Invasion Inhibitors of Human Fibrosarcoma HT 1080 Cells from the Rhizomes of *Zingiber cassumunar*: Structures of Phenylbutanoids, Cassumunols

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The methanolic extract and its EtOAc-soluble fraction from the rhizomes of *Zingiber cassumunar* inhibited invasion of human fibrosarcoma HT 1080 cells. From the EtOAc-soluble fraction, eight new phenylbutanoids, cassumunols A—H, were isolated together with 30 known constituents. The structures of new phenylbutanoids were elucidated on the basis of chemical and physicochemical evidence. Principal constituents were examined the inhibitory effects on the invasion of HT 1080 cells. Among them, phlain I and III, (*E*)-1-(3,4-dimethoxyphenyl)buta-1,3-diene, (*E*)-1-(2,4,5-trimethoxyphenyl)buta-1,3-diene, and (−)-β-sesquiphellandrene showed anti-invasion effects. Interestingly, (*E*)-1-(2,4,5-trimethoxyphenyl)buta-1,3-diene [inhibition (%) 46.8±7.2 ($p<0.05$) at 30 μM] significantly inhibited the invasion, and only a weak cytotoxic effect was observed.

Key words *Zingiber cassumunar*; cassumunol; invasion inhibitor; human fibrosarcoma HT 1080; phlain; Zingiberaceae

In the course of our studies on bioactive constituents from Zingiberaceae plant,[1—12] we previously reported that the methanolic extract from the rhizomes of *Zingiber* (*Z.*) *cassumunar* showed inhibitory effects on lipopolysaccharide (LPS)-induced nitric oxide (NO) production in mouse peritoneal macrophages.[12] From the extract, then new conjugated phenylbutanoids named phlains I—VI were isolated and their structures as well as the inhibitory activities on NO production were characterized. As a continuing study, the methanolic extract and its EtOAc-soluble fraction from the rhizomes of *Z. cassumunar* were found to inhibit invasion of human fibrosarcoma HT 1080 cells through Matrigel-coated filters *in vitro*. Eight new phenylbutanoids designated cassumunols A (**1**), B (**2**), C (**3**), D (**4**), E (**5**), F (**6**), G (**7**), and H (**8**) were isolated from the EtOAc-soluble fraction together with 30 known constituetns. This paper deals with the structure elucidation of new phenylbutanoids (**1**—**8**) and the inhibitory effects of principle constituents on invasion of HT 1080 cells (Chart 1).

The methanolic extract from the rhizomes of *Z. cassumunar* cultivated in Thailand was partitioned into an EtOAc–H_2O (1 : 1, v/v) mixture to give an EtOAc-soluble fraction and an H_2O-soluble fraction. The methanolic extract and EtOAc-soluble fraction were found to show potent inhibitory effects on the invasion of HT 1080 cells through Matrigel-coated filters (Table 1), whereas the H_2O-soluble fraction showed no activity. The EtOAc-soluble fraction was subjected to normal phase and reversed-phase column chro-

