Chemical Constituents of *Calophyllum brasiliense.* 2. Structure of Three New Coumarins and Cancer Chemopreventive Activity of 4-Substituted Coumarins

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Continuing our search for cancer chemopreventive agents from natural sources, we examined constituents of the stem bark of *Calophyllum brasiliense*. Three new 4-substituted coumarins named brasimarins A (**2**), B (**3**), and C (**4**) were isolated and characterized, along with 11 known coumarins belonging to the calanolides or inophyllums. We also discuss the inhibitory effects of these coumarins on Epstein−Barr virus early antigen (EBV-EA) activation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in Raji cells.

Introduction

In 1992, the research group of the National Cancer Institute reported that (+)-calanolide A (**1**), one of the 4-propyldipyranocoumarins isolated from *Calophyllum* plants, showed strong activity against human immunodeficiency virus type 1 (HIV-1).[1] Since then, the chemical constituents of *Calophyllum* species have been actively studied.[2] Previously, in Part 1 of this series, we reported the first study of the constituents of *Calophyllum brasiliense* Camb. (Guttiferae), including the isolation and identification of xanthones.[3] In further studies of the constituents of this plant, three new 4-substituted coumarins, together with 11 known coumarins belonging to calanolides or inophyllums, were characterized. This report deals with the isolation and characterization of three new coumarins named brasimarins A (**2**), B (**3**), and C (**4**) (calanolides and inophyllums), from the stem bark of *C. brasiliense* collected in Brazil. In addition, we have previously shown that xanthones and 4-phenylcoumarins from *Calophyllum* plants might be valuable as potential cancer chemopreventive agents (antitumor-promoters), using the short-term in vitro assay of 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced Epstein−Barr virus early antigen (EBV-EA) activation in Raji cells and an in vivo two-stage mouse skin carcinogenesis test.[3,4] We also describe the inhibitory effects of the 4-substituted coumarins isolated in this study on EBV-EA activation induced by TPA in Raji cells.

Results and Discussion

The acetone extract of the stem bark of the plant was treated successively with silica gel column and thin-layer chromatography to obtain three new coumarins named brasimarins A (**2**), B (**3**), and C (**4**) along with known coumarins and xanthones.[3]

Brasimarin A (**2**) was isolated as a pale yellow oil. The molecular formula was established as $C_{24}H_{22}O_5$ by HRE-IMS. The UV spectrum showed a strong absorption at λ_{max} 276 nm accompanied by a shoulder at λ_{max} 322 nm, and the IR spectrum exhibited bands at ν_{max} 3520, 1730, and 1650 cm^{-1}. The ^{1}H NMR spectrum revealed signals assignable to a hydrogen-bonded OH, an *n*-propyl group, a dimethylpyran ring, and an unsubstituted phenyl group, along with a lone isolated vinyl proton at δ_H 5.89. Good similarity of chemical shift values in the ^{13}C NMR of brasimarin A with those of calanolide A (**1**)[1] except for those of the D ring carbons in formula **1** indicated the presence of a partial structure of 4-propylcoumarin having a [5,6-*b*]-oriented dimethylpyran ring in the molecule. This partial structure was supported by HMBC, NOE, and MS data as follows: (1) The 4-*n*-propyl side chain was deduced by observations of a MS fragment ion at *m*/*z* 347 arising from cleavage of the allylic bond of the *n*-propyl moiety after loss of a methyl radical on the dimethylpyran ring from the molecular ion, NOE between H-1′ and a singlet due to H-3 on the coumarin skeleton, and HMBC correlations from C-4a to H-1′ and H-3, which correlated with the carbon at both C-2 and C-1′. (2) A [5,6-*b*]-oriented dimethylpyran ring was also indicated by HMBC correlations from C-3″ to H-1″, which also correlated with C-5. Further, HMBC correlations from C-6 to both H-2″ and a hydrogen-bonded OH (δ_H 12.16) also suggested the location of OH at C-7. The presence of a benzoyl group at C-8 was confirmed by C−H long-range correlations from another carbonyl carbon (δ_C 198.9, C-1‴) to *ortho*-located phenyl protons (H-3‴, H-7‴) and from C-8 to 7-OH (ν_{max} 3520 cm^{-1}, δ_H 12.16) hydrogen-bonded with the carbonyl group. From these spectral data coupled with other HMBC data, structure **2** was proposed for brasimarin A.

