Cytotoxic 3,4-*seco*-Cycloartane Triterpenes from the Exudate of *Gardenia tubifera*

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Four new 3,4-*seco*-cycloartanes, gardenoins A−D (**1**−**4**), together with the known compound secaubryenol (**5**), were isolated from the exudate of *Gardenia tubifera*. The structures of **1**−**4** were elucidated on the basis of spectroscopic analysis. The cytotoxic activity of compounds **1**−**4** was evaluated against five human tumor cell lines.

Plants belonging to the genus *Gardenia* have proven to be a rich source of cycloartane triterpenoids, some of which display interesting biological activities including cytotoxic and anti-HIV effects.[1−5] Previous investigations have shown that the extracts of various *Gardenia* species exhibited anti-implantation and abortifacient effects,[6] and antiulcer,[7] antibacterial,[8] diuretic,[9] analgesic,[9] hypertensive, and larvicidal activities.[10] Recently, we reported the isolation and structural elucidation of five 3,4-*seco*-cycloartanes from the EtOAc extract of the apical buds of *G. sootepensis*.[11] In a continuation of our project on the discovery of anticancer agents from plants in the genus *Gardenia*, we report herein the isolation and identification of four new 3,4-*seco*-cycloartane triterpenes, gardenoins A−D (**1**−**4**), and the known compound secaubryenol (**5**). Compounds **1**−**4** were evaluated for cytotoxic activity against human breast (BT474), lung (CHAGO), gastric (KATO-3), colon (SW-620), and liver (Hep-G2) cancer cell lines.

Results and Discussion

The exudate collected on the aerial parts of *G. tubifera* was dissolved in a 1:1 mixture of CH_2Cl_2 and MeOH, which was then subjected to silica gel column chromatography using EtOAc−hexane mixtures of increasing polarity as eluent. Further purification by repeated normal column chromatography gave four new 3,4-*seco*-cycloartane triterpenes (**1**−**4**) and the known compound secaubryenol (**5**).[5] The structure of **5** was determined by comparison of its NMR spectroscopic data with those in the literature.

Gardenoin A (**1**) was obtained as a white, amorphous solid. Its molecular formula was determined as $C_{30}H_{40}O_5$ from the HRESIMS ion at *m*/*z* 503.2771 $[M + Na]^+$ (calcd 503.2773), which indicated 11 degrees of unsaturation. The IR spectrum showed absorption bands for hydroxy (3350 cm^{-1}) and carbonyl (1732 cm^{-1}) groups. Analysis of ^{13}C and HSQC spectra revealed the presence of 30 nonequivalent carbons including two carbonyl carbons (δ_C 177.9 and 170.7), two sp^2 oxygenated carbons [one quaternary C (δ_C 155.2) and one CH (δ_C 137.4)], four sp^2 carbons [two quaternary C (δ_C 139.1 and 120.4), one CH (δ_C 109.1), and one CH_2 (δ_C 123.2)], one sp^3 oxgenated methine (δ_C 74.4), nine sp^3 methylenes (δ_C 35.0, 34.8, 32.8, 31.0, 30.7, 27.8, 27.2, 26.5, and 23.0), four sp^3 methines (δ_C 51.3, 39.0, 38.3, and 36.3), four quaternary carbons (δ_C 48.7, 45.8, 28.1, and 25.1), and four methyl carbons (δ_C 20.1, 18.8, 15.9, and 9.8). The ^1H NMR spectrum (Table 1) displayed a pair of doublets at δ_H 0.18 and 0.44 (J = 5.3 Hz), characteristic of the C-19 methylene protons of the cyclopropane ring of a cycloartane triterpene.[11−16] A pair of doublets at δ_H 5.74 and 6.34 (J = 1.6 Hz) was ascribed to H-28a and H-28b in the exocyclic methylene γ-lactone ring, and signals of the β- and γ-methine protons of the lactone ring appeared at δ_H 3.24 (H-5) and 4.76 (H-6), respectively. An observed HMBC correlation from H-6 to C-29 (δ_C 170.7) (Figure 1) was used to confirm the lactonization of C-4 to C-6. These data suggested that **1** is a 3,4-*seco*-cycloartane triterpenoid. Additionally, the NMR spectra indicated the presence of a disubstituted furan ring (δ_H 5.86 and 7.06; δ_C 155.2, 137.4, 120.4, and 109.1). On the basis of HMBC data, the cross-peak observed from Me-27 to C-25 and C-24 and from H_2-22 to C-23 and C-24 allowed H_2C-22 and Me-27 to be connected to C-23 and C-25 of the furan ring, respectively. These results, together with the lack of coupling between the two hydrogen atoms of the furan at δ_H 5.86 and 7.06, demonstrated that the furan ring is 2,4-disubstituted. The above data were closely related to those previously reported for dikamaliartane E,[17] with the only

