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Galtonioside A, a novel cytotoxic cholestane glycoside from *Galtonia candicans*

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Abstract

A novel polyoxygenated 5β-cholestane diglycoside, named galtonioside A, was isolated from the bulbs of *Galtonia candicans* by way of a cytotoxicity-guided fractionation procedure against HL-60 leukemia cells. Galtonioside A showed differential cytotoxicity in the Jpn. Fdn. for Cancer Res. 38 cell line assay.

Keywords: steroid; glycoside; cytotoxic; cytotoxic compound; structure–activity.

Galtonia candicans (Liliaceae) is a perennial plant native to the Cape Province of South Africa. As part of our search for potent antitumor substances from natural sources,[1] we have found that a methanolic extract of the bulbs of *G. candicans* exhibited significant cytotoxicity against HL-60 human leukemia cells. Thus, a phytochemical investigation was undertaken for the isolation of active compounds from the plant by way of a cytotoxicity-guided fractionation procedure, combined with the MTT assay method for cytotoxicity evaluation,[2] which led to the isolation of a novel polyoxygenated 5β-cholestane diglycoside, named galtonioside A (**1**; 0.00099%, fresh weight), as an active constituent responsible for HL-60 cells cytostasis.

Galtonioside A (**1**) was obtained as an amorphous powder, $[\alpha]_D$ −60.0° (MeOH). An accurate $[M+Na]^+$ ion at *m/z* 975.4580 in the HR FABMS corresponded to the molecular formula $C_{48}H_{72}O_{19}$ (Δ +1.4 mmu of calcd.). Alkaline hydrolysis of **1** with 4% KOH in EtOH produced 3,4,5-trimethoxybenzoic acid and a deacyl derivative (**1a**), while acid treatment with 1 M HCl in dioxane:H_2O (1:1) gave D-glucose and L-arabinose in a ratio of 1:1.[3] The above chemical evidence, along with the ^1H and ^{13}C NMR spectral data (the right-hand column of Table 1), indicated the presence of a 3,4,5-trimethoxybenzoyl ester, a β-D-glucopyranosyl, and an α-L-arabinopyranosyl group in the molecule. The ^{13}C NMR spectrum of **1** showed a total of 48 resonance lines, eleven of which were due to the two monosacchrides and ten to the

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3,4,5-trimethoxybenzoyl moiety. This led to a $C_{27}H_{44}O_6$ composition for the aglycone. The existence of a trisubstituted double bond was shown by a pair of ^{13}C NMR signals at δ 136.9 (C) and 125.6 (CH), which accounted for one of the six degrees of unsaturation. From this data and the ^1H NMR signals due to four methyls in the high field section, in which one was secondary [δ 1.46 (d, J=6.4 Hz)] and three were tertiaries [δ 1.82, 1.77, and 0.98 (each 3H, s)], the aglycone of **1** was assumed to have a polyoxygenated C_{27} steroid skeleton with a five-ring system.

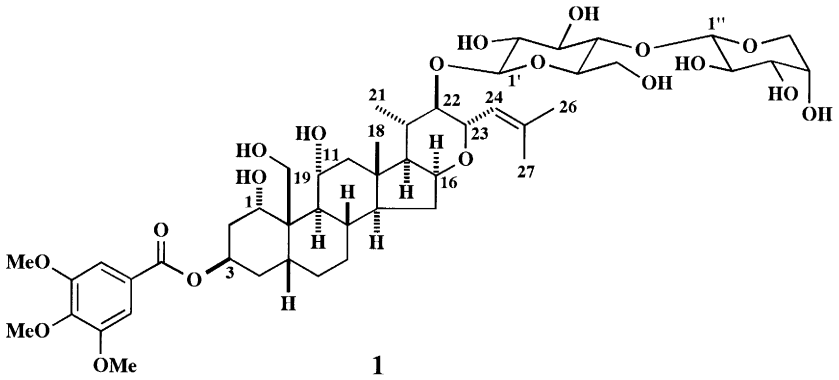

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Table 1
^1H and ^{13}C NMR spectral data for compound **1**[a]

