	d	7.7	98.3					
24	4.85	d	9.7	126.2					
25	-			134.5					
26	1.66	s		26.1					
27	1.74	s		19.0					

[a] 600 MHz for ^1H NMR. [b] $W_{1/2}$

1D-TOCSY[2] led to the sequential assignments of the ^1H resonances of the aglycone moiety, which were correlated to the corresponding one-bond coupled ^{13}C signals (Table 1). The distinctive ^1H signal at δ 5.03 (d, J = 7.7 Hz), which was correlated to the ^{13}C signal at δ 98.3 (CH) in the HMQC spectrum, was shifted downfield by 1.02 ppm to be observed at δ 6.05 on acetylation of **1**, indicating the presence of an hemiacetal group in **1**. The connectivity of each ^1H network through quaternary carbons was established by interpretation of the HMBC spectrum, leading to the fundamental structure of **1** as a 22-homo-23-norcholestane (Fig. 1). The methyl signal at δ 0.76 (s) showed $^2J_{C,H}$ correlation peak with the quaternary carbon signal at δ 36.9 due to C-10 of steroid, and was assigned to 19-Me. The aldehyde group was assigned to be linked to C-13 by $^3J_{C,H}$ correlations from $\delta_{12a\text{-}H}$ 1.00 and $\delta_{14\text{-}H}$ 1.28 to δ_{CHO} 207.5. Further $^3J_{C,H}$ cross peak between $\delta_{C\text{-}23}$ 98.3 and $\delta_{16\text{-}H}$ 4.82 resulted in the formation of a six-membered hemiacetal ring between C-16 and C-23.

The configuration of the C-3 hydroxyl group bearing saccharide moiety was determined to be β from the multiplicity of the 3-H proton ($W_{1/2}$ = 20 Hz). The NOE correlations, 8-H/15β-H, 18-CHO and 19-Me, 19-Me/18-CHO, 14-H/9-H and 15α-H, 15α-H/16-H, 16-H/17-H and 22-H, 17-H/21-Me and 22-H, 21-Me/12β-H and 22-H, and 20-H/23-H and 24-H in the phase-sensitive NOESY and ROESY spectra provided evidence for the B/C *trans*, C/D *trans* and D/E *cis* ring junctions, and 16*S**, 17*R**, 20*S**, 22*S** and 23*R** configurations (Fig. 2).

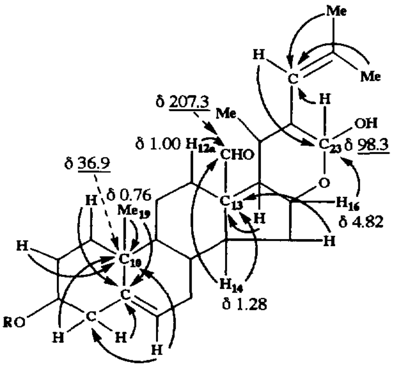

Fig. 1. HMBC Correlations

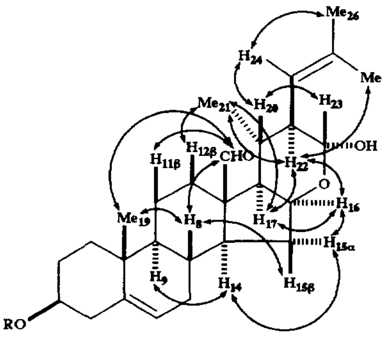

Fig. 2. NOE Correlations

The presence of a terminal α-L-rhamnopyranosyl ($^1C_4$) unit and two 2-substituted β-D-glucopyranosyl ($^4C_1$) units in the molecule was shown by comparison of the ^{13}C-NMR resonances for each monosaccharide. The ^1H-^{13}C long-range correlation from each anomeric proton across the glycosidic bond to the carbon of another substituted monosaccharide or the aglycone confirmed the sugar sequence. From the data presented above, the structure of **1** was elucidated.

The NMR data of **2** ($C_{39}H_{60}O_{13}$) and **3** ($C_{46}H_{64}O_{15}$) showed that they had identical aglycone structure to **1** but differed from it in terms of the saccharide structures. The ^1H NMR of **2** displayed two anomeric proton signals at δ 6.32 (br s) and 5.02 (d, J = 7.2 Hz), and acid hydrolysis gave D-glucose and L-rhamnose in a ratio of 1 : 1. These data proved **2** to possess disaccharide composed of D-glucose and L-rhamnose. The sequence was easily determined as α-L-rha*p*-(1→2)-β-D-glc*p*, for which confirmatory evidence was inferred from the comparison of the ^{13}C NMR data of **2** with those of authentic methyl glycosides.[3] Compound **3** appeared to be *p*-hydroxybenzoyl ester of **2** by spectral data and alkaline hydrolysis. The ester linkage in the rhamnose C-4 hydroxy position was formed from *p*-hydroxybenzoic acid as was evident in the ^1H NMR paramagnetic chemical shift due to acylation; the 4-H proton of the rhamnose of **3** was deshielded by 1.70 ppm in comparison with that of **2** to resonate at δ 6.00. Thus, the structures of **2** and **3** were revealed.

The E-ring conformation of the rearranged cholestanes was shown to be almost a boat-form 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 100ps and following by minimizing random structures sampled after multiple 1ps intervals. In this run, two conformers were obtained; the most stable conformer, whose boltzmann population was 94.5% at 296 K, showed 176.0° for the H_{20}-C_{20}-C_{22}-H_{22}, 153.9° for the H_{22}-C_{22}-C_{23}-H_{23} and 152.8° for the H_{22}-C_{22}-C_{24}-H_{24} torsion angles. The observed proton coupling constants, $^3J_{20\text{-H}, 22\text{-H}}$ = 11.0 Hz, $^3J_{22\text{-H}, 23\text{-H}}$ = 7.7 Hz and $^3J_{22\text{-H}, 24\text{-H}}$ = 9.7 Hz, almost corresponded to those (12.5, 7.4 and 9.7 Hz, respectively) calculated through the application of the given dihedral angles to the advanced Karplus-type equation proposed by Altona *et al.*4)

Compounds **1** - **3** have a rare type of rearranged carbon skeleton based on cholestane; the C-24 - C-27 moiety of the side-chain migrated to C-22 (22-homo-23-norcholestane) to form a six-membered hemiacetal ring between C-16 and C-23.

Compounds **1** - **3** were evaluated for their cytostatic activity on leukemia HL-60 cells. Compound **3** strongly suppressed the growth of HL-60 cells in a dose-dependent manner and its IC_{50} was calculated from the dose-dependent curve as 21nM, which is as potent as several antileukemia agents used clinically such as etoposide, doxorubicin, vincristine and methotrexate with IC_{50} ranging from 2.9 - 25 nM. The activity of **3** on HL-60 cells appeared to be mediated partially through induction of apoptosis by the presence of DNA fragmentation.5) This was well supported by the facts that the growth-inhibiting activity of **3** was modified by Ca^{2+} and Zn^{2+}; Ca^{2+} enhanced the activity in a dose-dependent manner and in contrast, Zn^{2+} attenuated the activity.6) Compound **3** also showed potent cytostatic activity on leukemia MOLT-4 cells (IC_{50} 18 nM). Compounds **1** and **2** have little activities. The acyl moiety attached to the saccharide part of **3** must be essential for the cytostatic activities.

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