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# TWO NEW BICOUMARINS FROM *CLAUSENA EXCAVATA*

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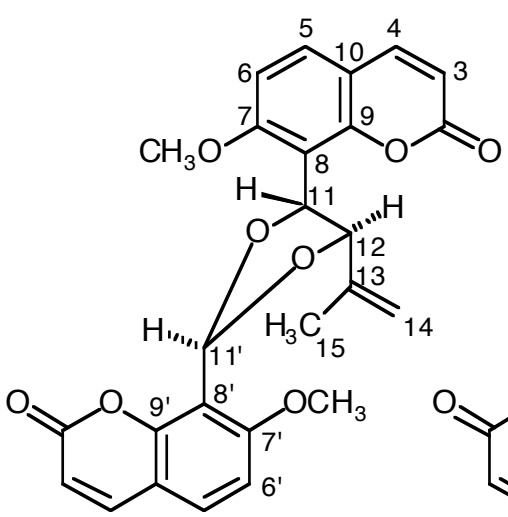
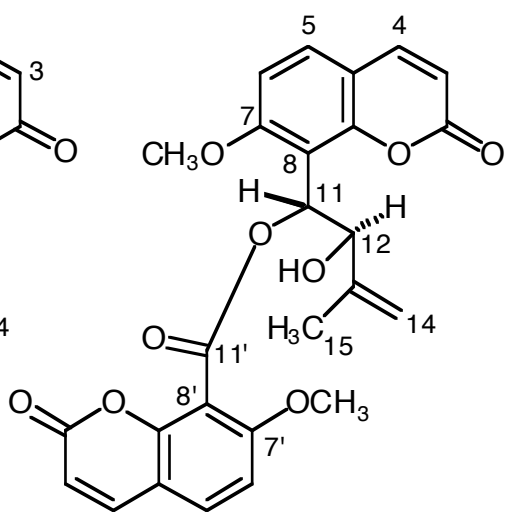
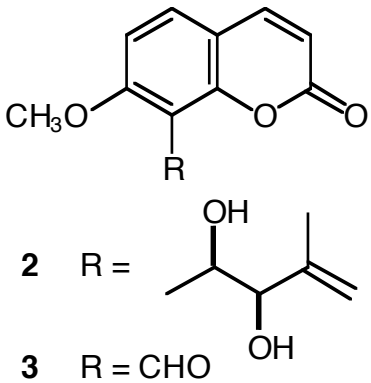
**Abstract** - Two novel type bicoumarins, named cladimarins A (**1**) and B (**4**), were isolated from the branch of *Clausena excavata* (Rutaceae) collected in Indonesia. The structures were elucidated on the basis of spectroscopical data especially by using HMBC method. Naturally occurring bicoumarin, 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 $\delta_H$ 7.63, 6.27 (each 1H, d, *J* = 9.5 Hz) and 7.41, 6.91 (each 1H, d, *J* = 8.8 Hz) [$\delta_H$ 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 $\delta_H$ 4.03, 4.01 (each 3H, s) in the $^1$H-NMR and $\delta_C$ 56.5 and 56.4 in the $^{13}$C-NMR spectra. In the NOE experiment, on irradiation of the signals at $\delta_H$ 4.03 and 4.01, each 16% and 17% increment of the signals at $\delta_H$ 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 [$\delta_H$ 2.00 (3H, br s)], exo-methylene [$\delta_H$ 4.98 (1H, br s), 4.93 (1H, t, *J* = 1.5 Hz), vicinal methine protons [$\delta_H$ 6.01 (1H, d, *J* = 8.8 Hz), 5.01 (1H, d, *J* = 8.8 Hz)] and a singlet proton [$\delta_H$ 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 $\delta_H$ 6.01 (H-11) showed cross peaks with the carbon signals at $\delta_C$ 161.4 (C-7), 153.9 (C-9) and 140.8 (C-13). The proton signal at $\delta_H$ 5.01 (H-12) showed cross peaks with the carbon signals at $\delta_C$ 113.9 (C-8), 17.1 (C-15) and 115.2 (C-14). The acetal proton signal at $\delta_H$ 7.29 (H-11') showed cross peaks with the carbon signals at $\delta_C$ 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 $\delta_H$ 5.01 (H-12) showed 8% increment of the signal at $\delta_H$ 7.29 (H-11') and no increment was observed on any protons on irradiation of the signal at $\delta_H$ 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]