Table 1. ^1H NMR Data of Compounds **1**−**4** (400 MHz, δ in ppm, J in Hz)

position	**1**[a]	**2**[a]	**3**[b]	**4**[a]
1	1.61 m	1.60 m	1.26 m	1.35 m
	2.26 m	2.04 m	1.94 m	2.13 m
2	2.46 m	2.46 m	2.13 m	2.30 m
	2.56 m	2.50 m		2.50 m
5	3.24 d (8.1)	3.23 d (8.3)	2.60 d (8.8)	2.50 m
6	4.76 dd (7.6, 7.8)	4.75 dd (6.9, 8.0)	4.18 dd (3.5, 8.8)	0.93 m
				1.67 m
7	1.54 m	1.50 m	3.44 dd (3.8, 4.9)	1.30 m
	1.81 m	1.75 m		
8	2.24 m	2.15 t (5.5)	2.01 d (6.2)	1.52 m
11	1.55 m	1.54 m	1.26 m	1.25 m
	1.79 m	1.78 m	1.42 m	2.10 m
12	1.61 m	1.60 m	1.42 m	1.67 m
15	1.37 m	1.36 m	1.40 m	1.31 m
16	1.37 m	1.42 m	1.40 m	1.91 m
	1.98 m	2.04 m		
17	1.61 m	1.60 m	1.39 m	1.57 m
18	0.96 s	0.95 s	0.71 s	0.96 s
19	0.18 d (5.3)	0.16 d (5.3)	−0.08 d (4.7)	0.47 d (3.9)
	0.44 d (5.3)	0.42 d (5.3)	0.89 m	0.71 d (3.9)
20	1.77 m	1.80 m	1.72 m	1.34 m
21	0.86 d (6.3)	0.86 d (6.5)	0.89 m	0.85 d (6.3)
22	2.15 m	2.25 m	2.22 m	1.73 m
	2.0 m	2.70 dd (2.6, 14.6)	2.72 d (2.4, 14.6)	2.17 m
23				5.59 br s
24	5.86 s	5.85 s	5.84 s	5.59 br s
26	7.06 s	7.06 s	7.01 s	1.31 br s
27	1.99 s	1.98 s	1.85 s	1.31 br s
28	5.74 br s	5.73 d (1.8)	5.17 d (1.9)	5.08 br s
	6.34 br s	6.33 d (1.8)	6.18 d (1.9)	
29				4.12 br s
30	0.91 s	0.91 s	0.71 s	0.91 s
OMe		3.69 s	3.39 s	

[a] Recorded in $CDCl_3$. [b] Recorded in C_6D_6.

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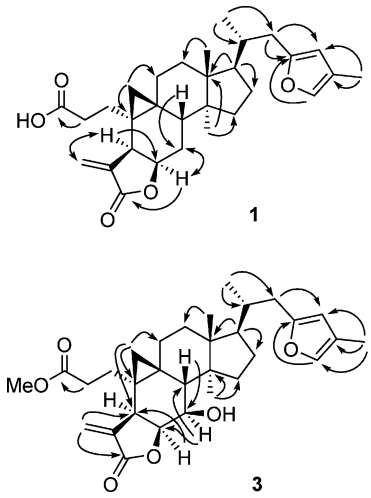

Figure 1. Key HMBC correlations of **1** and **3**.