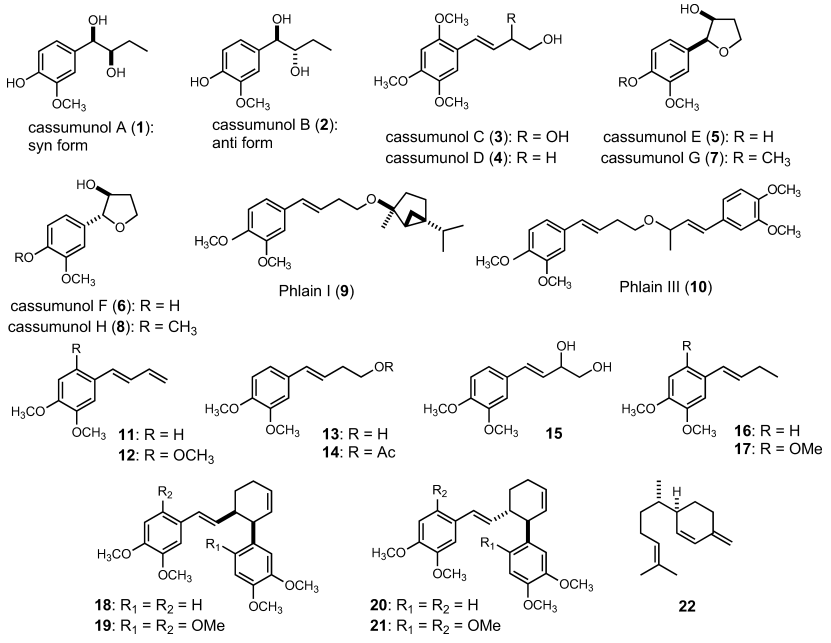

Chart 1. Structures of Constituents from the Rhizomes of *Zingiber cassumunar*

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Table 1. Inhibitory Effects of MeOH Extract and EtOAc- and H_2O-Soluble Fractions from *Z. cassumunar* on Invasion of HT1080 Cells

Concentration (μg/ml)	Inhibition			
	0	10	30	100
MeOH extract	0.0±7.1	12.6±6.1	23.4±2.4*	51.6±1.7**
EtOAc-soluble fraction	0.0±4.2	24.6±2.6**	49.9±1.3**	83.1±2.0**
H_2O-soluble fraction	0.0±13.1	−16.1±5.6	−23.1±11.7	−0.5±7.3

Each value represents the mean±S.E.M. (n=4). Significantly different from the control, * $p<0.05$, ** $p<0.01$.

matographies, and finally HPLC to give cassumunols A (**1**, 0.0093% from the dried rhizomes), B (**2**, 0.0026%), C (**3**, 0.0016%), D (**4**, 0.013%), E (**5**, 0.0044%), F (**6**, 0.017%), G (**7**, 0.0021%), and H (**8**, 0.024%) together with phlasins I (**9**),[12] II,[12] III (**10**),[12] IV,[12] V,[12] and VI,[12] (*E*)-1-(3,4-dimethoxyphenyl)buta-1,3-diene (**11**),[12,13] (*E*)-1-(2,4,5-trimethoxyphenyl)buta-1,3-diene (**12**),[12,13] (*Z*)-1-(2,4,5-trimethoxyphenyl)buta-1,3-diene (0.0057%),[14] (*E*)-4-(3,4-dimethoxyphenyl)but-3-en-1-ol (**13**),[12,15] (*E*)-4-(3,4-dimethoxyphenyl)but-3-enyl acetate (**14**),[12,16] (*E*)-4-(3,4-dimethoxyphenyl)but-3-ene-1,2-diol (**15**, 0.021%),[16] (*E*)-1-(3,4-dimethoxyphenyl)but-1-ene (**16**),[12,17] (*E*)-1-(2,4,5-trimethoxyphenyl)but-1-ene (**17**),[12,17] (±)-*cis*-3-(3,4-dimethoxyphenyl)-4-[(*E*)-3,4-dimethoxystyryl]cyclohex-1-ene (**18**),[12,17] (±)-*cis*-3-(2,4,5-trimethoxyphenyl)-4-[(*E*)-2,4,5-trimethoxystyryl]cyclohex-1-ene (**19**),[12,17] (±)-*trans*-3-(3,4-dimethoxyphenyl)-4-[(*E*)-3,4-dimethoxystyryl]cyclohex-1-ene (**20**, 0.013%),[17] (±)-*trans*-3-(2,4,5-trimethoxyphenyl)-4-[(*E*)-2,4,5-trimethoxystyryl]cyclohex-1-ene (**21**, 0.067%),[17] (*E*)-3-(3,4-dimethoxyphenyl)propenal (0.0055%),[18] (*E*)-3-(2.4,5-trimethoxyphenyl)propenal (0.0012%),[19] cassumunaquinones 1[12,20] and 2,[12,20] curcumin,[12,21,22] cassumunarins A (0.0080%)[23] and C (0.0043%),[23] 3,4-dimethoxybenzaldehyde (0.027%),[22] 2,4,5-trimethoxybenzaldehyde (0.010%),[24] vanillic acid (0.071%),[22,25] (−)-β-sesquiphellandrene (**22**),[12,26] and *b*-sitosterol (0.073%).[22,27]

