Chemical Constituents of *Clausena excavata*: Isolation and Structure Elucidation of Novel Furanone-Coumarins with Inhibitory Effects for Tumor-Promotion[1]

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A study of the chemical constituents of the leaves of *Clausena excavata* cultivated in a greenhouse led to the isolation and identification of 10 new furanone-coumarins named clauslactones A (**1**), B (**2**), C (**3**), D (**4**), E (**5**), F (**6**), G (**7**), H (**8**), I (**9**), and J (**10**), together with a known carbazole, clauszoline M, and a coumarin, umbelliferone. The new coumarins contain a C_{10} terpenoid side chain with a furanone (γ-lactone) moiety. Further, in clauslactones A−D (**1**−**4**), the terpenoid side chain was shown to be linked to the 7,8-dioxygenated coumarin skeleton through a 1,4-dioxane ring system. This is the first example of coumarins with these structural characteristics in nature. These furanone-coumarins were found to exhibit inhibitory activity against 12-*O*-tetradecanoylphorbol-13-acetate-induced Epstein−Barr virus early antigen activation in Raji cells.

In our previous paper[2,3] on the chemical constituents of *Clausena excavata* Burm. f. (Rutaceae), the isolation and structure elucidation of some carbazole alkaloids and coumarins from the roots and stem bark were reported. We now describe the isolation and structure elucidation of seven new coumarins having a C-10 terpenoid γ-lactone side chain, named clauslactones A (**1**), B (**2**), C (**3**), D (**4**), E (**5**), F (**6**), and G (**7**) from the leaves of *C. excavata*. Three additional coumarins, named clauslactones H (**8**), I (**9**), and J (**10**) were first reported by us in 1997.[4] Recently, Thuy and co-workers reported the isolation of coumarins named excavatins A−M from *C. excavata* collected in Vietnam.[5] Among them, excavatins J, L, and M were shown to have the same structure as clauslactones I, J, and H, respectively.[4,5] Among the coumarins reported herein, clauslactones A−D (**1**−**4**) were shown to have a C-10 terpenoid side chain containing a γ-lactone linked to the 7,8-oxygenated coumarin skeleton through 1,4-dioxide linkage. This is the first example of this type of coumarin to be found in nature.

In a primary screening test of inhibitory effects for tumor-promotion, it was found that linear and angular types of furocoumarins and angular-type pyranocoumarins suppressed 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-stimulated ^{32}Pi-incorporation into phospholipids of cultured cells and inhibited two-stage mouse-skin carcinogenesis.[6−9] In addition, the inhibitory effects of pyranocoumarins[9] and 8-substituted 7-methoxycoumarins[10] on TPA-induced Epstein−Barr virus early antigen (EBV-EA) activation, as evidence of potential inhibition of tumor-promoting activity, have been studied. This paper also describes the results of assays examining the inhibitory effects on TPA-induced EBV−EA activation of nine furanone-coumarins isolated from *C. excavata*.

Results and Discussion

The dried leaves of the plant were extracted with acetone at room temperature. The acetone extract was fractionated by a combination of Si gel column chromatography and preparative TLC to give 10 furanone-coumarins, along with a known carbazole and coumarin.

Clauslactones A−D (**1**−**4**) showed analogous UV spectra, with typical high-, medium-, and low-intensity absorption bands at λ_{max} 206−212, 261−262, and 319 nm, respectively, accompanied with some minor bands. This feature was similar to that of daphnetin (7,8-dihydroxycoumarin).[11] The presence of a 7,8-oxygenated coumarin nucleus as a common structural unit in these new coumarins was further deduced by the presence of ^{1}H NMR signals (Table 1) for two pairs of 1H doublets at δ_H 6.22−6.28 and 7.62−7.86 (each J = 9.5 Hz) and δ_H 6.97−7.10 and 6.83−6.87 (each J = 8.8 Hz), which were easily assignable to H-3 and H-4 and to H-5 and H-6 on the coumarin skeleton, respectively.[11] The IR spectra showed an additional C=O stretching band at ν_{max} 1764−1772 cm^{-1}, along with the coumarin carbonyl band in the vicinity of ν_{max} 1725−1727 cm^{-1} in molecules of compounds **1**−**3**, and an overlapping strong band at ν_{max} 1739 in **4**, indicating the presence of a γ-lactone ring in these molecules. The presence of the same partial structure in the side chain was evident from the close similarity of the chemical shifts, multiplicities, and coupling constants of signals in the ^{1}H NMR spectra, and the similarity of the chemical shifts corresponding to carbons C-1′−C-5′ in the ^{13}C NMR spectra (Table 2).

Clauslactone A (**1**) was obtained as a colorless oil, and its molecular formula was determined as $C_{19}H_{18}O_7$ by HRMS. IR bands at ν_{max} 3553 cm^{-1} indicated the presence of a hydroxyl group. Analyses of the ^{1}H and ^{13}C NMR spectra, including COSY and HMQC, suggested the presence of a C_{10} terpenoid side chain consisting of a methyl group (δ_C 23.2; δ_H 1.42) attached to a quaternary carbon (δ_C 72.0) bearing a hydroxyl group, a methylene adjacent to a methine linked to two oxygen functions [δ_C 65.0; δ_H 4.10, 4.64; δ_C 77.6; δ_H 4.18], a methine linked to two

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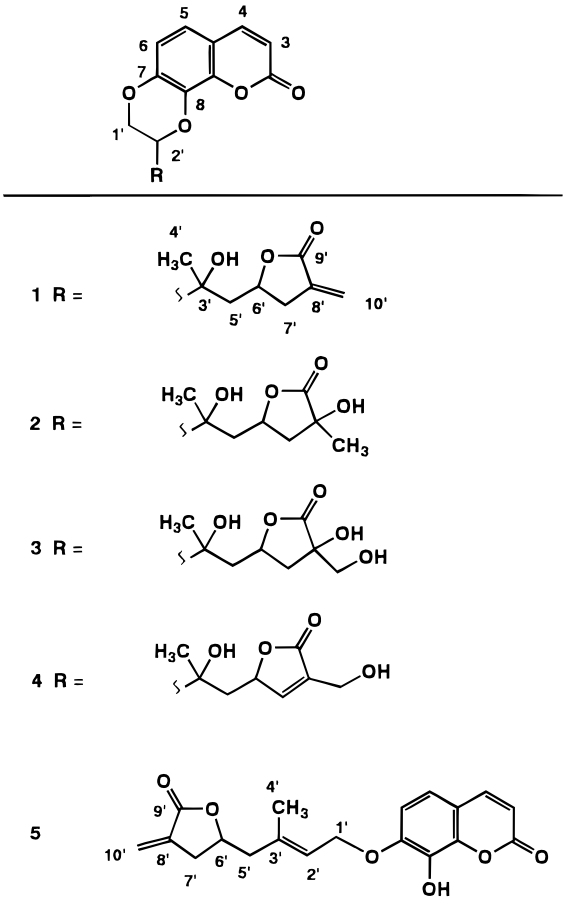