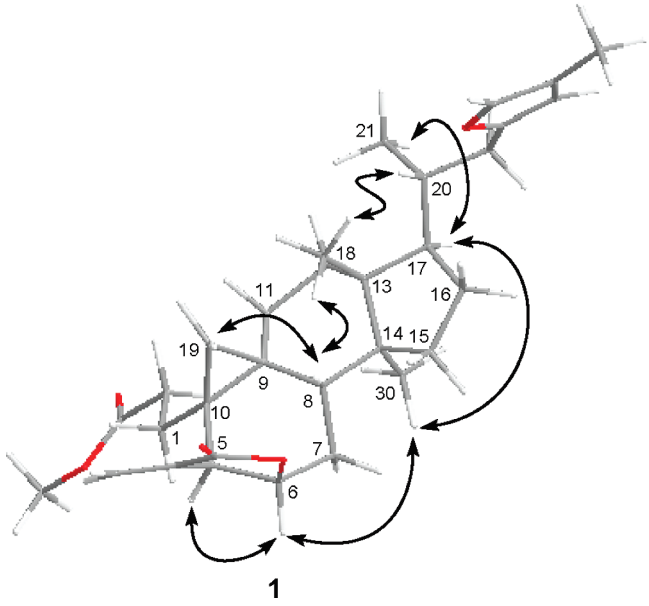

Figure 2. Key NOESY correlations of **1**.

difference being the absence of the hydroxy group at C-7 in dikamaliartane E.

The relative stereochemistry of **1** was elucidated by NOESY experiment (Figure 2). The 5,6-*cis*-configuration has been found to be exclusively β in all known 3,4-*seco*-cycloartanes.[1,3,5,11,17] Consistent with this, for compound **1**, the cross-peaks in the NOESY spectrum from H-5 to H-6, H-6 to Me-30, Me-30 to H-17, and H-17 to Me-21 indicated an α-orientation of these protons. Additionally, NOESY correlations of H-8 with H-19β and Me-18, and Me-18 with H-20, suggested that H-8, Me-18, and H-20 are in a β-orientation. These were in good agreement with the relative configurations at C-5, C-6, C-8, C-9, C-10, C-13, C-14, C-17, and C-20, long-established for the cycloartane core.[1,3,5,11]

Gardenoin B (**2**) was isolated as a white, amorphous solid with the molecular formula $C_{31}H_{42}O_5$ as determined by the HRESIMS ion at *m*/*z* 517.2929 $[M + Na]^+$ (calcd 517.2930). In the ^1H NMR spectrum, the typical signals for a cyclopropane methylene proton appeared as two doublets at δ_H 0.16 and 0.42 ($J = 5.3$ Hz), and its NMR data were almost the same those of **1**, except for the presence of a methoxy group at C-3 in **2**. This was confirmed by the HMBC correlation of the singlet methoxy protons at δ_H 3.69 to the carbonyl carbon at δ_C 173.5, suggesting that **2** is the methyl ester derivative of **1**. The relative configuration was determined to be the same as **1** from the NOESY spectrum.

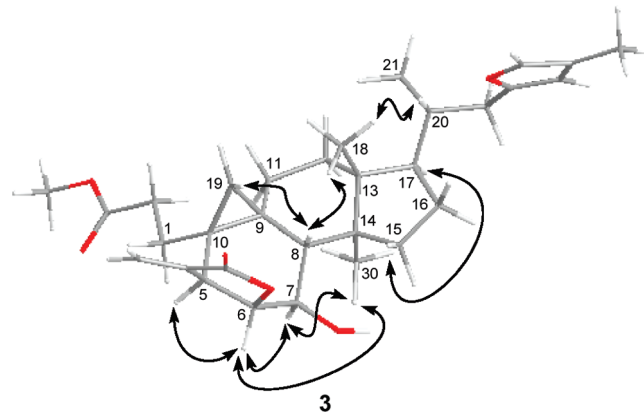

Figure 3. Key NOESY correlations of **3**.

