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Blepharocalyxins A and B, Novel Diarylheptanoids from *Alpinia blepharocalyx*, and their Inhibitory Effect on NO Formation in Murine Macrophages

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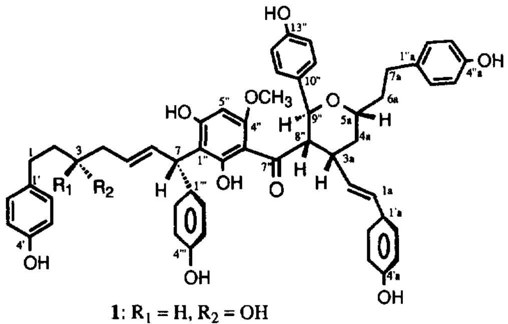
Abstract: *Blepharocalyxins A and B (1 and 2), novel natural products having two diarylheptanoids and a chalcone moiety were isolated from the seeds of Alpinia blepharocalyx K. Schum. The structures of these compounds were elucidated by intensive study of the 2D NMR spectroscopy. These compounds inhibited nitric oxide production in endotoxin-activated murine macrophages, J 774.1.* Copyright © 1996 Elsevier Science Ltd

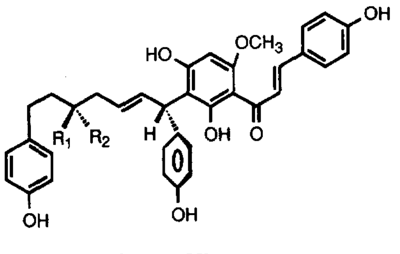
In a preceding communication,1) we reported the isolation and structure determination of three diarylheptanoids from the seeds of *A. blepharocalyx*. In continuing investigation of the ether soluble fraction of the EtOH extract of *A. blepharocalyx*, we isolated two novel diarylheptanoids, named blepharocalyxins A (**1**) and B (**2**), which showed inhibitory activity against nitric oxide (NO) production in activated murine macrophages. These compounds presented a very unique structure having two diarylheptanoids combined with a chalcone. We herein report the isolation and structure elucidation of these compounds based on analysis of the 2D NMR spectroscopy and their inhibitory effect on NO production in activated murine macrophages.

The ether soluble fraction of the EtOH extract of *A. blepharocalyx* yielded **1** (0.0013%) and **2** (0.008%), along with several other known compounds after a series of chromatographic separations together with chiral HPLC.

Blepharocalyxin A (**1**), a light yellow amorphous solid, showed $[\alpha]_D$ -56.4° (MeOH, c = 0.17) whose molecular formula was determined to be $C_{54}H_{54}O_{11}$ [*m/z*; 879.3768 $(M+H)^+$, calcd. 879.3745] by high-resolution FABMS. In the IR spectrum (KBr), absorption bands attributable to the hydroxyl (3350 cm^{-1}) and carbonyl (1680 cm^{-1}) groups appeared. Extensive analyses of ^1H- and ^{13}C-NMR spectra including



1: $R_1 = H, R_2 = OH$
2: $R_1 = OH, R_2 = H$



3: $R_1 = H, R_2 = OH$
4: $R_1 = OH, R_2 = H$

DEPT, off-resonance and quaternary carbon observation indicated the presence of a ketone carbonyl, six methylenes, six sp^3 methines, a methoxy group, two sets of *trans* double bonds, twenty *ortho* coupling aromatic protons, a singlet aromatic proton and fifteen quaternary carbons (see Table 1).

The ^{1}H- and ^{13}C-NMR spectra of **1** were closely similar in part to those of calyxin B (**3**)[1)] except for some chalcone signals, and therefore it was supposed that part of its structure could be similar to that of **3**. However, lack of a conjugated double bond and the presence of two methine signals (δ_H 4.65 dd, J = 4.5, 1.5 Hz and δ_H 5.22 brs) having correlation with ^{13}C signals at δ_C 51.27 and 76.64 in the HMQC spectrum suggested the substitution at the α- and β-positions of the conjugated ketone group of a chalcone moiety. Furthermore, another set of signals in ^{1}H- and ^{13}C-NMR spectra assignable to diarylheptanoid was also observed whose attachment to the chalcone part was revealed by the ^{1}H-^{1}H COSY. Thus, the NMR signals together with the molecular formula of **1** were in accord with the presence of two diarylheptanoids and a chalcone moiety.

Next, we measured the HMBC spectrum of **1** to elucidate the total structure. As can be seen in Fig. 1a, the ^{13}C-NMR signal at δ_C 32.08 (C-1), 31.89 (C-7a), 127.84 (C-5), 130.76 (C-1a), 136.55 (C-1''') and 163.53 (C-6''), showed long-range correlation with the ^{1}H-signals at δ_H 6.94 (2'-H), 6.95 (2''a-H), 5.12 (7-H), 7.08 (2'a-H) 5.12 (7-H), and 5.12 (7-H), respectively. These long-range correlations allowed us to define the attachment of the aromatic rings to the heptanoid chains. Further, the $^3J_{C,H}$ cross peak between δ_C 70.94 (C-5a) and δ_H 5.22 (9''-H) resulted in the formation of a tetrahydropyran ring between C-5a and C-9''. Thus, these long-range correlations together with others shown by arrows in Fig.1a, enabled us to establish the planar structure of **1**.