Structures of Cassumunols (1—8) Cassumunols A (**1**) and B (**2**) were isolated as a colorless oil with positive optical rotation (**1**: $[\alpha]_D^{26}$ +12.8; **2**: $[\alpha]_D^{27}$ +57.1 in $CHCl_3$), respectively. The IR spectra of **1** and **2** showed absorption bands assignable to hydroxyl functions and an aromatic ring (**1**, **2**: 3470, 1509 cm^{-1}), whereas their UV spectra indicated an absorption maximum to a phenyl function (**1**, **2**: 280 nm). In the electron ionization-mass spectra (EI-MS) spectra of **1** and **2**, the common molecular ion peak was observed at *m/z* 212 (M^+) and the molecular formula $C_{11}H_{16}O_4$ was determined by high-resolution (HR) MS measurement. The proton and carbon signals in the ^{1}H- and ^{13}C-NMR spectra of **1** and **2** were very similar to each other, except for the signals due to the methine protons with an oxygen function. Thus, the ^{1}H-NMR ($CDCl_3$) and ^{13}C-NMR (Table 2) spectra of **1** and **2**, which were assigned by various NMR experiments,[28] showed signals due to a methyl [**1**: δ 0.94 (3H, t, J=7.3 Hz, H_3-4); **2**: δ 0.97 (3H, t, J=7.3 Hz, H_3-4)], a methoxyl [**1**: δ 3.90 (3H, s); **2**: δ 3.91 (3H, s)], two methines bearing an oxygen function [**1**: δ 3.58 (1H, m, H-2), 4.38 (1H, d, J=6.7 Hz, H-1); **2**: δ 3.72 (1H, m, H-2), 4.59 (1H, d, J=6.9 Hz, H-1)], and a trisubstituted benzene ring [**1**: δ 6.81 (1H, dd, J=1.2, 8.0 Hz, H-6′), 6.88 (1H, d, J=8.0 Hz, H-5′), 6.89 (1H, d, J=1.2 Hz, H-2′); **2**: δ 6.83 (1H, dd like, J=1.0, 8.0 Hz, H-6′), 6.90 (1H, d, J=8.0 Hz, H-5′), 6.94 (1H, d like, J=1.0 Hz, H-2′)]. As shown in Fig. 1, the double quantum filter correlation spectroscopy (DQF-COSY) experiment on **1** and **2** indicated the presence of partial structures written in bold lines, and in the heteronuclear multiple bond connectivity spectroscopy (HMBC) experiment, long-range correlations were observed between the following protons and carbons: H-1 and C-2, 3, 2′, 6′; H-2 and C-1, 4; H-3 and C-1, 2, 4; H-4 and C-2, 3; H-2′ and C-1; H-5′ and C-1′, 3′, 4′; H-6′ and C-1; OCH_3 and C-3′. The positions of the methoxyl group in **1** and **2** were confirmed by difference nuclear Overhauser effect (dif.-NOE), which showed a NOE correlation between the methoxyl proton and the 2′-proton. On the basis of this evidence, the common plane structures of **1** and **2** were characterized. The relative stereostructures of 1- and 2-positions in **1** were clarified by dif.-NOE experiment on the 1,2-acetonide derivative (**1a**). Namely, **1** was treated with 2,2-dimethoxypropane in the presence of a trace of Amberlyst 15 ion-exchange resin to give the 1,2-acetonide (**1a**). The dif.-NOE experiment on **1a** exhibited NOE correlations between the 1,2-protons and acetonide methyls as shown in Fig. 1. Those finding led to us to formulate the relative stereostructures at the 1- and 2-positions in **1**, so that the relative stereostructure of **2**, which was the stereoisomer at the 1- and 2-positions in **1**, was also elucidated as shown.

