Cytotoxic C-Benzylated Dihydrochalcones from *Uvaria acuminata*

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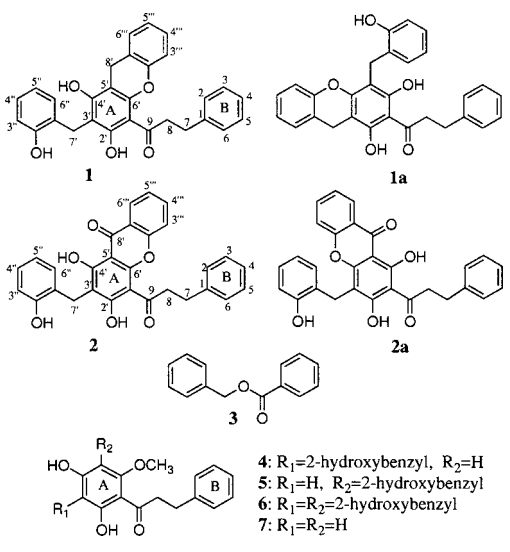
Two new C-benzylated dihydrochalcones, isochamuvaritin (1) and acumitin (2), have been isolated from the African medicinal plant *Uvaria acuminata*, together with the previously reported benzylbenzoate (3), uvaretin (4), isouvaretin (5), diuvaretin (6), and uvangoletin (7). The structural elucidation of compounds 1 and 2 in spectroscopic studies is described. C-Benzylated dihydrochalcones, especially 1, 2, 4, and 6, showed considerable cytotoxicity toward human promyelocytic leukemia HL-60 cells.

Key words *Uvaria acuminata*; C-benzylated dihydrochalcone; cytotoxicity against HL-60 cell

Uvaria acuminata OLIV. (Annonaceae) is an aromatic shrub distributed in East Africa. The root of this plant is an important folk medicine in Kenya and has been used as remedies for menstrual pain, dysentery, snakebite, and chest diseases.[1)]

Previously, we reported the alkaloidal constituents from a methanol extract of *U. acuminata*[2)] which appears closely related to the pharmacologic effects of this plant, but the content of the alkaloids was relatively low. On the other hand, from the fat-soluble fraction, several antitumor agents, *e.g.*, uvaretin (**4**),[3)] uvaricin,[4)] and desacetyluvaricin[5)] have been isolated.

When we examined HPLC profiles of the petroleum ether extract of the root of *U. acuminata*, many peaks were recognized which could not be attributed to compounds already reported. We therefore tried to obtain the compounds by a combination of column chromatography and preparative HPLC, and isolated two new C-benzylated dihydrochalcones called isochamuvaritin (**1**) and acumitin (**2**), along with benzylbenzoate (**3**), uvaretin (**4**), isouvaretin (**5**), diuvaretin (**6**), and uvangoletin (**7**). This paper deals with the structural elucidation of the two new C-benzylated dihydrochalcones isolated from the root of *U. acuminata*. Furthermore, we investigated the cytotoxic activities of the isolated dihydrochalcones against HL-60 cells, paying special attention to the cytotoxicity of the new compounds, since it was reported that dihydrochalcons of *Uvaria* like uvaretin, isouvaretin, and diuvaretin have cytotoxic activities against both PS and KB cell cultures.[6)]



The petroleum ether extract of the root of *U. acuminata* was separated by a combination of chromatographic procedures to yield seven compounds. The first new compound (**1**) was obtained as white crystals from $CHCl_3/Me_2CO$, mp 157—159 °C, and had a molecular formula of $C_{29}H_{24}O_5$ based on high resolution electron impact mass spectrum (HR-EI-MS). Its IR bands indicated the presence of hydroxyl (3380 cm^{-1}, broad) and conjugated carbonyl (1628 cm^{-1}) groups. The UV spectrum of **1** obtained using a HPLC photodiode array detector [λ_{max}: 283 (band II), 345 (shoulder, band I) nm][7)] was similar to **4**—**7**, and accordingly **1** was consistent with a dihydrochalcone. Confirmation followed from the ^{1}H-NMR spectrum, which showed two two-proton signals of the A_2B_2 pattern centered at δ 3.09 and 3.58 as expected for a β-propiophenone moiety. The ^{1}H-NMR spectrum also contained a sharp singlet signal (δ 14.38) for an intramolecularly hydrogen-bonded phenolic hydroxyl group. Thus compound **1** is a 2′-hydroxydihydrochalcone. Further, the ^{1}H- and ^{13}C-NMR spectral data of **1** (Tables 1, 2) were very similar to those of diuvaretin (**6**), except that a methoxyl signal was absent from **1**. The ^{1}H-NMR spectrum showed two sets of signals due to a 2-hydroxybenzyl group, which were found to be attached to the 3′ and 5′ of ring A of dihydrochalcone skeleton on the basis of the heteronuclear multiple-bond connectivity (HMBC) spectrum (Fig. 1). The ^{1}H- and ^{13}C-NMR chemical shifts of one of the two 2-hydroxybenzyl groups (group X) were almost the same as the values for diuvaretin, while the values of the other (group Y) were shifted. Based on the molecular formula, it was suggested that group Y was dehydrated intramolecularly with the phenolic hydroxyl group of ring A forming a xanthen skeleton. Two possible structures, *i.e.*, **1** and **1a** (chamuvaritin),[8)] are present due to the position of dehydration. When dehydration occurs between the 2′-hydroxyl and the 3′-(2-hydroxy)benzyl group, or the 6′-hydroxyl and the 5′-(2-hydroxy)benzyl group, the structure is that of **1**, and on the other hand,