Gardenoin C (**3**) was isolated as a white, amorphous solid with an evaluated molecular formula of $C_{31}H_{42}O_6$ by the HRESIMS ion at *m*/*z* 533.2878 $[M + Na]^+$ (calcd 533.2879). Comparison of the ^1H and ^{13}C NMR spectra of **3** with those of **2** revealed these to be very similar. Significant differences appeared only in the resonances corresponding to position C-7. The replacement in **3** of the two multiplets (δ_H 0.16 and 0.42) in **2** by a double doublet at δ_H 3.44 ($J = 3.8$, 4.9 Hz), coupled in the HSQC spectrum to a newly appearing oxymethine resonance at δ_C 69.1, indicated the occurrence of a hydroxy group at C-7. This was also confirmed by the HMBC correlations (Figure 1) of H-7/C-5, H-7/C-8, and H-8/C-7. The similar NOESY correlations between **3** (Figure 3) and **1** (Figure 2) were indicative of the same stereochemistry of the core skeleton of **3** as compared to **1**. The key NOE cross-peak for **3** between H-7 and H-6 and between H-7 and Me-30 confirmed the α-orientation of H-7.

Gardenoin D (**4**) was isolated as a white, amorphous solid, and its molecular formula was deduced as $C_{30}H_{48}O_4$ from the HRESIMS data (*m*/*z* 495.3452 $[M + Na]^+$, calcd 495.3450), suggesting seven degrees of unsaturation. The ^1H NMR spectrum displayed the typical signals associated with a 3,4-*seco*-cycloartane triterpene, including a characteristic pair of doublets at δ_H 0.47 and 0.71 ($J =$ 3.9 Hz), attributable to the C-19 methylene protons in the cyclopropane ring, two tertiary methyl singlets at δ_H 0.91 and 0.96, and one secondary methyl doublet at δ_H 0.85 ($J = 6.3$ Hz). Allylic coupling observed in the COSY spectrum between a two-proton broad singlet at δ_H 4.12, accounting for a primary alcoholic group and a two-proton broad singlet of a terminal alkene at δ_H 5.08, was suggestive of the structure of a 29-hydroxy-3,4-*seco*-cycloartane. Both ^1H and ^{13}C NMR signals of **4** were very similar to those of secaubrytriol,[5] with the marked differences being the appearance of a two-proton broad singlet due to a disubstituted alkene moiety between C-23 and C-24 at δ_H 5.59, coupled to the carbon resonances at δ_C 139.2 and 125.6 in the HSQC spectrum, and the absence of a hydroxy group attached to C-24 in secaubrytriol. Furthermore, the NMR signals attributable to the side chain of **4** closely resembled those previously described for cucurbita-5,23(*E*)-diene-3β,7β,25-triol,[18] giving evidence for a (3*E*)-2-hydroxy-2-methylhept-3-en-6-yl unit attached at C-17. Therefore, the structure of **4** was established as shown. The same NOESY correlations of H-8/Me-18, H-8/H-19β, H-17/Me-21, H-17/Me-30, and H-20/Me-18 as for secaubryenol (**5**) allowed the relative stereochemistry of **4** to be depicted as shown.

The cytotoxicity of compounds **1**−**4** was tested against five human tumor cell lines (Table 3). Compound **1** exhibited cytotoxicity against the CHAGO and Hep-G2 cancer cell lines, with IC_{50} values of 1.6 and 4.5 μg/mL, respectively. Compound **3** was also active against the CHAGO, Hep-G2, and SW-260 cancer cell lines, with IC_{50} values of 4.4, 2.8, and 2.5 μg/mL, respectively, whereas compounds **2** and **4** did not show cytotoxicity for any of the cell

