Kaempulchraols I–O: New isopimarane diterpenoids from *Kaempferia pulchra* rhizomes collected in Myanmar and their antiproliferative activity

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ABSTRACT

The isolation of the $CHCl_3$ soluble extract of *Kaempferia pulchra* rhizomes afforded seven new isopimarane diterpenoids, kaempulchraols I–O, together with one known analogue. The structures of these compounds were elucidated using 1D and 2D NMR and X-ray diffraction analyses. The antiproliferative activity of the isolated compounds was evaluated against a panel of five human cancer cell lines. Kaempulchraol L exhibited weak antiproliferative activity against PANC-1 and PSN-1 cells with IC_{50} values of 39.9 and 22.6 μM, respectively.

Keywords: *Kaempferia pulchra*, isopimarane diterpenoids, kaempulchraols, antiproliferative activity

1. Introduction

Natural products have played important roles as drug resources for humans since time immemorial. Most people in the developing countries rely on traditional medicine for their primary health care. In particular, the people of Myanmar have used traditional formulations which are mainly composed of medicinal plants. Natural products derived from plants have contributed significantly to the development of anticancer drugs. For instance, vinblastine, vincristine, podophyllotoxin, etoposide, indirubins, flavopiridol, rohitukine, paclitaxel and irinotecan are the anticancer agents derived from plants used in traditional medicines.[1,2] In our ongoing research for the discovery of anticancer agents from Myanmar medicinal plants,[3-7] we screened the crude extracts and found that the $CHCl_3$ soluble fraction of rhizomes from *Kaempferia pulchra* Ridl. (Zingiberaceae) exhibited reasonable antiproliferative activity against a panel of five human cancer cell lines including A549 (human lung cancer), HeLa (human cervix cancer), PANC-1 and PSN-1 (human pancreatic cancer), and MDA-MB-231 (human breast cancer), and TIG-3 (normal human primary fibroblast cells). A series of chromatographic separations furnished eight new isopimarane diterpenoids, kaempulchraols A–H, together with five known ones [9α-hydroxyisopimara-8(14),15-dien-7-one,[8] 7β,9α-dihydroxypimara-8(14),15-diene,[9] (1*S*,5*S*,9*S*,10*S*,11*R*,13*R*)-1,11-dihydroxypimara-8(14),15-diene,[10] sandaracopimaradien-1α,2α-diol,[11] and (2*R*)-*ent*-2-hydroxyisopimara-8(14),15-diene][12] in our previous study.[7] As a continuation of that work, the isolation of the remaining fractions obtained from the $CHCl_3$ extract of *K. pulchra* afforded seven new diterpenoids, kaempulchraols I–O (**1**–**7**) together with one known compound (Fig. 1), (1*R*,2*S*,5*S*,9*S*,10*S*,11*R*,13*R*)-1,2,11-trihydroxypimara-8(14),15-diene (**8**).[10] Herein, we report the structure elucidation of the new compounds and the antiproliferative activity of the isolated diterpenoids.

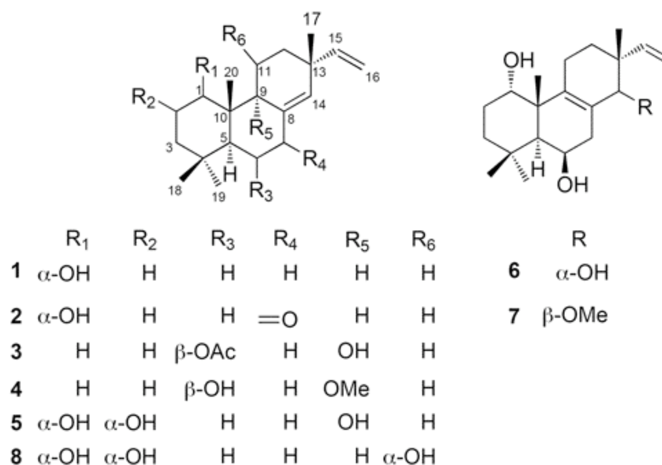

Figure 1. Structures of compounds **1–8** isolated from *K. pulchra* rhizomes.

2. Results and discussion

2.1. Structure elucidation of new compounds

Compound **1** was obtained as an amorphous solid, and its molecular formula was determined as $C_{20}H_{32}O$ from the HREIMS and ^{13}C NMR data. The IR spectrum of **1** showed absorption bands due to hydroxy and olefinic groups at 3386 and 1635 cm^{-1}, respectively. The ^{1}H NMR spectrum (Table 1) displayed signals due to terminal vinyl protons [δ_H 5.79, dd, (J = 17.4, 10.7 Hz, H-15), 4.93, dd (J = 17.4, 1.4 Hz, H-16a), 4.90, dd (J = 10.7, 1.4 Hz, H-16b)], four methines including olefinic and oxygenated ones [δ_H 5.27, br s (H-14), 3.72, br s (H-1β), 2.28, t (J = 7.2 Hz, H-9α), 1.43, m (H-5α)], six methylene signals, and four tertiary methyls [δ_H 1.06 (H_3-17), 0.88 (H_3-18), 0.93 (H_3-19), 0.80 (H_3-20)]. The ^{13}C NMR spectrum (Table 2) revealed 20 signals including four olefins (δ_C 149.0, 137.4, 129.0, 110.1), one oxygenated methine (δ_C 71.9), two methines (δ_C 47.7, 43.4), three quaternary carbons (δ_C 41.8, 37.3, 33.0), six methylenes (δ_C 35.6, 34.4, 34.0, 25.8, 22.5, 18.1) and four methyls (δ_C 33.5, 26.0, 22.3, 15.4). These data were similar to those of kaempulchraol E,[7] except for the absence of one of the oxygenated methines of kaempulchraol E, suggesting that **1** was a $\Delta^{8(14),15}$ monohydroxyisopimaradiene. The hydroxy group was determined to be at C-1, due to

the $^{1}H–^{13}C$ HMBC correlations of H-1β (δ_H 3.72, br s) to C-3 (δ_C 34.0) and C-5 (δ_C 47.7), and of H-3α (δ_H 1.71, m), H-3β (δ_H 1.19, m), and H_3-20 (δ_H 0.80, s) to C-1 (δ_C 71.9) (Fig. 2). The NOESY correlations (Fig. 3) between H-1β and H-2β (δ_H 1.87, m)/H-11β (δ_H 1.54, m)/H_3-20 suggested that the C-1 hydroxy group adopts the α orientation. Furthermore, the NOESY correlations between H-11β (δ_H 1.54, m) and H_3-17 (δ_H 1.06, s)/H_3-20 (δ_H 0.80, s), and between H_3-18 (δ_H 0.88, s) and H_3-20 suggested that they all exist in the axial positions. Interestingly, the spectroscopic data of **1** were very similar to those of the reported marginatol (6α-hydroxyisopimara-8(14),15-diene).[13] However, careful analyses of the 1D and 2D NMR data as mentioned above, revealed that the structure of **1** is not a 6α-hydroxy derivative. Thus, the structure of **1** was assigned as 1α-hydroxyisopimara-8(14),15-diene, and the compound was named kaempulchraol I.