Position	^1H		J (Hz)	^{13}C	Position	^1H		J (Hz)	^{13}C
1	5.15	dd	12.8, 3.3	68.1	1'	4.81	d	7.8	105.1
2 eq	2.45	br dd	13.3, 3.3	35.0	2'	3.99	dd	7.8, 8.6	75.4
ax	2.81	br dd	13.3, 12.8		3'	4.22	dd	8.6, 8.6	76.5
3	5.73	br s	7.6[b]	72.4	4'	4.24	dd	8.6, 8.6	81.5
4 eq	1.80			31.4	5'	3.82	ddd	8.6, 3.7, 3.1	76.0
ax	2.30	ddd	13.6, 13.6, 2.5		6' a	4.49	dd	11.7, 3.7	62.5
5	2.96	br d	13.6	32.0	b	4.41	dd	11.7, 3.1	
6 eq	2.14			26.5					
ax	1.31				1''	5.01	d	7.6	105.8
7 eq	1.44			26.9	2''	4.50	dd	9.2, 7.6	72.3
ax	1.23				3''	4.11	dd	9.2, 3.4	74.6
8	1.74			34.4	4''	4.23	br s		69.6
9	2.02	dd	10.1, 10.1	47.8	5'' a	4.26	dd	12.4, 2.1	67.9
10	-			47.0	b	3.76	br d	12.4	
11	4.30	ddd	10.1, 10.1, 5.0	65.6					
12 eq	2.59	dd	12.3, 5.0	51.1	1'''	-			126.7
ax	1.64				2'''	7.61	s		107.5
13	-			42.5	3'''	-			153.6
14	1.17			52.8	4'''	-			142.9
15 α	2.09			34.4	5'''	-			153.6
β	1.27				6'''	7.61	s		107.5
16	4.18	m		72.5	7'''	-			165.5
17	1.20			58.8	OMe	3.92	s		56.0
18	0.98	s		16.0		3.70 [c]	s		60.6
19 a	4.78	d	11.0	59.7					
b	4.38	d	11.0						
20	2.19	m		33.5					
21	1.46	d	6.4	17.1					
22	3.58	dd	11.2, 7.2	86.0					
23	4.61	dd	7.5, 7.2	76.0					
24	5.88	d	7.5	125.6					
25	-			136.9					
26	1.82	s		26.1					
27	1.77	s		18.9					

a) Spectra were measured in pyridine-d_5. b) $W_{1/2}$ c) 3H x 2

The proton coupling connectivities from C-1 to C-8, C-8 to C-12, and C-8 to C-23 were revealed by detailed interpretation of the ^1H–^1H COSY and TOCSY spectra of **1** (the left-hand column of Table 1),

giving evidence for the locations of oxygen atoms at the C-1, C-3, C-11, C-16, C-22, and C-23 positions. Further information was obtained from the HMBC data (Fig. 1). The quaternary carbon signal at δ 42.5 was assigned to C-13 by the observation of long-range correlations from δ 42.5 to δ 2.59 (H-12β), 1.20 (H-17), and 2.19 (H-20). The three-proton singlet signal at δ 0.98 was attributed to Me-18 by a $^2J_{C,H}$ correlation between δ_H 0.98 and δ_C 42.5. Signals for a geminal pair of protons due to an oxygen-linked methylene group were observed at δ 4.78 and 4.38 as an ABq spin system with a *J* value of 11.0 Hz, which were correlated to a one-bond coupled carbon at δ 59.7. The other quaternary carbon at δ 47.0 assignable to C-10 was correlated to one of the oxymethylene protons at δ 4.78, as well as to H-9 at δ 2.02, indicating the linkage of an oxymethylene group at C-10. The H-23 proton had a $^3J_{H,H}$ spin-coupling link with the H-24 olefinic proton. The H-24 proton showed long-range correlations with both the methyl groups geminally attached to C-25. This resulted in the presence of a double bond at C-24 (Δ^{24}).

