HETEROCYCLES, Vol. 63, No. 1, 2004, pp. 115 - 121
Received, 8th September, 2003, Accepted, 24th October, Published online, 31st October, 2003

TWO NEW BICOUMARINS FROM *CLAUSENA EXCAVATA*

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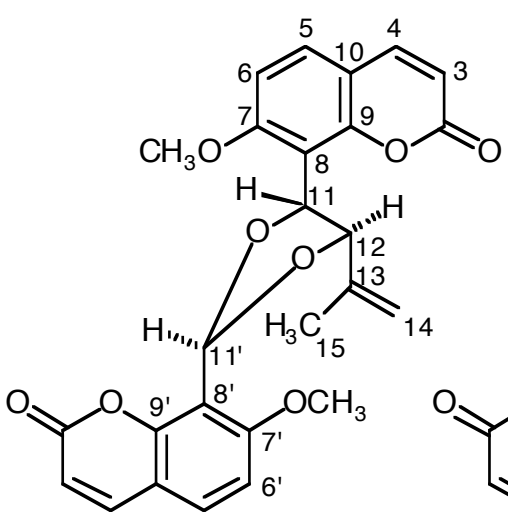
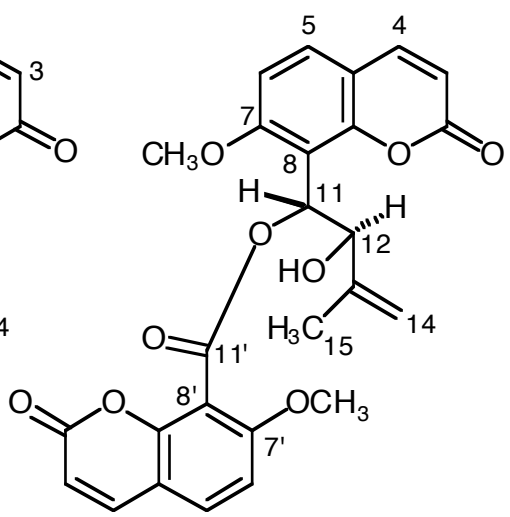
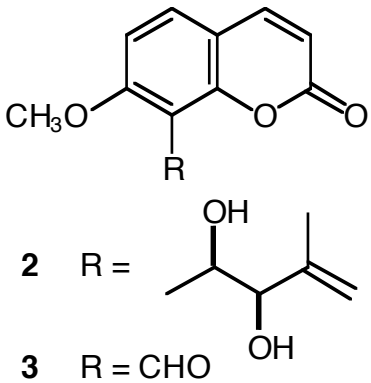
Abstract - Two novel type bicoumarins, named cladimarins A (**1**) and B (**4**), were isolated from the branch of *Clausena excavata* (Rutaceae) collected in Indonesia. The structures were elucidated on the basis of spectroscopical data especially by using HMBC method. Naturally occurring bicoumarin, connected two coumarin moieties by acetal ring as in **1** and by ester bond as in **4**, is rare type. Inhibitory effects of new bicoumarins on EBV-EA activation induced by TPA in Raji cells were also demonstrated.

Clausena excavata (Rutaceae) is growing wild and cultivated from India to southern China through the south-east Asia[1] and in some areas it has been used as folk medicines.[2,3] The constituents of this plant have been well studied and many carbazole alkaloids,[4] coumarins[5] and limonoids[6] were isolated. It is noteworthy that the constituents of this plant differ significantly according to a growing district, that is, plants rich in coumarins contain little or no carbazole alkaloids, whereas those rich in carbazole alkaloids scarcely contain coumarins. We have already reported[7] the isolation of new coumarins from the twigs and leaves of *Clausena excavata* collected in Sumatra, Indonesia. In continuing our investigation of Indonesian medicinal plants, we investigated the constituents of branches of this plant collected in Komandaru, Flores Island. The decoction of bark and branch is used as folk medicine "Ndawa laki" to cure a breast-ache. This paper describes the isolation and structure elucidation of two new type bicoumarins and the result of their inhibitory effects assay on 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced Epstein-Barr virus early antigen (EBV-EA) activation in Raji cells.