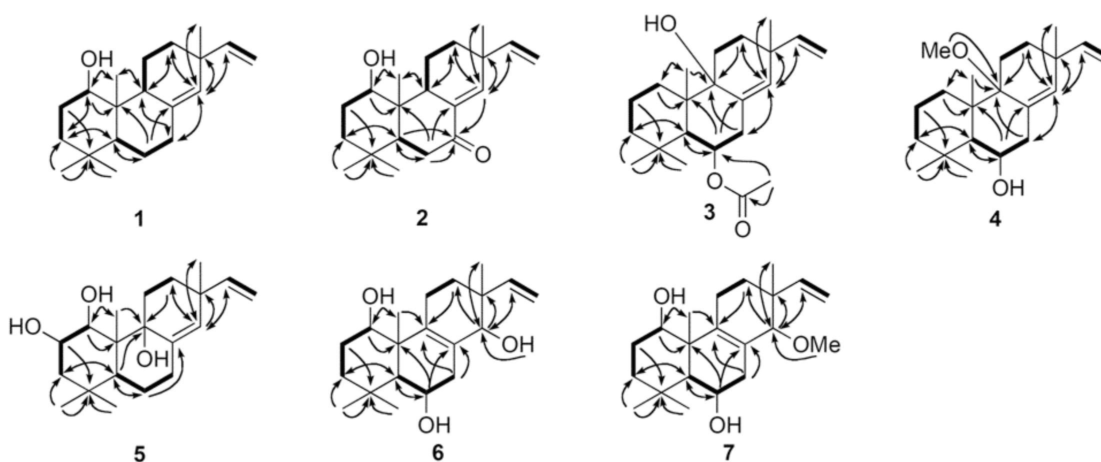

Figure 2. COSY (bold lines) and key HMBC ($^{1}H \rightarrow ^{13}C$) (arrows) correlations in compounds **1–7**.

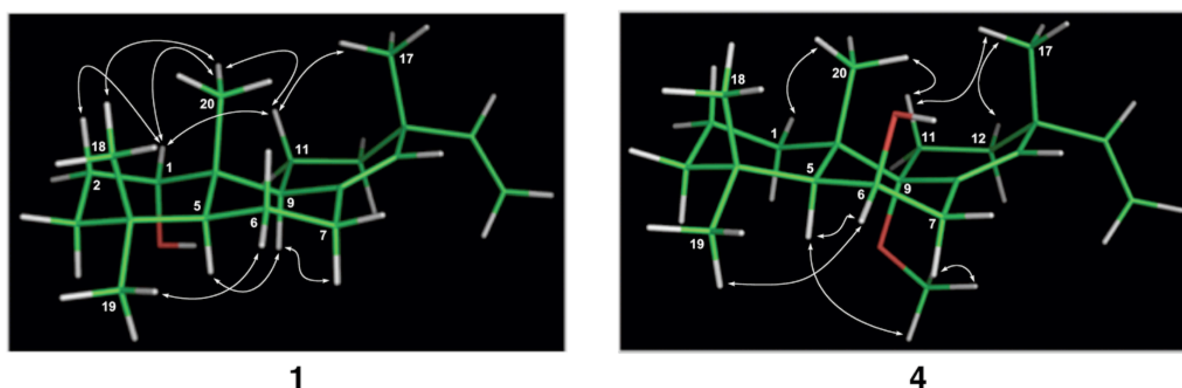

Figure 3. Key NOESY correlations (white arrows) in compounds **1** and **4**.

Table 1
^{1}H NMR spectroscopic data (600 MHz, $CDCl_3$) for kaempulchraols I–M (**1–5**), (δ in ppm and J values in (Hz) in parentheses)

Position	**1**	**2**	**3**	**4**	**5**
1α			1.74, td (12.9, 3.9)	1.83, m	
1β	3.72, br s	3.79, br t (2.5)	1.42, m	1.31, m	3.99, br d (1.5)
2α	1.59, m	1.62, m	1.52[a], m	1.46, m	
2β	1.87, m	1.96, tdd (14.5, 3.5, 2.2)	1.61, m	1.58, m	3.86, m
3α	1.71, m	1.75, td (14.0, 3.5)	1.26, m	1.22, m	1.79, t (12.3)
3β	1.19, m	1.27, dt (14.0, 3.5)	1.37, m	1.32, m	1.41, ddd (12.3, 4.2, 0.8)
5α	1.43, m	1.91, dd (13.7, 5.2)	2.01, d (2.2)	1.90, d (2.0)	2.20, dd (12.7, 3.1)
6α	1.61, m	2.58, dd (18.5, 5.2)	5.37, quin (2.3)	4.30, br s	1.61, m
6β	1.34, m	2.31, dd (18.5, 13.7)			1.35, qdd (17.7, 12.7, 4.8)
7α	2.25, m		2.67, dddd (15.4, 6.4, 4.0, 2.3)	2.46, dddd (13.9, 5.9, 3.8, 2.1)	2.09, ddd (14.4, 5.7, 2.4)
7β	2.04, m		2.20, dd (15.4, 2.4)	2.04, dd (13.9, 2.8)	2.48, tdd (14.1, 5.9, 2.4)
9α	2.28, t (7.2)	2.69, m			
11α	1.69, m	1.87, m	1.56, m	1.66, m	1.84, dt (14.1, 3.7)
11β	1.54, m	1.47, m	2.12, m	1.85, m	2.03, ddd (17.7, 14.1, 3.7)
12α	1.42, m	1.57, m	1.57, m	1.58, m	1.73, td (13.5, 3.5)
12β	1.49, m	1.64, m	1.52[a], m	1.64, m	1.48, dt (13.2, 3.7)
14	5.27, br s	6.73, br t (1.6)	5.35, br t (1.7)	5.72, br d (1.8)	5.32, br t (1.7)
15	5.79, dd (17.4, 10.7)	5.82, dd (17.5, 10.5)	5.81, dd (17.5, 10.6)	5.83, dd (17.5, 10.5)	5.82, dd (17.5, 10.6)
16-a	4.93, dd (17.4, 1.4)	5.02, dd (17.5, 0.9)	4.99, dd (17.5, 1.2)	4.99, dd (17.5, 1.3)	4.99, dd (17.5, 1.2)
16-b	4.90, dd (10.7, 1.4)	4.99, dd (10.5, 0.9)	4.96, dd (10.6, 1.2)	4.96, dd (10.5, 1.3)	4.94, dd (10.6, 1.2)
17	1.06, s	1.11, s	1.07, s	1.08, s	1.04, s
18	0.88, s	0.93, s	1.02, s	1.24, s	0.94, s
19	0.93, s	0.91, s	1.00, s	1.01, s	1.01, s
20	0.80, s	0.85, s	1.21, s	1.25, s	0.89, s
OH-1					4.13, br s
O-CO-*Me*-6			2.02, s		
OH-9			3.47, s		3.59, s
OMe-9				3.10, s	
OMe-14					

[a]Overlapping resonances within the same column. δ values were measured from the HMQC spectrum.