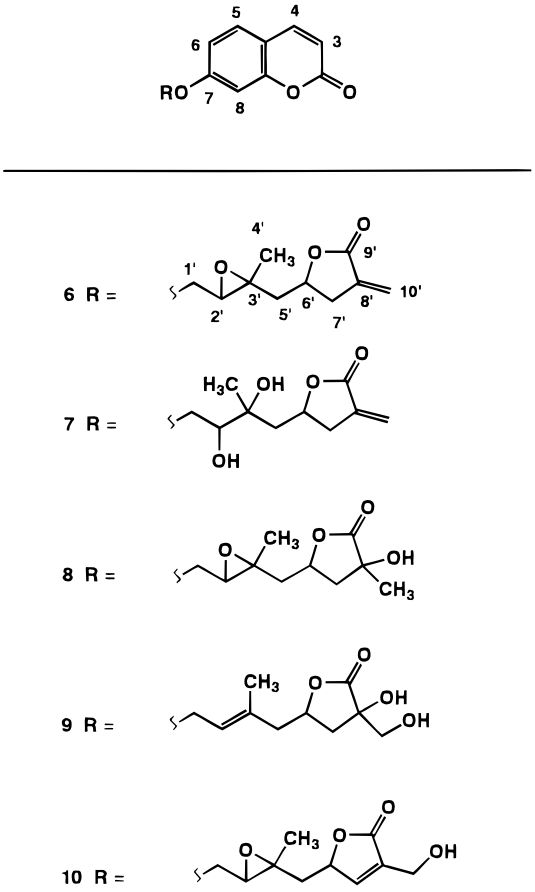

methylenes and an oxygen atom [δ_C 44.1; δ_H 2.32, 1.91; δ_C 74.2; δ_H 4.93; δ_C 34.7; δ_H 3.22, 2.69], an *exo*-methylene [δ_C 122.9; δ_H 6.28, 5.70; δ_C 133.5], and a lactone carbonyl group [δ_C 169.5]. The linked arrangement of these partial structural units was elucidated by HMBC spectroscopy. Significant C−H long-range correlations useful in the structure determination are shown as bold lines in Figure 1. Long-range correlations of a methyl carbon signal at δ_C 23.2 (C-4′) with proton signals at δ_H 4.18 (H-2′), 1.91 (H-5′), and 2.32 (H-5′), together with correlations of C-2′ (δ_C 77.6) with H-5′ (δ_H 2.32) and H-4′ (methyl, δ_H 1.42), allowed linkage of structural units as shown in the structure **1**. A correlation between the lactonic carbonyl carbon at δ_C 169.5 (C-9′) and *exo*-methylene protons at δ_H 6.28 (H-10′) and 5.70 (H-10′) revealed the structure of an α-methylene γ-lactone having a substituent at C-6′. Further, the linking orientation of the side chain with the 7,8-dioxygenated coumarin nucleus was proposed by the observation of a three-bond hetero correlation of the carbon signal at δ_C 146.6 (C-7) on the coumarin skeleton with the methylene proton signal at δ_H 4.64 (H-1′) on the side chain. These data, together with observed significant mass fragment ions (EIMS) at *m*/*z* 204 and 246 resulting from cleavage at C-2′/C-3′ and C-3′/C-5′ with a hydrogen transfer, respectively, led to the assignment of structure **1** to clauslactone A.

Clauslactone B (**2**) has a molecular formula of $C_{19}H_{20}O_8$, a difference of H_2O compared with **1**. The ^1H and ^{13}C NMR spectra were very similar to those of **1**, except for signals due to a quaternary methyl [δ_H 1.37 (H-10′); δ_C 22.3 (C-10′)] attached to an oxygenated carbon [δ_C 74.0 (C-8′)] instead of those of *exo*-methylene in **1** [δ_H 6.28, 5.70 (H-10′); δ_C 133.5 (C-8′), 122.9 (C-10′)], and other small chemical shift differences in some signals due to difference of the solvent (Table 1). These data, coupled with HMBC results, suggested structure **2** for clauslactone B.

Clauslactone C (**3**) was isolated as a colorless oil with molecular formula $C_{19}H_{20}O_9$, a difference of an oxygen atom compared with **2**. In the ^1H NMR spectrum the appearance of AB-type doublets at δ_H 3.53 and 3.75 (each J = 11 Hz) assignable to protons of an isolated methylene linked with a hydroxyl group, instead of a methyl singlet at δ_H 1.37 in the spectrum of **2**, and the close similarity of the remaining signal patterns (Table 1) suggested the structure of clauslactone C to be represented by formula **3**. The HMBC spectrum also supported structure **3** for this coumarin.

Clauslactone D (**4**) was obtained as a colorless powder. The molecular formula $C_{19}H_{18}O_8$, a difference of H_2O compared with **3**, suggested this coumarin as a dehydroxy analogue of **3**. In comparing the ^{13}C NMR spectrum of **4** and **3**, two additional sp^2 carbons at δ_C 150.1 and 133.3 in the spectrum of **4** were observed, along with the disappearance of signals due to a quaternary sp^3 carbon (δ_C 77.3, C-8′) and a methylene carbon (δ_C 40.5, C-7′) in that of **3**. In the ^1H NMR spectrum of **4** only one olefinic proton [δ_H 7.45 (1H, q, J = 1.5 Hz)] and a lone 2H-broad singlet (δ_H 4.44) were observed in place of signals for two methylenes (H-7′, 10′) in **3**. These data indicated the presence of a 3-hydroxymethyl-3,4-unsaturated γ-lactone moiety in the molecule. From these data, together with the results of COSY, ^{13}C NMR (Table 2), and HMBC, the structure of clauslactone D was proposed as **4**.

These four coumarins are the first examples of 7,8-dioxygenated coumarins having this unique side chain and ether linkage to be found in nature. The stereochemistry has not been established for any of these compounds.