2 R =

3 R = CHO

5 R = COOH

1 **4**

Cladimarin A (**1**) was obtained as amorphous powder, $[\alpha]_D$ -12.3° ($CHCl_3$). The molecular formula $C_{26}H_{22}O_8$ was obtained by HR-EI-MS which showed a molecular ion peak at *m/z* 462.1309. The UV (250, 260, 320 nm) and IR (1730, 1610 cm^{-1}) spectral absorptions indicated the presence of coumarin skeleton.[8] The presence of two 7, 8-substituted coumarin moieties was suggested by the ^{1}H-NMR spectrum which showed characteristic signals of H-4, H-3, H-5 and H-6 [H-4', H-3', H-5' and H-6'] of coumarin at δ_H 7.63, 6.27 (each 1H, d, *J* = 9.5 Hz) and 7.41, 6.91 (each 1H, d, *J* = 8.8 Hz) [δ_H 7.61, 6.25 (each 1H, d, *J* = 9.5 Hz) and 7.44, 6.89 (each 1H, d, *J* = 8.8 Hz)]. The presence of two methoxyl groups was suggested by the signals at δ_H 4.03, 4.01 (each 3H, s) in the ^{1}H-NMR and δ_C 56.5 and 56.4 in the ^{13}C-NMR spectra. In the NOE experiment, on irradiation of the signals at δ_H 4.03 and 4.01, each 16% and 17% increment of the signals at δ_H 6.91 (H-6) and 6.89 (H-6') were induced, respectively, thus these methoxyl groups were assigned to locate at C-7 and C-7', respectively. The presence of an allyl methyl [δ_H 2.00 (3H, br s)], exo-methylene [δ_H 4.98 (1H, br s), 4.93 (1H, t, *J* = 1.5 Hz), vicinal methine protons [δ_H 6.01 (1H, d, *J* = 8.8 Hz), 5.01 (1H, d, *J* = 8.8 Hz)] and a singlet proton [δ_H 7.29 (1H, s)] were also suggested. The full assignment of the ^{1}H- and ^{13}C-NMR resonances of cladimarin A was made using HMQC and HMBC experiments. In the HMBC experiments (Figure 1), the proton signal at δ_H 6.01 (H-11) showed cross peaks with the carbon signals at δ_C 161.4 (C-7), 153.9 (C-9) and 140.8 (C-13). The proton signal at δ_H 5.01 (H-12) showed cross peaks with the carbon signals at δ_C 113.9 (C-8), 17.1 (C-15) and 115.2 (C-14). The acetal proton signal at δ_H 7.29 (H-11') showed cross peaks with the carbon signals at δ_C 162.0 (C-7') and 154.5 (C-9'). These connectivities established the partial structure containing acetal ring between two coumarin nuclei. The relative stereochemistry

around the acetal ring was assigned on the basis of NOE experiment. Irradiation of the signal at δ_H 5.01 (H-12) showed 8% increment of the signal at δ_H 7.29 (H-11') and no increment was observed on any protons on irradiation of the signal at δ_H 6.01 (H-11). From the results mentioned above, the structure of cladimarin A was determined as **1** leaving the absolute stereochemistry undetermined. Previously, a novel type bicoumarin arising from the condensation of meranzin hydrate with 7-methoxy-8-(2-formyl-2-methylpropyl)coumarin have been reported.[9] Cladimarin A is the second bicoumarin linked by acetal ring other than lactone carbonyl carbon. Because of cladimarin A corresponds to a dimer of murrangatin (**2**)[10] and paniculal (**3**),[9] which also cooccurred in the same plant,[11] the possibility of an artifact formed during extraction can not be excluded.[11]