Table 2
^{13}C NMR spectroscopic data (150 MHz, $CDCl_3$) for kaempulchraols I–O (**1**–**7**), (δ in ppm)

Position	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1	71.9	71.1	33.8	33.7	76.7	72.9	73.1
2	25.8	25.9[b]	18.7	18.9	67.0	25.1	24.4
3	34.0	33.8	43.6	43.6	42.3	35.7	35.7
4	33.0	32.9	33.9	34.2	33.9	33.6	33.6
5	47.7	43.3	46.6	48.0	38.9	46.3	46.3
6	22.5	37.1	70.2	68.8	21.8	65.8	65.7
7	35.6	200.4	37.6	42.5	31.2	40.5	40.5
8	137.4	135.6	134.0	132.2	136.2	127.8	127.8
9	43.4	43.7	74.6	79.4	77.2	136.9	137.4
10	41.8	39.7	42.5	44.4	44.4	42.6	42.6
11	18.1	18.4	27.0	22.1[a]	27.0	19.8	20.6
12	34.4	33.9	31.9	34.6	31.1	29.6	29.0
13	37.3	38.5	37.9	37.4	37.6	40.1	40.7
14	129.0	144.3	133.8	137.8	131.8	77.3	84.3
15	149.0	146.3	147.9	147.5	148.3	143.2	144.8
16	110.1	111.7	110.7	111.1	110.5	114.6	111.7
17	26.0	25.8[b]	24.2	26.8	23.6	22.8	24.1
18	22.3	21.3	23.8	22.1[a]	23.5	23.8	23.5
19	33.5	32.4	33.7	33.9	33.7	33.5	33.5
20	15.4	14.2	21.2	24.4	17.9	21.6	21.3
O-CO-*Me*-6			21.8				
O-*CO*-Me-6			170.5				
OMe-9				50.8			
OMe-14							62.0

[a]Overlapping resonances within the same column. [b]These assignments may be reversed. δ values were measured from the HMQC spectrum.

Compound **2** was obtained as an amorphous solid, and its molecular formula was determined as $C_{20}H_{30}O_2$ by the HREIMS and ^{13}C NMR data. The absorption bands at 3453, 1681, and 1634 cm^{-1} in the IR spectrum indicated the presence of hydroxy, conjugated carbonyl, and olefinic groups in **2**. The ^{1}H and ^{13}C NMR spectroscopic data (Tables 1 and 2) of **2** were similar to those of **1**, except for the presence of a carbonyl carbon (δ_C 200.4) and the downfield shift of H-14 (δ_H 6.73) in **2**. Thus, **2** was considered to be a derivative of **1** with an additional carbonyl group, which may be in the proximity of C-14. The HMBC correlations (Fig. 2) of H-1β (δ_H 3.79) to C-3/C-5/C-9, and those of H-5α (δ_H 1.91), H_2-6 (δ_H 2.31, 2.58), H-9α (δ_H 2.69), and H-14 (δ_H 6.73) to the carbonyl carbon (δ_C 200.4) suggested the attachment of these hydroxy and carbonyl groups to C-1 and C-7, respectively. The

relative orientation of the C-1 hydroxy group was deduced as the α configuration since an oxygenated methine (δ_H 3.79, H-1β) provided NOESY correlations with H_3-20 (δ_H 0.85, s) and H-11β (δ_H 1.47). Accordingly, the structure of **2** was deduced as 1α-hydroxy-7-oxoisopimara-8(14),15-diene, and the compound was named kaempulchraol J.

Compound **3** was obtained as an amorphous solid and its molecular formula, $C_{22}H_{34}O_3$, was determined via the HREIMS and ^{13}C NMR data. The ^{1}H and ^{13}C NMR spectroscopic data (Tables 1 and 2) suggested that **3** was a $\Delta^{8(14),15}$ monohydroxyisopimaradiene with an acetoxy group [δ_H 2.02, s (-O-CO-*Me*), δ_C 21.8 (-O-CO-*Me*), 170.5 (-O-*CO*-Me)]. The downfield shift of an oxygenated methine (δ_H 5.37, quin, J = 2.3 Hz) suggested that it was an acetoxy group bearing proton. These data were similar to those of the reported 6β-acetoxysandaracopimaradiene-9α-ol-1-one,[14] an isolate from the same plant native to Thailand, except for the lack of a carbonyl group in **3**. The HMBC correlations of H-6α (δ_H 5.37, quin, J = 2.3 Hz) to C-5/C-8 and of H_2-11 (δ_H 1.56, 2.12, both m), H-14 (δ_H 5.35, br t, J = 1.7 Hz), and H_3-20 (δ_H 1.21, s) to C-9 (δ_C 74.6) revealed the location of the acetoxy group at C-6 and the presence of a hydroxy group at C-9, respectively (Fig. 2). The NOESY correlations of H-6α and H-5α/H-7α confirmed the relative configuration of the C-6 acetoxy as β orientation. However, C-9 hydroxy group was assumed as α orientation based on the biogenetic point of view. Hence, **3** was established as 6β-acetoxy-9α-hydroxyisopimara-8(14),15-diene, and was named kaempulchraol K.

Compound **4** was isolated as a colorless oil. Its molecular formula was determined to be $C_{21}H_{34}O_2$ from the HREIMS and ^{13}C NMR data. Its ^{1}H and ^{13}C NMR spectroscopic data along with the COSY and HMQC data (Tables 1 and 2, Fig. 2) indicated that **4** was a $\Delta^{8(14),15}$ monohydroxyisopimaradiene with a methoxy group (δ_H 3.10, s; δ_C 50.8). The unusual high field ^{13}C NMR chemical shift of this methoxy group suggested that it may be attached to the quaternary carbon. The HMBC correlations (Fig. 2) of an oxygenated methine (δ_H 4.30, br s)

to C-5/C-7/C-8/C-10, of H-5α/H_2-7/H-14 to C-9, and of methoxy protons (δ_H 3.10, s) to C-9 (δ_C 79.4) allowed us to confirm the presence of these hydroxy and methoxy groups at C-6 and C-9, respectively. The NOESY correlations described in Figure 3 confirmed that **4** was 6β-hydroxy-9α-methoxyisopimara-8(14),15-diene, and the compound was named kaempulchraol L. Interestingly, this is the first report of the presence of a methoxy group at C-9 of a $\Delta^{8(14),15}$ isopimarane diterpene skeleton.

