New Carbazole Alkaloids from *Clausena anisata* with Antitumor Promoting Activity

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Four new carbazole alkaloids, named clausamine D (**1**), E (**2**), F (**3**), and G (**4**), were isolated from *Clausena anisata* as inhibitors of Epstein−Barr virus early antigen activation induced by 12-*O*-tetradecanoylphorbol-13-acetate in Raji cells.

Systematic studies of the chemical constituents of the plant genus *Clausena* have been conducted by our group,[1−3] and in our previous report we showed that *Clausena anisata* (Willd.) Oliv. (Rutaceae) collected in Thailand contained three novel lactonic carbazole alkaloids named clausamines A (**13**), B (**9**), and C (**11**).[3] This paper describes the results of a further examination of the constituents of *C. anisata*.

The acetone extract of the dried branches of the plant was subjected successively to Si gel column and preparative thin-layer chromatographies (TLC) to give new carbazoles named clausamines D (**1**), E (**2**), F (**3**), and G (**4**), along with some known carbazoles. All new carbazole alkaloids isolated on this occasion belong to the class of 1-oxygenated 3-carbomethoxy carbazoles having a prenyl or analogous moiety at C-4.

In a preliminary screening test for antitumor-promoting agents, we found that some coumarins[4] and phenylpropanoids[5] isolated from Rutaceous plants showed potent inhibitory effects on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced Epstein−Barr virus early antigen (EBV-EA) activation in Raji cells. The activity of carbazole alkaloids, one of the major constituents of Rutaceous plants,[6] is also of interest. This paper describes the isolation and structural characterization of four new carbazoles, clausamines D (**1**), E (**2**), F (**3**), and G (**4**), and the results of assays examining the inhibitory effects on TPA-induced EBV-EA activation of nine carbazole alkaloids isolated from *Clausena anisata*.

Clausamine D (**1**) was obtained as a colorless powder. The molecular formula was determined as $C_{20}H_{21}NO_3$ by HR MS. The UV spectrum was similar to that of 1-oxygenated 3-carbomethoxy carbazole, clausine F (**5**).[2,7] The IR spectrum showed an absorption band due to an NH group at ν_{max} 3467 cm^{-1}. In the ^{1}H NMR spectrum, a set of four-spin proton signals at δ_H 8.14 (1H, d, J = 8.1 Hz, H-5), 7.27 (1H, t, J = 8.1 Hz, H-6), 7.44 (1H, t, J = 8.1 Hz, H-7), and 7.49 (1H, d, J = 8.1 Hz, H-8); a lone singlet at δ_H 7.46 (1H, s, H-2) in the aromatic proton region; and two OMe signals [δ 4.03 and 3.93] were observed. These spectral data, coupled with biogenetic considerations, suggested the presence of a 1-oxygenated 3-substituted carbazole skeleton having no substituent on the A ring.[6,8,9] An IR band at ν_{max} 1709 cm^{-1} and two significant mass fragments at *m*/*z* 292 [$M^+ - \cdot OCH_3$] and 264 [$M^+ - \cdot COOCH_3$] in the EIMS suggested the presence of a carbomethoxy group on the carbazole nucleus. Further, the presence of a prenyl moiety in the molecule was indicated by ^{1}H NMR signals at δ 4.30 (2H, d, J = 4.8 Hz), 5.29 (m), 1.91 (3H, s), and 1.70 (3H, s) and an observation of a fragment peak at *m*/*z* 268 [$M^+ - \cdot CH{=}C(CH_3)_2$] in the EIMS. In NOE experiments, a 1H singlet at δ 7.46 (H-2) showed 21% NOE enhancement on irradiation of the methoxy signal at δ 4.03 (C-1), and the methoxy signal at δ 4.03 showed 7% enhancement on irradiation of the 1H singlet at δ 7.46. Irradiation of the signal at δ 4.30 (H-1′) on the prenyl moiety gave 22% increases of the signal at δ 8.14 (H-5). On the other hand, no NOE enhancement was observed at any proton signal on irradiation of another methoxy signal at δ 3.93. Based on these results, we proposed the structure **1** to be clausamine D. *O*-Methylation of clausine F (**5**), which had previously been isolated by us from the same plant, with methyl iodide in acetone in the presence of anhydrous potassium carbonate, gave a colorless powder. It was found to be identical with natural **1** by IR and ^{1}H NMR spectroscopic comparisons.