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Table 1. ^{1}H-NMR Chemical Sift Values (δ) of Compounds **1**, **2** and **6**[a]

Proton	Compound		
	1	**2**	**6**
2,6	7.28	7.29	7.19—7.28
3,5	7.31	7.31—7.35	
4	7.22 (m)	7.25 (m)	7.18 (m)
7	3.09 (t-like)	3.14 (t-like)	2.98 (t-like)
8	3.58 (t-like)	3.68 (t-like)	3.38 (t-like)
7′	3.95 (s)	3.99 (s)	3.91 (s)
8′	3.83 (s)		3.84 (s)
3″	6.81 (dd, J=8.0, 1.2)	6.88 (dd, J=8.0, 1.0)	6.80 (dd, J=8.0, 1.0)
4″	7.10 (t-like)	7.11 (t-like)	7.07 (t-like)
5″	6.93 (td, J=7.5, 1.2)	6.84 (td, J=7.5, 1.0)	6.86 (m)
6″	7.59 (dd, J=7.5, 1.8)	7.55 (dd, J=7.5, 2.0)	7.57 (dd, J=7.5, 1.5)
3‴	6.86 (dd, J=8.0, 1.2)	7.33[b]	6.80 (dd, J=8.0, 1.0)
4‴	7.13 (t-like)	7.74 (t-like)	7.07 (t-like)
5‴	7.04 (td, J=7.5, 1.2)	7.47 (t-like)	6.86 (m)
6‴	7.16 (dd, J=7.5, 1.5)	8.29 (dd, J=8.0, 1.5)	7.40 (dd, J=7.5, 2.0)
OMe			3.71
2′-OH	14.38	14.80 (s)	13.56
4′-OH		15.56 (s)	

a) Spectra were recorded at 499.84 MHz in $CHCl_3$. Coupling constants (J) are given in Hz. *b*) Overlapped with 3, 5-protons.

Table 2. ^{13}C-NMR Chemical Sift Values (δ) of Compounds **1**, **2** and **6**[a]

Carbon	Compound		
	1	**2**	**6**
1	141.4	140.7	141.2
2,6	128.4	128.3	128.5
3,5	128.5	128.7	128.5
4	126.0	126.4	126.1
7	30.8	30.3	31.1
8	45.8	45.8	44.0
9	204.6	204.0	205.2
1′	104.6	102.6	109.0
2′	162.2	164.6	161.3
3′	108.7	111.2	111.9
4′	158.6	169.1	158.9
5′	99.8	102.9	113.5
6′	152.2	157.6	159.1
7′	22.4	22.4	23.1
8′	22.1	180.6	23.7
1″	126.5	125.2	126.3
2″	151.8	154.5	152.8
3″	115.3	116.7	115.6[b]
4″	127.9	128.1	127.9
5″	122.0	120.2	121.2[c]
6″	131.9	132.0	132.2
1‴	120.0	120.1	126.1
2‴	150.5	154.9	152.8
3‴	116.1	117.4	115.8[b]
4‴	127.7	135.8	128.0
5‴	123.9	125.5	121.3[c]
6‴	129.2	126.1	131.6
OCH_3			63.7

a) Spectra were recorded at 125.7 MHz in $CHCl_3$. *b*, *c*) May be interchanged.

