Cytotoxic 20,22-Dihydrodigitoxigenin Glycosides and Other Constituents of *Vallaris glabra* Stems

Sudarat Kruakaew, Chonticha Seeka, Jantana Yahuafai, Pongpun Siripong, and Somyote Sutthivaiyakit

Supporting Information

Structural Identification of 7-10

Compound **7** was isolated as an amorphous solid with molecular formulas of $C_{42}H_{68}O_{18}$ based on its HRESIMS data. The ^{1}H and ^{13}C NMR spectra of **7** exhibited resonances rather similar to those of **3**, except that **7** possesses an acofriopyranosyl unit instead of a vallarosyl group, together with recognizable resonances of two glucopyranosyl units in **7** (Table S1, Supporting Information), respectively. On the basis of previous study[2] and HMBC spectra of **7** showing cross-peaks between H-1′/C-3, H-1″/C-4′ and H-1‴/C-6″, compound **7** was 20,22-dihydrodigitoxigenin-3-*O*-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranosyl-(1→4)-*α*-L-acofriopyranoside.

Compound **8** was isolated as an amorphous solid exhibiting a molecular formula of $C_{44}H_{70}O_{19}$. The ^{1}H and ^{13}C NMR spectra showed resonances similar to those of **7** (Table S1, Supporting Information), but with additional resonances of an acetyl group (δ_H 2.10, s) and a less-shielded H-2′ resonance at δ_H 5.17 (dd, J = 3.2 and 1.8 Hz). Based on the 2-D NMR data, **8** was thus proposed as 20,22-dihydrodigitoxigenin-3-*O*-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranosyl-(1→4)-*α*-L-2′-*O*-acetylacofriopyranoside.

Compound **9** was obtained as an amorphous solid, its HRESIMS exhibited a $[M+Na]^+$ ion at *m/z* 897.4056 (calcd for $C_{42}H_{66}O_{19}Na$, 897.4077). The ^{1}H and ^{13}C NMR spectra showed rather complex sets of resonances, although the characteristic resonances of a gitoxigenin and vallarosyl groups could be recognized (Table S2, Supporting Information). Additional resonances, particularly of the two anomeric [δ_H 4.39 (d, J = 7.8 Hz, H-1″, and δ_C 102.5, C-1″) and δ_H 4.39 (d, J = 7.8 Hz, H-1‴, and δ_C 105.0, C-1‴)] and two oxymethylene groups [δ_H 4.14 (dd, J =11.8 and 1.9 Hz), δ_C 70.2, C-6″, and δ_H 3.86 (dd, J = 11.5 and 1.8 Hz), and 3.66 (dd, J = 11.5 and 5.3 Hz) and δ_C 62.8, C-6‴], indicated the presence of two glucosyl groups in **9**. The connectivities of C-3-*O* to C-1′, C-4′-*O* to C-1″ and C-6″-*O* to C-1‴ were based on the HMBC cross-peaks between H-1′/C-3; H-1″/C-4′, and H-1‴/C-6″, respectively. On the basis of previous report which provided L-vallarose, L-acofriose and D-glucose after acid hydrolyses of oleandrigenin-3-*O*-*α*-L-2′-*O*-acetylvallaropyranoside and oleandrigenin-3-*O*-*β*-D-glucopyranosyl-(1→4)-*α*-L-2′-*O*-acetylacofriopyranoside,[2] **9** was thus proposed as gitoxigenin-3-*O*-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranosyl-(1→4)-*α*-L-vallaropyranoside.

Compound **10** was isolated as an amorphous colorless solid with same molecular mass as of **9**. The ^{1}H and ^{13}C NMR spectra exhibited resonances similar to those of **9** (Table S2, Supporting Information), although the resonances for a vallarosyl moiety were replaced by those of an

acofriosyl group, showing an indicative resonance for H-5′ at somewhat higher-field than that of a vallarosyl group in **9**. Based on its spectroscopic data and a previous study, **10** was therefore concluded to be gitoxigenin-3-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→4)-α-L-acofriopyranoside.

Isolation of Compounds 7-16

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were measured using an Electrothermal melting point apparatus and are uncorrected. Optical rotations were recorded on a JASCO DIP 1020 polarimeter. The IR spectra were obtained on a Perkin-Elmer 1760x FT-IR spectrophotometer. The ^{1}H and ^{13}C NMR spectra were recorded with a Bruker AVANCE III HD 400 MHz NMR spectrometer. Chemical shifts are referenced to the residual solvent signals (MeOH-d_4: δ_H 3.30 and δ_C 49.0 ppm). HRESIMS were recorded on a Bruker DaltonicsmicroTOF mass spectrometer.