Clauslactone E (**5**) was obtained as a colorless powder, and its molecular formula, $C_{19}H_{18}O_6$, was determined by HRMS. Strong UV bands at λ_{max} 261 and 319 nm, an IR band at ν_{max} 1722 cm^{-1}, and two pairs of doublets at δ_H 6.27, 7.64 (each 1H, d, J = 9.5 Hz) and δ_H 6.99, 6.85 (each

Table 1. ^{1}H NMR Data for **1**−**10**[a]

	1	**2**[b]	**3**[b]	**4**	**5**	**6**	**7**	**8**	**8**[c]	**9**[d]	**10**[d]
H-3	6.28 (d, J = 9.5)	6.22 (d, J = 9.5)	6.26 (d, J = 9.5)	6.27 (d, J = 9.5)	6.27 (d, J = 9.5)	6.25 (d, J = 9.5)	6.27 (d, J = 9.5)	6.27 (d, J = 9.5)	6.29 (d, J = 9.5)	6.20 (d, J = 9.5)	6.22 (d, J = 9.5)
H-4	7.64 (d, J = 9.5)	7.82 (d, J = 9.5)	7.86 (d, J = 9.5)	7.62 (d, J = 9.5)	7.64 (d, J = 9.5)	7.62 (d, J = 9.5)	7.64 (d, J = 9.5)	7.65 (d, J = 9.5)	7.99 (d, J = 9.5)	7.89 (d, J = 9.5)	7.90 (d, J = 9.5)
H-5	6.98 (d, J = 8.8)	7.06 (d, J = 8.8)	7.10 (d, J = 8.8)	6.97 (d, J = 8.4)	6.99 (d, J = 8.8)	7.38 (d, J = 8.8)	7.39 (d, J = 8.8)	7.40 (d, J = 8.4)	7.63 (d, J = 8.8)	7.57 (d, J = 8.4)	7.59 (d, J = 8.8)
H-6	6.85 (d, J = 8.8)	6.83 (d, J = 8.8)	6.87 (d, J = 8.8)	6.83 (d, J = 8.4)	6.85 (d, J = 8.8)	6.88 (dd, J = 8.8, 2.2)	6.88 (dd, J = 8.8, 2.2)	6.90 (dd, J = 8.4, 2.2)	7.00 (br d, J = 8.8)	6.92 (dd, J = 8.4, 2.6)	6.99 (dd, J = 8.8, 2.6)
H-8						6.84 (d, J = 2.2)	6.86 (d, J = 2.2)	6.86 (d, J = 2.2)	7.07 (br)	6.90 (d, J = 2.6)	6.98 (d, J = 2.6)
H-1′	4.10 (dd, J = 11.0, 9.2)	4.10 (dd, J = 9.5, 9.1)	4.13 (dd, J = 11.4, 9.1)	4.06 (dd, J = 11.0, 8.0)	4.72 (2H, d, J = 6.2)	4.04 (dd, J = 11.4, 6.6)	4.11 (dd, J = 9.5, 7.3)	4.08 (dd, J = 11.4, 6.6)	4.11 (dd, J = 11.0, 7.0)	4.75 (2H, d, J = 6.2)	4.17 (dd, J = 11.4, 7.0)
	4.64 (dd, J = 11.0, 2.2)	4.60 (br d, J = 9.5)	4.65 (dd, J = 11.4, 2.2)	4.57 (dd, J = 11.0, 2.0)		4.34 (dd, J = 11.4 3.7)	4.28 (dd, J = 9.5, 3.7)	4.34 (dd, J = 11.4, 3.7)	4.44 (dd, J = 11.0, 2.6)		4.52 (dd, J = 11.4, 2.9)
H-2′	4.18 (dd, J = 9.2, 2.2)	3.98 (br d, J = 9.1)	4.03 (dd, J = 9.1, 2.2)	4.15 (dd, J = 8.0, 2.0)	5.62 (m)	3.18 (dd, J = 6.6, 3.7)	4.00 (br)	3.22 (dd, J = 6.6, 3.7)	3.17 (dd, J = 7.0, 2.6)	5.64 (m)	3.24 (dd, J = 7.0, 2.9)
H-4′	1.42 (3H)	1.38 (3H)	1.43 (3H)	1.45 (3H)	1.83 (3H)	1.46 (3H)	1.37 (3H)	1.52 (3H)	1.38 (3H)	1.86 (3H)	1.50 (3H)
H-5′	2.32 (dd, J = 15.0, 3.3)	2.11 (m)	2.17 (m)	2.33 (dd, J = 14.7, 4.0)	2.50 (dd, J = 14.3, 7.7)	1.91 (2H, m)	2.14 (dd, J = 15.0, 9.9)	1.92 (2H, m)	1.90 (dd, J = 14.3, 4.4)	2.52 (dd, J = 14.2, 7.7)	2.13 (dd, J = 14.3, 4.8)
	1.91 (dd, J = 15.0, 9.9)	1.92 (m)	2.03 (dd, J = 15.0 3.3)	1.73 (dd, J = 14.7, 9.2)	2.39 (dd, J = 14.3, 5.5)		1.93 (dd, J = 15.0, 2.9)		1.83 (m)	2.43 (dd, J = 14.2, 5.9)	1.82 (dd, J = 14.3, 8.8)
H-6′	4.93 (m)	4.96 (m)	5.02 (m)	5.36 (m)	4.69 (m)	4.76 (m)	4.92 (m)	4.88 (m)	4.72 (m)	4.78 (m)	5.26 (m)
H-7′	3.22 (ddt, J = 17.2, 7.3, 2.6)	2.36 (dd, J = 13.6, 5.1)	2.29 (2H, m)	7.45 (q, J = 1.5)	3.05 (ddt, J = 17.2, 7.7, 2.9)	3.14 (ddt, J = 17.1, 7.6, 2.6)	3.17 (dd, J = 16.9, 7.3)	2.53 (dd, J = 13.6, 5.5)	2.27 (dd, J = 13.2, 5.5)	2.25 (2H, m)	7.50 (m)
	2.69 (ddt, J = 17.2, 7.0, 2.6)	1.95 (m)			2.62 (ddt, J = 17.2, 5.9, 2.9)	2.64 (ddt, J = 17.1, 6.3, 2.6)	2.65 (dd, J = 16.9, 7.3)	1.84 (dd, J = 13.6, 9.5)	1.83 (m)		
H-10′	6.28 (t, J = 2.6)	1.37 (3H)	3.75 (d, J = 11.0)	4.44 (2H, br s)	6.24 (t, J = 2.9)	6.24 (t, J = 2.6)	6.26 (t, J = 2.6)	1.48 (3H)	1.30 (3H)	3.76 (d, J = 10.6)	4.27 (2H, br s)
	5.70 (t, J = 2.6)		3.53 (d, J = 11.0)		5.64 (t, J = 2.9)	5.66 (t, J = 2.6)	5.69 (t, J = 2.6)			3.55 (d, J = 10.6)	
Others	2.93 (br, OH)			2.41 (br, OH)	6.09 (br, 8-OH)				5.90 (br, 8′-OH)	4.91 (br, 8′-OH)	
				2.98 (br, OH)						4.20 (br, 10′-OH)	

[a] Values in (δ) ppm. The coupling constants (J) in parentheses are in Hz. All signals corresponding to 1H were observed as a singlet, unless otherwise stated. [b] Spectra were taken in MeOD-d_4. [c] A spectrum was taken in DMSO-d_6. [d] Spectra were taken in acetone-d_6.