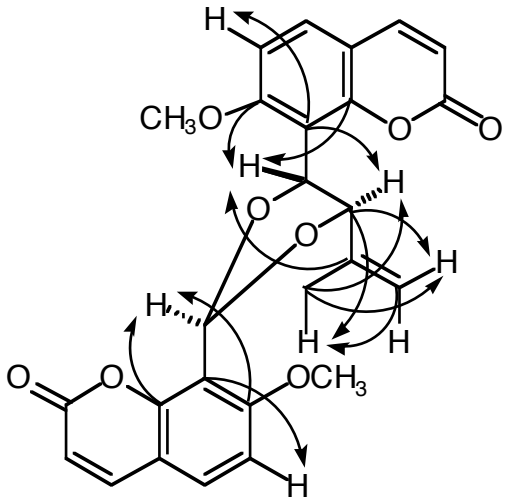


Figure 1 Key CH Long-Range Correlations in the HMBC Spectrum of **1**

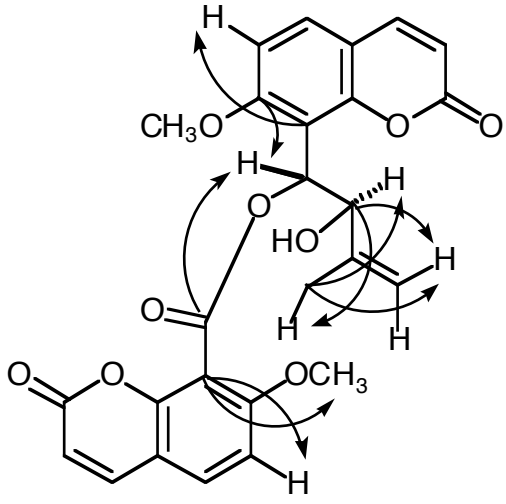


Figure 2 Key CH Long-Range Correlations in the HMBC Spectrum of **4**

Cladimarin B (**4**), $[\alpha]_D$ -33.7° ($CHCl_3$), was isolated as a colorless amorphous powder and had the molecular formula $C_{26}H_{22}O_9$ (HR-FAB-MS: $[M+H]^+$ at *m/z* 479.1335). The UV (258, 320 nm) and IR (1730, 1610 cm^{-1}) spectra suggested the presence of 7, 8-disubstituted coumarin.[8] The ^{1}H-NMR spectrum of **4** showed the presence of four AB type aromatic protons [δ_H 7.62, 6.23 (each 1H, d, J = 9.5 Hz), 7.59, 6.21 (each 1H, d, J = 9.5 Hz), 7.41, 6.89 (each 1H, d, J = 8.8 Hz), 7.46, 6.87 (each 1H, d, J = 8.8 Hz)] and two methoxyl groups [δ_H 3.98, 3.89 (each 3H, s)]. In the NOE experiment, irradiation of the methoxyl signals at δ_H 3.98 (7-OMe) and 3.89 (7'-OMe) induced 14% and 15% enhancement of the signals at δ_H 6.89 (H-6) and 6.87 (H-6'), respectively, suggesting the location of methoxyl groups at C-7 and C-7', respectively. Thus, the linkage of two coumarin nuclei was concluded between C-8 and C-8'. The presence of a -CH(O)-CH(O)-C(CH_3)=CH_2 side chain was suggested by the ^{1}H-NMR [δ_H 6.73 (1H, d, J = 8.4 Hz), 5.14 (1H, d, J = 8.4 Hz), 4.76 (1H, br s), 4.71 (1H, t, J = 1.8 Hz), 1.80 (3H, br s)] and