Compound **5** was obtained as colorless needles from acetone. The molecular formula of **5** was determined to be $C_{20}H_{32}O_3$, from the quasi-molecular ion peak at *m/z* 343 in the FAB (fast atom bombardment) mass spectrum, and this was further supported by HRFABMS (*m/z* 343.2233, calcd 343.2249, $[C_{20}H_{32}O_3Na]^+$). The ^{1}H NMR spectrum (Table 1) of **5** displayed signals for a $\Delta^{8(14),15}$ isopimaradiene skeleton including terminal vinyl protons [δ_H 5.82, dd (J = 17.5, 10.6 Hz, H-15), 4.99, dd (J = 17.5, 1.2 Hz, H-16a), 4.94, dd (J = 10.6, 1.2 Hz, H-16b)], an olefinic methine [δ_H 5.32, br t (J = 1.7 Hz, H-14)], two oxygenated methines [δ_H 3.86, m (H-2β), 3.99 br d (J = 1.5 Hz, H-1β)], two hydroxy protons [δ_H 4.13, br s (OH-1α), 3.59, s (OH-9α)], five methylenes and four methyl singlets [δ_H 1.04 (H_3-17), 0.94 (H_3-18), 1.01 (H_3-19), 0.89 (H_3-20)]. The detailed analysis of the HMQC and ^{13}C NMR spectroscopic data (Table 2) of **5** revealed the presence of four olefins, three methines including two oxygenated ones, four quaternary carbons including an oxygenated one, five methylenes and four methyl groups. These data revealed that **5** was a $\Delta^{8(14),15}$ trihydroxyisopimaradiene. The COSY correlations between the two oxygenated methines and the HMBC correlations (Fig. 2) of H-1β (δ_H 3.99, br d, J = 1.5 Hz) to C-2/C-3/C-5/C-9/C-20, of H-2β (δ_H 3.86, m) to C-1/C-3/C-4/C-10, and of OH-9α (δ_H 3.59, s) to C-8/C-9/C-10 confirmed the attachment of these hydroxyl groups at C-1, C-2, and C-9, respectively. The 2D NOESY correlations of H-1β and H-2β to H_3-20 and of OH-9α to H-5α/H-11α/H-12α (Fig. 4a), and the X-ray diffraction analysis (Fig. 4b) revealed the orientations of the C-1, C-2, and C-9 hydroxy

groups as the α configurations. Consequently, its structure was established as 1α,2α,9α-trihydroxyisopimara-8(14),15-diene and was named kaempulchraol M.

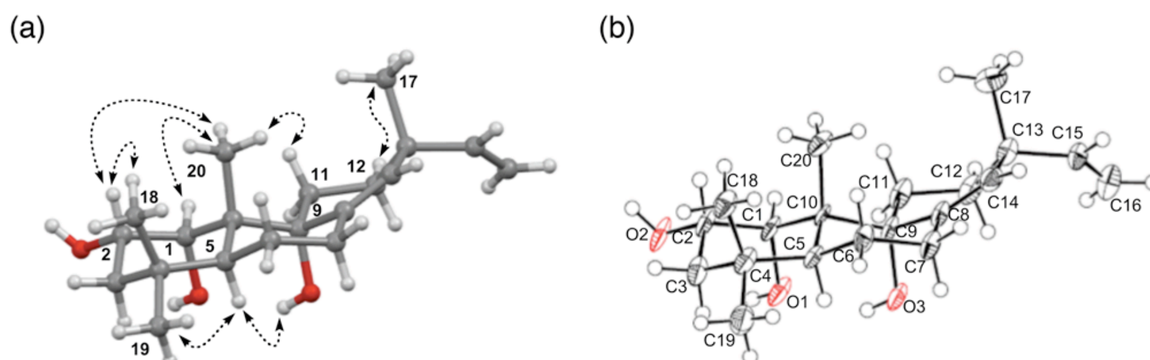

Figure 4. Key NOESY correlations (dashed arrows) (a) and ORTEP drawing (b) of compound **5**.

Compound **6** was isolated as colorless needles. The HREIMS analysis of **6** afforded a molecular ion at *m/z* 320.2342 consistent with the molecular formula of $C_{20}H_{32}O_3$. The ^{1}H NMR spectrum of **6** displayed protons of a terminal vinyl [δ_H 6.00, dd (J = 17.7, 10.9 Hz, H-15), 5.14, dd (J = 17.7, 1.5 Hz, H-16a), 5.18, dd (J = 10.9, 1.5 Hz, H-16b)], four methines including three oxygenated ones [δ_H 3.80, t (J = 2.8 Hz, H-1β), 1.63, br s (H-5α), 4.58, d (J = 4.8 Hz, H-6α), 3.56, br s (H-14)], five methylenes and four singlets of methyls [δ_H 1.04 (H_3-17), 1.23 (H_3-18), 1.03 (H_3-19), 1.37 (H_3-20)]. The ^{13}C NMR spectrum showed four olefinic carbons (δ_C 143.2, 136.9, 127.8, 114.6), four methines including three oxygenated ones (δ_C 77.3, 72.9, 65.8, 46.3), five methylenes (δ_C 40.5, 35.7, 29.6, 25.1, 19.8), three quaternary carbons (δ_C 42.6, 40.1, 33.6), and four methyls (δ_C 33.5, 23.8, 22.8, 21.6). The absence of an olefinic methine proton of C-14 and the presence of four olefinic carbons and three oxygenated methines suggested that **6** was a trihydroxy analogue of a $\Delta^{8(9),15}$ isopimaradiene. Careful analyses revealed that, its ^{1}H and ^{13}C NMR data (Tables 2 and 3) were very similar to those of a previously reported compound, kaempulchraol C,[7] except for the presence of an oxygenated methine group [δ_H 3.80, t (J = 2.8 Hz), δ_C 72.9]. The key HMBC correlations of H-1β (δ_H 3.80) to C-5/C-9/C-20, of H-6α [δ_H 4.57, d (J = 4.8 Hz)] to C-8/C-10, and of H-14 [δ_H 3.56, br s] to C-7/C-9/C-13/C-15/C-17 revealed that these

hydroxy groups were located at C-1, C-6, and C-14, respectively. The relative configurations at the C-6 and C-14 hydroxy groups were found to be similar to those of kaempulchraol C,[7] while that of the C-1 hydroxy group was found to be in the α orientation, which was supported by the NOESY correlations between H-1β and H_3-20 as well as by its X-ray diffraction pattern as shown in Figure 5. Hence, the structure of **6** was established as 1α,6β,14α-trihydroxyisopimara-8(9),15-diene, and the compound was named kaempulchraol N.