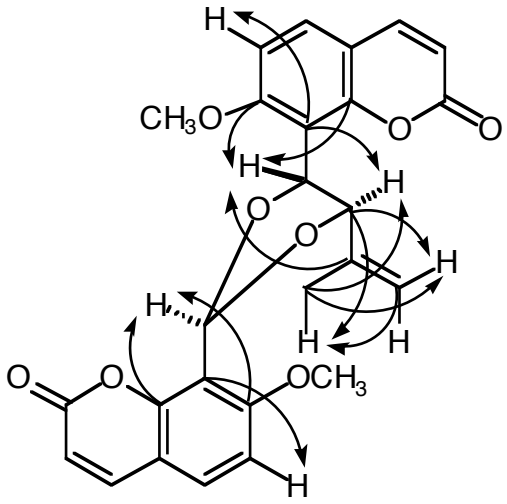


Figure 1 Key CH Long-Range Correlations in the HMBC Spectrum of **1**

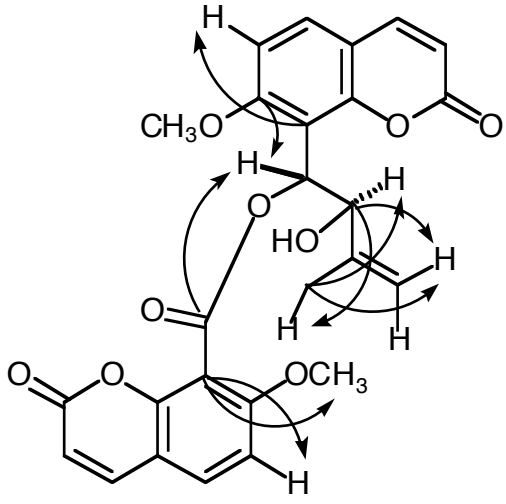


Figure 2 Key CH Long-Range Correlations in the HMBC Spectrum of **4**

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 [$\delta_H$ 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 [$\delta_H$ 3.98, 3.89 (each 3H, s)]. In the NOE experiment, irradiation of the methoxyl signals at $\delta_H$ 3.98 (7-OMe) and 3.89 (7'-OMe) induced 14% and 15% enhancement of the signals at $\delta_H$ 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 [$\delta_H$ 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 | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ |
| 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 [$\delta_C$ 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 $\delta_H$ 6.73 (H-11) caused cross peaks with the carbon signals at $\delta_C$ 160.5 (C-7), 153.6 (C-9) and 163.0 (C-11'). In the NOE experiment, irradiation of the signal at $\delta_H$ 5.14 (H-12) showed 4% increment of the signal at $\delta_H$ 4.71 (H-14) and no increment of the signal at $\delta_H$ 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 $\alpha$-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 $\delta_H$ 5.0, while in the case of murrangatin esters, it resonates approximatelly at $\delta_H$ 4.7. Since the exo-methylene signals of cladimarin B (**4**) were observed at $\delta_H$ 4.71 and 4.76, the relative stereochemistry of the $\alpha$-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, $\beta$-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 $\beta$-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 |
| $\beta$-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 $\delta$ 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 $\lambda_{max}$ (EtOH, nm) : 250, 260, 320. IR $\nu_{max}$ ($CHCl_3$, $cm^{-1}$) : 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 ($CDCl_3$, $\delta$): see Table 1. Differential NOE: irradiation at $\delta_H$ 4.03 (7-OMe) gave 16% enhancement of the signal at $\delta_H$ 6.91 (H-6); irradiation at $\delta_H$ 4.01 (7'-OMe) gave 17% enhancement of the signal at $\delta_H$ 6.89 (H-6'); irradiation of the signal at $\delta_H$ 5.01 (H-12) gave 8% enhancement of the signal at $\delta_H$ 7.29 (H-11'). No NOE was observed on irradiation of the signal at $\delta_H$ 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 $\lambda_{max}$ (EtOH, nm) : 205, 258, 320. IR $\nu_{max}$ ($CHCl_3$, $cm^{-1}$) : 1730, 1610; $^1$H- and $^{13}$C-NMR ($CDCl_3$, $\delta$) : see Table 1. Differential NOE: irradiation of the signal at $\delta_H$ 3.98 (7-OMe) showed 14% increment of the signal at $\delta_H$ 6.89 (H-6); irradiation of the signal at $\delta_H$ 3.89 (7'-OMe) showed 15% increment of the signal at $\delta_H$ 6.87 (H-6'); irradiation of the signal at $\delta_H$ 5.14 (H-12) showed 4% increment of the signal at $\delta_H$ 4.71 (H-14); irradiation of the signal at $\delta_H$ 6.73 (H-11) showed no increment of any proton signals.

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