Plant Material. The plant investigated, *Vallaris glabra*, was obtained as previously reported. A voucher specimen (SSVG-1/2012) is maintained at the Department of Chemistry, Ramkhamhaeng University.[2]

Extraction and Isolation. Dried *V. glabra* stems (4.5 kg) were ground and extracted successively with hexanes (8 L), CH_2Cl_2 (8 L) and MeOH (8 L), respectively, using a Soxhlet extractor. The resultant hexanes (70.0 g), CH_2Cl_2 (59.9 g) and MeOH (95.2 g) extracts were obtained after removal of solvent.

The CH_2Cl_2 extract (59.9 g) was fractionated by column chromatography (CC, silica gel, hexanes- CH_2Cl_2 65:35 to CH_2Cl_2-MeOH 85:15) to obtain ten fractions. Fraction 3 (3.65 g) was fractionated by CC (Sephadex LH-20, MeOH) to give four subfractions (3.1-3.4). Subfraction 3.3 (1.37 g) was fractionated (CC, silica gel, hexanes-EtOAc 70-30) to give seven subfractions (3.3.1-3.3.7). Subfraction 3.3.1 provided ursolic acid (30.8 mg) and subfraction 3.3.2 provided 3,27-dihydroxyursolic acid (19.1 mg) after recrystallization from CH_2Cl_2/MeOH. Subfraction 3.3.3 (134.0 mg) was purified using CC (silica gel, hexanes-EtOAc 70:30) to give three subfractions (3.3.3.1-3.3.3.3), and subfraction 3.3.3.2 (42.4 mg) was further purified by CC (silica gel, CH_2Cl_2-MeOH 99:1) to afford **15** (8.3 mg), **11** (4.2 mg) and **13** (13.8 mg). Subfraction 3.3.5 (190.3 mg) was subjected to CC (silica gel, hexanes-EtOAc 70:30) to give **16** (22.0 mg). Fraction 4 (1.65 g), after fractionation (CC, Sephadex LH-20, MeOH), provided three subfractions (4.1-4.3). Subfraction 4.2 (703.6 mg) was further fractionated (Sephadex LH-20, MeOH, then CC, silica gel, CH_2Cl_2-MeOH 98:2) to give additional amount of **11** (10.9 mg) and **13** (3.8 mg), and also **14** (39.5 mg) and **12** (31.7 mg).

The MeOH extract (95.2 g) was fractionated by CC (Dianion HP-20, MeOH-H_2O, 0:100 to 100:0) to obtain five fractions. The water-soluble fractions 1-3, containing mostly sugars, were not investigated further. The less polar fraction 5 (6.35 g) was fractionated by reversed-phase CC (RP-18, MeOH-H_2O 30:70 to 100:0) to obtain six subfractions (5.1-5.6). Subfraction 5.2 (820.3 mg) was purified by CC (silica gel, CH_2Cl_2-MeOH, 88:12 to 85:15) and provided three subfractions (5.2.1- 5.2.3). Subfraction 5.2.2 (79.2 mg) after reversed-phase CC (RP-18, MeOH-H_2O 40:60 to 100:0) furnished oleandrigenin-3-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→4)-α-L-2′-*O*-acetylacofriopyranoside (48.3 mg) and **8** (4.2 mg). Subfraction 5.4 (1.29 g) was fractionated by CC (RP-18, MeOH-H_2O 10:90 to 100:0) to give eight

subfractions (5.4.1-5.4.8). Oleandrigenin-3-*O*-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranosyl-(1→4)-*α*-L-vallaropyranoside[2] (38.1 mg) was obtained from subfraction 5.4.3.4. Subfraction 5.4.5 (147.1 mg) was CC (silica gel, CH_2Cl_2-MeOH 88:12 to 86:14, then RP-18, MeOH-H_2O 45:55 to 100:0) to give oleandrigenin-3-*O*-*β*-D-glucopyranosyl-(1→4)-*α*-L-*O*-acofriopyranoside (11.2 mg) and oleandrigenin-3-*O*-*β*-D-glucopyranosyl-(1→4)-*α*-L-*O*-vallaropyranoside (2.3 mg). Subfraction 5.4.6 (243.5 mg) was purified by CC (silica gel, CH_2Cl_2-MeOH 88:12 to 86:14) to give five subfractions (5.4.6.1-5.4.6.5). Subfraction 5.4.6.5 (33.9 mg) after further purification (CC, RP-18, MeOH-H_2O 40:60 to 100:0) provided **7** (4.7 mg). Subfraction 5.4.8 (270.5 mg) was subjected to CC (silica gel, CH_2Cl_2-MeOH 85:15 to 80:20) to give four subfractions (5.4.8.1-5.4.8.4). Subfraction 5.4.8.1 (12.6 mg) afforded **3** (2.9 mg) and **4** (1.7 mg) after CC (RP-18, MeOH-H_2O 50:50 to 100:0). Subfraction 5.4.8.3 (29.0 mg) was further purified by CC (RP-18, MeOH-H_2O 55:50 to 100:0) to give additional quantity of **8** (6.3 mg). The polar fraction 4 (2.25 g) was fractionated using CC (silica gel, CH_2Cl_2-MeOH 88:12 to 84:16) to obtain eleven subfractions (4.1-4.11). Selection of compounds based on TLC identity led to **9** (5.3 mg) and **10** (4.0 mg) being obtained from subfraction 4.11 (45.2 mg) after reversed-phase CC (RP-18, MeOH-H_2O 40:60 to 100:0).