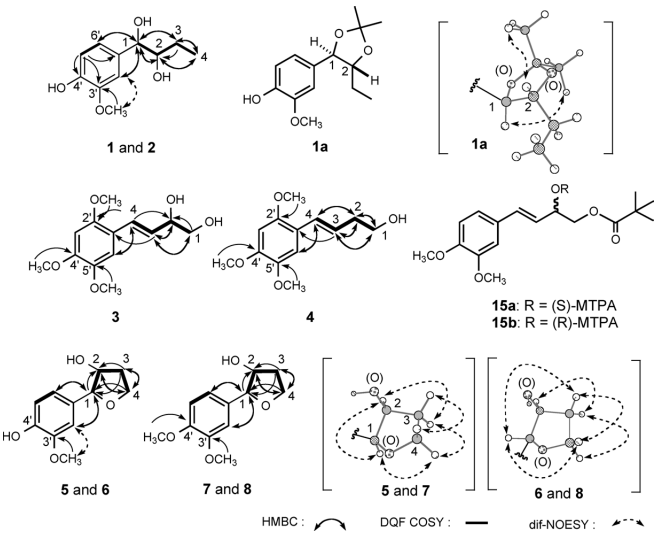

Fig. 1. Selected HMBC and NOE Correlations

Cassumunol C (**3**) was obtained as a pale yellow oil with very low optical activity. The IR spectrum of **3** indicated the presence of hydroxyl functions and an aromatic ring, whereas the UV spectrum showed an absorption maximum suggestive of styryl group. The ^{1}H-NMR ($CDCl_3$) and ^{13}C-

Table 2. ^{13}C-NMR (150 MHz) Data for **1**—**8** ($CDCl_3$)

Position	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
1	77.6	76.8	66.6	62.2	85.0	87.3	84.9	87.3
2	77.5	76.6	73.8	36.8	73.6	78.7	73.7	78.8
3	25.7	24.9	125.9	124.6	34.7	34.2	34.7	34.3
4	10.0	10.2	126.9	127.0	66.9	67.0	66.9	67.0
2-OMe								
1′	133.2	132.5	116.9	118.1	128.6	132.6	129.2	133.2
2′	109.1	109.3	151.6	151.0	109.3	108.1	109.9	108.7
3′	146.7	146.6	97.5	97.8	146.8	146.6	149.2	149.1
4′	145.5	145.4	149.9	149.2	145.3	145.1	148.7	148.5
5′	114.2	114.2	143.3	143.3	114.5	114.3	111.3	111.1
6′	120.0	120.0	109.9	109.7	119.5	118.5	118.8	117.8
2′-OMe			56.6[a)]	56.6[b)]				
3′-OMe	56.0	56.0			56.0	56.0	55.9	55.9
4′-OMe			56.1[a)]	56.1[b)]			55.9	55.9
5′-OMe			56.5[a)]	56.5[b)]				

a, *b*) Interchangeable.

NMR (Table 2) spectra[28)] of **3** showed signals due to three methoxyl groups [δ 3.83, 3.86, 3.90 (all s, CH_3O-2′, 5′, 4′, interchangeable)], a methylene [δ 3.62 (dd, J=7.3, 11.0 Hz), 3.76 (dd, J=3.7, 11.0 Hz), H_2-1] and a methine [δ 4.43 (m, H-2)] with an oxygen function, and a tetrasubstituted benzene ring [δ 6.50, 6.97 (both s, H-3′, 6′)]. On the basis of the DQF-COSY and HMBC experiments (Fig. 1), the plane structure of cassumunol D (**3**) was determined to be as shown in Fig. 1. On the other hand, the 2-(−) and (+)-MTPA esters (**15a**, **15b**), which were derived from **15** with very low optical rotation as well as **3** *via* the 1-pivaloyl derivatives, were found to be a diastereomeric mixture (*ca.* 1 : 1) on the basis of their ^{1}H- and ^{13}C-NMR spectra, respectively. From this result, cassumunol C (**3**), which have the same structure as **15** except for the methoxyl group at the 2′ position, was indicated to be a racemic mixture.