Table 2. ^{13}C NMR Data for **1**−**10**[a]

	1	**2**[b]	**3**[b]	**4**	**5**	**6**	**7**	**8**[c]	**9**[d]	**10**[d]
C-2	160.7 (s)	162.9 (s)	162.9 (s)	160.7 (s)	160.3 (s)	161.1 (s)	161.0 (s)	160.2 (s)	160.9 (s)	160.9 (s)
C-3	113.5 (d)	113.6 (d)	113.6 (d)	113.5 (d)	113.6 (d)	113.4 (d)	113.5 (d)	112.7 (d)	113.6 (d)	113.8 (d)
C-4	143.9 (d)	146.3 (d)	146.3 (d)	144.0 (d)	143.8 (d)	143.3 (d)	143.3 (d)	144.3 (d)	144.5 (d)	144.5 (d)
C-4a	113.4 (s)	114.8 (s)	114.9 (s)	113.5 (s)	113.7 (s)	112.9 (s)	113.0 (s)	112.6 (s)	113.5 (s)	113.8 (s)
C-5	119.8 (d)	121.1 (d)	121.1 (d)	119.9 (d)	118.6 (d)	128.9 (d)	128.9 (d)	129.5 (d)	130.1 (d)	130.2 (d)
C-6	113.7 (d)	114.8 (d)	114.9 (d)	113.7 (d)	109.2 (d)	112.8 (d)	122.7 (d)	112.8 (d)	113.7 (d)	113.6 (d)
C-7	146.6 (s)	148.2 (s)	148.2 (s)	146.4 (s)	148.6 (s)	161.5 (s)	161.5 (s)	161.3 (s)	163.0 (s)	162.7 (s)
C-8	131.3 (s)	132.8 (s)	132.9 (s)	131.2 (s)	133.3 (s)	101.8 (d)	101.8 (d)	101.5 (d)	102.2 (d)	102.4 (d)
C-8a	143.8 (s)	145.0 (s)	145.0 (s)	143.8 (s)	142.2 (s)	155.8 (s)	155.8 (s)	155.3 (s)	156.9 (s)	156.8 (s)
C-1′	65.0 (t)	66.4 (t)	66.3 (t)	64.9 (t)	66.0 (t)	67.3 (t)	69.2 (t)	67.7 (t)	66.1 (t)	68.7 (t)
C-2′	77.6 (d)	80.1 (d)	80.2 (d)	77.9 (d)	122.8 (d)	60.9 (d)	74.7 (d)	60.3 (d)	123.2 (d)	61.8 (d)
C-3′	72.0 (s)	72.5 (s)	72.5 (s)	71.8 (s)	136.8 (s)	58.1 (s)	73.1 (s)	58.1 (s)	137.7 (s)	58.7 (s)
C-4′	23.2 (q)	23.4 (q)	22.4 (q)	23.2 (q)	17.3 (q)	17.0 (q)	23.0 (q)	16.9 (q)	17.1 (q)	17.4 (q)
C-5′	44.1 (t)	45.1 (t)	45.3 (t)	41.1 (t)	45.6 (t)	45.1 (t)	44.8 (t)	43.0 (t)	45.7 (t)	43.0 (t)
C-6′	74.2 (d)	75.7 (d)	75.7 (d)	78.5 (d)	75.5 (d)	74.3 (d)	74.3 (d)	74.5 (d)	76.4 (d)	79.7 (d)
C-7′	34.7 (t)	45.7 (t)	40.5 (t)	150.1 (d)	33.1 (t)	33.9 (t)	34.7 (t)	43.3 (t)	38.9 (t)	150.1 (d)
C-8′	133.5 (s)	74.0 (s)	77.3 (s)	133.3 (s)	134.1 (s)	133.7 (s)	133.5 (s)	72.0 (s)	77.2 (s)	135.2 (s)
C-9′	169.5 (s)	179.6 (s)	178.7 (s)	171.9 (s)	170.1 (s)	169.8 (s)	169.5 (s)	177.2 (s)	177.0 (s)	172.3 (s)
C-10′	122.9 (t)	22.3 (q)	64.6 (t)	57.1 (t)	122.5 (t)	122.7 (t)	122.9 (t)	23.1 (q)	64.7 (t)	56.9 (t)

[a] Values in (δ) ppm. [b] Spectra were taken in MeOD-d_4. [c] A spectrum was taken in DMSO-d_6. [d] Spectra were taken in acetone-d_6.

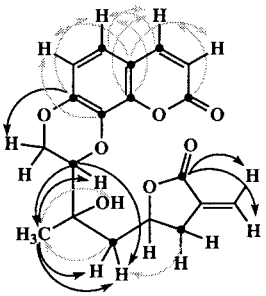

Figure 1. C−H long-range correlations in the HMBC spectrum of clauslactone A (**1**). Bold line: more significant correlations in the structure determinations.

