Eupatozansins A – C, Sesquiterpene Lactones from *Eupatorium chinense* var. *tozanense*

by **Chia-Ching Liaw**[a]), **Yao-Haur Kuo**[b]), **Tsong-Long Hwang**[c]), and **Ya-Ching Shen***[d])

[a]) Department of Marine Biotechnology and Resources, National Sun Yat-Sen University, Kaohsiung 804, Taiwan, Republic of China

[b]) National Research Institute of Chinese Medicine, Taipei 112, Taiwan, Republic of China

[c]) Graduate Institute of Natural Products, Chang Gung University, Taoyuan 333, Taiwan, Republic of China

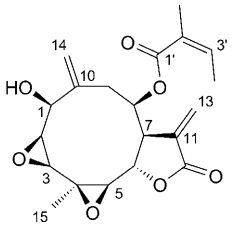
[d]) School of Pharmacy, College of Medicine, National Taiwan University, No. 1, Jen-Ai Road, Sec. 1, Taipei, Taiwan, Republic of China (phone: 886-2-2312-3456 ext. 2226; fax: 886-2-02-2391-9098; e-mail: ycshen@ntu.edu.tw)

Phytochemical investigation of *Eupatorium chinense* var. *tozanense* has resulted in the isolation of three new germacranolides, designated as eupatozansins A – C (**1** – **3**), along with five known compounds, (5*S*,6*R*,7*R*,8*R*)-8-angeloyloxy-2-oxoguaia-1(10),3,11(13)-trien-12,6-olide (**4**), costunolide (**5**), leptocarpin (**6**), 2α-hydroxyeupatolide 8-*O*-angelate (**7**), and quercetin (**8**). The structures of the new compounds were identified by 1D and 2D NMR experiments, as well as high-resolution mass spectrometry. The *in vitro* cytotoxic activities of compounds **1** – **8** were evaluated.

Introduction. – Plants belonging to the genus *Eupatorium* are well known as a rich source of sesquiterpene lactones of the germacranolide type [1][2]. They have been used for long time in traditional Chinese medicine for the treatment of several diseases [3][4]. These sesquiterpene lactones are well-known for their diverse biological activities, especially cytotoxic [5][6] and antitumor effects [7]. In previous studies, eupahualins A – E [8] and eupakirunsins A – F [9] isolated from *E. hualienense* and *E. kiirunense,* respectively, showed potent inhibitory activity against several human tumor cell lines. *E. chinense* var. *tozanense* is an endemic herb commonly grown in mountainous area in Taiwan from 1000 to 2500 m above sea level [10]. Herein, we report a chemical investigation of *E. chinense* var. *tozanense*, which has resulted in the isolation of three new sesquiterpene lactones, eupatozansins A – C (**1** – **3**) together with five known compounds, (5*S*,6*R*,7*R*,8*R*)-8-angeloyloxy-2-oxoguaia-1(10),3,11(13)-trien-12,6-olide (**4**), costunolide (**5**) [11], leptocarpin (**6**) [12], 2α-hydroxyeupatolide 8-*O*-angelate (**7**) [13][14], and quercetin (**8**) [15]. The cytotoxicities of **1** – **8** against four tumor cell lines were tested and evaluated.

Results and Discussion. – The aerial parts of *E. chinense* var. *tozanense* were extracted with acetone to give a residue which was chromatographed on a silica gel column to afford seven sesquiterpene lactones (**1** – **7**) and quercetin (**8**).

Eupatozansin A (**1**) had a molecular formula of $C_{20}H_{24}O_7$, as determined by the HR-EI-MS signal at *m*/*z* 358.3589 ($[M - H_2O]^+$), inferring ten degrees of unsaturation.