Clausamine E (**2**) was isolated as a colorless oil, $C_{20}H_{21}NO_4$. The UV spectrum (see Experimental Section) was similar to that of **1**. The ^{1}H NMR spectrum also showed a signal pattern similar to that of **1**, except for the lack of signals due to a prenyl side chain [$-CH_2CH{=}C(CH_3)_2$]. AB-type signals at δ 7.37 and 6.00 (each 1H, d), having a large coupling constant (J = 16.1 Hz) assignable to protons due to an (*E*)-disubstituted double bond, and a 6H singlet at δ 1.55 due to two methyls attached to a carbon atom bearing an oxygen atom were observed. These data, coupled with two significant mass fragment ion peaks at *m*/*z* 321 (4%) and 280 (100%) ascribed to ions [$M^+ - H_2O$] and [$M^+ - \cdot C(CH_3)_2OH$], respectively, showed the presence of the side chain [(*E*)−CH=CH−$C(CH_3)_2$OH]. On the basis of these results, the structure of clausamine B was proposed for **2**.

Clausamine G (**4**) was obtained as a pale yellow oil. The molecular formula $C_{20}H_{21}NO_5$, a difference of a single oxygen compared with **2**, was established by HRFABMS. The UV and ^{1}H NMR spectra showed good similarity with those of **2**. Observation of a broad singlet at δ 9.56, which

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Table 1. ^{1}H NMR Spectral Data for the New Carbazole Alkaloids in $CDCl_3$[a]

	1	2	3	4	6
1-OCH_3	4.03 (3H, s)	4.05 (3H, s)		4.06 (3H, s)	4.04 (3H, s)
2	7.46 (s)	7.44 (s)	7.44 (s)	7.54 (s)	7.48 (s)
3-$COOCH_3$	3.93 (3H, s)	3.90 (3H, s)	3.87 (3H, s)	3.92 (3H, s)	3.97 (3H, s)
5	8.14 (d, 8.1)	8.34 (d, 8.1)	8.33 (d, 8.1)	8.28 (d, 8.1)	8.06 (d, 8.1)
6	7.27 (t, 8.1)	7.20 (t, 8.1)	7.20 (t, 8.1)	7.21 (t, 8.1)	7.27 (t, 8.1)
7	7.44 (t. 8.1)	7.42 (t, 8.1)	7.43 (t, 8.1)	7.44 (t, 8.1)	7.46 (t, 8.1)
8	7.49 (d, 8.1)	7.48 (d, 8.1)	7.48 (d, 8.1)	7.49 (d, 8.1)	7.51 (d, 8.1)
NH	8.51 (br s)	8.49 (br s)	8.53 (br s)	8.54 (br s)	8.54 (br s)
1′	4.30 (2H, d, 4.8)	7.37 (d, 16.1)	7.35 (d, 16.1)	7.47 (d, 16.5)	4.25 (dd, 10.0, 14.4) 3.83 (dd, 3.2, 14.4)
2′	5.29 (m)	6.00 (d, 16.1)	5.99 (d, 16.1)	5.92 (d, 16.5)	4.75 (dd, 10.0, 3.2)
3′-CH_3	1.91 (3H, s) 1.70 (3H, s)	1.55 (6H, s)	1.55 (6H, s)	1.60 (6H, s)	1.98 (3H, s)
other			5.83 (br s, OH) 4.56 (br s, OH)	9.56 (br s, OOH)	9.70 (br s, OOH) 5.21 (2H, s, 3′-CH_2)

[a] Values in parts per million (ppm). The coupling constants (J) in parentheses are in Hertz. All signals correspond to 1H and were observed as singlets, unless otherwise stated.

disappeared on addition of D_2O, together with a significant mass fragment ion base peak at m/z 281, corresponding to $[M^+ - \cdot C(CH_3)_2OOH + \cdot H]$ in the EIMS, suggested the presence of a side chain [−CH═CH−$C(CH_3)_2$OOH] on the carbazole nucleus.