Brasimarin B (**3**) was obtained as a colorless oil, $[\alpha]_D$ +8.1 (MeOH), $C_{25}H_{26}O_6$. A UV band appeared at λ_{max} 299 nm and IR bands at ν_{max} 3563 and 1729 cm^{-1}. Close resemblance of ^{13}C-chemical shift values (Table 1) of C-2−C-7 including C-4a and C-8a between **2** and brasimarin B together with a singlet at δ_H 6.07, typical of H-3 on the coumarin nucleus, in ^{1}H NMR suggested that brasimarin B could have a 5,7-oxygenated 4,6,8-substituted coumarin skeleton, the same as that of **2**. Structures and arrangements of substituents on this nucleus were determined by NMR and HMBC analyses. A nonsubstituted phenyl moiety at C-4 was assigned by NMR signals at δ_H 7.44 (3H,

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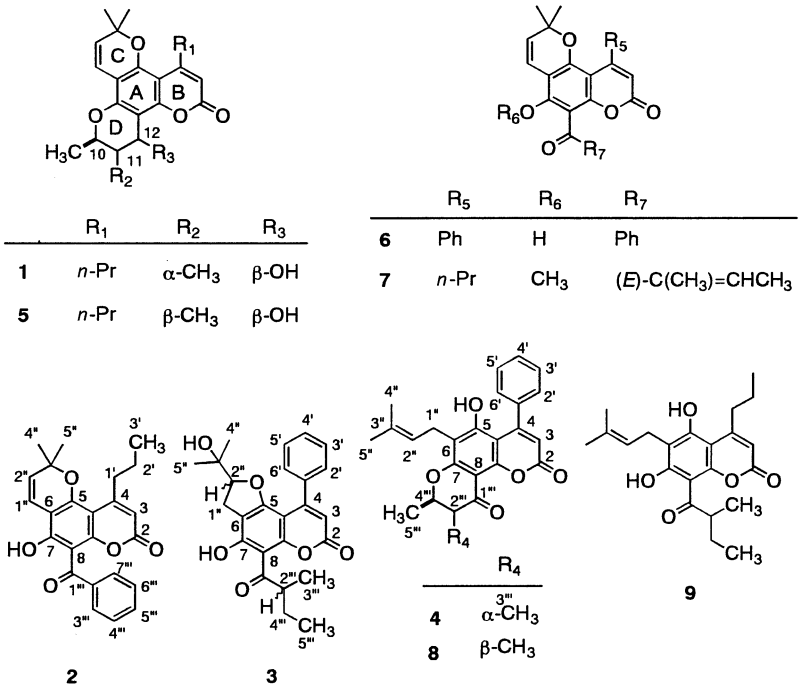
Scheme 1. Structures of 4-Substituted Coumarins from *Calophyllum brasiliense*



m) and 7.28 (2H, m) and HMBC correlations from C-1′ to H-3′, H-5′, and H-3, which further correlated with a carbonyl carbon (C-2) and C-4a, together with the appearance of H-3 as a singlet (δ_H 6.07). A [5,6-*b*]-oriented dihydrofuran ring bearing a 1-hydroxy-1-methylethyl moiety at C-2″ was proposed by observations of signals assignable to two quaternary methyls linked to an oxygenated carbon (δ_H 0.93 and 1.01; δ_C 71.6) and ABC-type protons due to an oxymethine (δ_H 4.51; δ C 92.7) linked to a benzylic CH_2 (δ_H 3.07; 2.93; δ C 26.9) in NMR and HMBC correlations from C-2″ to quart-CH_3, from C-3″ to H-1″, and from C-5 to H-1″. Further, the presence of a 2-methylbutyryl group at C-8 and 7-OH was determined by a hydrogen-bonded carbonyl and OH (ν_{max} 1729 cm^{-1}, δ_C 210.5; ν_{max} 3563 cm^{-1}, δ_H 14.30), a deshielded methine (δ_H 3.97, δ_C 46.8) both bearing a methyl (δ_H 1.29) and an ethyl group (δ_H 1.02; 1.94, 1.51), and HMBC correlations from C-2‴ to H-5‴ and from C-1‴ to H-3‴, which correlated with C-4‴. The location of the remaining OH at C-7 was also supported by HMBC both from C-6 and C-8 to OH. Thus, we assigned structure **3** to brasimarin B.