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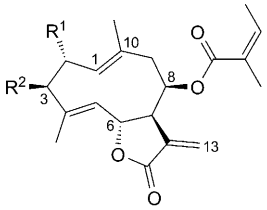
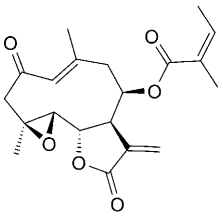


2 R^1 = OH, R^2 = OH
3 R^1 = AcO, R^2 = H
5 R^1 = H, R^2 = H
7 R^1 = OH, R^2 =H



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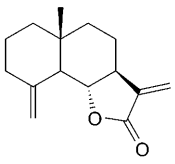

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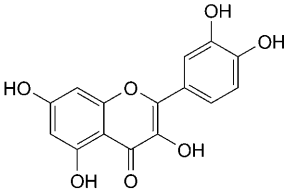

8

The IR spectrum displayed absorption bands diagnostic of a OH group (3430 cm^{-1}), an α,β-unsaturated γ-lactone (1761 cm^{-1}), an α,β-unsaturated ester (1710 cm^{-1}), and C=C-bond (1645 cm^{-1}) functionalites. The ^{1}H-NMR spectrum of **1** (*Table 1*) exhibited typical signals at δ(H) 6.30 and 5.50 (H–C(13β) and H–C(13α)) indicating the

Table 1. *^{1}H-NMR Spectral Data* ($CDCl_3$) *for Eupatozansins A–C* (**1**–**3**). Chemical shifts in ppm, *J* values in Hz are in parentheses. Assignments were made using COSY and HMBC techniques.

	1	**2**	**3**
H–C(1)	4.34 (br. *s*)	5.06 (*d*, *J* = 9.9)	5.01 (*d*, *J* = 9.9)
H–C(2)	2.91–2.97 (*m*)	4.49 (*dd*, *J* = 9.9, 7.5)	5.60 (*ddd*, *J* = 10.2, 10.2, 6.3)
H–C(3)	3.34 (*s*)	4.12 (*d*, *J* = 7.5)	2.19 (*dd*, *J* = 10.2, 10.2), 2.74 (*dd*, *J* = 10.2, 6.3)
H–C(5)	2.34 (*m*)	5.09 (*d*, *J* = 9.6)	5.05 (*d*, *J* = 9.9)
H–C(6)	4.18 (*dd*, *J* = 9.0, 10.5)	5.13 (*dd*, *J* = 9.6, 8.4)	5.10 (*dd*, *J* = 9.9, 9.0)
H–C(7)	3.15 (*dd*, *J* = 8.5, 3.3)	2.93–2.98 (*m*)	2.92–2.98 (*m*)
H–C(8)	5.46–5.51 (*m*)	5.79 (*d*, *J* = 3.6)	5.71 (*d*, *J* = 7.2)
CH_2(9)	2.88–2.94 (*m*)	2.89 (*dd*, *J* = 15.0, 3.3), 2.39 (*dd*, *J* = 15.0, 3.3)	2.29 (*dd*, *J* = 15.0, 2.4), 2.85 (*dd*, *J* = 15.0, 6.6)
CH_2(13)	6.30 (*d*, *J* = 2.4), 5.50 (*d*, *J* = 2.4)	6.36 (*d*, *J* = 2.4), 5.61 (*d*, *J* = 2.4)	6.32 (*d*, *J* = 2.4), 5.61 (*d*, *J* = 2.4)
CH_2(14) or Me(14)	5.05 (*s*)	1.56 (*s*)	1.68 (*s*)
Me(15)	1.67 (*s*)	1.88 (*s*)	1.82 (*s*)
H–C(3′)	6.05 (*q*, *J* = 6.9)	6.08 (*q*, *J* = 6.9)	6.10 (*q*, *J* = 7.2)
Me(4′)	1.90 (*d*, *J* = 6.6)	1.83 (*d*, *J* = 7.2)	1.85 (*d*, *J* = 7.2)
Me(5′)	1.78 (*s*)	1.79 (*s*)	1.80 (*s*)
COMe			2.06 (*s*)