For confirmation of the structures of clausamine E and G, the following chemical reactions were carried out: (a) The hematoporphyrin-sensitized photooxygenation[10] of clausamine D (**1**) in oxygen gas gave two isomeric peroxygenated products. One of them was found to be identical with natural **4** by IR, UV, and ^{1}H NMR spectroscopic comparisons. The structure of the other reaction product was assigned as formula **6** on the basis of spectroscopic analyses (see Experimental Section). (b) Treatment of clausamine G (**4**) with triphenylphosphine gave a colorless oil that was found to be identical with clausamine E (**2**). Consequently, the structures of clausamine E and G were established as formulas **2** and **4**, respectively. Clausamine G (**4**), containing a hydroperoxy moiety in the molecule, is the first example of the isolation of a peroxygenated carbazole alkaloid in nature.

Clausamine F (**3**) was isolated as a pale yellow oil having the molecular formula $C_{19}H_{19}NO_4$, a difference of CH_2 compared with **2**. The UV and IR spectra (see Experimental Section) indicated the characteristic absorptions of the 1-oxygenated 3-carbomethoxy carbazole skeleton.[2,6] The ^{1}H NMR spectrum of **3** also showed a signal pattern similar to that of **2**, except for the appearance of an additional broad D_2O exchangeable signal assignable to the hydroxyl group, instead of the singlet of the methoxy group due to 1-OCH_3 in the ^{1}H NMR spectrum of **2**. These spectral data led us to assign the structure **3** to clausamine F. The ^{13}C NMR data of the new carbazole alkaloids (**1**−**3**) are shown in Table 2.

Nine carbazole alkaloids (**1**, **5**, and **7**−**13**) isolated from *C. anisata* were tested for antitumor-promoting activity in a short-term in vitro assay of TPA-induced EBV-EA activation in Raji cells (Table 2). All of the test compounds inhibited EBV activation even at 1×10^2 mol ratio and showed significant inhibitory effect at high concentration (1×10^3 mol ratio). On the other hand, all compounds showed only weak cytotoxicity on Raji cells even at 1×10^3 mol ratio. Five of the carbazole alkaloids (**1**, **5**, **7**, **11**, **13**) showed weak inhibitory activity even at 1×10 mol ratio/TPA (4.5−9.7%). Among these compounds, ekeberginine (**7**) was found to be more effective than the others on EBV-EA inhibition (89.5%, 77.6%, 36.5%, and 9.7% inhibition of activation at 1×10^3, 5×10^2, 1×10^2, 10 mol ratio/TPA, respectively). On the other hand, the compounds **8**, **10**, and **12**, lacking the prenyl side chain, were less activities (no activity at 1×10 mol ratio/TPA) than **7** having a prenyl group at C-4. In the structure−activity relationship analysis of antitumor carbazole alkaloids, it seems that a prenyl group at C-4 plays an important role for inhibitory activity. These results suggest that ekeberginine (**7**) might be valuable as antitumor promoters in chemical carcinogenesis. Investigation into the inhibitory mechanisms of these compounds on the tumor-promoting stage is now in progress.

Table 2. ^{13}C NMR Spectral Data for the New Carbazole Alkaloids in $CDCl_3$[a]

	1	2	3
1	142.94 (s)	143.71 (s)	149.48 (s)
1-OCH_3	55.69 (q)	55.76 (q)	
2	107.97 (d)	107.50 (d)	112.22 (d)
3	123.14 (s)	121.91 (s)	122.80 (s)
3-$COOCH_3$	168.94 (s)	168.86 (s)	168.67 (s)
	51.91 (q)	51.82 (q)	51.83 (q)
4	133.67 (s)	130.35 (s)	130.42 (s)
4a	120.70 (s)	120.93 (s)	120.80 (s)
4b	123.92 (s)	124.14 (s)	124.13 (s)
5	123.37 (d)	123.27 (d)	123.29 (d)
6	120.15 (d)	119.75 (d)	119.77 (d)
7	126.64 (d)	125.95 (d)	126.13 (d)
8	111.01 (d)	111.09 (d)	111.12 (d)
8a	139.46 (s)	139.56 (s)	139.80 (s)
9a	132.26 (s)	132.24 (s)	132.76 (s)
1′	29.38 (t)	124.62 (d)	124.56 (d)
2′	122.91 (d)	141.14 (d)	141.16 (d)
3′	132.37 (s)	71.20 (s)	71.24 (s)
3′-CH_3	18.43 (q) 25.68 (q)	29.62 (q × 2)	29.61 (q × 2)

[a] Values in (δ_H and δ_C) ppm.