*20,22-Dihydrodigitoxigenin-3-O-β-D-glucopyranosyl-(1→6)- β-D-glucopyranosyl-(1→4)- α-L-acofriopyranoside (**7**):* $[\alpha]^{25}_D$ −42.3 (*c* 0.23, MeOH); FT-IR (ATR) ν_{max} 3366, 2922, 2873, 2855, 1746, 1450, 1379, 1234, 1199, 1105, 1068, 1045, 1016, 986 cm^{-1}; ^1H NMR (MeOH-d_4, 400 MHz) and ^{13}C NMR (MeOH-d_4, 100 MHz) data see Table S1, Supporting Information; HRESIMS *m/z* 883.4315 $[M + Na]^+$ (calcd for $C_{42}H_{68}NaO_{18}$, 883.4284).

*20,22-Dihydrodigitoxigenin-3-O-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→4)-α-L-2'-O-acetylacofriopyranoside (**8**):* $[\alpha]^{25}_D$ −40.7 (*c* 0.34, MeOH); FT-IR (ATR) ν_{max} 3369, 2928, 2884, 2865, 1732, 1723, 1447, 1376, 1236, 1214, 1122, 1094, 1067, 1038, 1019, 987 cm^{-1}; ^1H NMR (MeOH-d_4, 400 MHz) and ^{13}C NMR (MeOH-d_4, 100 MHz) data see Table S1, Supporting Information; HRESIMS *m/z* 925.4407 $[M + Na]^+$ (calcd for $C_{44}H_{70}NaO_{19}$, 925.4389).

*Gitoxigenin-3-O-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→4)-α-L-vallaropyranoside (**9**):* $[\alpha]^{25}_D$ −35.9 (*c* 0.26, MeOH); FT-IR (ATR) ν_{max} 3333, 2922, 2882, 2858, 1732, 1627, 1603,1453, 1349, 1269, 1165, 1068, 1027, 1014 cm^{-1}; ^1H NMR (MeOH-d_4, 400 MHz) and ^{13}C NMR (MeOH-d_4, 100 MHz) data see Table S2, Supporting Information; HRESIMS *m/z* 897.4056 $[M + Na]^+$ (calcd for $C_{42}H_{66}NaO_{19}$, 897.4077).

*Gitoxigenin-3-O-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→4)-α-L-acofriopyranoside (**10**):* $[\alpha]^{25}_D$ −26.4 (*c* 0.22, MeOH); FT-IR (ATR) ν_{max} 3359, 2922, 2854, 1730, 1627, 1449, 1377, 1288, 1243, 1164, 1103, 1068, 1023, 987 cm^{-1}; ^1H NMR (MeOH-d_4, 400 MHz) and ^{13}C NMR (MeOH-d_4, 100 MHz) data see Table S2, Supporting Information; HRESIMS *m/z* 897.4062 $[M + Na]^+$ (calcd for $C_{42}H_{66}NaO_{19}$, 897.4077).