1H, d, J = 8.8 Hz) in the ^{1}H NMR spectrum indicated the presence of a 7,8-dioxygenated coumarin nucleus, as in **1**−**4**. The structure of the side chain of this coumarin was elucidated by comparing its ^{13}C and ^{1}H NMR spectra with those of **1**. A carbonyl carbon signal at δ_C 170.1 and *exo*-methylene signals at δ_C 122.5 and δ_H 6.24, 5.64 (each t, J = 2.9 Hz) in the ^{13}C and ^{1}H NMR spectra and an IR band at ν_{max} 1759 cm^{-1} suggested the presence of an α-methylene γ-lactone moiety in the side chain, the same as in **1**. Significant differences in the NMR spectra of **5** compared with those of **1** are as follows: appearance of (a) two sp^2 carbon signals at δ_C 122.8 (d) and 136.8 (s) instead of an oxygenated sp^3 carbon at δ_C 77.6 (d) and 72.0 (s) in **1**; (b) only one olefinic proton signal at δ_H 5.62 (m); and (c) a 3H-singlet at δ_H 1.83 due to an allylmethyl instead of 3H-singlet at δ_H 1.42 in **1**. These observations, together with the result of a NOE experiment and ^{13}C chemical shift values of the C-4′ (δ_C 17.3) and C-1′ (δ_C 66.0), showed the presence of an *E*-oriented trisubstituted double bond having a methyl group in the side chain. Further, COSY analyses revealed two spin systems, H-1′−H-4′ and H-5′−H-10′, in the side chain. The connectivity of these two partial structures was established by three-bond long-range hetero correlations between one of the sp^2 carbons (C-2′) at δ_C 122.8 and methylene protons at δ_H 2.50 and 2.39 (each a double doublet, H-5′), and between the methylene carbon (C-5′) at δ_C 45.6 and an olefinic proton at δ_H 5.62 (m, H-2′) and the allyl methyl proton at δ_H 1.83 (H-4′). The location of the ether linkage of the side chain at C-7 was based on the three-bond correlation between the 2H doublet (H-1′) at δ_H 4.72 and C-7 (δ_C 148.6) on the coumarin nucleus observed in the HMBC spectrum. These results, together with C−H long-range correlations in the HMBC spectrum, support structure **5** for clauslactone E.

Clauslactones F−J (**6**−**10**)[4] show common features in their UV and ^{1}H NMR spectra (Table 1). UV absorptions were observed as a broad strong band at λ_{max} 321−323 nm and a sharp band at λ_{max} 204−218 nm, similar to that of umbelliferone (7-hydroxycoumarin)[12] and typical of 7-oxygenated coumarins.[11] The ^{1}H NMR spectra of **6**−**10** also show typical signals assignable to a 7-oxygenated coumarin nucleus[11] as follows: δ_H 6.20−6.29 and δ_H 7.62−7.99 (each 1H, J = 9.5 Hz, H-3 and H-4, respectively) and δ_H 7.38−7.63 (1H, d, J = 8.4−8.8 Hz, H-5), δ_H 6.88−7.00 (1H, dd, J = 8.4−8.8, 2.2−2.6 Hz, H-6), and δ_H 6.84−7.07 (1H, d, J = 2.2−2.6 Hz, H-8). Among these coumarins, clauslactones I, J, and H[4,5] were later isolated by others and named excavatins J, L, and M, respectively.[5] Thus, we only report here the structure assignments of other coumarins. The structure of each side chain bonded through an ether linkage at C-7 on the coumarin skeleton was deduced as described below.

Clauslactone F (**6**) was isolated as a pale yellow powder and had the molecular formula $C_{19}H_{18}O_6$. IR bands at ν_{max} 1761 and ^{1}H and ^{13}C NMR signal patterns [δ_H 4.76 (m), 3.14 (ddt, J = 17.1, 7.6, and 2.6 Hz), 2.64 (ddt, J = 17.1, 6.3, and 2.6 Hz), 5.66 (t, J = 2.6 Hz), 6.24 (t, J = 2.6 Hz); δ_C 74.3 (d), 33.9 (t), 133.7 (s), 169.8 (s), 122.7 (t)] were in good agreement with those of a 5-substituted α-methylene γ-lactone ring, similar to the side chain of **1**, except for some chemical shift differences. Further, correlations of these protons with a 2H multiplet at δ_H 1.91 in the COSY spectrum revealed the linkage of C-5′ to the γ-lactone ring. The remaining proton signals for the side chain appeared as a 3H-singlet at δ_H 1.46 and an ABC pattern at δ_H 4.04 (dd, J = 11.4, 6.6 Hz, H-1′), 4.34 (dd, J = 11.4, 3.7 Hz, H-1′), and 3.18 (dd, J = 6.6, 3.7 Hz, H-2′). The ^{1}H NMR chemical shifts of the 3H-singlet (δ_H 1.46) and the methine double doublet (δ_H 3.18) coupled with ^{13}C NMR chemical shifts of the methine carbon at δ_C 60.9 (d, C-2′) and a fully substituted carbon at δ_C 58.1 (s, C-3′) suggested the presence of a trisubstituted oxirane ring in the molecule. The linked arrangement of these signals was elucidated by HMBC spectral analysis. A methine carbon (C-2′) showed a three-bond correlation with a methylene proton signal at δ_H 1.91 (2H, d, H-5′) along with a quaternary methyl proton signal at δ_H 1.46 (s, H-4′). Further, a correlation between this methylene proton signal (H-5′) and

Table 3. Inhibitory Effects of Furanone-Coumarins on TPA-Induced EBV−EA Activation[a]

	EBV−EA positive cells (% viability)			
	compound concentration (mol ratio/32 pmol TPA)			
compound	1000	500	100	10
clauslactone A (**1**)	26.4 ± 0.8 (60)	59.2 ± 0.8 (>80)	85.2 ± 2.4 (>80)	100.0 ± 0.3 (>80)
clauslactone B (**2**)	17.1 ± 0.5 (60)	50.5 ± 2.0 (>80)	82.7 ± 1.9 (>80)	100.0 ± 0.5 (>80)
clauslactone C (**3**)	10.3 ± 0.2 (60)	46.9 ± 1.5 (>80)	80.1 ± 2.1 (>80)	95.3 ± 0.2 (>80)
clauslactone D (**4**)	19.6 ± 0.6 (60)	52.3 ± 1.9 (>80)	84.8 ± 3.0 (>80)	100.0 ± 0.7 (>80)
clauslactone E (**5**)	22.4 ± 0.6 (60)	58.8 ± 1.5 (>80)	86.2 ± 2.2 (>80)	100.0 ± 0.2 (>80)
clauslactone F (**6**)	29.3 ± 0.4 (60)	62.7 ± 1.3 (>80)	89.6 ± 2.0 (>80)	100.0 ± 0.1 (>80)
clauslactone H (**8**)	25.1 ± 0.7 (60)	60.4 ± 0.8 (>80)	88.4 ± 2.1 (>80)	100.0 ± 0.3 (>80)
clauslactone I (**9**)	20.6 ± 0.3 (60)	54.3 ± 1.1 (>80)	82.5 ± 1.7 (>80)	100.0 ± 0.5 (>80)
clauslactone J (**10**)	27.4 ± 0.7 (60)	65.8 ± 0.9 (>80)	85.2 ± 2.1 (>80)	100.0 ± 0.7 (>80)
7-methoxycoumarin	0.0 ± 0.2 (30)	71.7 ± 1.4 (60)	100.0 ± 1.5 (>80)	100.0 ± 0.3 (>80)