Table 3

^{1}H NMR spectroscopic data (600 MHz, $CDCl_3$) for kaempulchraols N (**6**) and O (**7**), (δ in ppm and J values in (Hz) in parentheses)

Position	**6**	**7**
1α		
1β	3.80, t (2.8)	3.71, t (2.8)
2α	1.56, dq, (14.4, 2.8)	1.57, m
2β	1.92, tq (14.4, 2.2)	1.87, m
3α	1.65, ddd (17.7, 13.3, 4.0)	1.65, dd (13.5, 4.2)
3β	1.15, dq (13.3, 2.8)	1.13, dq (14.9, 4.2)
5α	1.63, br s	1.60, br s
6α	4.58, d (4.8)	4.55, d (4.5)
7α	2.76, m	2.35[a], m
7β	2.01, m	2.35[a], m
11α	2.23, m	1.92, m
11β	2.10, m	2.14, m
12α	1.82, m	1.48, m
12β	1.50, m	1.70, m
14α		3.01, s
14β	3.56, br s	
15	6.00, dd (17.7, 10.9)	5.75, dd (17.7, 11.0)
16-a	5.14, dd (17.7, 1.5)	4.90, dd (17.7, 1.2)
16-b	5.18, dd (10.9, 1.5)	4.99, dd (10.9, 1.2)
17	1.04, s	1.10, s
18	1.23, s	1.22, s
19	1.03, s	1.02, s
20	1.37, s	1.36, s
OMe-14		3.53, s

[a]Overlapping resonances within the same column. δ values were measured from the HMQC spectrum.

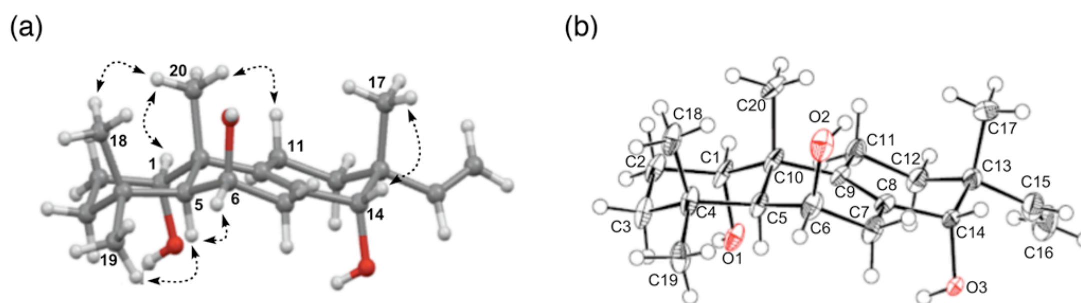

Figure 5. Key NOESY correlations (dashed arrows) (a) and ORTEP drawing (b) of compound **6**.

Compound **7** was isolated as colorless needles and its molecular formula, $C_{21}H_{34}O_3$, was determined from the HREIMS and ^{13}C NMR data. The ^{1}H and ^{13}C NMR spectroscopic data (Tables 2 and 3) of **7** were similar to those of **6**, except for the presence of a methoxy group (δ_H 3.53, s, δ_C 62.0) instead of the hydroxyl group of **6**. This methoxy group was located at C-14, since an oxygenated methine (δ_H 3.01, s) provided HMBC correlations to C-7/C-8/C-9/C-13/C-15/C-17. On the basis of the NOESY correlations (Fig. 6a) between H_3-20 (δ_H 1.36, s) and H-1β (δ_H 3.71, t, J = 2.8)/H-11β (δ_H 2.14, m), between H-6α (δ_H 4.55, d, J = 4.5) and H-5α (δ_H 1.60, br s)/H_3-19 (δ_H 1.02, s), and between OMe-14β (δ_H 3.53, s) and H_3-17 (δ_H 1.10, s)/H-12β (δ_H 1.70, m), the relative configurations of the C-1 and C-6 hydroxy groups, and the C-14 methoxy groups were assigned as the α, β, and β orientations, respectively. The X-ray diffraction analysis data (Fig. 6b) additionally supported the structure of **7** as 1α, 6β-dihydroxy-14β-methoxyisopimara-8(9),15-diene, and the compound was named kaempulchraol O.

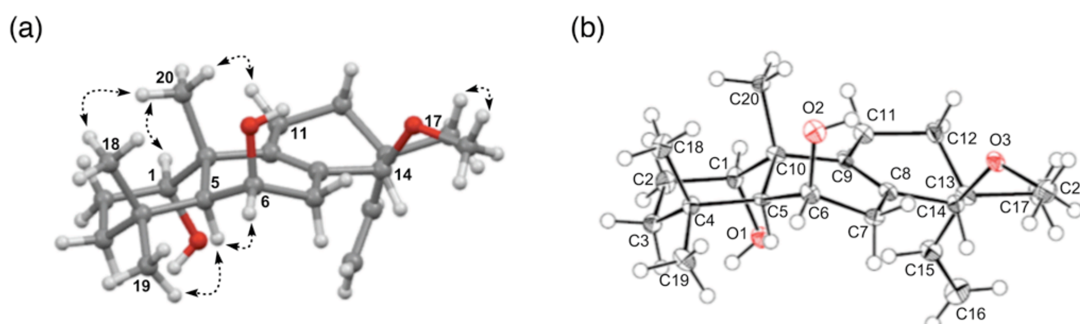

Figure 6. Key NOESY correlations (dashed arrows) (a) and ORTEP drawing (b) of compound **7**.

The structure of the known compound (1*R*,2*S*,5*S*,9*S*,10*S*,11*R*,13*R*)-1,2,11-trihydroxypimara-8(14),15-diene (**8**) was identified by comparison of its observed and reported NMR data.[10] Based on the present study, 1α-hydroxy as well as 6β-hydroxy bearing $\Delta^{8(9),15}$ and/or $\Delta^{8(14),15}$ isopimarane diterpenoids are abundant in *K. pulchra* rhizomes collected from Myanmar.