when generated between the 4′-hydroxyl and the 3′-(2-hydroxy)benzyl group, or the 4′-hydroxyl and the 5′-(2-hydroxy)benzyl group, the structure is that of **1a**. HMBC correlations between the methylene protons of group X and the joint carbon (C-2′) of the chelated hydroxyl group were observed, and thus group X was found to be attached to C-3′ (Fig. 1). Furthermore, in the nuclear Overhauser enhancement and exchange spectroscopy (NOESY) (Fig. 1), an aromatic proton of H-3‴ (H-3 of group Y) had NOE correlations with H-7 and H-8. These correlations can be explained reasonably, since the distance between H-3‴ and H-7/H-8 can approach about 2—2.5 Å in the case of the molecular model of **1**. Therefore the structure **1** was assigned. In conclusion, the new compound (**1**) was found to be 1-[1,3-dihydroxy-2-[(2-hydroxyphenyl)methyl]-9*H*-xanthen-4-yl]-3-phenyl-1-propanone, and called isochamuvaritin.

The second new compound (**2**) was obtained as colorless crystals from $CHCl_3$, mp 186—187 °C, and had the molecular formula of $C_{29}H_{22}O_6$ by HR-EI-MS. Its IR bands indicated the presence of hydroxyl (3440 cm^{-1}) and conjugated carbonyl (1641, 1620 cm^{-1}) groups. The UV spectrum of **2** obtained by HPLC (λ_{max}: 296, 345 nm) showed a bathochromic shift of band II in comparison with **1**. The ^{1}H- and ^{13}C-NMR spectral data of **2** (Tables 1, 2) were similar to those of **1**, except that a methylene signal was absent and an additional carbonyl signal (δ 180.6) in the ^{13}C-NMR and an additional chelated hydroxyl signal (δ 15.56) in the ^{1}H-NMR spectrum were evident in **2**. The ^{1}H-NMR spectral data (Table 1) showed signals due to a dihydrochalcone moiety lacking substituents in ring B, two chelated phenolic hydroxyl groups, two units of the 2-hydroxyphenyl group, and one methylene of the benzyl group, and thus only one 2-hydroxybenzyl group was found to exist in **2**. The ^{1}H-NMR chemical shifts of the aromatic protons belonging to one of the 2-hydroxyphenyl groups appeared at a considerably lower field than the values of the other one. Based on the above, **2** must have a benzoyl fragment and thus form a xanthon skeleton. Two possible structures, *i.e.*, **2** and **2a**, are present. The HMBC spectrum of **2** (Fig. 1) showed that the methylene protons of the 2-hydroxybenzyl group were correlated with both carbons, which were joined to the chelated hydroxyl group, respectively. Further, in the NOESY spectrum, an aromatic proton of H-3‴ had NOE correlations with H-7 and H-8,[9] as in the case of **1**. Hence the possibility of the structure **2a** was excluded. Consequently, the new compound

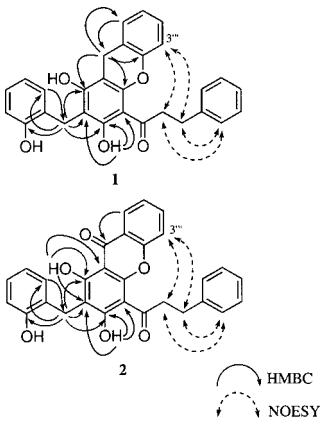

Fig. 1. Significant Correlations Observed in the HMBC and NOESY Spectra of Isochamuvaritin (**1**) and Acumitin (**2**)

Table 3. Cytotoxic Activity of Dihydrochalcones Isolated from *U. acuminata* against HL-60

Compound	IC_{50} (μM)
Isochamuvaretin (**1**)	8.2
Acumitin (**2**)	4.1
Uvaretin (**4**)	9.3
Isouvaretin (**5**)	24.7
Diuvaretin (**6**)	6.1
Uvangoletin (**7**)	>50

(**2**) was determined to be 1-[1,3-dihydroxy-2-[(2-hydroxyphenyl)methyl]-9*H*-xanthen-9-one-4-yl]-3-phenyl-1-propanone, and called acumitin.

Compounds **3**—**7** were determined to be benzylbenzoate (**3**),[10] uvaretin (**4**),[3,6,11] isouvaretin (**5**),[6,11] diuvaretin (**6**),[6,11] and uvangoletin (**7**),[12] respectively, by comparing these spectral data with values reported in the literature. Compounds **3**, **5**, **6**, and **7** were isolated for the first time from *U. acuminata*.