Experimental Section

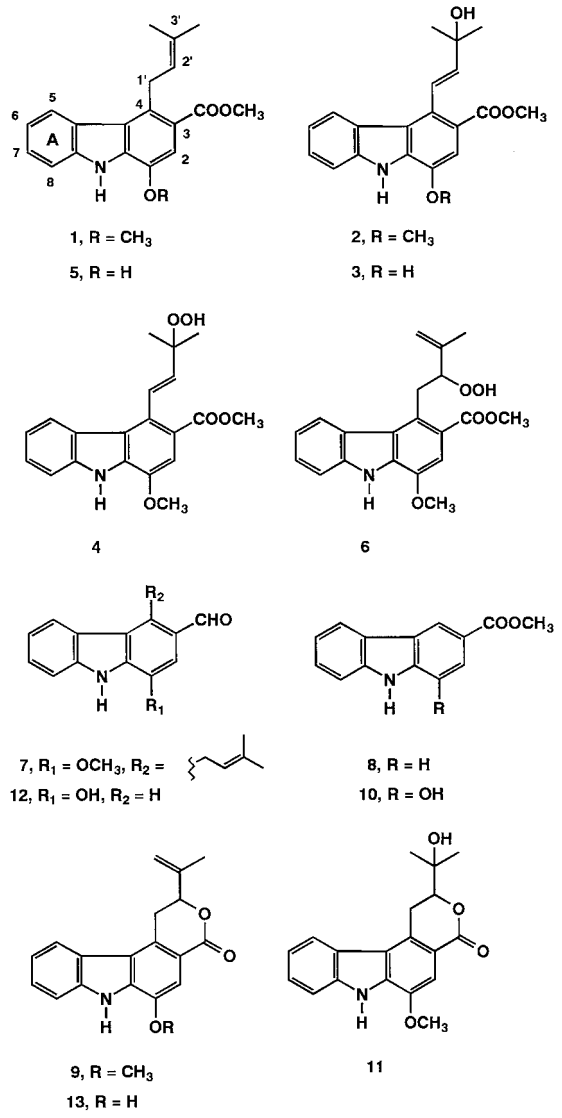
General Experimental Procedures. ^{1}H NMR and NOE spectra were recorded on an A-400 or A-600 (JEOL) spectrometer, in $CDCl_3$. Chemical shifts are shown in δ values (ppm) with tetramethylsilane (TMS) as an internal reference. All mass spectra were taken under electron impact (EI) conditions, unless otherwise stated, using an M-80 (Hitachi) spectrometer having a direct inlet system. UV spectra were recorded on a V-550 UV/VIS spectrophotometer (JASCO) in MeOH, IR spectra on an IR-230 (JASCO) in $CHCl_3$. Preparative TLC was done on Kieselgel 60 F_{254} (Merck).

Plant Materials. The plant materials used in this study, *Clausena anisata* (Willd.) Oliv., were collected during January−February 1996, in Kanchanaburi Province, Thailand. Authentication was achieved by comparison with the herbarium specimen at the Royal Forest Department, Ministry

Table 3. Inhibitory Effects of Carbazole Alkaloids on TPA-induced EBV-EA Activation[a]