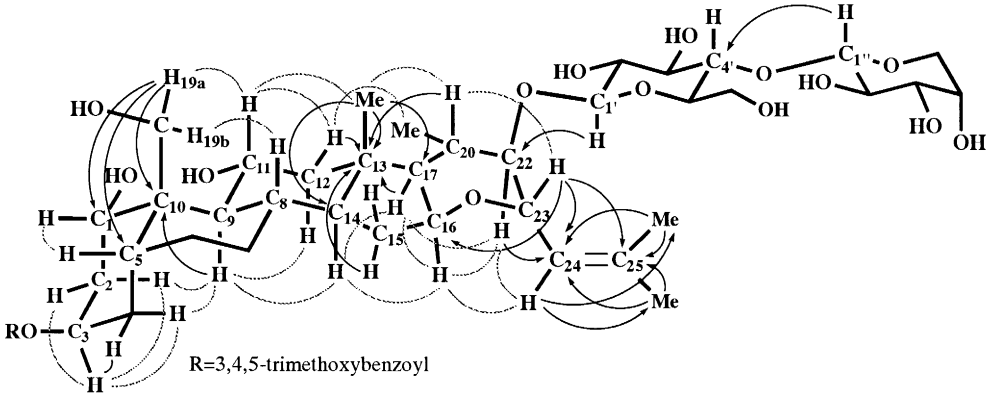

Fig. 1. HMBC (arrows) and NOE (curve lines) correlations for compound **1**

Among the oxygen-linked carbons, C-1, C-11, and C-19 had free hydroxyl groups as was evident from the fact that acetyl groups were introduced into C-1, C-11, and C-19 by the treatment of **1** with Ac_2O in pyridine. An HMBC correlation from H-23 to C-16 was consistent with the formation of a six-membered ether ring between C-16 and C-23. Consequently, C-3 and C-22 were substituted. The trimethoxybenzoyl ester linkage at C-3 was shown by the ^{1}H NMR paramagnetic chemical shift of H-3 due to *O*-acylation. The H-3 proton was deshielded by 1.19 ppm in comparison of the ^{1}H NMR spectrum of **1** with that of the deacyl derivative (**1a**), and was observed at δ 5.73. Long-range correlations from the respective anomeric protons at δ 5.01 (arabinosyl) and 4.81 (glucosyl) to the δ 81.5 (C-4 of glucose) and δ 86.0 (C-22 of aglycone) resonances in the HMBC spectrum led to the construction of the arabinosyl-(1→4)-glucosyl structure and its linkage to C-22 of the aglycone. Thus, the structure of **1**, except for the stereochemistry of the aglycone moiety, was shown to be 16,23-epoxycholest-24-ene-1,3,11,19,22-pentol embracing a trimethoxybenzoyl ester group at C-3 and an α-L-arabinosyl-(1→4)-β-D-glucosyl moiety at C-22.

The stereostructure of **1** was determined by the following NOE correlations (Fig. 1) and ^{1}H NMR coupling values. NOE correlations from H-9 to H-2ax, H-4ax, H-12ax and H-14, H-14 to H-16 and H-17, H-16 to H-17, and H-8 to H-19b in the phase-sensitive NOESY spectrum were consistent with the A/B *cis* (H-5β), B/C *trans*, C/D *trans*, and D/E *cis* (H-16α/H-17α) ring fusions. Further NOEs from H-12eq to Me-21, H-16 to H-24, H-20 to Me-18 and H-23, and H-22 to H-16, H-17 and H-24 confirmed the 20*S*, 22*R*, and 23*S* configurations. The oxygen atoms at C-1, C-3, and C-11 were revealed to be present in the equatorial, axial, and equatorial orientations, respectively, by the coupling constants of the hydrogens, H-1: dd, *J*=12.8, 3.3 Hz, H-3: br s $W_{1/2}$=7.6 Hz, and H-11: ddd, *J*=10.1, 10.1, 5.0 Hz.

From the foregoing data, the complete structure of **1** was elucidated. Galtonioside A (**1**) is very unique in structure, having a polyoxygenated pentacyclic 5β-steroid framework and the linkages of a 3,4,5-trimethoxy-benzoyl group at C-3 and a diglycoside composed of one mol each of glucose and arabinose at C-22. The E-ring conformation of the aglycone of **1** was presumed to be boat-form by the NOE correlations as shown in Fig. 1. This was also indicated by the following molecular mechanics (MM) calculation study using the MM2* force field as implemented in Macro-model 6.0. 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 (MD) calculations in vacuo at 300 K with a path length of 100 ps and followed by minimizing random structures sampled after multiple 1 ps intervals. The most stable conformer thus obtained, whose boltzmann population was 97.5% at 300 K, showed 177.5° for the H_{20}–C_{20}–C_{22}–H_{22} torsion angle and 138.8° for the H_{22}–C_{22}–C_{23}–H_{23} torsion angle. The observed proton coupling constants, $^3J_{H\text{-}20,H\text{-}22}$=11.2 Hz and $^3J_{H\text{-}22,H\text{-}23}$=7.2 Hz, almost corresponded to those (10.8 Hz and 7.1 Hz, respectively) calculated through the application of the given dihedral angles to the advanced Karplus-type equation proposed by Altona et al.[4]