2.2. Antiproliferative activity

All of the isolated compounds (**1–8**) were evaluated for their antiproliferative activity against five human cancer cell lines (Table 4). The anticancer drug 5-fluorouracil was used as a positive control. Compound **1** exhibited mild antiproliferative activity against all of the cell lines, with IC_{50} values ranging from 39.3 to 87.5 μM. Compound **3** was the most active compound against A549 cells (IC_{50} 33.1 μM), but it lacked activity against other cell lines. Compound **4** had good activity against all of the tested cell lines except for HeLa cells, for which it lacked activity. The good antiproliferative activity of compound **4** might be due to

Table 4

Antiproliferative activity (IC_{50} μM) of compounds **1–8** isolated from *K. pulchra* against a panel of five human cancer cell lines and normal human primary fibroblast cells

Compounds	Cell line[a]					
	A549	HeLa	PANC-1	PSN-1	MDA-MB-231	TIG-3
1	55.8	58.2	70.2	39.9	79.6	87.5
2	71.6	>100	>100	70.3	>100	>100
3	33.1	>100	>100	>100	>100	>100
4	72.3	>100	39.9	22.6	73.6	57.1
5	45.8	28.4	>100	45.5	>100	>100
6	>100	>100	>100	>100	>100	>100
7	>100	>100	>100	>100	>100	>100
8	93.1	59.4	91.7	99.3	>100	>100
[b]5-Fluorouracil	2.8	5.8	3.7	4.4	5.2	8.4

[a]A549, human lung cancer; HeLa, human cervix cancer; PANC-1, PSN-1, human pancreatic cancer; MDA-MB-231, human breast cancer; TIG-3, normal human primary fibroblast cell

[b]Positive control

the presence of the methoxy group at C-9, which is apparently different from the substituents of the other isolates. Compounds **6** and **7** had no activity up to 100 μM against all of the tested cell lines.

3. Experimental section

3.1. General experimental procedures

The melting points were determined with a Yanaco micro melting point apparatus and are uncorrected. Optical rotations were recorded on a JASCO P2100 polarimeter. Infrared spectra were recorded as KBr pellets on a Jasco FT/IR-460 Plus spectrometer. NMR spectra were recorded at 600 MHz (^{1}H NMR) and 150 MHz (^{13}C NMR) on a Varian UNITY 600 spectrometer. Chemical shift values were expressed in δ (ppm) downfield from TMS as an internal standard. The mass spectra, including high-resolution mass spectra, were recorded on a JEOL MStation JMS-700 spectrometer. Open column chromatography was performed with normal-phase silica gel (silica gel 60N, spherical, neutral, 40–50 μm, Kanto Chemical Co., Inc, Tokyo, Japan) and Cosmosil 75C18-OPN (Nacalai Tesque Inc., Kyoto, Japan). MPLC was performed with a Büchi Sepacore system (Büchi Labortechnik AG, Flawil, Switzerland). TLC was carried out on precoated silica gel $60F_{254}$ and RP-18 F_{254} plates (Merck, 0.25 or 0.50 mm thickness). The cell lines A549 (human lung cancer), HeLa (human cervix cancer), PANC-1 and PSN-1 (human pancreatic cancer), MDA-MB-231 (human breast cancer), and TIG-3 (normal human primary fibroblast) were available and maintained in our laboratory. Cell culture flasks and 96-well plates were purchased from Corning Inc. (Corning, NY, USA). The SH-1200 microplate reader (Corona, Hitachinaka, Japan) was used to measure the absorbance of the cells in the antiproliferative activity assay.

3.2. Plant material

Kaempferia pulchra Ridl. rhizomes were collected from Pindaya Township, Shan State, Myanmar in September 2013, and were identified by an authorized botanist from the Department of Botany, University of Yangon. A voucher specimen (TMPW 28301) was deposited at the Museum for Materia Medica, Analytical Research Center for Ethnomedicines, Institute of Natural Medicine, University of Toyama, Japan.

3.3. Extraction and isolation

The *K. pulchra* rhizomes (500 g) were extracted with $CHCl_3$ with sonication (1L, 90 min, ×3) at 35 °C, and the solvent was evaporated under reduced pressure to yield 30 g of extract.

The chloroform extract (30 g) was chromatographed on silica gel with an EtOAc−*n*-hexane solvent system to yield seven fractions [1: EtOAc−*n*-hexane (10:90) eluate, 0.96 g; 2: EtOAc−*n*-hexane (15:85) eluate, 1.99 g; 3: EtOAc−*n*-hexane (20:80) eluate, 6.43 g; 4: EtOAc−*n*-hexane (25:75) eluate, 3.52 g; 5: EtOAc−*n*-hexane (30:70) eluate, 3.84 g; 6: EtOAc−*n*-hexane (40:60) eluate, 1.34 g; 7: EtOAc−*n*-hexane (50:50) eluate, 3.94 g].

Fraction 2 (1.99 g) was rechromatographed on Cosmosil 75C18-OPN with MeCN−acetone−MeOH−H_2O (2:2:2:1) to give three subfractions [2-1: 645 mg; 2-2: 890 mg; 2-3: 315 mg]. Subfraction 2-2 (890 mg) was subjected to MPLC with *n*-hexane−EtOAc (95:5) [Column: polypropylene (i.d. 40 mm × 150 mm); flow rate: 20 mL/min] to yield kaempulchraols I (**1**, 300 mg), and J (**2**, 10 mg). Subfraction 2-3 (315 mg) was purified by normal phase preparative TLC with C_6H_6−CH_2Cl_2−EtOAc (80:80:3) to yield kaempulchraols K (**3**, 10 mg) and L (**4**, 67 mg).

Isolation and purification of fractions 3−6 by various chromatographic techniques afforded kaempulchraols A−H and five known compounds.[7]

The repeated column chromatography of fraction 7 (3.94 g) on Cosmosil 75C18-OPN using MeCN−acetone−MeOH−H_2O (1:1:1:1) afforded three subfractions [7-1: 350 mg; 7-2:

448 mg; 7-3: 2.02 g]. Recrystallization of subfraction 7-1 by acetone furnished kaempulchraol M (**5**, 275 mg). Repeated column chromatography of subfraction 7-3 by MPLC [Column: polypropylene (i.d. 40 mm × 150 mm); flow rate: 20 mL/min] with *n*-hexane−CH_2Cl_2−acetone (4:4:1) and purification by reverse phase preparative TLC with MeCN−acetone−MeOH−H_2O (1:1:1:2) afforded kaempulchraols N (**6**, 87 mg) and O (**7**, 34 mg), and (1*R*,2*S*,5*S*,9*S*,10*S*,11*R*,13*R*)-1, 2, 11-trihydroxypimara-8(14),15-diene[10] (**8**, 10 mg).