Brasimarin C (**4**) was isolated as a colorless oil. The molecular formula was determined to be $C_{25}H_{24}O_5$ by HREIMS. The UV spectrum showed the typical absorptions of a 4-phenylcoumarin nucleus at λ_{max} 228, 286, and 324 nm, the same as that of calocoumarin A (**8**).[4] The NMR spectrum revealed signals assignable to a monosubstituted phenyl, a lone proton at H-3, an OH group, and a prenyl moiety. Further, an analysis of the H−H COSY spectrum of **4** indicated the presence of the partial structure [−O−CH(CH_3)−CH(CH_3)−C═O−]. Arrangements of these moieties on the 4-phenylcoumarin nucleus were determined by C−H long-range coupling in the HMBC spectrum as follows: correlation from the carbon signal at δ_C 102.8 to the singlet at δ_H 6.04 (H-3) and an OH signal at δ_H 5.87 placed this carbon at C-4a and the location of a OH at C-5. Further, correlations to H-1″ (δ_H 3.27) from oxygenated carbons at C-5 (δ_C 156.5) and C-7 (δ_C 162.8) suggested the location of the prenyl moiety at C-6 and the presence of a [2,3-*h*]-fused 5,6-dimethyldihydro-4-pyrone ring on the coumarin nucleus. A *trans*-relative stereochemistry was suggested by the presence of a large coupling constant (J = 11.0 Hz) between H-2‴ and H-4‴. Thus, the structure of brasimarin C was determined to be **4**, except for the absolute stereochemistry.

Recently we also isolated the 2‴-epimer (**8**) of **4**, named calocoumarin A, from this plant and *C. inophyllum* L.[4] Full details of the structure **8** will be reported elsewhere. Other coumarins isolated from this plant were fully characterized as calanolide A (**1**),[1] calanolide C (**5**),[1] inophyllum D,[5] inophyllum A,[5] inophyllum C,[5] inophyllum E,[5] calanone (**6**),[6] calophyllolide,[7,8] 5-methoxy-2,2-dimethyl-6-(2-methyl-1-oxo-2-butenyl)-10-propyl-2*H*,8*H*-benzo[1,2-*b*;3,4-*b*′]dipyran-8-one (**7**),[9] and mammea B/BB (**9**)[10] by comparison of the ^{1}H NMR and IR spectra with spectral data reported in the literature.

Inhibitory Effects of 4-Substituted Coumarins on EBV-EA Induction. Eight natural 4-substituted coumarins, three 4-phenylcoumarins (**3**, **4** and **6**), and five 4-propylcoumarins (**1**, **2**, **5**, **7**, and **9**), were tested for their inhibition of tumor-promoting activity by using a short-term in vitro assay for TPA-induced EBV-EA activation in Raji cells. Their inhibitory effects on the activation of the virus-genome, the viabilities of Raji cells, and the 50% inhibitory concentration (IC_{50}) values are shown in Table 2. All the test compounds showed inhibitory activity on the EBV-EA activation even at 1 × 10 mol ratio/TPA (5.7−15.8%) and fully blocked EBV-EA activation at high concentration (1 × 10^3 mol ratio/TPA) without causing a decrease in viability of the Raji cells. These values corresponded to an IC_{50} of 170−351 mol ratio/TPA. The IC_{50} values of all the test compounds were lower than that of β-carotene, a vitamin A precursor commonly used in cancer prevention studies.[11] In previous studies, we reported that the 4-phenylcoumarins might be valuable as potential cancer chemopreventive agents, and the prenyl side chain may enhance the antitumor-promoting effect.[4] Among 4-propylcoumarins, calanolide A (**1**), 5-methoxy-2,2-dimethyl-6-(2-methyl-1-oxo-2-butenyl)-10-propyl-2*H*,8*H*-benzo[1,2-*b*;3,4-*b*′]dipyran-8-one (**7**) and mammea B/BB (**9**) showed the more significant activities (IC_{50} 170−290) when compared with 4-phenylcoumarins, brasimarin B (**3**), brasima-

Table 1. ^{13}C and ^{1}H NMR Spectral Data of 4-Substituted Coumarins[a]