presence of an exocyclic α-methylene-γ-lactone group in the germacranolide, commonly encountered in *Eupatorium* species. The COSY spectrum of **1** showed correlations of H–C(13)/H–C(7) (δ(H) 3.15, *dd*, $J = 8.5, 3.3$), H–C(7)/H–C(6) (δ(H) 4.1, *dd*, $J = 9.0, 10.5$)/H–C(8) (δ(H) 5.46–5.51, *m*), H–C(8)/H–C(9) (δ(H) 2.88–2.94 (*m*)). The large coupling constants of $J_{5,6}$ and $J_{6,7}$ were consistent with a *trans*-fused lactone ring and an α-oriented H–C(7). The ^{13}C-NMR data of **1** exhibited two exomethylene C-atoms (*Table 2*). One of the exomethylene C-atoms resonated at δ(C) 120.4 and an olefinic CH_2 group appeared as a *singlet* at δ(H) 5.05. Both signals were assigned to CH_2(14). The presence of an angeloyl ester was supported by signals at δ(C) 167.2 (C(1′)), 127.4 (C(2′)), 138.9 (C(3′)), 15.9 (C(4′)), and 20.7 (C(5′)), along with a base peak at *m*/*z* 83 ($[C_5H_7O]^+$) in the EI-MS. The oxygenated H-atom at δ(H) 4.34 showed an HMQC correlation to δ(C) 76.6 (C(1)) in addition to HMBC correlation with the exomethylene (C(14)). The Me group at δ(H) 1.67 (*s*, Me(15)) showed HMBC correlations with both the oxygenated quaternary C-atom at δ(C) 66.2 (C(4)) and the oxygenated CH group at δ(C) 65.2 (C(3)), along with the presence of two oxygenated CH groups (δ(C) 51.8 and 49.8), which implied the presence of two epoxy rings close to the Me(15) group. The epoxy rings were located at the 4,5- and 2,3-positions as a result of the observed correlations between H–C(7)/C(5), H–C(6)/C(4), H–C(5)/C(3), H–C(3)/C(1) and C(5), and H–C(2)/C(10) in the HMBC spectrum. The magnitudes of $J_{5,6}$ (10.5 Hz) and $J_{6,7}$ (8.5 Hz), together with the small $J_{7,8}$ (3.3 Hz) were consistent with *trans*-fusion of the lactone ring and α-orientation of both H–C(5) and H–C(8). The proposed relative configuration of **1** was determined by

Table 2. *^{13}C-NMR Spectral Data* ($CDCl_3$) *for Eupatozansins A–C* (**1**–**3**). DEPT and HMQC Experiments were used for assignment.

	1	**2**	**3**
C(1)	76.6 (*d*)	126.7 (*d*)	129.6(*d*)
C(2)	49.8 (*d*)	74.8 (*d*)	70.4 (*d*)
C(3)	65.2 (*d*)	83.4 (*d*)	45.3 (*t*)
C(4)	66.2 (*s*)	143.2 (*s*)	140.5 (*s*)
C(5)	51.8 (*d*)	131.2 (*d*)	129.5 (*d*)
C(6)	77.0 (*d*)	75.1 (*d*)	74.8 (*d*)
C(7)	48.5 (*d*)	52.2 (*d*)	52.7 (*d*)
C(8)	68.2 (*d*)	71.3 (*d*)	70.5 (*d*)
C(9)	37.9 (*t*)	43.8 (*t*)	43.2 (*t*)
C(10)	140.8 (*s*)	136.1 (*s*)	136.7 (*s*)
C(11)	134.3 (*s*)	136.3 (*s*)	137.1 (*s*)
C(12)	169.3 (*s*)	170.6 (*s*)	169.3 (*s*)
C(13)	122.3 (*t*)	121.6 (*t*)	121.2 (*t*)
C(14)	120.4 (*t*)	13.8 (*q*)	19.7 (*q*)
C(15)	18.4 (*q*)	19.8 (*q*)	18.6 (*q*)
C(1′)	167.2 (*s*)	166.2 (*s*)	166.7 (*s*)
C(2′)	127.4 (*s*)	127.3 (*s*)	127.5 (*s*)
C(3′)	138.9 (*d*)	139.1 (*d*)	139.4 (*d*)
C(4′)	15.9 (*q*)	15.7 (*q*)	15.6 (*q*)
C(5′)	20.7 (*q*)	20.9 (*q*)	20.7 (*q*)
COMe			170.1 (*s*), 21.2 (*q*)

NOESY correlations (*Fig. 1*) of H–C(7)/H–C(5)/H–C(8), H–C(5)/Me(15) and H–C(2), H–C(3)/Me(15) and H–C(1), and H–C(2)/H–C(1), proving the β-orientation of the OH group of C(1) and of the epoxy rings. On the basis of the above analysis, compound **1** was assigned as 8α-angeloyloxy-1α-hydroxy-2α,3-epoxy-4α,5-epoxy-6βH,7αH-germacra-10(14),11(13)-dien-12,6-olide.