3.4. Kaempulchraol I (1)

Amorphous solid; $[\alpha]_D^{25}$ +81 (*c* 0.14, MeOH); IR (KBr) ν_{max} 3386, 2948, 2360, 1635, 1463, 1380, 1211, 1044, 908 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; EIMS *m/z* 288 $[M]^+$ (8); HREIMS *m/z* 288.2461 $[M]^+$ (calcd for $C_{20}H_{32}O$, 288.2453).

3.5. Kaempulchraol J (2)

Amorphous solid; $[\alpha]_D^{25}$ +26 (*c* 0.1, MeOH); IR (KBr) ν_{max} 3453, 2958, 2361, 1681, 1634, 1597, 1456, 1207, 1047, 919 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; EIMS *m/z* 302 $[M]^+$ (14); HREIMS *m/z* 302.2244 $[M]^+$ (calcd for $C_{20}H_{30}O_2$, 302.4510).

3.6. Kaempulchraol K (3)

Amorphous solid; $[\alpha]_D^{25}$ +35 (*c* 0.1, MeOH); IR (KBr) ν_{max} 3512, 2944, 2362, 1713, 1363, 1219, 1029, 912 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; EIMS *m/z* 346 $[M]^+$ (9); HREIMS *m/z* 346.2486 $[M]^+$ (calcd for $C_{22}H_{34}O_3$, 346.2508).

3.7. Kaempulchraol L (4)

Colorless oil; $[\alpha]_D^{25}$ +40 (*c* 0.1, MeOH); IR (KBr) ν_{max} 3494, 2917, 1464, 1718, 1636,

1464, 1074, 912 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; EIMS *m/z* 318 [M]$^{+}$ (18); HREIMS *m/z* 318.2561 [M]$^{+}$ (calcd for $C_{21}H_{34}O_2$, 318.2559).

3.8. Kaempulchraol M (5)

Colorless needles (acetone); mp 135−140°; $[\alpha]_D^{25}$ +61 (*c* 0.13, MeOH); IR (KBr) ν_{max} 3314, 2953, 2362, 1633, 1366, 1248, 1044, 909 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; FABMS *m/z* 343 [M + Na]$^{+}$ (98); HRFABMS *m/z* 343.2233 [M + Na]$^{+}$ (calcd for $C_{20}H_{32}O_3Na$, 343.2249).

3.9. Kaempulchraol N (6)

Colorless needles (*n*-hexane−$CHCl_3$, 7:3); mp 145−148°; $[\alpha]_D^{25}$ +60 (*c* 0.12, MeOH); IR (KBr) ν_{max} 3361, 2932, 2364, 1638, 1463, 1224, 1067, 1010, 907 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 2 and 3; EIMS *m/z* 320 [M]$^{+}$ (12); HREIMS *m/z* 320.2342 [M]$^{+}$ (calcd for $C_{20}H_{32}O_3$, 320.2351).

3.10. Kaempulchraol O (7)

Colorless needles (acetone); mp 100−103°; $[\alpha]_D^{25}$ +145 (*c* 0.13, MeOH); IR (KBr) ν_{max} 3402, 2907, 1638, 1459, 1216, 922 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 2 and 3; EIMS *m/z* 334 [M]$^{+}$ (27); HREIMS *m/z* 334.2514 [M]$^{+}$ (calcd for $C_{21}H_{34}O_3$, 334.2508).

3.11. X-ray crystallographic analysis of kaempulchraols M−O (5−7)

X-ray data for compounds **5−7** were collected on a Bruker APEX 2 CCD area detector diffractometer with a Helios multi-layered confocal mirror (ϕ−ω scans) using Mo *Kα* radiation (λ = 0.71069 Å) from a Bruker TXS fine-focus rotating anode. Bruker *APEX 2* was

used for cell refinement and data reduction. The programs *SHELXL*-97, *SHELXL*-2014, and *PLATON* were used for the structure solution, the structure refinement, and the ORTEP plot, respectively.[15–17] Crystallographic data for the structures of compounds **5–7** have been deposited in the Cambridge Crystallographic Data Centre (deposition numbers: CCDC 1060925−1060927). Copies of these data can be obtained, free of charge, upon application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

3.12. Crystal data of kaempulchraol M (5)

$C_{20}H_{32}O_3$, M = 320.46, monoclinic, space group *C2*, a = 26.401 (15) Å, b = 10.034(5) Å, c = 7.896(4) Å, β = 103.475°, V = 2034.3(19) Å^3, Z = 4, D_{calcd} = 1.046 g/cm^3, T = 100 K, $F(000)$ = 704, and μ (Mo $K\alpha$) = 0.068 mm^{-1}. A total of 5069 reflections (3542 unique, R_{int} = 0.0498) were collected from 1.58° to 27.70° in θ and index ranges $34 \geq h \geq -23$, $13 \geq k \geq -11$, $10 \geq l \geq -10$. The final stage converged to R_1 = 0.1205 (wR_2 = 0.3455) for 3228 observed reflections [with $I > 2\sigma(I)$] and 215 variable parameters, and R_1 = 0.1261 (wR_2 = 0.3526) for all unique reflections and GoF = 1.599.

3.13. Crystal data of kaempulchraol N (6)

$C_{20}H_{32}O_3$, M = 320.46, monoclinic, space group *P2₁*, a = 11.1771 (17) Å, b = 23.538 (4) Å, c = 18.307 (3) Å, β = 106.809° (2), V = 4610.6 (12) Å^3, Z = 2, D_{calcd} = 1.165 g/cm^3, T = 100 K, $F(000)$ = 1760, and μ (Mo $K\alpha$) = 0.241mm^{-1}. A total of 24589 reflections (17061 unique, R_{int} = 0.0328) were collected from 1.162° to 26.262° in θ and index ranges $12 \geq h \geq -13$, $29 \geq k \geq -29$, $20 \geq l \geq -22$. The final stage converged to R_1 = 0.0978 (wR_2 = 0.2691) for 15633 observed reflections [with $I > 2\sigma(I)$] and 993 variable parameters, and R_1 = 0.1036 (wR_2 = 0.2770) for all unique reflections and GoF = 1.047.