[a] Mole ratio/TPA (32 pmol = 20 ng/mL), 1000 mol ratio = 32 nmol, 500 mol ratio = 16 nmol, 100 mol ratio = 3.2 nmol, and 10 mol ratio = 0.32 nmol. Values are EBV−EA activation (%) ± s.d. in the presence of the test compound relative to the positive control (100%). Values in parentheses represent the viability % of Raji cells measured through Trypan Blue staining. At least 60% viability of Raji cells 2 days after treatment with the compounds is required for an accurate result.

the methyl carbon at δ_C 17.0 (C-4′) was also observed. The location of the linkage of the side chain at C-7 on the coumarin nucleus was revealed by observation of a three-bond correlation between the methylene proton signals (H-1′) and the carbon C-7 at δ_C 161.5 on 7-oxycoumarin nucleus. From the HMBC data, together with the observation of significant mass fragment ions at *m/z* 162 and 175, which result from cleavage at O-7/C-1′ with hydrogen transfer and C-1′/C-2′, the structure of clauslactone F is proposed as shown in **6**.

Clauslactone G (**7**) was obtained as a colorless powder, having the molecular formula $C_{19}H_{20}O_7$, a difference of H_2O compared with **6**. The presence of an α-methylene γ-lactone moiety in the molecule, the same as in **6**, was suggested by the similarities of the ^{1}H and ^{13}C NMR signals for the 6′−10′ positions of **7** with those of **6** (Tables 1 and 2). The proton signals for the methylene (H-5′) attached to the γ-lactone ring were observed clearly as double doublets at δ_H 2.14 (J = 15.0, 9.9 Hz) and 1.93 (J = 15.0, 2.9 Hz). The remaining protons appeared as ABC-type signals at δ_H 4.11 (1H, dd, J = 9.5, 7.3 Hz), 4.28 (1H, dd, J = 9.5, 3.7 Hz), and 4.00 (1H, br), assignable to an oxygenated methylene (H-1′) and an adjacent hydroxymethine group (H-2′). The connectivities of these protons and carbons were deduced from analysis of the HMBC spectrum, and the structure of clauslactone G was assigned as **7**.

Clauslactones H (**8**), I (**9**), and J (**10**) were obtained as colorless powders, and analyzed for $C_{19}H_{20}O_7$, $C_{19}H_{20}O_7$, $C_{19}H_{18}O_7$, respectively, by HRMS. The chemical shifts in the ^{1}H and ^{13}C NMR spectra of **8**−**10** were in good agreement with those reported for excavatins M, J, and L,[5] respectively. The results of HMBC spectra of these coumarins also supported the assignment of these structures. The other compounds isolated from the same plant material were fully characterized as the carbazole clauszoline M[3] and the coumarin umbelliferone[12] by analyses of UV, IR, MS, and NMR spectra.

Nine furanone-coumarins (**1**−**6** and **8**−**10**) exhibited inhibitory activity against TPA-induced EBV−EA activation in a nonproducer Raji cell line (Table 3).[10] All of the test compounds inhibited EBV activation, even at 1 × 10^2 mol ratio/TPA (10.4−19.9%), and showed significant inhibitory effects at high concentration (1 × 10^3 mol ratio/TPA). However, all compounds showed only weak cytotoxicity to Raji cells, even at 1 × 10^3 mol ratio/TPA. In a previous study, the C-10 terpenoid side chain was found to be essential for inhibition of tumor-promoting activity by the 7-geranyloxycoumarin, auraptene.[13] In our present study, all of the C-10 terpenoid-substituted coumarins tested showed significant inhibitory effects for tumor-promotion. On the other hand, 7-methoxycoumarin (ayapanin), which lacks a C-10 terpenoid side chain, showed almost no inhibitory effect at 1 × 10^2 and 1 × 10 mol ratio/TPA and was cytotoxic at 1 × 10^3 mol ratio/TPA. Among the C-10 terpenoid-substituted coumarins, clauslactone C (**3**) was found to exhibit the most significant inhibitory activity (about 90% inhibition of induction at 1 × 10^3 mol ratio/TPA and > 50% inhibition at 5 × 10^2 mol ratio/TPA, see Table 3). Thus, clauslactone C (**3**), which has a C-10 terpenoid side chain containing a γ-lactone moiety, may be especially valuable as a potential cancer chemopreventive agent (antitumor promoter) to protect against chemical carcinogenesis.

Experimental Section

General Experimental Procedures. Melting points were measured on a micromelting point hot-stage apparatus (Yanagimoto) and are uncorrected. ^{1}H NMR spectra were recorded on a A-400 (JEOL) spectrometer in $CDCl_3$, unless otherwise stated. Chemical shifts are shown in δ values (ppm) with tetramethylsilane as an internal reference. All MS were obtained under electron impact (EI) conditions using an M-80 (Hitachi) with a direct inlet system. UV spectra were recorded on a UVIDEC-610C double-beam spectrophotometer (JASCO) in MeOH; IR spectra, on an IR-230 (JASCO) in $CHCl_3$; and optical rotations, on a DIP-370 (JASCO) in $CHCl_3$ at 25 °C. Preparative TLC was performed on Kieselgel 60 F_{254} (Merck).

Extraction and Isolation. The plant material used in this study, *C. excavata*, was grown in a greenhouse at the Okitsu Branch, Fruit Tree Research Station, Ministry of Agriculture, Forestry and Fisheries, Shimizu, Shizuoka (no. 84598). A voucher specimen has been deposited in the Okitsu Branch. The acetone extract (2.64 g) of the dried leaves (70 g) was subjected to Si gel chromatography. Elution with hexane−acetone (9:1, 4:1, 3:1, 7:3, 3:2, 2:3, and 1:4), acetone, and MeOH successively gave nine fractions. Each fraction was further subjected to Si gel column chromatography and preparative TLC (developed with an appropriate mixture of hexane, CH_2Cl_2, acetone, EtOAc, $CHCl_3$, benzene, iso-Pr_2O, and MeOH). From the hexane−acetone (7:3) eluate: umbelliferone[12] (3.2 mg) and clauszoline M[3] (1.0 mg). From the hexane−acetone (3:2) eluate: clauslactone A (**1**, 2.0 mg), clauslactone B (**2**, 2.6 mg), clauslactone F (**6**, 5.3 mg), clauslactone G (**7**, 1.5 mg), and clauslactone H (**8**, 4.9 mg). From the hexane−acetone (2:3) eluate: clauslactone C (**3**, 5.7 mg), clauslactone D (**4**, 3.0 mg), clauslactone I (**9**, 5.4 mg), and clauslactone J (**10**, 3.8 mg). From the hexane−acetone (1:4) eluate: clauslactone E (**5**, 4.5 mg). Known components were fully characterized by UV, IR, ^{1}H NMR, and MS analyses.

