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Blepharocalyxins C–E: three novel antiproliferative diarylheptanoids from the seeds of *Alpinia blepharocalyx*

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Abstract

Three novel diarylheptanoids, blepharocalyxins C–E (**1**–**3**), were isolated from an EtOH extract of seeds of *Alpinia blepharocalyx*, and their structures have been elucidated by the use of spectroscopic techniques. Blepharocalyxins D (**2**) and E (**3**) exhibited potent antiproliferative activity against murine colon 26-L5 carcinoma and human HT-1080 fibrosarcoma cells, with ED_{50} values of 3.61 and 9.02 μM, respectively.

Alpinia blepharocalyx K. SCHUM. is a Zingiberaceous plant and its seeds are used for treatment of stomach disorders in the People's Republic of China. This plant contains many diarylheptanoids having a chalcone or a flavanone moiety in the molecule,[1–4] i.e. calyxin As with a chalcone or a flavanone moiety at the C-5 position,[2,4] calyxin Bs with a chalcone or a flavanone moiety at the C-7 position,[1] and blepharocalyxins with two diarylheptanoid units in the molecule.[3] In a continuing study on the constituents of the seeds of *A. blepharocalyx*, we have recently isolated three novel diarylheptanoids, blepharocalyxins C–E (**1**–**3**, Fig. 1), from a residual fraction of the 95% EtOH extract of the seeds, after a series of chromatographic separations on Sephadex LH-20, silica gel and ODS columns, followed by normal- and reversed-phase preparative TLC. Their structures have been determined by the use of spectroscopic techniques to be a diarylheptanoid dimer with a novel carbon framework. In this communication, we report the structure elucidation of these novel diarylheptanoids, together with their antiproliferative activity against murine colon 26-L5 carcinoma and human HT-1080 fibrosarcoma cells.

Blepharocalyxin C (**1**) was obtained as a light-yellow amorphous solid and showed $[\alpha]_D^{25}$ +63.5 (*c* 0.035; MeOH). The molecular formula of **1** was determined by negative ion HR-FABMS to be $C_{38}H_{42}O_7$ [*m*/*z* 609.2849; calcd for $C_{38}H_{41}O_7$ $(M-H)^-$, 609.2852], and its IR spectrum showed hydroxyl absorption at 3350 cm^{-1}. The ^1H and ^{13}C NMR spectra indicated the presence of four

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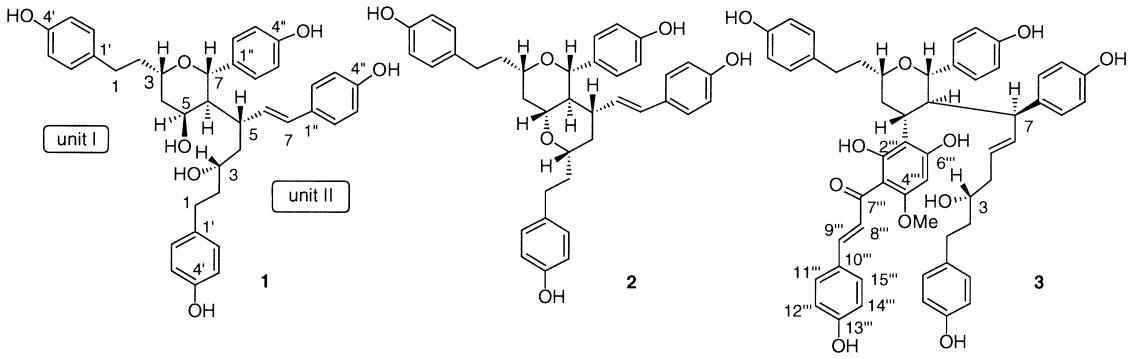