Table **1** ^{1}H- and ^{13}C-NMR spectral data of **1** and **4**

	1		**4**	
Position	δ_H	δ_C	δ_H	δ_C
2		160.3 (s)[b]		160.7 (s)[f]
3	6.27 (1H , d , J = 9.5)[a]	113.4 (d)[c]	6.23 (1H , d , J = 9.5)	113.3 (d)
4	7.63 (1H , d , J = 9.5)	143.5 (d)	7.62 (1H , d , J = 9.5)	143.6 (d)
5	7.41 (1H , d , J = 8.8)	128.9 (d)	7.41 (1H , d , J = 8.8)	129.4 (d)
6	6.91 (1H , d , J = 8.8)	108.1 (d)[d]	6.89 (1H , d , J = 8.8)	108.1 (d)
7		161.4 (s)		160.5 (s)[f]
7-OMe	4.03 (3H , s)	56.5 (q)	3.98 (3H , s)	56.5 (q)
8		113.9 (s)		112.8 (s)
9		153.9 (s)		153.6 (s)
10		113.0 (s)[e]		113.1 (s)
11	6.01 (1H , d , J = 8.8)	72.5 (d)	6.73 (1H , d , J = 8.4)	73.1 (d)
12	5.01 (1H , d , J = 8.8)	84.6 (d)	5.14 (1H , d , J = 8.4)	75.8 (d)
13		140.8 (s)		142.7 (s)
14	4.98 (1H , br s)	115.2 (t)	4.76 (1H , br s)	114.5 (t)
	4.93 (1H , t , J = 1.5)		4.71 (1H , t , J = 1.8)	
15	2.00 (3H , br s)	17.1 (q)	1.80 (3H , br s)	17.4 (q)
2'		160.3 (s)[b]		159.5 (s)[g]
3'	6.25 (1H , d , J = 9.5)[a]	113.3 (d)[c]	6.21 (1H , d , J = 9.5)	113.8 (d)
4'	7.61 (1H , d , J = 9.5)	143.3 (d)	7.59 (1H , d , J = 9.5)	142.9 (d)
5'	7.44 (1H , d , J = 8.8)	130.0 (d)	7.46 (1H , d , J = 8.8)	130.3 (d)
6'	6.89 (1H , d , J = 8.8)	107.9 (d)[d]	6.87 (1H , d , J = 8.8)	107.9 (d)
7'		162.0 (s)		159.4 (s)[g]
7'-OMe	4.01 (3H , s)	56.4 (q)	3.89 (3H , s)	56.6 (q)
8'		112.9 (s)[e]		111.6 (s)
9'		154.5 (s)		152.2 (s)
10'		112.8 (s)[e]		112.9 (s)
11'	7.29 (1H , s)	97.7 (d)		163.0 (s)

a-g) Signal assignment may be interchangeable. Values are in ppm. Figures in parentheses are coupling constants (J) in Hz.

^{13}C-NMR [δ_C 73.1 (d), 75.8 (d), 142.7 (s), 114.5 (t), 17.4 (q)] signals. In the HMBC spectrum of **4** (Figure 2), the methine proton at δ_H 6.73 (H-11) caused cross peaks with the carbon signals at δ_C 160.5 (C-7), 153.6 (C-9) and 163.0 (C-11'). In the NOE experiment, irradiation of the signal at δ_H 5.14 (H-12) showed 4% increment of the signal at δ_H 4.71 (H-14) and no increment of the signal at δ_H 6.73 (H-11). These data led us to conclude the structure of cladimarin B to be **4**, connected by ester bond between the C-11 hydroxyl group of murrangatin (**2**) and the carboxyl group of 7-methoxy-8-carboxycoumarin (**5**). This is the first bicoumarin linked by an ester bond. Previously, we determined[12] the absolute stereochemistry of the α-glycol part of murrangatin (**2**). In that study, the remarkable differences of exo-methylene proton signals of ester derivatives were observed in the ^{1}H-NMR spectrum. In the case of minumicrolin esters, the exo-methylene proton signals resonate approximatelly at δ_H 5.0, while in the case of murrangatin esters, it resonates approximatelly at δ_H 4.7. Since the exo-methylene signals of cladimarin B (**4**) were observed at δ_H 4.71 and 4.76, the relative stereochemistry of the α-glycol function in cladimarin B (**4**) was concluded to be *threo* configuration, the same as that of murrangatin (**2**).

The inhibitory effects of new compounds (**1, 4)** and the positive control, β-carotene, on EBV-EA activation induced by TPA were performed according to the short term *in vitro* assay.[13] As shown in Table 2, cladimarin A (**1**) showed almost equal inhibitory activity to that of β-carotene and it might be valuable as anti-tumor promoter in chemical carcinogenesis.

Table 2 Inhibitory effects of **1** and **4** on TPA-induced EBV-EA activation

Compound	EBV-EA positive cells (% viability) Compound concentration (mol ratio/32 pmol TPA)			
	1000	500	100	10
Cladimerin A (**1**)	8.9 (40)	41.0 (60)	76.2	98.1
Cladimerin B (**4**)	10.0 (40)	45.2 (60)	79.3	100.0
β-Carotene	9.1 (60)	34.3	82.7	100.0

EXPERIMENTAL

^{1}H- and ^{13}C-NMR, NOE and HMBC (J = 8Hz) spectra were recorded on an A-400 or A-600 (JEOL) spectrometer in $CDCl_3$. Chemical shifts are shown in δ values (ppm) with tetramethylsilane (TMS) as an internal reference. MS were taken with a HX-110 (JEOL) or JMS-700 (JEOL) spectrometer having a direct inlet system. UV spectra were recorded on a Shimadzu UV 160A in EtOH, IR spectra on a Shimadzu IR-450 in $CHCl_3$. and optical rotations on a DIP-370 (JASCO) in $CHCl_3$ at 25°C. Preparative TLC was carried out on Kieselgel 60 F_{254} (Merck).