Clauslactone A (1): colorless oil, $[\alpha]_D$ +92° (*c* 0.15, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 212 (4.49), 225sh (4.19), 252 (3.72),

261 (3.80), 319 (4.02) nm; IR ($CHCl_3$) ν_{max} 3553, 1764, 1727, 1615 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; EIMS *m/z* (%) 358 (M^+, 19), 246 (5), 204 (100), 189 (23), 178 (17), 175 (74), 149 (20), 147 (33); HRMS *m/z* $[M]^+$ 358.1052 (calcd for $C_{19}H_{18}O_7$, 358.1051).

Clauslactone B (2): colorless powder, $[\alpha]_D$ +76° (*c* 0.20, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 206 (4.36), 227sh (3.90), 253 (3.53), 262 (3.58), 319 (3.84) nm; IR ($CHCl_3$) ν_{max} 3518 (br), 1772, 1725, 1614 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HMBC C−H correlations C-2 → H-4; C-4a → H-3, H-6; C-5 → H-4; C-7 → H-5, H-1′; C-8 → H-6; C-8a → H-4, H-5; C-2′ → H-4′; C-5′ → H-4′; C-9′ → H-7′, H-10′; EIMS *m/z* (%) 376 (M^+, 13), 289 (4), 247 (10), 204 (100), 189 (18), 178 (75), 175 (76), 162 (16), 149 (12), 147 (27); HRMS *m/z* $[M]^+$ 376.1158 (calcd for $C_{19}H_{20}O_8$, 376.1157).

Clauslactone C (3): colorless oil, $[\alpha]_D$ +50° (*c* 0.44, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 206 (4.37), 227sh (3.91), 253 (3.58), 262 (3.63), 319 (3.87) nm; IR ($CHCl_3$) ν_{max} 3566, 1766, 1726, 1614 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HMBC C−H correlations C-2 → H-4; C-4a → H-3, H-6; C-5 → H-4; C-7 → H-5, H-1′; C-8 → H-6; C-8a → H-4, H-5; C-2′ → H-4′, H-5′; C-4′ → H-2′, H-5′; C-5′ → H-4′, H-7′; C-7′ → H-5′, H-10′; C-9′ → H-7′, H-10′; C-10′ → H-7′; EIMS *m/z* (%) 392 (M^+, 20), 362 (4), 316 (4), 289 (5), 247 (17), 204 (100), 189 (27), 178 (78), 175 (89); HRMS *m/z* $[M]^+$ 392.1135 (calcd for $C_{19}H_{20}O_9$, 392.1106).

Clauslactone D (4): colorless powder, $[\alpha]_D$ +27° (*c* 0.23, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 207 (4.48), 225sh (4.03), 254 (3.64), 261 (3.69), 319 (3.96) nm; IR ($CHCl_3$) ν_{max} 3568, 3421 (br), 1739, 1616 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HMBC C−H correlations C-2 → H-3, H-4; C-4 → H-5; C-4a → H-3, H-6; C-5 → H-4; C-7 → H-5, H-1′; C-8 → H-6; C-8a → H-4, H-5; C-2′ → H-1′, H-4′; C-3′ → H-4′; C-5′ → H-4′; C-6′ → H-5′, H-7′; C-7′ → H-10′; C-8′ → H-10′; C-9′ → H-7′, H-10′; EIMS *m/z* (%) 374 (M^+, 18), 356 (25), 247 (80), 204 (100), 189 (14), 175 (33), 171 (32), 162 (7); HRMS *m/z* $[M]^+$ 374.1007 (calcd for $C_{19}H_{18}O_8$, 374.1001).

Clauslactone E (5): colorless powder, $[\alpha]_D$ +22° (*c* 0.34, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 209 (4.59), 261 (3.92), 319 (4.09) nm; IR ($CHCl_3$) ν_{max} 3631, 3425 (br), 1759, 1722, 1620 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HMBC C−H correlations C-2 → H-3, H-4; C-4 → H-5; C-4a → H-3, H-6; C-5 → H-4; C-7 → H-5, H-1′; C-8 → H-6; C-8a → H-4, H-5; C-2′ → H-1′, H-4′, H-5′; C-3′ → H-1′, H-4′, H-5′; C-4′ → H-2′, H-5′; C-5′ → H-2′, H-4′, H-7′; C-6′ → H-5′; C-7′ → H-5′, H-10′; C-8′ → H-7′; C-9′ → H-7′, H-10′; C-10′ → H-7′; EIMS *m/z* (%) 342 (M^+, 5), 191 (4), 178 (100), 165 (33), 150 (35); HRMS *m/z* $[M]^+$ 342.1094 (calcd for $C_{19}H_{18}O_6$, 342.1102); differential NOE: irradiation at δ 1.83 (H-4′) gave 3% enhancement at δ 4.72 (H-1′) and 2% enhancement at δ 4.69 (H-6′).

Clauslactone F (6): pale yellow powder, $[\alpha]_D$ +37° (*c* 0.40, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 205 (4.45), 217sh (4.26), 243sh (3.46), 253sh (3.31), 294sh (3.89), 321 (4.12) nm; IR ($CHCl_3$) ν_{max} 3510 (br), 1761, 1730, 1614 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HMBC C−H correlations C-2 → H-3, H-4; C-4 → H-5; C-4a → H-3, H-6, H-8; C-5 → H-4; C-7 → H-5, H-8, H-1′; C-8 → H-6; C-8a → H-4, H-5, H-8; C-1′ → H-2′; C-2′ → H-1′, H-4′, H-5′; C-3′ → H-4′, H-5′; C-4′ → H-5′; C-5′ → H-7′; C-6′ → H-5′; C-7′ → H-5′, H-10′; C-8′ → H-7′; C-9′ → H-7′, H-10′; EIMS *m/z* (%) 342 (M^+, 100), 188 (20), 175 (34), 162 (91); HRMS *m/z* $[M]^+$ 342.1125 (calcd for $C_{19}H_{18}O_6$, 342.1103); differential NOE: irradiation at δ 1.46 (H-4′) gave 4, 2, and 7% enhancements at δ 4.04 (H-1′), 4.34 (H-1′), and 4.76 (H-6′), respectively.