Figure 1. Structures of compounds isolated from *Alpinia blepharocalyx*

para-substituted benzene rings, a *trans*-olefin, six (including oxygen-substituted four) methines and six methylenes (Table 1), suggesting **1** to be a diarylheptanoid dimer. The analyses of the DEPT, COSY, HMQC and HMBC spectra (Fig. 2a) suggested the diarylheptanoid units to be 5-hydroxy-4″-de-*O*-methylcentrolobin (unit I) and 1,7-bis(4-hydroxyphenyl)-5-hydroxy-1-heptene (unit II).† The COSY correlation between H-6 of unit I (H-I-6) and H-5 of unit II (H-II-5) and the HMBC correlations between C-5 of unit II (C-II-5) and H-I-6 indicated the two units to be connected through the C–C bond between C-I-6 and C-II-5. The stereochemistry at the chiral centers was determined by analyses of the coupling constants and the ROESY correlations (Fig. 2b). The axial nature of H-I-3, H-I-6 and H-I-7 was evident from the large coupling constants of H-I-3 with H-I-4ax ($J = 11.3$ Hz) and of H-I-6 with H-I-7 ($J = 11.0$ Hz), while H-I-5 must be equatorial from the small coupling constant ($J = 1.6$ Hz). In the ROESY spectrum, on the other hand, H-I-3 and H-I-5 showed ROESY correlations with H-I-4eq and H-I-7 and with H-I-4ax, H-I-4eq and H-I-6, indicating the configuration, on the pyran ring, as depicted in Fig. 2b. On the configuration at II-5, the coupling constant between the vicinal protons H-I-6 and H-II-5 ($J = 5.0$ Hz) and the ROESY correlations of H-II-5 with H-I-5 and H-I-6 revealed the two protons to

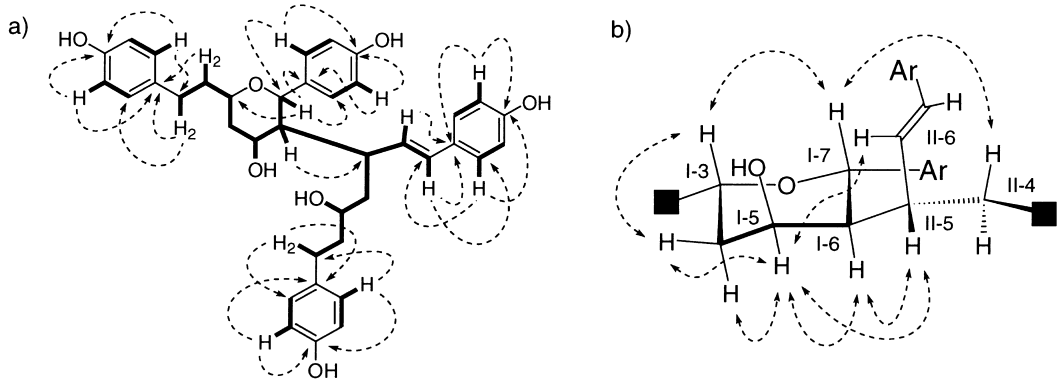

Figure 2. Significant HMBC (a) and ROESY correlations (b) of blepharocalyxin C (**1**). Bold line in (a) indicates the C–C bond deduced by the COSY and HMQC spectra

† 5,6-Dehydro-4″-de-*O*-methylcentrolobin was isolated from the same 95% EtOH extract[5] and 1,7-bis(4-hydroxyphenyl)-5-hydroxy-1-heptene was a plausible biogenetic intermediate for the novel diarylheptanoids.[1–3]

have a *gauche* relationship. Thus, the intense ROESY correlations between H-II-6 and H-I-5 and between H-II-4 and H-I-7 should indicate the configuration in Fig. 2b. Because the diarylheptanoids isolated so far from *A. blepharocalyx* had the *S* configuration at the C-3 position,[1–3] the absolute configuration at I-3 and II-3 was assumed to be *S*. Thus, the absolute configuration of blepharocalyxin C (**1**) was concluded to be (I-3)*S*, (I-5)*S*, (I-6)*S*, (I-7)*S*, (II-3)*S* and (II-5)*S*.