Cassumunol D (**4**), obtained as a pale yellow oil, showed absorption bands at 3520 and 1516 cm^{-1} due to hydroxyl functions and an aromatic ring in the IR spectrum. The molecular formula $C_{13}H_{18}O_4$ of **4** was determined by HR-MS measurement of the molecular ion peak at *m/z* 238 (M^+). The ^{1}H-NMR ($CDCl_3$) and ^{13}C-NMR (Table 2) spectra[28)] indicated the presence of three methoxyl groups [δ 3.83, 3.87, 3.89 (each 3H, all s, CH_3O-2′, 5′, 4′, interchangeable)], a methylene [δ 2.50 (2H, dt, J=6.2, 7.1 Hz, H_2-2)], a methylene with an oxygen function [δ 3.76 (2H, t, J=6.2 Hz, H_2-1)], an olefin [δ 6.07 (1H, dt, J=7.1, 15.8 Hz, H-3), 6.76 (1H, d, J=15.8 Hz, H-4)], and a tetrasubstituted benzene ring [δ 6.50, 6.97 (both s, H-3′, 6′)]. On the basis of the DQF-COSY and HMBC experiments (Fig. 1), the structure of cassumunol D was determined as **4**.

Cassumunols E (**5**) and F (**6**), obtained as a pale yellow oil with no optical activity, showed a common absorption maximum at 281 nm in their UV spectra, whereas the IR spectra indicated the presence of hydroxyl and ether functions and aromatic ring (**5**: 3450, 1509, 1038 cm^{-1}; **6**: 3450, 1510, 1034 cm^{-1}). The common molecular formula $C_{11}H_{14}O_4$ of **5** and **6** was determined by HR-MS measurement of the molecular ion peak at *m/z* 210 (M^+). The ^{1}H-NMR ($CDCl_3$) and ^{13}C-NMR (Table 2) spectra[28)] of **5** and **6**, showed singanls due to a methoxyl group [**5**: δ 3.91 (s, CH_3O-3′); **6**: δ 3.88 (s, CH_3O-3′)], a methylene [**5**: δ 2.18 (m, H-3β), 2.27 (m, H-3α); **6**: δ 1.94 (m, H-3β), 2.20 (m, H-3α)], a methylene [**5**: δ 4.02 (ddd like, J=4.3, 8.5, 8.5 Hz, H-4α), 4.26 (br dd like, J=8.5, 15.9 Hz, H-4β); **6**: δ 4.11 (br dd like, J=8.6, 15.9 Hz, H-4β), 4.18 (ddd like, J=4.3, 8.6, 8.6 Hz, H-4α)] and two methines [**5**: δ 4.36 (m, H-2), 4.84 (d, J=3.1 Hz, H-1); **6**: δ 4.24 (ddd like, J=3.7, 6.7, 6.7 Hz, H-2), 4.67 (d, J=3.7 Hz, H-1)] with an oxygen function, and a trisubstituted benzene ring [**5**: δ 6.83 (dd, J=1.9, 8.0 Hz, H-6′), 6.93 (d, J=8.0 Hz, H-5′), 6.95 (br d, J=1.9 Hz, H-2′); **6**: δ 6.82 (dd, J=1.8, 8.0 Hz, H-6′), 6.86 (d, J=1.8 Hz, H-2′), 6.88 (br d, J=8.0 Hz, H-5′)]. The common plane structures of **5** and **6** were clarified by DQF-COSY and HMBC experiments (Fig. 1). Furthermore, the positions of methoxyl group in **5** and **6** were confirmed by dif.-NOE experiment, which showed NOE correlation between the methoxyl proton and the 2′-proton. The relative stereostructure of **5** was elucidated by by dif.-NOE experiment, which showed NOEs between the following protons: H-1 and H-2, H-4α; H-2 and H-3α; H-3α and H-4α; H-3β and H-4β. On the other hand, the dif.-NOE experiment on **6** showed NOEs between the following protons: H-1 and H-3β, H-4β; H-2 and H-3α; H-3β and H-4β; H-3α and H-4α (Fig. 1). Consequently, the relative stereostructures of cassumunols E (**5**) and F (**6**) were characterized as shown.