Table 2
The GI_{50}, TGI and LC_{50} values of compound **1** against the 38 cell lines[a]

Panel/Cell Line	GI_{50} (μM)	TGI (μM)	LC_{50} (μM)	Panel/Cell Line	GI_{50} (μM)	TGI (μM)	LC_{50} (μM)
Breast Cancer				**Melanoma**			
HBC-4	1.55	—[b]	—	LOX IMVI	0.18	0.85	—
BSY-1	0.066	0.44	—				
HBC-5	0.29	2.05	—	**Ovarian Cancer**			
MCF-7	0.51	7.10	—	OVCAR-3	—	—	—
MDA-MB-231	2.21	—	—	OVCAR-4	2.00	—	—
				OVCAR-5	2.35	—	—
CNS Cancer				OVCAR-8	—	—	—
U251	0.11	—	—	SK-OV-3	7.97	—	—
SF-268	—	—	—				
SF-295	0.031	0.10	4.10	**Renal Cancer**			
SF-539	0.042	0.39	7.46	RXF-631L	0.39	3.32	—
SNB-75	0.019	0.093	—	ACHN	—	—	—
SNB-78	0.20	3.32	—				
				Stomach Cancer			
Colon Cancer				St-4	5.91	—	—
HCC-2998	—	—	—	MKN1	—	—	—
KM12	1.05	—	—	MKN7	2.89	—	—
HT29	3.99	—	—	MKN28	—	—	—
HCT-15	8.94	—	—	MKN45	7.27	—	—
HCT-116	—	—	—	MKN74	0.16	—	—
Lung Cancer				**Prostate Cancer**			
NCI-H23	—	—	—	DU-145	—	—	—
NCI-H226	2.52	—	—	PC-3	1.18	—	—
NCI-H522	0.59	8.15	—				
NCI-H460	0.10	—	—				
A549	0.83	—	—	**Mean Conc.**	1.55	23.0	74.4
DMS273	0.030	0.11	0.58				
DMS114	0.33	—	—				

a) The LC_{50} is the concentration at which only 50% of the cells are viable, the GI_{50} value is the concentration that yields 50% growth, and the total growth inhibition (TGI) is the concentration at which no growth was observed.
b) The value is more than 10 μM.

The cytotoxic activity of **1** on HL-60 human promyelocytic leukemia cells was evaluated. The cells were continuously treated with **1** for 72 h, and the cell growth was measured with an MTT assay procedure. The IC_{50} value was calculated from a dose-dependent curve as 0.057 μM, which was as potent as those of the clinically applied anticancer agents, etoposide (IC_{50} 0.025 μM) and methotrexate (IC_{50} 0.012 μM). The activity of the deacyl derivative (**1a**: IC_{50} 1.7 μM) was far less potent than that of **1**, indicating that the aromatic acid group attached to the aglycone plays an important role for the

appearance of the potent activity of **1** as a whole. Subsequent evaluation of **1** in the Jpn. Fdn. for Cancer Res. 38 cell line assay[5] showed that the mean concentrations required to achieve GI_{50}, TGI, and LC_{50} levels against the panel cells tested were 1.55, 23.0, and 74.4 μM, respectively (Table 2). Compound **1** displayed differential cytotoxicities, with breast cancer, CNS cancer, and lung cancer subpanel cell lines showing particular sensitivity but with colon cancer, ovarian cancer, and stomach cancer subpanel cell lines being relatively resistant to it. The pattern of differential cytotoxicity of **1** was evaluated by Compare Program and was revealed not to be correlated with that shown by any of the other compounds, including currently used anticancer drugs (correlation coefficient value is less than 0.5). This indicates that **1** may have a new mode of action. Some in vivo xenograft work on **1** is now in progress.

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