	brasimarin A (**2**)			brasimarin B (**3**)			brasimarin C (**4**)		
	δ_C	δ_H	HMBC	δ_C	δ_H	HMBC	δ_C	δ_H	HMBC
2	158.4 (s)		H-3	159.1 (s)		H-3	159.2 (s)		H-3
3	111.2 (d)	5.89 (s)	H-1′	111.0 (d)	6.07 (s)		113.8 (d)	6.04 (s)	
4	157.6 (s)		H-1′	155.0 (s)			152.8 (s)		
4a	103.0 (s)		H-3, H-1′	98.7 (s)		H-3	102.8 (s)		H-3, 5-OH
5	156.7 (s)		H-1″	161.9 (s)		H-1″	156.5 (s)		5-OH, H-1″
5-OH								5.87 (br s)	
6	105.9 (s)		7-OH, H-2″	110.1 (s)		7-OH, H-1″	112.4 (s)		5-OH, H-1″
7	160.7 (s)		7-OH	163.9 (s)		7-OH	162.8 (s)		H-1″
7-OH		12.26 (s)			14.30 (s)				
8	104.1 (s)		7-OH	104.6 (s)		7-OH	103.9 (s)		
8a	156.2 (s)			157.2 (s)			153.9 (s)		
1′	38.7 (t)	2.89 (2H, t, 7.6)	H-3, H-2′, H-3′	138.1 (s)		H-3, H-3′, H-5′	136.4 (s)		H-3, H-3′, H-5′
2′	23.2 (t)	1.66 (2H, sext, 7.6)	H-1′, H-3′	127.4 (d)	7.28 (m)	H-4′	127.6 (d)	7.42 (m)	H-4′
3′	14.0 (q)	1.04 (3H, t, 7.6)	H-1′, H-2′	127.9 (d)	7.44 (m)	H-5′	129.7 (d)	7.57 (m)	
4′				128.8 (d)	7.44 (m)	H-2′, H-6′	130.3 (d)	7.57 (m)	H-2′, H-6′
5′				127.9 (d)	7.44 (m)	H-3′	129.7 (d)	7.57 (m)	
6′				127.4 (d)	7.28 (m)	H-4′	127.6 (d)	7.42 (m)	H-4′
1″	115.9 (d)	6.77 (d, 10.1)		26.9 (t)	2.93 (dd, 8.8, 15.4) 3.07 (dd, 9.5, 15.4)		21.9 (t)	3.27 (2H, 7.0)	
2″	126.6 (d)	5.62 (d, 10.1)	H-4″, H-5″	92.7 (d)	4.51 (dd, 8.8, 9.5)	H-1″, H-4″, H-5″	120.9 (d)	5.08 (m)	H-1″, H-4″, H-5″
3″	79.7 (s)		H-1″, H-2″, H-4″, H-5″	71.6 (s)		H-1″, H-4″, H-5″	133.4 (s)		H-1″, H-4″, H-5″
4″	28.3 (q)	1.57 (3H, s)	H-5″	24.8 (q)	0.93 (3H, s)	H-5″	17.9 (q)	1.71 (3H, s)	H-2″, H-5″
5″	28.3 (q)	1.57 (3H, s)	H-4″	23.2 (q)	1.01 (3H, s)	H-4″	25.7 (q)	1.66 (3H, s)	H-2″, H-4″
1‴	198.9 (s)		H-3‴, H-7‴	210.5 (s)		H-2‴, H-3‴, H-4‴	190.5 (s)		H-2‴, H-3‴, H-4‴
2‴	140.3 (s)	7.61 (d, 7.6)	H-4‴, H-6‴	46.8 (d)	3.97 (dq, 6.6, 11.0)	H-3‴, H-4‴, H-5‴	47.2 (d)	2.57 (dq, 6.9, 11.0)	H-3‴, H-5‴
3‴	128.2 (d)	7.44 (t, 7.6)	H-7‴	16.5 (q)	1.29 (3H, d, 6.6)	H-2‴, H-4‴	10.6 (q)	1.25 (3H, d, 6.9)	H-2‴
4‴	128.1 (d)	7.56 (t, 7.6) 7.44 (t, 7.6)	H-6‴	27.2 (t)	1.94 (m) 1.51 (m)	H-2‴, H-3‴, H-5‴	79.5 (d)	4.30 (dq, 6.2, 11.0)	H-2‴, H-3‴, H-5‴
5‴	132.3 (d)	7.61 (d, 7.6)	H-3‴, H-7‴	11.8 (q)	1.02 (3H, t, 6.6)	H-4‴	19.6 (q)	1.55 (3H, d, 6.2)	
6‴	128.1 (d)		H-4‴						
7‴	128.2 (d)		H-3‴						

[a] Values in (δ_H and δ_C) ppm. All signals correspond to 1H, unless otherwise stated. Figures in parentheses are coupling constants (J) in Hz. Spectra were taken in $CDCl_3$.