compound	EBV-EA positive cells (% viability)			
	compound concentration (mol ratio/32 pmol TPA)			
	1000	500	100	10
clausamine D (**1**)	18.2 ± 0.7 (60)	34.2 ± 1.2 (70)	66.0 ± 2.0 (>80)	92.3 ± 0.7 (>80)
clausine F (**5**)	20.2 ± 0.7 (60)	37.5 ± 1.5 (>80)	69.8 ± 1.1 (>80)	94.6 ± 0.5 (>80)
ekeberginine (**7**)	10.5 ± 0.4 (60)	22.4 ± 0.6 (>80)	63.5 ± 1.4 (>80)	90.3 ± 1.0 (>80)
methyl carbazole-3-carboxylate (**8**)	21.7 ± 0.4 (60)	46.3 ± 1.5 (>80)	81.4 ± 2.0 (>80)	100.0 ± 0.2 (>80)
clausamine B (**9**)	18.3 ± 0.3 (60)	39.2 ± 2.0 (70)	78.2 ± 1.5 (>80)	100.0 ± 0.5 (>80)
clausine E (**10**)	27.4 ± 0.6 (60)	42.4 ± 1.1 (>80)	74.6 ± 1.4 (>80)	100.0 ± 0.9 (>80)
clausamine C (**11**)	12.4 ± 0.7 (60)	27.2 ± 1.3 (70)	72.0 ± 1.4 (>80)	92.3 ± 0.9 (>80)
O-demethylmurrayanine (**12**)	25.2 ± 0.5 (60)	40.3 ± 1.4 (>80)	69.4 ± 1.6 (>80)	100.0 ± 0.4 (>80)
clausamine A (**13**)	15.4 ± 0.5 (60)	28.3 ± 1.5 (70)	73.7 ± 1.6 (>80)	95.5 ± 1.0 (>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 compounds is required for accurate result.



of Agriculture and Cooperative, Bangkok, Thailand. A voucher specimen has been deposited in the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Extraction and Isolation. The dried branches (120 g) of *C. anisata* were extracted with acetone (800 mL × 3) at room temperature. The acetone extract (1.02 g) was subjected to Si gel column chromatography eluted with hexane, hexane−acetone (9:1, 4:1, 3:1, 3:2, 1:1), acetone, $CHCl_3$−MeOH (3:1), and MeOH, successively, to give 9 fractions. The hexane−acetone (4:1) eluate was subjected to preparative Si gel TLC developed with hexane−$CHCl_3$ (3:7) to afford clausamine D (**1**) (4.0 mg), ekeberginine (**7**)[11] (0.3 mg), methyl carbazole 3-carboxylate (**8**)[12] (0.5 mg), and clausamine G (**4**) (1.9 mg). The hexane−acetone (3:1) eluate was subjected to preparative TLC developed with $CHCl_3$ to afford clausamine B (**9**)[3] (1.1 mg) and clausine F (**5**)[7] (27.6 mg). The hexane−acetone (3:2) eluate was subjected to preparative TLC developed with CH_2Cl_2−acetone (9:1) to afford six fractions 1−6. Fraction 4 was also subjected to preparative TLC developed with $CHCl_3$−MeOH (99:1) to give clausine E (**10**)[13] (3.7 mg). Fraction 5 was subjected to preparative TLC developed with $CHCl_3$−MeOH (49:1) to give clausamine E (**2**) (1.3 mg), clausamine C (**11**)[3] (1.0 mg), and *O*-demethylmurrayanine (**12**)[14] (1.6 mg). Fraction 6 was subjected to preparative TLC developed with iso-Pr_2O−MeOH (19:1) to afford clausamine A (**13**)[3] (1.0 mg) and clausamine F (**3**) (1.0 mg).

Clausamine D (1): colorless powder; UV λ_{max} nm 222, 240, 248, 256, 269, 310, 321, 335; IR ν_{max} cm^{-1} 3467, 1709, 1610, 1587; EIMS *m/z* (%) 323 (M^+, 100), 292 (M^+-OCH_3, 19), 291 (48), 280 (64), 276 (28), 268 (16), 264 ($M^+-COOCH_3$, 17), 252 (15), 248 (15), 234 (15), 204 (18). HRMS calcd for $C_{20}H_{21}NO_3$, 323.1520; found: 323.1525.

Clausamine E (2): colorless oil; UV λ_{max} nm: 224, 239, 273, 322, 337; IR ν_{max} cm^{-1} 3465, 3300 (br), 1701, 1608, 1583, 1508; EIMS *m/z* (%) 339 (M^+, 18), 321 (4), 280 (100), 262 (12), 250 (20), 241 (26), 210 (12); NOE irradiation of 1-OCH_3 (δ 4.05) gave 14% NOE at H-2 (δ 7.44); irradiation of H-5 (δ 8.34) gave 5% NOE at H-1′ (δ 7.37) and 4% NOE at H-2′ (δ 6.00); irradiation of 3-$COOCH_3$ (δ 3.90) gave no NOE enhancement at any proton signal; HRMS calcd for $C_{20}H_{21}NO_4$, 339.1470; found 339.1473.