Table 1
^{1}H and ^{13}C NMR data for the diarylheptanoid moiety of blepharocalyxins C–E (**1**–**3**) in CD_3OD[a)]

		1		**2**		**3**	
Unit	No.	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
I	1	2.54 m (2H)	31.73	2.57 m (2H)	31.74	2.32 m; 2.56 m	32.38
	2	1.61 m; 1.71 m	39.26	1.82 m (2H)	39.11	1.58 m (2H)	39.39[b)]
	3	3.89 dtd (11.3, 5.5, 1.6)	72.08	3.54 m	76.40	3.27 m	71.09
	4	1.50 m	41.54	1.49 m	41.76	1.64 m	41.50[c)]
		1.80 dt (13.0, 1.6)		1.97 br d (12.0)		1.91 br t (11.2)	
	5	4.31 q (1.6)	67.96	3.32 m[d)]	80.64	3.04 dt (11.0, 4.9)	28.81
	6	1.89 ddd (11.0, 5.0, 1.6)	51.69	1.57 q (10.0)	52.22	2.52 td (11.0, 9.5)	47.44
	7	4.60 d (11.0)	79.58	3.95 d (10.0)	84.39	5.25 d (11.0)	83.82
	1'		134.36		134.20		134.72
	2',6'	6.94 d (8.5)	130.34	6.98 d (8.5)	130.32	6.89 d (8.5)	130.14
	3',5'	6.64 d (8.5)	116.00	6.67 d (8.5)	116.10	6.69 d (8.5)	116.02
	4'		156.12		156.30[b)]		155.94
	1"		133.60		133.51		133.60
	2",6"	7.19 d (8.5)	130.97	6.96 d (8.5)	130.36	7.13 d (8.5)	131.09
	3",5"	6.73 d (8.5)	115.97	6.66 d (8.5)	116.06	6.75 d (8.5)	116.02
	4"		158.13		158.11		158.62
II	1	2.40 m (2H)	32.09	2.62 m (2H)	31.61	2.24 m; 2.37 m	31.37
	2	1.57 m (2H)	41.41	1.71 m (2H)	39.32	1.47 m (2H)	39.06[b)]
	3	3.31 m	69.60	3.48 m	77.35	3.19 m	71.18
	4	1.50 m (2H)	40.68	1.25 m	39.32	1.50 dt (13.0 6.0)	38.51[c)]
				1.60 m		1.75 dt (13.0, 5.6)	
	5	2.46 m	40.91	2.17 m	43.14	4.87 m[e)]	125.57
	6	5.60 dd (15.9, 9.3)	131.56	5.00 dd (15.9, 8.5)	132.73	4.78 dd (15.5, 9.5)	137.97
	7	6.00 d (15.9)	131.11	5.74 d (15.9)	128.64	3.32 m[f)]	51.51
	1'		134.41		134.25		134.46
	2',6'	6.87 d (8.5)	130.26	6.98 d (8.5)	130.32	6.73 d (8.5)	130.22
	3',5'	6.58 d (8.5)	115.97	6.67 d (8.5)	116.10	6.62 d (8.5)	115.99
	4'		156.21		156.35[b)]		156.06
	1"		130.89		130.98		135.44
	2",6"	6.97 d (8.5)	128.22	6.62 d (8.5)	128.03	6.98 d (8.5)	130.03
	3",5"	6.66 d (8.5)	116.06	6.53 d (8.5)	115.77	6.73 d (8.5)	116.61
	4"		157.38		157.02		156.84