3.14. Crystal data of kaempulchraol O (7)

$C_{21}H_{34}O_3$, M = 334.49, triclinic, space group $P1$, a = 7.6010 (10) Å, b = 15.114 (2) Å, c = 17.819 (2) Å, α = 103.9984° (17), β = 96.4188° (17), γ = 97.4604° (16), V = 1947.5 (5) Å^3, Z = 4, D_{calcd} = 1.141g/cm^3, T = 100 K, F(000) = 736, and μ (Mo $K\alpha$) = 0.074 mm^{-1}. A total of 11980 reflections (10340 unique, R_{int} = 0.0207) were collected from 1.191° to 29.217° in θ and index ranges $10 \geq h \geq -9$, $20 \geq k \geq -18$, $24 \geq l \geq -13$. The final stage converged to R_1 = 0.1309 (wR_2 = 0.3660) for 10159 observed reflections [with $I > 2\sigma(I)$] and 894 variable parameters, and R_1 = 0.1319 (wR_2 = 0.3705) for all unique reflections and GoF = 1.949.

3.15. In vitro antiproliferative activity

The in vitro antiproliferative activity of the crude extracts and the isolated compounds against the A549 (human lung cancer), HeLa (human cervix cancer), TIG-3 (normal human primary fibroblast cell), PANC-1 and PSN-1 (human pancreatic cancer), MDA-MB-231 (human breast cancer), and TIG-3 (normal human primary fibroblast) cell lines was evaluated by the procedure as described previously.[7] Briefly, each cell line was seeded in 96-well plates (2 × 10^3 per well) and cultured either in α-MEM or DMEM at 37 °C under a 5% CO_2 and 95% air atmosphere for 24 h. After the cells were washed with PBS (Nissui Pharmaceuticals), serial dilutions of the samples to be tested were added. After 72 h incubation, the cells were washed with PBS, and 100 μL of α-MEM or DMEM containing 10% WST-8 cell counting kit solution (Dojindo; Kumamoto, Japan) was added to the wells. After 2 h incubation, the absorbance at 450 nm was measured. The different concentrations of the serial dilutions of the tested samples were 100−3.125 μg/mL for the crude extract, 100−3.125 μM for the isolated compounds, and 10−0.3125 μM for the positive control, respectively. Cell viability was calculated from the mean values of data from three wells by using the following equation,

and the antiproliferative activity was expressed as the IC_{50} (50% inhibitory concentration) value.

$$(\%)\ \text{Cell viability} = 100 \times [\{Abs_{(test\ samples)} - Abs_{(blank)}\}/\{Abs_{(control)} - Abs_{(blank)}\}]$$

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Supplementary material

The ^{1}H and ^{13}C NMR, $^{1}H-^{1}H$ COSY, HMQC, HMBC, and NOESY spectra of compounds **1–7** (Figure S1 – Figure S42). This material is available free of charge via the internet at ----.

References and notes

1. Cragg, G. M.; Grothaus, P. G.; Newman, D. J. *Chem. Rev.* **2009**, *109*, 3012−3043.
2. Rocha, A. B.; Lopes, R. M.; Schwartsmann, G. *Curr. Opin. Pharmacol.* **2001**, *1*, 364−369.
3. Win, N. N.; Awale, S.; Esumi, H.; Tezuka, Y.; Kadota, S. *J. Nat. Prod*. **2007**, *70*, 1582−1587.
4. Win, N. N.; Awale, S.; Esumi, H.; Tezuka, Y.; Kadota, S. *Chem. Pharm. Bull*. **2008**, *56*, 491−496.
5. Win, N. N.; Awale, S.; Esumi, H.; Tezuka, Y.; Kadota, S. *Bioorg. Med. Chem. Lett*. **2008**, *18*, 4688−4691.
6. Win, N. N.; Awale, S.; Esumi, H.; Tezuka, Y.; Kadota, S. *Bioorg. Med. Chem*. **2008**, *16*, 8653−8660.
7. Win, N. N.; Ito, T.; Aimaiti, S.; Imagawa, H.; Ngwe, H.; Abe, I.; Morita, H. DOI: 10.1021/acs.jnatprod.5b00108.
8. Chang, C. I.; Tseng, M. H.; Kuo, Y. H. *Chem. Pharm. Bull.* **2005**, *53*, 286−289.
9. Xia, X.; Zhang, J.; Zhang, Y.; Wei, F.; Liu, X.; Jia, A.; Liu, C.; Li, W.; She, Z.; Lin, Y. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 3017−3019.
10. Thongnest, S.; Mahidol, C.; Sutthivaiyakit, S.; Ruchirawat, S. *J. Nat. Prod.* **2005**, *68*, 1632−1636.
11. Tuchinda, P.; Udchachon, J.; Reutrakul, V.; Santisuk, T.; Skelton, B. W.; White, A. H.; Taylor, W. C. *Phytochemistry* **1994**, *36*, 731−734.
12. Nagashima, F.; Murakami, M.; Takaoka, S.; Asakawa, Y. *Phytochemistry* **2003**, *64*, 1319−1325.
13. Jingguang, Y.; Donglei, Y.; Lan, S.; Shu, Z.; Caicheng, Z.; Yuheng, C. *J. Chin. Pharm. Sci.* **2001**, *10*, 61−64.

14. Prawat, U.; Tuntiwachwuttikul, P.; Taylor, W. C.; Engelhardt, L. M.; Skelton, B. W.; White, A. H. *Phytochemistry* **1993**, *32*, 991−997.

15. Sheldrick, G. M. *Acta Crystallogr.* **1990**, *A46*, 467−473.

16. Sheldrick, G. M. *SHELXL2014*. University of Göttingen, Germany.

17. Spek, A. L. *Acta Crystallogr.* **2009**, *D65*, 148−155.

Figure Legends

Figure 1. Structures of compounds **1−8** isolated from *K. pulchra* rhizomes.

Figure 2. COSY (bold lines) and key HMBC ($^{1}H \rightarrow {}^{13}C$) (arrows) correlations in compounds **1−7**.

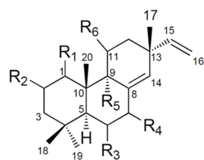
Figure 3. Key NOESY correlations (white arrows) in compounds **1** and **4**.

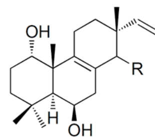
Figure 4. Key NOESY correlations (dashed arrows) (a) and ORTEP drawing (b) of compound **5**.

Figure 5. Key NOESY correlations (dashed arrows) (a) and ORTEP drawing (b) of compound **6**.

Figure 6. Key NOESY correlations (dashed arrows) (a) and ORTEP drawing (b) of compound **7**.

Graphical Abstract





	R_1	R_2	R_3	R_4	R_5	R_6
1	α-OH	H	H	H	H	H
2	α-OH	H	H	=O	H	H
3	H	H	β-OAc	H	OH	H
4	H	H	β-OH	H	OMe	H
5	α-OH	α-OH	H	H	OH	H
8	α-OH	α-OH	H	H	H	α-OH

	R
6	α-OH
7	β-OMe