Table 2. Inhibitory Effects of 4-Substituted Coumarins on TPA-Induced EBV-EA Activation[a]

	EBV-EA-positive cells (% viability)				
	compound concentration (mol ratio/32 pmol TPA)				IC_{50} [b]
compound	1000	500	100	10	(mol ratio/32 pmol TPA)
calanolide A (**1**)	0.0±0.5 (60)	27.7±1.3 (>80)	66.6±2.1 (>80)	92.5±0.5 (>80)	290
brasimarin A (**2**)	0.0±0.5 (70)	33.3±1.5 (>80)	72.1±1.7 (>80)	93.8±0.5 (>80)	349
brasimarin B (**3**)	0.0±0.3 (70)	32.6±1.5 (>80)	71.6±2.4 (>80)	92.5±0.5 (>80)	342
brasimarin C (**4**)	0.0±0.5 (70)	33.3±1.4 (>80)	70.2±1.8 (>80)	93.8±0.4 (>80)	348
calanolide C (**5**)	0.0±0.6 (70)	35.1±1.7 (>80)	74.0±2.1 (>80)	94.3±0.2 (>80)	351
calanone (**6**)	0.0±0.5 (70)	32.3±1.1 (>80)	73.1±1.9 (>80)	93.3±0.3 (>80)	347
compound **7**	0.0±0.3 (60)	19.5±1.1 (>80)	62.5±2.1 (>80)	90.8±0.3 (>80)	268
mammea B/BB (**9**)	0.0±0.3 (60)	13.7±1.5 (>80)	50.6±1.9 (>80)	84.2±0.6 (>80)	170
β-carotene[c]	9.1±0.5 (60)	34.3±1.1 (>80)	82.7±1.8 (>80)	100.0±0.2 (>80)	400

[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 (%) ± SD in the presence of the test compound relative to the positive control (100%). Values in parentheses represent the surviving Raji cells measured by Trypan Blue staining. A minimum 60% survival rate of Raji cells 2 days after treatment with the compounds is required for an accurate result. [b] IC_{50} represents the mol ratio to TPA that inhibits 50% of positive control (100%) activated with 32 pmol of TPA. [c] Positive control substance.

rin C (**4**), and calanone (**6**) (IC_{50} 342−348). Mammea B/BB (**9**), 4-propylcoumarins with a prenyl side chain, exhibited the most potent inhibitory activity (IC_{50} 170). The *trans*-10,11-dimethyldihydropyran-12-ol ring, calanolide A (**1**), was more potent (IC_{50} 290) than the 10,11-*cis* derivative, calanolide C (**5**) (IC_{50} 351). These results fit the patterns of anti-HIV activity observed previously,[1,5] that the functional groups at carbons 10, 11, and 12 and their relatives are critical for their anti-HIV activity. In view of the present findings, 4-propylcoumarins might also be valuable

as potential cancer chemopreventive agents (antitumor-promoter) similarly to 4-phenylcoumarins in our previous studies.[4] A study examining the tumor-promoting inhibitory activity of these compounds in vivo is now in progress.

Experimental Section

^{1}H and ^{13}C NMR, COSY, HMQC, HMBC (J = 8 Hz), and NOE were measured on JNM A-400, A-600, and/or ECP-500 (JEOL) spectrometers. Chemical shifts are shown in δ ppm with tetramethylsilane (TMS) as an internal reference. All mass spectra were taken under EI conditions, unless otherwise stated, using an M-80 (Hitachi), HX-110 (JEOL), and/or JMS-700 (JEOL) spectrometer having a direct inlet system. UV spectra were recorded on a UVIDEC-610C double-beam spectrophotometer (JASCO) in MeOH, and IR spectra on an IR-230 (JASCO) in $CHCl_3$. Preparative TLC was done on silica gel 60 F_{254} (Merck).

Plant Materials. *Calophyllum brasiliense* Camb. was collected in the garden of Federal University of Santa Catarina, Brazil, in March 1998. Plant materials were classified by Dr. Ademir Reis. A voucher specimen has been deposited at Barbosa Rodrigues Herbarium under number VC Filho 007.