[a)] Data for the chalcone moiety of **3**: δ_H 5.98 (1H, s, H-I-5'''), 7.79 (1H, d, J = 16.0 Hz, H-I-8'''), 7.69 (1H, d, J = 16.0 Hz, H-I-9'''), 7.50 (2H, d, J = 8.5 Hz, H-I-11''',15'''), 6.82 (2H, d, J = 8.5 Hz, H-I-12''',14'''), 3.86 (3H, s, OMe); δ_C 110.95 (C-I-1'''), 164.31 (C-I-2'''), 106.69 (C-I-3'''), 162.79 (C-I-4'''), 93.19 (C-I-5'''), 162.46 (C-I-6'''), 194.20 (C-I-7'''), 125.46 (C-I-8'''), 143.92 (C-I-9'''), 128.39 (C-I-10'''), 131.35 (C-I-11''',15'''), 116.89 (C-I-12''',14'''), 161.14 (C-13'''), 56.37 (OMe). [b,c)] May be interchanged. [d,f)] Overlapped with the solvent signal but in acetone-d_6 appeared at δ 3.37 (td, J = 10.2, 4.5 Hz) and 3.42 (t, J = 9.5 Hz), respectively. [e)] Overlapped with H_2O signal but in acetone-d_6 appeared at δ 5.04 (ddd, J = 15.5, 9.5, 5.3 Hz).

Blepharocalyxin D (**2**), $[\alpha]_D^{25}$ +18.5 (*c* 0.025; MeOH), was obtained as a light-yellow amorphous solid, and its molecular formula was determined by negative ion HR-FABMS to be $C_{38}H_{40}O_6$, a water molecule less than that of **1**. Its IR spectrum showed a broad absorption band at 3400 cm^{-1}, indicating the presence of a hydroxyl group. The ^{1}H and ^{13}C NMR spectra of **2** resembled those of **1** and indicated the presence of two diarylheptanoid units, which was confirmed by the COSY, HMQC and HMBC spectra. The molecular formula and the low-field shift of C-I-5 (**2**: δ

80.64; **1**: δ 67.96) and C-II-3 (**2**: δ 77.35; **1**: δ 69.60) indicated the presence of an ether linkage between I-5 and II-3. The coupling pattern of H-I-6 with a large coupling constant ($J = 10.0$ Hz) and the ROESY correlations among H-I-3, H-I-5 and H-I-7, and among H-I-5, H-II-3 and H-II-5, indicated their relative configuration. From these data and the assumption that I-3 and II-3 have the *S* configuration, the stereochemistry of **2** was concluded to be (I-3)*S*, (I-5)*R*, (I-6)*S*, (I-7)*S*, (II-3)*S* and (II-5)*S*.

Blepharocalyxin E (**3**), $[\alpha]_D^{25}$ +145.5 (*c* 0.025; MeOH), was also obtained as a light-yellow amorphous solid, and its molecular formula was determined to be $C_{54}H_{54}O_{11}$ [*m*/*z* 877.3566; calcd for $C_{54}H_{53}O_{11}$ $(M-H)^-$, 877.3588] by negative ion HR-FABMS. In the IR spectrum, an absorption band attributable to a hydroxyl group was observed at 3300 cm^{-1}. The ^1H and ^{13}C NMR spectra were partially identical with those of epicalyxin F (**4**), previously isolated from the same extract,[4] but they indicated the presence of one more diarylheptanoid unit. Extensive analyses of the DEPT, COSY, TOCSY and HMQC spectra indicated the two units to be connected through the C–C bond between C-6 of unit I (I-6) and C-7 of unit II (II-7), which was confirmed by the HMBC correlations H-I-7/C-II-7 and H-II-7/C-I-6 (Fig. 3a). The stereochemistry at the chiral centers in unit I (I-3, I-5, I-6, I-7) was determined to be the same as that of **2** by ROESY experiments (Fig. 3b). The large coupling constant between the vicinal protons H-I-6 and H-II-7 ($J = 9.5$ Hz) indicated the two protons to have an *anti* relationship. On the other hand, the intense ROESY correlations of H-II-2″(6″) with H-I-5 and H-I-7 and of H-II-6″(2″) with H-I-6 in CD_3OD, together with those of H-I-6 with H-II-5 and H-II-6 in acetone-d_6, revealed the relative configuration depicted in Fig. 3b. From these data and the assumption that I-3 and II-3 have the *S* configuration, the stereochemistry of **3** was concluded to be (I-3)*S*, (I-5)*R*, (I-6)*S*, (I-7)*S*, (II-3)*S* and (II-7)*S*.