Clausamine F (3): pale yellow oil; UV λ_{max} nm 223, 242, 272, 324, 341; IR ν_{max} cm^{-1} 3456, 3300 (br), 1699, 1558, 1508; EIMS *m/z* (%) 325 (M^+, 8), 307 (13), 280 (16), 266 (100), 262 (11), 248 (76), 241 (16), 236 (38), 220 (15), 218 (11), 210 (22), 204 (19); NOE irradiation of H-5 (δ 8.33) gave 4% NOE at H-1′ (δ 7.35) and 4% NOE at H-2′ (δ 5.99); irradiation of 3-$COOCH_3$ (δ 3.87) gave no NOE enhancement at any proton signal; HRMS calcd for $C_{19}H_{19}NO_4$, 325.1313; found 325.1315.

Clausamine G (4): pale yellow oil; UV λ_{max} nm 223, 236, 270, 313, 321, 333; IR ν_{max} cm^{-1} 3465, 3300 (br), 1699, 1608, 1508; EIMS *m/z* (%) 340 (M^+-CH_3, 14), 324 (9), 298 (6), 281 (100), 269 (13), 266 (16), 253 (19), 251 (24), 237 (15), 234 (10), 224 (9), 206 (11); FABMS *m/z* 356 $[M + H]^+$; NOE irradiation of 1-OCH_3 (δ 4.06) gave 5% NOE at H-2 (δ 7.54); irradiation of H-5 (δ 8.28) gave 4% NOE at H-1′ (δ 7.47) and 3% NOE at H-2′ (δ 5.92); irradiation of 3-$COOCH_3$ (δ 3.92) gave no NOE enhancement at any proton signal; HRFABMS calcd for $C_{20}H_{22}NO_5$, 356.1498; found 356.1479.

***O*-Methylation of Clausine F (5).** A mixture of clausine F (**5**) (20 mg), anhydrous K_2CO_3 (30 mg), and methyl iodide (15 mg) in acetone (3.0 mL) containing one drop of H_2O was refluxed for 3 h. K_2CO_3 was filtered off, and the filtrate was concentrated to dryness. The residue was dissolved in water. Diluted HCl was added, and the solution was extracted with

CH_2Cl_2. The extract was dried over anhydrous $MgSO_4$ and concentrated to dryness. The residue was subjected to preparative TLC with hexane−acetone (7:3) to give a colorless powder (7.2 mg, 48% yield), which was found to be identical with natural clausamine D (**1**) by spectroscopic comparisons (IR and ^{1}H NMR).

Photooxygenation of Clausamine D (1). Oxygen gas was bubbled through a solution of clausamine D (**1**) (6.2 mg) in pyridine (6 mL) containing hematoporphyrin (2 mg), and the solution was irradiated with a high-pressure Hg lamp using a Pyrex glass filter for 1 h. Then, the solvent was evaporated off. The residue was subjected to preparative TLC with $CHCl_3$−MeOH (49:1) to afford 0.3 mg and 0.7 mg of hydroperoxide **4** and **6**, respectively. Compound **4** was found to be identical with natural clausamine G (**4**) by spectroscopic comparisons (UV, IR, and ^{1}H NMR).

Compound 6: pale yellow oil; UV λ_{max} nm 223, 239, 270, 312, 322, 335; IR ν_{max} cm^{-1} 3465, 3307 (br), 1699, 1610; FABMS *m*/*z* (%) 356 [M + H]$^+$.

Treatment of Clausamine G (4) with Triphenylphosphine. A methanolic solution (0.7 mL) of **4** (0.3 mg) and Ph_3P (0.3 mg) was stirred for 4 h at room temperature. The solvent was evaporated off in vacuo. The residue was subjected to preparative TLC with hexane−acetone (7:3) to afford a colorless oil (**2**) (0.27 mg, 99% yield) that was found to be identical to clausamine E (**2**) by comparisons of the IR and ^{1}H NMR.

***In Vitro* EBV-EA Activation Experiments.** The inhibition of EBV-EA activation was assayed using the same method described previously.[4,5]

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