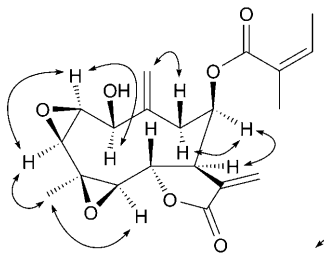

Fig. 1. *NOESY Correlations for compound* **1**

Eupatozansin B (**2**) had a molecular formula $C_{20}H_{26}O_6$ as deduced from HR-EI-MS. The ^{1}H- and ^{13}C-NMR data (*Tables 1* and *2*) indicated that compound **2** was a germacranolide similar to **1**. Its ^{1}H-NMR spectrum exhibited typical signals of an α-methylene-γ-lactone moiety at δ(H) 6.36 (*d*, $J = 2.4$) and 5.61 (*d*, $J = 2.4$) and an angeloyl ester at δ(H) 6.08 (*q*, $J = 6.9$), 1.83 (*d*, $J = 7.2$), and 1.79 (*s*). The HMBC of **2** showed a correlation between the lactone C=O group (δ(C) 170.6) and H–C(8) (δ(H) 5.79). In the COSY spectrum, cross-peaks between H–C(1)/H–C(2)/H–C(3) were observed. The olefinic H–C(5) at δ(H) 5.09 (*d*, $J = 9.6$) exhibited HMBC correlations with C(6) (δ(C) 75.1), C(5) (δ(C) 131.2), C(7) (δ(C) 52.2), C(15) (δ(C) 19.8), and C(3) (δ(C) 83.4). The relative configuration of **2** was determined by a NOESY experiment as illustrated in *Fig. 2*. Thus, the structure of **2** was identified as 8β-angeloyloxy-2α,3β-dihydroxy-6βH,7αH-germacra-1(10),11(13)-dien-12,6-olide.

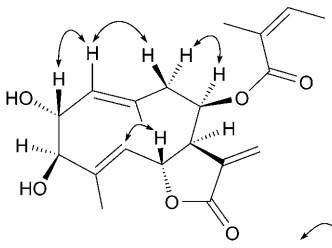

Fig. 2. *NOESY Correlations for compound* **2**

The molecular formula $C_{20}H_{22}O_6$ of eupatozansin C (**3**) was obtained from the HR-EI-MS signal at *m*/*z* 388.4549. The EI-MS showed a molecular ion peak at *m*/*z* 388 (M^+), and a base peak at *m*/*z* 83, indicating that **3** contains a C_5-unsaturated ester side chain. The NMR together with the MS data established the presence of an angeloyl ester. The ^{1}H-NMR spectrum (*Table 1*) of **3** exhibited typical signals of an α-methylene-γ-lactone (δ(H) 6.32, *d*, $J = 2.4$; 5.61, *d*, $J = 2.4$), which coupled to H–C(7) (δ(H) 2.92–2.98). Furthermore, the ^{1}H,^{1}H-COSY spectrum revealed correlations of

H–C(7)/H–C(6) and H–C(6)/H–C(5), as well as H–C(3) (δ(H) 2.19 and 2.74)/H–C(2) (δ(H) 5.60)/H–C(1) (δ(H) 5.01). The ^{13}C-NMR data of **3** indicated the presence of an AcO group (δ(C) 170.1, 21.2). Moreover, HMBC correlations between C(1′) of the angeloyl moiety and H–C(8), and between C(1″) of the AcO group and H–C(2) located the positions of the two ester substituents. The relative configuration of **3** was determined by acetylation of compound **7**, which yielded a product identical to compound **3**. In conclusion, the structure of **3** was determined as 8β-angeloyloxy-2α-acetyloxy-6βH,7αH-germacra-1(10),11(13)-dien-12,6-olide.