Table S1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR Spectroscopic Data of **7** and **8** (in MeOH-d_4)

	7		**8**	
position	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type
1	1.74, 1.38	30.9, CH_2	1.83, 1.55	31.0, CH_2
2	1.78, 1.15	27.9, CH_2	1.90, 1.25	27.4, CH_2
3	3.83 brs ($W_{1/2}$ = 7.5 Hz)	74.0, CH	3.93 ($W_{1/2}$ = 8.0 Hz)	74.7, CH
4	1.42, 1.30	31.6, CH_2	1.39	31.6, CH_2
5	1.51	38.2, CH	1.65	38.2, CH
6	1.48	27.4, CH_2	1.58	27.8, CH_2
7	1.66	22.4, CH_2	1.77	22.4, CH_2
8	1.56	42.3, CH	1.64	42.4, CH
9	1.60	36.8, CH	1.64	36.4, CH
10	-	36.5, C	-	36.4, C
11	1.28, 1.15	22.1, CH_2	1.25, 1.35	22.1, CH_2
12	1.33, 1.25	42.0, CH_2	1.33, 1.42	42.0, CH_2
13	-	48.5, C	-	48.3, C
14	-	86.8, C	-	86.8, C
15	1.88, 1.45	32.4, CH_2	1.98, 1.56	32.4, CH_2
16	1.83, 1.43	25.3, CH_2	1.93, 1.51	26.2, CH_2
17	1.62	55.3, CH	1.73	55.3, CH
18	0.88 s	16.9, CH_3	0.94 s	16.9, CH_3
19	0.87 s	24.4, CH_3	0.96 s	24.4, CH_3
20	2.77 quint-like (8.7)	40.4, CH	2.86 quint-like (8.8)	40.3, CH
21	4.33 t (8.7), 4.00 t (9.1)[a]	73.9, CH_2	4.41 t (8.8), 4.08 t (9.1)	73.9, CH_2
22	2.57 dd (17.6, 8.5), 2.15 dd (17.6, 10.0)	36.5, CH_2	2.65 dd (17.6, 9.9), 2.23 dd (17.6, 8.5)	36.5, CH_2
23	-	180.8, C	-	180.7, C
1′	4.71 d (2.0)	99.8, CH	4.79 d (1.8)	97.3, CH
2′	3.87 dd (2.0, 3.1)	68.5, CH	5.17 dd (3.2, 1.8)	70.2, CH
3′	3.50 dd (3.1, 8.9)	82.6, CH	3.72 dd (9.4, 3.2)[d]	81.0, CH
4′	3.64 t (8.9)[b]	79.0, CH	3.62 t (9.4)	79.4, CH
5′	3.62 dq (8.9, 5.8)[b]	68.7, CH	3.75 dq (6.3, 9.4)[d]	68.8, CH
6′	1.18 d (5.4)	18.3, CH_3	1.29 d (6.3)	18.3, CH_3
OCH_3	3.34 s	56.3, CH_3	3.39 s	57.7, CH_3
1″	4.51 d (7.8)	104.9, CH	4.58 d (7.8)	104.9, CH
2″	3.11 t (8.4)	75.2, CH	3.14 t (8.6)	75.6, CH
3″	3.25	77.9, CH	3.36 t (8.9)[e]	77.7, CH
4″	3.24	71.6, CH	3.34 t (9.2)[e]	71.8, CH
5″	3.29[c]	77.0, CH	3.42 ddd (5.7, 3.5, 1.9)	76.9, CH

6″	4.04 dd (11.8, 1.8)[a], 3.67[b]	70.5, CH_2	4.13 dd (11.7, 1.9), 3.78 dd (11.7, 5.7)[d]	70.5, CH_2
1‴	4.28 d (7.8)	105.0, CH	4.37 d (7.8)	105.0, CH
2‴	3.08 t (8.1)	75.7, CH	3.21 dd (8.9, 7.8)	75.2, CH
3‴	3.25[c]	77.8, CH	3.36 t (8.9)[e]	78.0, CH
4‴	3.24 t (6.9)	71.8, CH	3.27 t[f]	71.6, CH
5‴	3.17	77.9, CH	3.26 [f]	78.0, CH
6‴	3.77 d (11.1), 3.58 dd (5.0, 11.7)	62.7, CH_2	3.86 dd (12.1, 1.7), 3.66 dd (11.9, 5.1)	62.8, CH_2
$OCOCH_3$-2′			2.10 s	20.9, CH_3 172.2, C

[a-f]Overlapped signals.

Table S2. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR Spectroscopic Data of **9** and **10** (in MeOH-d_4)