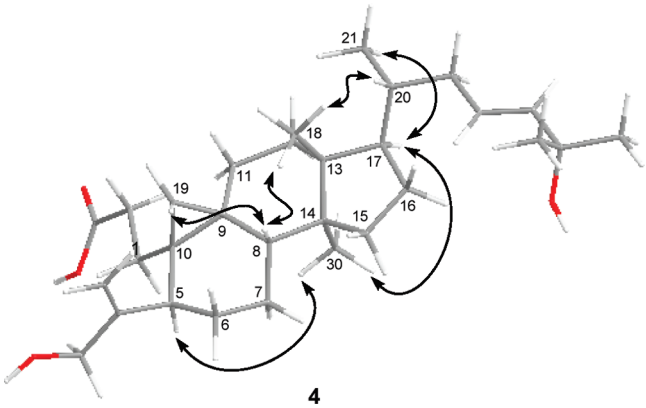

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Figure 4. Key NOESY correlations of **4**.

Table 2. ^{13}C NMR Data of Compounds **1**−**4** (400 MHz, δ in ppm)

position	**1**[a]	**2**[a]	**3**[b]	**4**[a]
1	30.7	30.9	31.5	28.7
2	31.0	31.2	35.2	31.5
3	177.9	173.5	173.0	179.0
4	139.1	139.1	140.1	152.1
5	39.0	39.0	39.6	41.9
6	74.4	74.4	77.1	28.9
7	27.2	27.2	69.1	25.2
8	38.3	38.3	46.0	47.9
9	25.1	25.0	24.7	21.8
10	28.1	28.2	28.3	27.3
11	26.5	26.5	26.8	26.9
12	32.8	32.8	32.6	32.8
13	45.8	45.8	46.2	45.1
14	48.7	48.7	47.6	48.8
15	34.8	34.8	36.4	35.7
16	27.8	27.8	28.4	28.0
17	51.3	51.3	51.2	51.9
18	15.9	15.8	16.4	18.2
19	23.0	22.9	25.9	30.2
20	36.3	36.3	36.7	36.3
21	18.8	18.8	19.0	18.2
22	35.0	35.0	35.5	39.0
23	155.2	155.2	155.6	125.6
24	109.1	109.0	109.5	139.2
25	120.4	120.3	120.6	70.9
26	137.4	137.3	138.0	29.8
27	9.8	9.8	9.9	29.8
28	123.2	123.2	120.3	110.6
29	170.7	170.7	170.2	64.6
30	20.1	20.1	20.1	19.3
OMe		51.8	51.3	

[a] Recorded in $CDCl_3$. [b] Recorded in C_6D_6.

Table 3. Cytotoxic Data for Compounds **1**−**4**

	IC$_{50}$ (μg/mL)/cell line				
compound	BT-474	KATO-3	CHAGO	SW-620	Hep-G2
1	>5	>5	1.6	>5	4.5
2	>5	>5	>5	>5	>5
3	>5	>5	4.4	2.5	2.8
4	>5	>5	>5	>5	>5

lines tested. Thus, this result revealed that an exomethylene γ-lactone ring system is required for the cytotoxicity of the compounds in this group, as previously reported.[5,11]

Experimental Section

General Experimental Procedures. Melting points were measured using a Fisher-Johns melting point apparatus. Optical rotations were measured on a Perkin-Elmer 341 polarimeter using a sodium lamp at wavelength 589 nm, and UV data were recorded on a Shimadzu UV-160 spectrophotometer. IR spectra were recorded on a Bruker vector22 Fourier transform infrared spectrophotometer. The NMR spectra were recorded on a Varian YH400 spectrometer at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR using TMS (trimethylsilane) as the internal standard. HRESIMS were obtained using a Bruker micrOTOF mass spectrometer.

Plant Material. The exudate was manually collected on the fresh aerial parts of *G. tubifera* from Bangkhan, Bangkok, Thailand, from April to June 2009. A voucher specimen (BKF 159044) has been deposited at the Forest Herbarium, Royal Forest Department, Bangkok, Thailand.