In addition to the new compounds **1**–**3**, five known compounds were determined as (5*S*,6*R*,7*R*,8*R*)-8-angeloyloxy-2-oxoguaia-1(10),3,11(13)-trien-12,6-olide (**4**), costunolide (**5**), leptocarpin (**6**), 2α-hydroxyeupatolide 8-*O*-angelate (**7**) and quercetin (**8**) by comparison of their spectral data with those in the literature [11–15].

All isolated sesquiterpene lactones **1**–**7** were evaluated in a cytotoxicity assay against human Hepa59T/VGH, Daoy, HeLa, and WiDr tumor cell lines (*Table 3*). A preliminary result revealed that compounds **1**, **6**, and **7** exhibit moderate cytotoxicity against the four tumor cells.

Table 3. *Cytotoxicity Data of* **1**–**7** *Against Human Tumor Cells*

	IC_{50} [μg/ml]			
	HeLa[a])	WiDr[a])	Daoy[a])	Hepa59T/VGH[a])
1	7.5	5.1	4.5	3.5
2	5.4	5.6	–[b])	7.1
3	–	12.2	10.7	15.6
4	–	3.7	–	4.1
5	5.6	–	5.1	3.3
6	3.9	4.4	3.9	5.1
7	4.9	3.7	2.9	4.7
Mitomycin-C	0.11	0.09	0.07	0.13

[a]) Key to cell lines used: Hepa59T/VGH: Human liver carcinoma, HeLa: Human cervical epitheloid carcinoma, WiDr: Human colon carcinoma, Daoy: human medulloblastoma. [c]) –: Inactive, $IC_{50} > 20$ μg/ml.

Experimental Part

General. Silica gel *60* (*Merck*) was used for column chromatography (CC), and pre-coated silica gel plates (*Merck, Kieselgel 60 F-254*, 1 mm) were used for preparative TLC. *LiChrospher® 100 RP-18e* (5 μm, *250–10*, *Merck*) was used for RP-HPLC. Optical rotation: *Jasco DIP-1000* polarimeter. IR and UV Spectra: *Hitachi T-2001* and *Hitachi U-3210* spectrophotometers, resp. The ^{1}H-, ^{13}C-NMR, COSY, HMQC, HMBC, and NOESY spectra: *Bruker FT-300* spectrometer, with TMS as internal standard; the chemical shifts are given in δ [ppm] and coupling constants *J* in Hz. Low-resolution EI-MS and HR-ESI-MS: *JEOL JMS-HX 110* mass spectrometer.

Plant Material. The aerial parts of *E. chinense* var. *tozanense* were collected at the mountain Ali, Taiwan in September, 2005. The material was identified by one of the authors (*Y. C. S.*). A voucher specimen was deposited with the School of Pharmacy, College of Medicine, National Taiwan University, Taipei, Taiwan.

Extraction and Isolation. The dried arial parts of *E. chinense* var. *tozanense* (1.2 kg) was ground to powder and extracted with acetone (10 l) three times, and the combined extracts were evaporated under

vacuum. The dark green crude extract (140 g) was partitioned between AcOEt/H_2O (1:1, each 3 l) to yield an AcOEt-soluble fraction (70 g). The latter was partitioned between hexane/MeOH/H_2O (4:3:1), successively, to give an aq. MeOH layer and a hexane layer. The aq. MeOH-soluble layer was extracted with AcOEt to give an AcOEt-soluble layer (14.7 g), which was chromatographed on a *Sephadex LH-20* (4.7 × 75 cm) column using MeOH/$CHCl_2$ (1:1) as eluent to yield four fractions, *Fr. 1–4*. *Fr. 4* was insoluble in MeOH and gave pure **8** (20 mg). *Fr. 2* (1.3 g) was separated by SiO_2 CC with hexane/AcOEt (9:1–0:1) to afford 5 subfractions, *Fr. 4-1–4-5*. *Fr. 4-2* (350 mg) was subjected to a prep. RP-HPLC (10 × 250 mm, flow rate 2 ml/min) column, using 65% MeOH/H_2O to afford compounds **3** (12 mg), **5** (27 mg) and **6** (11 mg). *Fr. 4-3* (230 mg) was chromatographed on a prep. RP-HPLC column (10 × 250 mm, flow rate 2 ml/min), eluted with 65% MeOH/H_2O to yield **1** (34 mg) and **7** (12.5 mg). *Fr. 4-4* (95 mg) was repeatedly chromatographed on a prep. RP-HPLC column (10 × 250 mm, flow rate 2 ml/min) using 60% MeOH/H_2O to yield **2** (7.6 mg) and **4** (10 mg).