Clauslactone G (7): colorless powder, $[\alpha]_D$ +21° (*c* 0.11, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 205 (4.44), 218sh (4.21), 244sh (3.47), 253sh (3.30), 292sh (3.78), 323 (4.04) nm; IR ($CHCl_3$) ν_{max} 3579, 3406 (br), 1765, 1728, 1614 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HMBC C−H correlations C-2 → H-3, H-4; C-4 → H-5; C-4a → H-3, H-6, H-8; C-5 → H-4; C-7 → H-5, H-8, H-1′; C-8 → H-6; C-8a → H-4, H-5, H-8; C-1′ → H-2′; C-2′ → H-1′, H-4′, H-5′; C-3′ → H-4′; C-4′ → H-5′; C-5′ → H-4′; C-6′ → H-5′; C-7′ → H-10′; C-8′ → H-7′, H-10′; C-9′ → H-7′, H-10′; EIMS *m/z* (%) 360 (M^+, 9), 249 (6), 213 (7), 205 (16), 187 (9), 175 (13), 162 (100); HRMS *m/z* $[M]^+$ 360.1207 (calcd for $C_{19}H_{20}O_7$, 360.1203).

Clauslactone H (8): colorless powder, $[\alpha]_D$ +43° (*c* 0.38, MeOH); UV (MeOH) λ_{max} (log ϵ) 204 (4.35), 217sh (4.03), 242sh (3.47), 251sh (3.30), 291sh (3.75), 321 (3.99) nm; IR ν_{max} ($CHCl_3$) 3568, 3350 (br), 1772, 1732, 1616 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HMBC C−H correlations C-2 → H-3, H-4; C-4 → H-5; C-4a → H-3, H-6, H-8; C-7 → H-5, H-8, H-1′; C-8 → H-6; C-8a → H-4, H-5, H-8; C-2′ → H-1′, H-4′, H-5′; C-3′ → H-1′, H-4′, H-5′; C-4′ → H-2′, H-5′; C-5′ → H-2′, H-4′, H-7′; C-6′ → H-5′, H-7′; C-7′ → H-5′, H-10′; C-9′ → H-7′, H-10′; C-10′ → H-7′; EIMS *m/z* (%) 360 (M^+, 90), 273 (29), 199 (22), 188 (15), 175 (32), 162 (100); HRMS *m/z* $[M]^+$ 360.1188 (calcd for $C_{19}H_{20}O_7$, 360.1207); differential NOE (DMSO-d_6): irradiation at δ 1.38 (H-4′) gave 4, 3, and 6% enhancements at δ 4.11 (H-1′), 4.44 (H-1′), and 4.72 (H-6′), respectively.

Clauslactone I (9): colorless powder, $[\alpha]_D$ +27° (*c* 0.41, MeOH); UV (MeOH) λ_{max} (log ϵ) 205 (4.44), 217sh (4.10), 244sh (3.49), 252sh (3.36), 295sh (3.77), 323 (4.01) nm; IR ($CHCl_3$) ν_{max} 3545, 3400 (br), 1766, 1730, 1614 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HMBC C−H correlations C-2 → H-3, H-4; C-4 → H-5; C-4a → H-3, H-6, H-8; C-5 → H-4; C-7 → H-5, H-8, H-1′; C-8 → H-6; C-8a → H-4, H-5, H-8; C-2′ → H-1′, H-4′, H-5′; C-3′ → H-1′, H-4′, H-5′; C-4′ → H-2′, H-5′; C-5′ → H-2′, H-4′, H-7′; C-6′ → H-5′, H-7′; C-7′ → H-5′, H-10′; C-9′ → H-7′, H-10′; C-10′ → H-7′; EIMS *m/z* (%) 360 (M^+, 12), 204 (14), 198 (11), 175 (9), 171 (12), 162 (100); HRMS *m/z* $[M]^+$ 360.1210 (calcd for $C_{19}H_{20}O_7$, 360.1208); differential NOE (acetone-d_6): irradiation at δ 1.86 (H-4′) gave 4% enhancement at δ 4.75 (H-1′) and 2% enhancement at δ 4.78 (H-6′).

Clauslactone J (10): colorless powder, $[\alpha]_D$ +15° (*c* 0.29, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 205 (4.37), 216sh (4.18), 242sh (3.46), 252sh (3.29), 293sh (3.80), 322 (4.02) nm; IR ($CHCl_3$) ν_{max} 3597, 3510 (br), 1753, 1732, 1614 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HMBC C−H correlations C-2 → H-3, H-4; C-4 → H-5; C-4a → H-3, H-4, H-6, H-8; C-5 → H-4; C-6 → H-8; C-7 → H-5, H-8, H-1′; C-8 → H-6; C-8a → H-4, H-5, H-8; C-2′ → H-1′, H-4′, H-5′; C-3′ → H-1′, H-4′, H-5′; C-4′ → H-5′; C-5′ → H-4′; C-6′ → H-5′, H-7′; C-7′ → H-5′, H-10′; C-8′ → H-10′; C-9′ → H-7′, H-10′; EIMS *m/z* (%) 342 (12), 199 (10), 188 (12), 175 (14), 162 (100); FABMS *m/z* $[M + H]^+$ 359; HRFABMS *m/z* $[M]^+$ 359.1137 (calcd for $C_{19}H_{19}O_7$, 359.1131); differential NOE (DMSO-d_6): irradiation at δ 1.50 (H-4′) gave 3, 1, and 5% enhancements at δ 4.17 (H-1′), 4.52 (H-1′), and 5.26 (H-6′), respectively.

In Vitro EBV−EA Activation Experiments. The inhibition of EBV−EA activation was assayed using the same method described previously.[10] In brief, Raji cells were grown to a density of 10^6 cells/mL, harvested by centrifugation, and resuspended in RPMI 1640 medium (Nakalai Tesque, Kyoto, Japan) with 10% fetal calf serum containing 4 mM *n*-butyric acid as inducer, 32 pmol of TPA (20 ng/mL in DMSO), and 32, 3.2 or 0.32 nmol of the test compound (DMSO solutions). The cells were incubated at 37 °C for 48 h. Cell number and viability were determined after 48 h by means of a hemocytometer (Trypan Blue staining method). Untreated cultures served as the controls. EBV−EA inhibitory activity of the test compounds was estimated on the basis of the percentage of the number of positive cells compared to that observed in the case of a control without the test product. In each assay, at least 500 cells were counted, and the results were read blind.

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References and Notes

(1) A part of this paper was presented at the 116th and 117th Annual Meetings of the Pharmaceutical Society of Japan (Kanazawa, March 1996, and Tokyo, March, 1997).

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