The relative stereochemistry at six chiral centres (3, 7, 8'', 9'', 3a, 5a) within **1** was elucidated on the basis of the coupling constants of each proton and a ROESY experiment. The relative stereochemistry at positions 3 and 7 was assigned similarly as those of **3** in view of their similar splitting pattern in ^{1}H-NMR and nOe correlations. The determination of the relative stereochemistry at positions C-8'', C-9'', 3a and 5a was found to be ambiguous mainly because of the possibility of the existence of the compound **1** in two diastereomeric forms. However, one possible configuration was proposed on the basis of ROESY correlations. An intense correlation peak between 8''-H and 3a-H in the ROESY spectrum implied that both of them project on the same side. As can be seen in Fig.1b, 8''-H, 9''-H, 3a-H and 5a-H displayed mutual correlation with 11''-H in the 2D ROESY spectrum. The observed correlations in 2D ROESY were further supported by 1D ROESY differences as similar effects were obtained on irradiating 8''-H, 9''-H, 3a-H and 5a-H. Use of a Dreiding stereo model suggested that the correlation among 8''-H, 9''-H, 3a-H, 5a-H and 11''-H would be possible if the benzene ring attached to 9'' together with 8''-H, 3a-H and 5a-H were in β configuration. Based on this consideration, the relative stereochemistry at chiral centres 8'', 9'', 3a and 5a was elucidated. The conformation of the tetrahydropyran ring was determined to be a quasi chair-form through the Dreiding stereo model using the observed coupling constants, corresponding dihedral angels and ROESY correlations. The absolute configuration has not yet been determined. These findings enabled us to propose one of the diastereomeric stereo-structures of blephocalyxin A as shown in **1**.

Blepharocalyxin B (**2**), a light yellow amorphous solid, showed $[\alpha]_D$ -97.7° (MeOH, c = 0.16). The FABMS of **2** and **1** were identical to each other. The ^{1}H- and ^{13}C-NMR spectra of **2** were almost the same as those of **1** (Table 1) but differed from it in the ^{1}H-NMR spectrum splitting patterns at 4-H which was quartet

Table 1. ^{1}H- and ^{13}C-NMR Spectral Data of Blepharocalyxin A (1) and B (2)

	1		2	
Position	δH	δC	δH	δC
1	2.52, 2.61 m	32.08 t	2.51, 2.60 m	32.09 t
2	1.62, 1.78 m	39.62 t	1.61, 1.76 m	39.63 t
3	3.61 m	71.83 d	3.59 m	71.75 d
4	2.26 t (6.5)	41.44 t	2.26 q (6.5)	41.55 t
5	5.55 dt (16.0,7.5)	127.84 d	5.53 dt (16.0, 7.5)	127.92 d
6	6.30 dd (16.0, 7.5)	135.45 d	6.30 dd (16.0, 7.5)	135.62 d
7	5.12 d (7.5)	43.23 d	5.12 d (7.5)	43.01 d
1'	——	134.60 s	——	134.61 s
2'	6.94 d (8.0)	130.28 d	6.91 d (8.0)	130.28 d
3'	6.63 d (8.0)*	116.06 d	6.63 d (8.0)*	116.07 d
4'	——	156.19 s*	——	156.23 s*
1"	——	112.46 s	——	112.43 s
2"	——	166.20 s	——	166.64 s
3"	——	106.22 s	——	106.16 s
4"	——	162.07 s	——	162.11 s
5"	5.91 s	91.77 d	5.90 s	91.82 d
6"	——	163.53 s	——	163.53 s
7"	——	207.15 s	——	207.16 s
8"	4.65 dd (4.5, 1.5)	51.27 d	4.66 dd (4.5, 1.5)	51.30 d
9"	5.22 brs	76.64 d	5.22 brs	76.71 d
10"	——	131.65 s	——	131.67 s
11"	7.36 d (8.0)	129.70 d	7.36 d (8.0)	129.73 d
12"	6.86 d (8.0)	116.31 d	6.86 d (8.0)	116.33 d
13"	——	157.76 s	——	157.80 s
1"'	——	136.55 s	——	136.45 s
2"'	7.01 d (8.0)	129.46 d	7.01 d (8.0)	129.45 d
3"'	6.61 d (8.0)	115.46 d	6.61 d (8.0)	115.48 d
4"'	——	155.76 s	——	155.82 s
1a	6.31 d (16.0)	130.76 d	6.31 d (16.0)	130.79 d
2a	6.18 dd (16.0, 7.5)	130.93 d	6.18 dd (16.0, 7.5)	130.97 d
3a	2.84 m	38.84 d	2.84 m	38.88 d
4a	2.31, 1.50 m	35.49 t	2.31, 1.50 m	35.52 t
5a	3.56 m	70.94 d	3.56 m	70.94 d
6a	1.70, 1.84 m	39.37 t	1.70, 1.84 m	39.42 t
7a	2.48, 2.70 m	31.89 t	2.48, 2.70 m	31.90 t
1'a	——	130.76 s	——	130.79 s
2'a	7.08 d (8.0)	128.33 d	7.07 d (8.0)	128.34 d
3'a	6.66 d (8.0)*	116.24 d	6.66 d (8.0)*	116.26 d
4'a	——	157.62 s	——	157.65 s
1"a	——	134.41 s	——	134.41 s
2"a	6.95 d (8.0)	130.28 d	6.95 d (8.0)	130.28 d
3"a	6.64 d (8.0)*	116.06 d	6.64 d (8.0)*	116.07 d
4"a	——	156.12 s*	——	156.14 s*
-OCH3	3.53 (s)	56.17 q	3.53 (s)	56.17 q