Dihydrochalcones isolated from *U. acuminata* were evaluated for their cytotoxic activity against human promyelocytic leukemia HL-60 cells. Cytotoxicities of the compounds tested by WST-8 assay[13] are listed in Table 3. C-Benzylated dihydrochalcones, especially isochamuvaritin (**1**), acumitin (**2**), uvaretin (**4**), and diuvaretin (**6**), exhibited considerable cytotoxicity. New compound **2** showed cytotoxic activity stronger than that of **4** and **6**, which were reported to have cytotoxicities against other cells.[6] On the other hand, uvangoletin (**7**), lacking a benzyl group, was not cytotoxic. Thus the structure of the 2-hydroxybenzylated portions appeared to play an important role in the cytotoxicity against HL-60 cells.

Experimental

General Melting points were determined using a Yanaco MP-500D micro melting point apparatus and uncorrected. IR spectra were obtained on a Shimadzu 8200 FT-IR spectrometer, and UV spectra were measured with a photodiode array detector (Model 991J, Waters). EI-MS were measured with a Hitachi M-4100 spectrometer or Shimadzu QP-2000 spectrometer. HR-EI-MS were recorded with a Hitachi M-4100 spectrometer. All NMR experiments were performed on a Varian VXR-500 spectrometer equipped with 5 mm ^{1}H and ^{13}C probes operating at 499.84 and 125.7 MHz, respectively. Chemical shifts were referenced to internal TMS. HPLC conditions for analytical HPLC were as follows: column, Cosmosil 5C18-AR (5 μm, ODS-type), 150×6 mm i.d. (Nacalai Tesque); mobile phase (A) 0.2 M $NaClO_4$: 60% $HClO_4$ = 1000 : 0.2 and (B) CH_3CN [A/B = 50/50 to 0/100, 25 min, 1.5 ml/min]. For preparative scale HPLC the following conditions were used: column, Cosmosil 5C18-AR (5 μm, ODS-type), 250×20 mm i.d. (Nacalai Tesque); mobile phase, A/B = 50/50 to 0/100, 77 min, 9.0 ml/min. Kieselgel 60 (60—230 mesh, Merck) was used for column chromatography. The following reagents and instruments were obtained from the indicated companies: RPMI-1640 medium (Sigma, St. Louis, MO, U.S.A.); fetal bovine serum (FBS) (Sigma); 200 mM L-glutamine solution (Sigma); WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt) (Cell Counting Kit-8, Dojindo Co., Ltd.); and 96-well microplates (Iwaki Scitech Co., Ltd.). Absorbance was measured using Microplate Luminometer Anthos Lucy 2 (Aloka Co., Ltd.)

Plant Material Roots of *U. acuminata* were collected in the Kaloleni district of Kenya in 1995. The samples were identified and authenticated by Mr. S. G. Mathenge, Department of Botany, University of Nairobi, Nairobi, Kenya. Voucher specimens are deposited at both Kobe Pharmaceutical University and the University of Nairobi.

Extraction and Isolation The dried and finely cut roots (252 g) of *U. acuminata* were extracted with hot petroleum ether three times. The combined filtrates were evaporated *in vacuo* until dryness to yield a syrupy mass (2.0 g). The crude petroleum ether extract (1.6 g) was chromatographed on silica gel and eluted with a mixture of $CHCl_3$–Me_2CO and then $CHCl_3$–MeOH, and fractions I—VI were collected. Fraction I gave **3** (77 mg). Fractions II—VI were dissolved in DMSO and further submitted to preparative HPLC. Main peaks were collected and CH_3CN was evaporated from the collected effluent, $CHCl_3$ was poured into the remaining aqueous solution, and the compounds were then extracted with $CHCl_3$ to afford **1**, **2**, and **4**—**7**. The yields of compounds were as follows: **1** (5 mg, from fr. III, retention time (t_R) for analytical HPLC: 19.8 min), **2** (4.4 mg, from fr. II, t_R: 17.8 min), **4** (60 mg, from fr. III, IV, t_R: 11.2 min), **5** (5 mg, from fr. VI, t_R: 8.5 min), **6** (65 mg, from fr. IV, V, VI, t_R: 13.7 min), **7** (4 mg, from fr. V, t_R: 7.3 min).

Isochamuvaritin (**1**): White crystals, mp 157—159 °C ($CHCl_3$–Me_2CO). IR (KBr) cm^{-1}: 3380 (OH, broad), 1628 (CO), 1605, 1578, 1458, 1242, 754. EI-MS *m/z* (%): 452 $[M]^+$ (100), 347 (39), 253 (34), 91 (31). HR-EI-MS *m/z*: 452.1616 (calcd for $C_{29}H_{24}O_5$: 452.1623). ^{1}H- and ^{13}C-NMR ($CDCl_3$): see Tables 1 and 2.