Isolation of Brasimarins A (2), B (3), and C (4) from *C. brasiliense*. The dried stem bark (630 g) of *C. brasiliense* was extracted with acetone at room temperature, and the solvent was evaporated under reduced pressure to give the acetone extract. The acetone extract was chromatographed on a silica gel column eluted with hexane−acetone (19:1, 97:3, 9:1, 85:15, 4:1, 3:1, 7:3, 2:1, 3:2, 1:1), acetone, CH_2Cl_2−MeOH (3:1), and MeOH, successively, to separate 13 fractions. Successive treatment of each fraction with silica gel column and preparative TLC using appropriate combinations of solvents (hexane, EtOAc, $CHCl_3$, CH_2Cl_2, Et_2O, acetone, iPr_2O, benzene, and MeOH) as eluting or developing solvents yielded the following compounds. From fraction 4 (hexane−acetone, 85:15): brasimarin A (**2**, 5.2 mg), brasimarin B (**3**, 2.7 mg), mammea B/BB (**9**, 3.2 mg), calanone (**6**, 1.9 mg), calophyllolide (6.3 mg), 5-methoxy-2,2-dimethyl-6-(2-methyl-1-oxo-2-butenyl)-10-propyl-2*H*,8*H*-benzo[1,2-*b*;3,4-*b*′]dipyran-8-one (**7**, 137.8 mg) along with brasixanthone A (9.9 mg), brasixanthone B (44.8 mg), brasixanthone C (8.3 mg), brasixanthone D (2.4 mg), toxyloxanthone A (46.2 mg), latisxanthone C (2.8 mg), 8-desoxygartanin (9.9 mg), pyranojacareubin (10.6 mg), 6-deoxyjacareubin (7.5 mg), and 1,2-dimethoxyxanthone (2.1 mg). From fraction 5 (hexane−acetone, 4:1): brasimarin B (**3**, 2.9 mg), inophyllum C (10.7 mg), inophyllum A (2.1 mg), calanolide A (26 mg), calanolide C (2.4 mg), inophyllum D (1.9 mg) along with brasixanthone E (2.0 mg), brasixanthone F (2.8 mg), 8-desoxygartanin (6.1 mg), and 3,8-dihydroxy-1,2-dimethoxyxanthone (8.8 mg). From fraction 6 (hexane−acetone, 3:1): inophyllum E (2.4 mg), calocoumarin A (**8**, 12.6 mg), along with brasixanthone G (2.8 mg), garcinone B (2.3 mg), cudraxanthone F (28.0 mg), and 4-hydroxyxanthone (3.6 mg).

Brasimarin A (2): pale yellow oil; UV (MeOH) λ_{max} (log ϵ) 203 (4.18), 231sh (3.94), 276 (4.03), 322sh (3.77), 380sh (3.30) nm; IR ($CHCl_3$) ν_{max} 3520br, 1730, 1650 cm^{-1}; differential NOE, irradiation of H-3 (δ 5.89) − 6% enhancement of H-1′ (δ 2.89); EIMS m/z 390 (M^+, 79), 375 ($M^+ - CH_3$, 100), 347 ($M^+ - C_3H_7$, 26), 319 (10), 297 (10), 269 (17), 241 (6); HREIMS m/z 390.1438 (calcd for $C_{24}H_{22}O_5$, 390.1467).

Brasimarin B (3): colorless oil. $[\alpha]_D$ +8.1° (c 0.0765, MeOH). UV (MeOH) λ_{max} (log ϵ) 202 (4.26), 227 (4.22), 236sh (4.20), 299 (4.18), 330sh (3.92) nm; IR ($CHCl_3$) ν_{max} 3563br, 1729 cm^{-1}; EIMS m/z 422 (M^+, 29), 365 ($M^+ - C_4H_9$, 100), 347 (8), 307 (11), 293 (23); HREIMS m/z 422.1714 (calcd for $C_{25}H_{26}O_6$, 422.1729).

Brasimarin C (4): colorless oil; $[\alpha]_D$ +8.9° (c 0.135, MeOH); UV (MeOH) λ_{max} (log ϵ) 228 (4.38), 286 (4.15), 324 (4.13) nm; IR ($CHCl_3$) ν_{max} 3487, 1731, 1693 cm^{-1}; EIMS m/z 404 (M^+, 91), 387 (84), 361 (23), 347 (41), 331 (100), 320 (40), 305 (77), 293 (42), 277 (22); HREIMS m/z 404.1582 (calcd for $C_{25}H_{24}O_5$, 404.1623).

In Vitro EBV-EA Activation Experiments. The inhibition of EBV-EA activation was assayed using the same method described previously.[3,4] In brief, Raji cells were grown to a density of 10^6 cells/mL, harvested by centrifugation, and exposed to the RPMI 1640 medium (Sigma) with 10% FCS containing 4 mM *n*-butyric acid as inducer, 32 pmol of TPA (20 ng/mL in DMSO), and 32, 16, 3.2, or 0.32 nmol of the test compound (DMSO solutions). After cultivation at 37 °C for 48 h, cell number and viability were counted 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 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

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