Cassumunols G (**7**) and H (**8**) also obtained as a pale yellow oil with no optical activity, indicated the presence of hydroxyl and ether functions and an aromatic ring in the IR and UV spectra. The common molecular formula $C_{12}H_{16}O_4$ was clarified by HR-MS measurement of the molecular ion peak at *m/z* 224 (M^+). The proton and carbon signals in the ^{1}H- and ^{13}C-NMR data of **7** and **8** were superimposable on those of **5** and **6**, respectively, except for the 3′, 4′, and 5′-positions. Detailed comparison of the NMR data for **7** and **8** with those for **5** and **6** deduced the structure of **7** and **8** to be the 4′-methyl derivatives of **5** and **6**, respectively. Finally, on the basis of the DQF-COSY, HMBC, and dif.-NOE experiments on **7** and **8** (Fig. 1), the relative stereostructures of cassumunols G (**7**) and H (**8**) were elucidated as shown.

Previously, we reported the isolation of racemic isocoumarins[29,30)] and sesquiterpenes[8,9,31,32)] and their formation chemical process from the genuine compounds. Since cassumunols C (**3**), E (**5**), F (**6**), G (**7**), H (**8**), and **15** showed no significant optical activity. Those phenylbutanoids may be

Table 3. Inhibitory Effects of Constituents from *Z. cassumunar* on Invasion of HT1080 Cells

Concentration (μM)	Inhibition			
	0	10	30	100
Phlain I (**9**)	0.0±12.2	20.2±6.3	40.5±7.7**	69.4±3.0**
Phlain III (**10**)	0.0±9.5	20.7±5.4	33.0±0.8**	57.3±3.2**
11	0.0±6.2	−27.1±7.2	2.0±8.1	48.5±12.3**
12	0.0±10.4	3.9±9.9	46.8±7.2*	60.4±11.5**
13	0.0±20.2	−14.2±23.0	−25.1±7.6	−27.1±14.9
16	0.0±5.2	−9.3±3.7	−15.6±4.1	−21.7±6.2
17	0.0±15.3	13.6±12.7	11.8±11.2	−3.3±18.3
18	0.0±20.6	−0.7±19.5	−16.8±1.8	24.4±11.5
20	0.0±10.7	9.0±20.0	45.4±16.2	−0.3±13.1
22	0.0±10.4	12.2±5.6	29.0±9.0**	54.3±3.4**
Deguelin[a,34)]	0.0±7.3	58.0±7.5**	57.9±10.5**	—

Each value represents the mean±S.E.M. (n=4). Significantly different from the control, * $p<0.05$, ** $p<0.01$. *a*) Reference compound.

secondarily formed from achiral phenylbutanoid.

Inhibition of Effects of Principal Constituents from *Z. cassumunar* on Invasion of HT1080 Cells through Matrigel-Coated Filter Metastatis of cancer is the major cause of death in cancer patients. Therefore, its blockade has been considered to enhance survival of cancer patients. Invasion into the circulation from the primary tumor through the extracellular matrix (ECM) and basement membrane (BM) is an essential step in the metastasis. Recently, transwell chamber pre-coated with ECM components and a reconstituted BM (Matrigel)-coated filter has been used for *in vitro* assay of invasion of tumor cells.[33)] To develop the invasion inhibitors, we have reported the inhibitory effects of several rotenoids on invasion of HT 1080 cells.[34)] As a continuing study, effects of the principal constituents from the rhizomes of *Z. cassumunar* on the invasion of HT1080 cells through Matrigel-coated filters were examined (Table 3). Among them, phlain I (**9**) and III (**10**), (*E*)-1-(3,4-dimethoxyphenyl)buta-1,3-diene (**11**), (*E*)-1-(2,4,5-trimethoxyphenyl)-buta-1,3-diene (**12**), and (−)-β-sesquiphellandrene (**22**) showed anti-invasion effects. Particularly, **12** significantly inhibited the invasion [inhibition (%) 46.8±7.2 ($p<0.05$) at 30 μg/ml]. Next, the cytotoxicities of **9**, **10**, **11**, **12**, and **22** with anti-invasion effects were examined (Table 4). Interestingly, the cytotoxicity of **12** was found to be weak. On the other hand, (*E*)-4-(3,4-dimethoxyphenyl)but-3-en-1-ol (**13**), (*E*)-1-(3,4-dimethoxyphenyl)but-1-ene (**16**), and (*E*)-1-(2,4,5-trimethoxyphenyl)but-1-ene (**17**) showed no anti-invasion effects. With regard to structural requirements of phenylbutanoids **11**—**17** for the activity, the terminal olefins of the side chain moieties on **11** and **12** were suggested to be important.