*Values may be interchangeable. ^{1}H-and ^{13}C-NMR spectra were measured at 400 MHz and 100 MHz, respectively, in CD_3OD and coupling constants (parentheses) are in Hz. Multiplicities were established by DEPT data and quaternary carbon observation.

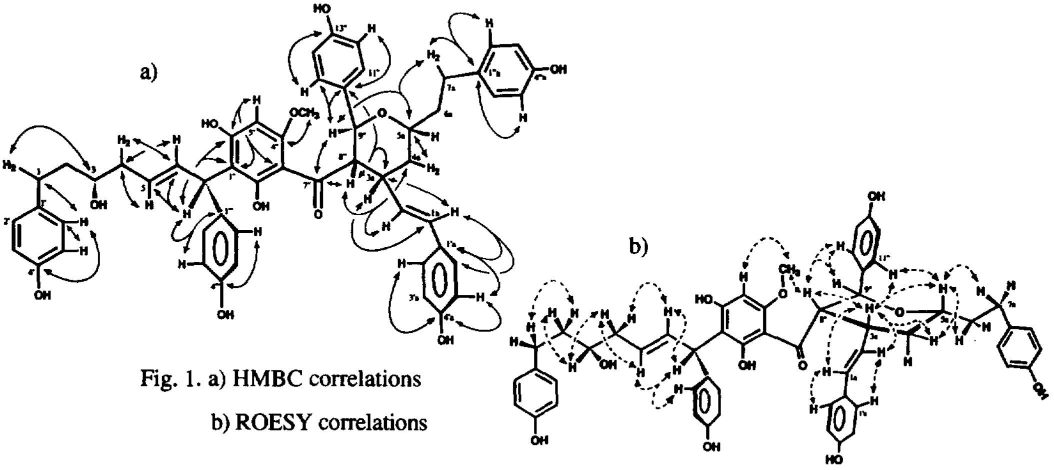


Fig. 1. a) HMBC correlations
b) ROESY correlations

in **2**, while it was triplet in **1**. This difference in the splitting pattern was taken as an important clue to distinguishing the structure between **1** and **2** as a similar difference was observed between **3** and **4**. It is, therefore, pertinent to describe the stereochemistry of 3-H in **2** and **1** as those of **4** and **3**, respectively. Similar ambiguity was observed in assignment of the relative configuration at the chiral centres 8"-H, 9"-H, 3a-H and 5a-H as those of **1** owing to the possibility of the existence of this compound in two diastereomeric forms. However, as in the previous case, the relative stereochemistry at the chiral centres 8"-H, 9"-H, 3a-H and 5a-H was assigned similarly as those of **1** because of their similar splitting pattern in ^{1}H-NMR and ROESY correlations. Thus, this compound was structurally similar to **1**, except for the stereochemistry at position 3. From the data presented above, one of the possible diastereomeric stereo-structures of blepharocalyxin B (**2**) was postulated.

Blepharocalyxins A and B (**1** and **2**) were evaluated for their role as an immunoregulator in activated murine macrophages, J774.1, by *in vitro* assay method.2-3) Nitric oxide produced by activated macrophages has been shown to be involved in TNF-induced shock, hypertension, and vasodilation including hepatotoxicity.4) These compounds at a concentration of 100 μg/ml inhibited the production of nitric oxide by 95%. The inhibitory activity of both compounds was comparable and showed concentration-dependent inhibition in the range of 1-100 μg/ml. These results suggest that **1** and **2** can modulate the immune responses by controlling production of nitric oxide. Studies as to whether the diarylheptanoids act as a direct inhibitor of the nitric oxide synthase or act upon a control system are in progress and will separately be reported.

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