Acumitin (**2**): Colorless crystals, mp 186—187 °C ($CHCl_3$). IR (KBr) cm^{-1}: 3440 (OH), 1641, 1620 (CO), 1610, 1578, 1225, 768. EI-MS *m/z* (%): 466 $[M]^+$ (75), 334 (28), 267 (100), 255 (53), 228 (48), 91 (42). HR-EI-MS *m/z*: 466.1429 (calcd for $C_{29}H_{22}O_6$: 466.1415). ^{1}H- and ^{13}C-NMR ($CDCl_3$): see Tables 1 and 2.

Benzylbenzoate (**3**): Oily compound. IR ($CHCl_3$) cm^{-1}: 1716 (CO), 1274, 1112. EI-MS *m/z* (%): 212 $[M]^+$ (14), 194 (10), 105 (100). HR-EI-MS *m/z*: 212.0846 (calcd for $C_{14}H_{12}O_2$: 212.0837). The IR spectrum was in close agreement with that reported in the literature.[10]

Uvaretin (**4**): Colorless crystals, mp 167—169 °C ($CHCl_3$). IR (KBr) cm^{-1}: 3320 (OH, broad), 1628 (CO), 1601, 1493, 1456, 752. EI-MS *m/z* (%): 378 $[M]^+$ (31), 273 (40), 179 (100), 91 (46). HR-EI-MS *m/z*: 378.1471 (calcd for $C_{23}H_{22}O_5$: 378.1466). ^{1}H- and ^{13}C-NMR ($CDCl_3$) spectra are in close agreement with values reported in the literature.[3,6,11]

Isouvaretin (**5**): Resin compound. IR (KBr) cm^{-1}: 3300 (OH, broad), 1626 (CO), 1595, 1491, 1456, 754. EI-MS *m/z* (%): 378 $[M]^+$ (100), 273 (89), 167 (41), 91 (37). HR-EI-MS *m/z*: 378.1463 (calcd for $C_{23}H_{22}O_5$: 378,1466). ^{1}H- and ^{13}C-NMR ($CDCl_3$) spectra are in close agreement with values reported in the literature.[6,11]

Diuvaretin (**6**): Colorless crystals, mp 127—131 °C ($CHCl_3$). IR (KBr) cm^{-1}: 3400 (OH, broad), 1612 (CO), 1489, 1456, 754. EI-MS *m/z* (%): 484 $[M]^+$ (24), 179 (89), 107 (69), 91 (100). HR-EI-MS *m/z*: 484.1870 (calcd for $C_{30}H_{28}O_6$: 484.1884). ^{1}H- and ^{13}C-NMR ($CDCl_3$): see Tables 1 and 2. These spectra are in close agreement with values reported in the literature.[6,11]

Uvangoletin (**7**): Colorless crystals, mp 185—187 °C ($CHCl_3$). IR (KBr) cm^{-1}: 3450 (OH, broad), 1625 (CO), 1601, 1497, 1456. EI-MS *m/z* (%): 272 $[M]^+$ (45), 167 (100). HR-EI-MS *m/z*: 272.1045 (calcd for $C_{16}H_{16}O_4$: 272.1047). ^{1}H- and ^{13}C-NMR ($CDCl_3$) spectra are in close agreement with values reported in the literature.[12]

Cell Culture HL-60 human promyelocytic leukemia cells were purchased from Dainippon Pharmaceutical Co. Ltd., and maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in RPMI-1640 medium, supplemented with 10% heat-inactivated FBS and 2 mM L-glutamine.

Assay for Cytotoxic Activity Test compounds were dissolved in DMSO and diluted with medium to their final concentrations in each 96-well microplate. The final concentration of DMSO in the medium was less than 0.1%. The *in vitro* cytotoxicity of the compounds was assessed using a tetrazolium-based colorimetric assay, Cell Counting Kit-8, containing WST-8 reagent.[13] HL-60 cells were inoculated at 5×10^3 (90 μl) per each 96-well microplate and preincubated. After 24 h, they were treated without or with test compounds (10 μl) (final concentration: 0.8—50 μM) and then incubated. After 48 h, 10 μl of the WST-8 solution was added to each well and the samples were incubated for a further 3 h. The relative viable cell number was determined by measuring the absorbance at 450 nm (reference at 650 nm). The IC_{50} value, which reduced the viable cell number by 50%, was determined from the seven-point dose–response curve using six-fold serial dilutions, and each point on the curve was tested in triplicate.

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9) Although the ^{1}H-NMR signal of H-3‴ (δ 7.33) overlapped with 3,5-protons, it was concluded that these NOE correlations were based on H-3‴ and H-7/H-8, because there was also no NOE correlation between H-3, 5 and H-7/H-8 in **1**.
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