In conclusion, eight new phenylbutanoids, cassumunols A—H (**1**—**8**), were isolated from the rhizomes of *Z. cassumunar* and their chemical structures were elucidated on the basis of chemical and physicochemical evidence. In addition, the principal constituent, (*E*)-1-(2,4,5-trimethoxyphenyl)buta-1,3-diene (**12**), significantly inhibited the invasion of human fibrosarcoma HT 1080 cells, and only a weak cytotoxic effect was observed.

Table 4. Cytotoxicities of Constituents from *Z. cassumunar* on HT1080 Cells[a)]

Concentration (μM)	Inhibition			
	0	10	30	100
Phlain I (**9**) 24 h	0.0±3.3	21.7±1.4**	31.4±0.6**	64.5±0.8**
48 h	0.0±1.2	22.6±3.4**	48.0±1.2**	74.7±1.7**
72 h	0.0±1.5	14.2±2.4**	52.1±2.1**	74.9±1.2**
Phlain III (**10**) 24 h	0.0±1.9	15.7±0.8**	31.3±1.3**	65.9±0.5**
48 h	0.0±0.6	13.3±2.0**	39.7±1.9**	74.9±1.3**
72 h	0.0±1.0	10.4±0.9**	44.6±2.2**	91.6±0.2**
11 24 h	0.0±2.2	8.9±2.1	14.6±2.6**	23.4±3.7**
48 h	0.0±2.5	1.3±1.0	11.7±5.5	40.1±1.3**
72 h	0.0±2.0	5.2±1.7	8.5±1.6**	39.9±1.3**
12 24 h	0.0±2.8	1.6±1.1	5.6±3.0	28.4±0.7**
48 h	0.0±1.3	4.6±1.2	5.4±1.4*	19.8±1.5**
72 h	0.0±1.7	2.1±1.1	−0.7±1.0	10.0±1.4**
22 24 h	0.0±1.6	5.7±1.6	0.9±2.8	22.6±2.5**
48 h	0.0±1.6	4.7±1.7	11.1±2.9**	39.9±1.8**
72 h	0.0±2.1	5.5±0.7	8.2±1.7*	46.1±2.9**
Deguelin[b,34)] 24 h	0.0±1.4	−1.8±5.1	17.0±1.4**	—
48 h	0.0±1.6	51.0±2.1**	40.4±2.7**	—
72 h	0.0±0.6	98.5±0.1**	98.5±0.1**	—

a) The effects were observed after 24 h, 48 h, or 72 h incubation. Each value represents the mean±S.E.M. (n=4). Significantly different from the control, * $p<0.05$, ** $p<0.01$. *b*) Reference compound.