Extraction and Isolation. The dried exudate (5.74 g) of *G. tubifera* was dissolved in a 1:1 mixture of CH_2Cl_2 and MeOH (10 mL). This solution was subsequently subjected to passage over a silica gel column eluted with a gradient system of hexane−EtOAc (from 1:0 to 3:2) to yield 12 fractions (I−XII). Precipitation from fraction V (3.27 g) led to the isolation of the pure compound **1** (26.9 mg) after filtration. Fraction IX (2.3 g) was rechromatographed on a silica gel column using a gradient system of acetone−hexane (from 1:4 to 3:7) to give **3** (24.2 mg). Fraction X (1.18 g) was further purified using a silica gel column eluting with acetone−hexane (1:3) to afford **2** (52.1 mg). Fraction XII (2.0 g) was subjected to silica gel column chromatography eluted with a gradient system of acetone−hexane (from 1:2 to 1:1) to yield **4** (56.7 mg) and **5** (61.8 mg).

Gardenoin A (1): white, amorphous solid; mp 92−93 °C; $[\alpha]^{20}_D$ +111 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 215 (4.07) nm; IR (KBr) ν_{max} 3350, 2918, 2851, 1732, 1457, 1379, 1270, 1178, 992 cm^{-1}; ^{1}H and ^{13}C NMR ($CDCl_3$), see Tables 1 and 2; HRESIMS *m*/*z* 503.2771 (calcd for $C_{30}H_{40}O_5Na$, 503.2773).

Gardenoin B (2): white, amorphous solid; mp 82−83 °C; $[\alpha]^{20}_D$ +41 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 210 (3.12) nm; IR (KBr) ν_{max} 2918, 2850, 1762, 1737, 1464, 1437, 1298, 1278, 1172, 942 cm^{-1}; ^{1}H and ^{13}C NMR ($CDCl_3$), see Tables 1 and 2; HRESIMS *m*/*z* 517.2929 (calcd for $C_{31}H_{42}O_5Na$, 517.2930).

Gardenoin C (3): white, amorphous solid; mp 98−100 °C; $[\alpha]^{20}_D$ +134 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 210 (4.04) nm; IR (KBr) ν_{max} 3456, 2934, 2875, 1712, 1654, 1458, 1376, 1272, 1143, 941 cm^{-1}; ^{1}H and ^{13}C NMR ($CDCl_3$), see Tables 1 and 2; HRESIMS *m*/*z* 533.2878 (calcd for $C_{31}H_{42}O_6Na$, 533.2879).

Gardenoin D (4): white, amorphous solid; mp 91−92 °C; $[\alpha]^{20}_D$ +113 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 210 (4.02) nm; IR (KBr) ν_{max} 3449, 2939, 2871, 1709, 1647, 1457, 1377, 1278, 1169, 1025, 899 cm^{-1}; ^{1}H and ^{13}C NMR ($CDCl_3$), see Tables 1 and 2; HRESIMS *m*/*z* 495.3452 (calcd for $C_{30}H_{48}O_4Na$, 495.3450).

In Vitro Cytotoxicity Bioassays.[19,20] All stock cultures were grown in T-25 flasks. Freshly trypsinized cell suspensions were seeded in 96-well microtiter plates at densities of 5000 cells per well with compounds added from DMSO-diluted stock. After three days in culture, attached cells were stained with MTT (3-[4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium] bromide). The absorbance at 540 nm was measured using a microplate reader after solubilizing the bound dye. The mean IC$_{50}$ is the concentration of agent that inhibits cell growth by 50% under the experimental conditions and is the average from at least six independent determinations that were reproducible and statistically significant. The following human tumor cell lines were used in the assay: human breast ductal carcinoma ATCC No. HTB 20 (BT474), undifferentiated lung carcinoma (CHAGO), liver hepatoblastoma (Hep-G2), gastric carcinoma ATCC No. HTB 103 (KATO-3), and colon adenocarcinoma ATCC No. CCL 227 (SW-620). All cell lines were obtained from the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, and were cultured in RPMI-1640 supplemented with 25 mM HEPES, 0.25% (w/v) sodium bicarbonate, 5% (v/v) fetal bovine serum, and 100 μg/mL kanamycin.

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra of compounds **1**−**4** are available free of charge via the Internet at http://pubs.acs.org.

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