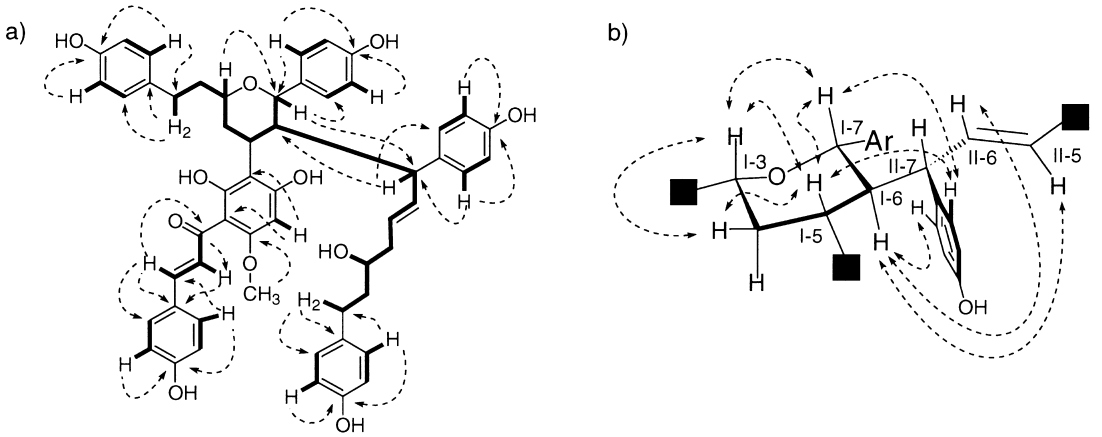

Figure 3. Significant HMBC (a) and ROESY correlations (b) of blepharocalyxin E (**3**). Bold line in (a) indicates the C–C bond deduced by the COSY and HMQC spectra

Blepharocalyxins C–E (**1**–**3**) have a novel carbon framework consisting of two diarylheptanoid units joined with a C–C bond between C-6 of one unit and C-5 or C-7 of the other unit. Moreover, blepharocalyxin E (**3**) possesses an additional chalcone moiety. Previously, we reported two diarylheptanoids, blepharocalyxins A and B, having two diarylheptanoid units joined by a chalcone unit from the 95% EtOH extract of the seeds of *A. blepharocalyx*.[3] However, there is no

precedent of dimeric diarylheptanoids having the C–C bond between two diarylheptanoid units, and thus **1**–**3** are the first examples of dimeric diarylheptanoids.

Finally, we examined the antiproliferative activity of blepharocalyxins C–E (**1**–**3**) against human HT-1080 fibrosarcoma[6] and highly liver-metastatic murine colon 26-L5 carcinoma[7] cells. Cellular viability in the presence and absence of experimental agents was determined using the standard MTT assay,[8] and the results are summarized in Table 2. Among the three compounds, blepharocalyxin D (**2**) showed the strongest antiproliferative activity against colon 26-L5 carcinoma cells, with an ED_{50} value of 3.61 μM, whereas blepharocalyxin E (**3**) exhibited the strongest activity against HT-1080 fibrosarcoma cells, with an ED_{50} value of 9.02 μM. The cytotoxicity of **3** against human HT-1080 fibrosarcoma cells is identical with that of 5-fluorouracil (ED_{50} 8.0 μM), a clinically used drug for the treatment of human tumor,[9] and falls within the range of a potent cytotoxic agent ($ED_{50} < 4$ μg/mL) made by Geran et al.[10]

Table 2
Antiproliferative activity of isolated compounds from residual fraction of *Alpinia blepharocalyx* (ED_{50} values are in μM)[a]

Compounds	Colon 26-L5	HT-1080
Blepharocalyxin C (**1**)	29.55	54.29
Blepharocalyxin D (**2**)	3.61	25.72
Blepharocalyxin E (**3**)	32.17	9.02
Curcumin	23.23	23.42
5-Fluorouracil (**5-FU**)	0.53	8.00

[a] ED_{50} values were calculated from the mean of data of six determinations.

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