Extraction and Isolation *Clausena excavata* BURUM. f. (Rutaceae) was collected in Komandaru,

Flores Island, Indonesia. A voucher specimen was deposited in the laboratory of Mukogawa Women's University. The dried branches (426 g) were extracted with acetone (1 L) at rt for 1 week and refluxed with acetone and MeOH (each 51 h x 2). The combined extract was evaporated under reduced pressure to yield extract (33.57 g). The extract was subjected to silica gel column chromatography eluting with hexane, acetone—hexane [(1:4), (3:7) and (1:1) increasing polarity], acetone and MeOH. The acetone — hexane (3:7) eluate was further separated with PTLC using solvent systems [acetone— $CHCl_3$ (1:1) and acetone—benzene (3:7)] to yield cladimarin A (**1**) (3 mg). From the acetone—hexane (1:1) eluate, cladimarin B (**4**) (23.4 mg) was obtained by repeated PTLC using solvent systems [acetone—benzene (4:6 and 3:7), acetone—$CHCl_3$ (1:9), ethyl acetate—hexane (7:3) and EtOH—ethyl acetate—hexane (1:15:4).

Cladimarin A (1): Colorless amorphous powder, $[\alpha]_D$ -12.3° (*c* = 0.2, $CHCl_3$). UV λ_{max} (EtOH, nm) : 250, 260, 320. IR ν_{max} ($CHCl_3$, cm^{-1}) : 1730, 1610. EI-MS *m/z*: 462 $[M]^+$, 392, 258 (base peak), 213, 189. HR-EI-MS Calcd for $C_{26}H_{22}O_8$: 462.1315. Found: 462.1309. ^{1}H- and ^{13}C-NMR ($CDCl_3$, δ): see Table 1. Differential NOE: irradiation at δ_H 4.03 (7-OMe) gave 16% enhancement of the signal at δ_H 6.91 (H-6); irradiation at δ_H 4.01 (7'-OMe) gave 17% enhancement of the signal at δ_H 6.89 (H-6'); irradiation of the signal at δ_H 5.01 (H-12) gave 8% enhancement of the signal at δ_H 7.29 (H-11'). No NOE was observed on irradiation of the signal at δ_H 6.01 (H-11).

Cladimarin B (4): Colorless amorphous powder, $[\alpha]_D$ -33.7° (*c* = 0.042, $CHCl_3$); HR-FAB-MS Calcd for $C_{26}H_{23}O_9$: 479.1342 $(M+H)^+$. Found: 479.1335. UV λ_{max} (EtOH, nm) : 205, 258, 320. IR ν_{max} ($CHCl_3$, cm^{-1}) : 1730, 1610; ^{1}H- and ^{13}C-NMR ($CDCl_3$, δ) : see Table 1. Differential NOE: irradiation of the signal at δ_H 3.98 (7-OMe) showed 14% increment of the signal at δ_H 6.89 (H-6); irradiation of the signal at δ_H 3.89 (7'-OMe) showed 15% increment of the signal at δ_H 6.87 (H-6'); irradiation of the signal at δ_H 5.14 (H-12) showed 4% increment of the signal at δ_H 4.71 (H-14); irradiation of the signal at δ_H 6.73 (H-11) showed no increment of any proton signals.

ACKNOWLEDGEMENTS

We are grateful to Emeritus Prof. I. Kitagawa (Osaka University) and Prof. H. Shibuya (Fukuyama University) for collecting the plant material. Thanks are also due to Ms. K. Suwa, S. Horiyama and C. Honda of Mukogawa Women's University for measurements of MS and NMR spectra. This work was partly supported by Ministry of Education, Culture, Sports, Science and Technology of Japan for Grants-in-aid of High-Tech Research Center Project and Scientific Research (C).

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