Eupatozansin A (=*8α-Angeloyloxy-1α-hydroxy-2α,3-epoxy-4α,5-epoxy-6β*H,*7α*H-*germacra-10(14),11(13)-dien-12,6-olide*; **1**). Gum. $[\alpha]_D^{24} = -31.8$ ($c = 0.5$, CH_2Cl_2). IR (KBr): 3430, 2927, 1761, 1710, 1645. ^{1}H- (300 MHz, $CDCl_3$) and ^{13}C- (75 MHz, $CDCl_3$) NMR: *Tables 1* and *2*, resp. EI-MS: 358 ($[M - H_2O]^+$), 283 ($[M - C_5H_7O]^+$), 83 (100, $[C_5H_7O]^+$). HR-EI-MS: 358.3589 ($[M - H_2O]^+$, $C_{20}H_{22}O_6^+$; calc. 358.3851).

Eupatozansin B (=*8β-Angeloyloxy-2α,3β-dihydroxy-6β*H,*7α*H-*germacra-1(10),11(13)-dien-12,6-olide*; **2**). Gum. $[\alpha]_D^{24} = +37.6$ ($c = 0.5$, CH_2Cl_2): IR (KBr): 3470, 2941, 1758, 1713, 1645. ^{1}H- (300 MHz, $CDCl_3$) and ^{13}C- (75 MHz, $CDCl_3$) NMR: *Tables 1* and *2*, resp. EI-MS: 344 ($[M - H_2O]^+$), 279 ($[M - C_5H_7O]^+$), 83 ($[C_5H_7O]^+$). HR-EI-MS: 344.4011 ($[M - H_2O]^+$, $C_{20}H_{24}O_5^+$; calc. 344.4016).

Eupatozansin C (=*8β-Angeloyloxy-2α-acetyloxy-6β*H,*7α*H-*germacra-1(10),11(13)-dien-12,6-olide*; **3**). Gum. $[\alpha]_D^{24} = +43.1$ ($c = 0.5$, CH_2Cl_2). IR (KBr): 1766, 1720, 1660. ^{1}H- (300 MHz, $CDCl_3$) and ^{13}C- (75 MHz, $CDCl_3$) NMR: *Tables 1* and *2*, resp. EI-MS: 388 (M^+), 370 ($[M - H_2O]^+$), 305 ($[M - C_5H_7O]^+$), 83 (100, $[C_5H_7O]^+$). HR-EI-MS: 388.4549 (M^+, $C_{20}H_{22}O_6^+$; calc. 388.4541).

Acetylation of **7**. Compound **7** (2 mg) was treated with a mixture of Ac_2O/pyridine at r.t. for 1 h. Usual workup gave a product (1.5 mg), which showed identical signals in the ^{1}H-NMR- spectra and in the MS, and an identical $[\alpha]$ as compound **3**.

Cytotoxicity Assay. Cytotoxicity was determined against HeLa (human cervical epitheloid carcinoma), Daoy (medulloblastoma), and WiDr (human colon adenocarcinoma), and Hepa59T/VGH (liver carcinoma) tumor cells. The assay procedure using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was carried out as previously described [16]. In brief, the cells were cultured in *RPMI-1640* medium. After seeding of the cells in a 96-well microplate for 4 h, 20 µl of sample was placed in each well and incubated at 37° for 3 d, and then 20 µl MTT was added. After 5 h, the medium was removed, and DMSO (200 µl/well) was put into the microplate with shaking for 10 min, the formazan crystals were redissolved, and their absorbance was measured on a microtiter plate reader (*Dynatech*, *MR 7000*) at a wavelength of 550 nm.

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