	9		**10**	
position	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type
1	1.87, 1.47	31.5, CH_2	1.87, 1.47	31.6, CH_2
2	1.60	27.9, CH_2	1.60	27.4, CH_2
3	3.92 ($W_{1/2}$ = 8.0 Hz)[a]	74.8, CH	3.94 ($W_{1/2}$ = 9.0 Hz)	74.1, CH
4	1.84, 1.52	31.5, CH_2	1.52, 1.40	31.0, CH_2
5	1.77	38.1, CH	1.64	38.2, CH
6	1.98, 1.26	27.6, CH_2	1.90	27.8, CH_2
7	1.43	22.5, CH_2	1.43, 1.24	22.1, CH_2
8	1.58	42.9, CH	1.60	42.9, CH
9	1.66	36.8, CH	1.66	36.8, CH
10	-	36.3, C	-	36.3, C
11	1.80, 1.24	22.1, CH_2	1.83	22.4, CH_2
12	1.56, 1.40	41.0, CH_2	1.55, 1.39	41.0, CH_2
13	-	51.3, C	-	51.3, C
14	-	85.7, C	-	85.6, C
15	2.61 dd (8.4, 14.8), 1.70 dd (2.3, 14.8)	43.8, CH_2	2.62 dd (8.5, 15.0), 1.70 dd (2.2, 15.0)	43.8, CH_2
16	4.64 dd (8.0, 2.0)[b]	73.2, CH	4.64 dt (7.9, 2.3)[e]	73.1, CH
17	3.12 d (7.8)	57.9, CH	3.12 d (7.9)	59.7, CH
18	0.91 s	17.1, CH_3	0.91 s	17.1, CH_3
19	0.94 s	24.4, CH_3	0.95 s	24.4, CH_3
20	-	173.6, C	-	173.6, C
21	5.16 dd (1.6, 16.8), 5.09 dd (1.6, 16.9)	77.9, CH_2	5.16 dd (18.4, 1.7), 5.09 dd (18.5, 1.6)	77.9, CH_2
22	5.93 t (1.6)	120.6, CH_2	5.93 t (1.6)	120.6, CH_2
23	-	177.3, C	-	177.3, C
1′	4.66 d (2.9)[b]	100.3, CH	4.80 d (1.7)	99.8, CH
2′	3.93 dd (5.4, 3.4)[a]	69.3, CH	3.97 dd (3.1, 1.9)	68.6, CH
3′	3.63 dd (5.2, 3.3)	79.0, CH	3.59 dd (8.9, 3.2)	82.7, CH
4′	3.95 dd (7.6, 3.2)	76.8, CH	3.72 [f]	79.1, CH
5′	4.20 quintet (7.1)	67.2, CH	3.71[f]	68.8, CH
6′	1.24 d (6.6)	17.9, CH_3	1.27 d (5.3)	18.3, CH_3
OCH_3	3.48 s	59.7, CH_3	3.43 s	59.9, CH_3
1″	4.39 d (7.8)[c]	102.5, CH	4.60 d (7.8)[e]	104.9, CH
2″	3.21 t (7.9)	75.2, CH	3.16 t (8.6)	75.8, CH

3″	3.36 t (8.8)[d]	77.7, CH	3.34 t (8.6)	77.9, CH
4″	3.31[d]	71.7, CH	3.33 t (9.5)	71.9, CH
5″	3.45 ddd (8.4, 6.3, 2.0)	77.3, CH	3.40 ddd (9.8, 4.9, 2.6)	77.0, CH
6″	4.14 dd (11.8, 1.9)	70.2, CH_2	4.13 dd (11.6, 1.9), 3.75 dd (11.7, 6.0)[f]	70.5, CH_2
1‴	4.39 d (7.8)[c]	105.0, CH	4.37 d (7.8)	105.0, CH
2‴	3.19 t (7.9)	75.3, CH	3.19 t (7.8)	75.2, CH
3‴	3.36[d]	77.8, CH	3.34 t (7.7)	77.9, CH
4‴	3.28	71.7, CH	3.28[g]	71.6, CH
5‴	3.26	78.0, CH	3.26[g]	78.0, CH
6‴	3.86 dd (11.5, 1.8), 3.66 dd (11.5, 5.3)	62.8, CH_2	3.85 dd (11.9, 1.8), 3.66 dd (11.9, 5.3)	62.8, CH_2

[a-g]Overlapped signals.

Table S3. Cytotoxic Activity of Compounds **11-16**[a]

compound	HT-29	A 549	HeLa	Vero
11	>10	2.3 ± 0.1	6.1 ± 0.8	>10
12	>10	2.2 ± 0.3	8.0 ± 1.6	>10
13	0.6 ± 0.1	>10	>10	>10
14	>10	6.5 ± 0.8	7.2 ± 0.5	>10
15	>10	>10	>10	>10
16	>10	6.8 ± 0.1	9.8 ± 0.01	>10
doxorubicin[b]	0.44 ± 0.15	1.0 ± 0.1	0.12 ± 0.05	3.5 ± 0.7

[a] Values indicated are IC_{50} values in μM, data are means ± SD of three independent experiments, each performed in six replicates.[b]Positive control