Experimental

General Experimental Procedures The following instruments were used to obtain physical data: specific rotations, Horiba SEPA-300 digital polarimeter (l=5 cm); UV spectra, Shimadzu UV-1600 spectrometer; IR spectra, Shimadzu FTIR-8100 spectrometer (Kyoto, Japan); EI-MS and HR-EI-MS, JEOL JMS-GCMATE mass spectrometer; ^{1}H-NMR spectra, JEOL JNM-EX 270 (270 MHz), JEOL JNM-LA 500 (500 MHz), and JEOL JNM-ECA 600 (600 MHz) spectrometers; ^{13}C-NMR spectra, JEOL JNM-EX 270 (68 MHz) JEOL JNM-LA (125 MHz), and JEOL JNM-ECA 600 (150 MHz) spectrometers with tetramethylsilane as an internal standard; HPLC detector, Shimadzu RID-10A refractive index detector; and HPLC column, YMC-Pack ODS-A (YMC, Inc., Kyoto, Japan, 250×4.6 mm i.d.) and (250×20 mm i.d.) columns were used for analytical and preparative purposes, respectively. The following experimental materials were used for chromatography: normal-phase silica gel column chromatography, Silica gel BW-200 (Fuji Silysia Chemical, Ltd., Aichi, Japan, 150—350 mesh); reversed-phase silica gel column chromatography, Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd., 100—200 mesh); TLC, precoated TLC plates with Silica gel 60F_{254} (Merck, Germany, 0.25 mm) (ordinary phase) and Silica gel RP-18 F_{254S} (Merck, 0.25 mm) (reversed phase); reversed-phase HPTLC, precoated TLC plates with Silica gel RP-18 WF_{254S} (Merck, 0.25 mm); and detection was achieved by spraying with 1% $Ce(SO_4)_2$–10% aqueous H_2SO_4 followed by heating.

Reagents for Bioassay Minimum essential medium Eagle's (MEM) and RPMI1640 were purchased from Sigma-Aldrich (MO, U.S.A.); fetal calf serum (FCS) was from Roche Diagnostics (Basel, Switzerland); Matrigel (BD Matrigel™) was from BD Biosciences (NJ, U.S.A.); Cell Culture Insert™ was from BD Falcon (NJ, U.S.A.); Cell Counting Kit-FTM was from Dojindo Lab. (Kumamoto, Japan); other reagents were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 24-well multiplates were purchased from Sumitomo Bakelite Co., Ltd. (Tokyo, Japan); 96-well black microplates were from Nunc (Roskild, Denmark).

Plant Material The rhizomes of *Zingiber cassumunar* were cultivated in Nakhon Si Thammarat of Thailand in 2007, and identified by one of authors (Y. P.). A voucher specimen is on file in our laboratory.

Extraction and Isolation The methanolic extract (340.0 g, 24.1%) was obtained from the rhizomes of *Zingiber cassumunar* (1.4 kg) as reported previously.[12)] The aliquot (277.7 g) from the extract was partitioned into an EtOAc–H_2O (1 : 1, v/v) mixture to furnish an EtOAc-soluble fraction (192.9 g, 16.7%) and an aqueous phase (84.8 g, 7.4%). The aliquot (80.9 g) from the EtOAc fraction was subjected to ordinary-phase silica gel column chromatography [2.4 kg, *n*-hexane–EtOAc (20 : 1→5 : 1→2 : 1→1 : 1, v/v)→EtOAc→MeOH] to give 6 fractions [Fr. 1, Fr. 2 (15.5 g), Fr. 3 (5.1 g), Fr. 4

(28.6 g), Fr. 5 (10.0 g), Fr. 6 (16.6 g)] as reported previously. The aliquot (7.8 g) of fraction 2 was subjected to reversed-phase silica gel column chromatography [234 g, MeOH–H_2O (70 : 30, v/v)→MeOH] to give 3 fractions [Fr. 2-1, Fr. 2-2 {=[(*E*)-1-(3,4-dimethoxyphenyl)buta-1,3-diene (**11**), 5.300 g, 2.17%]}, Fr. 2-3 (528 mg)]. The aliquot (295 mg) of fraction 2-3 was purified by HPLC [MeOH–H_2O (80 : 20, v/v)] to give *trans*-3-(3,4-dimethoxyphenyl)-4-[(*E*)-3,4-dimethoxystyryl]cyclohex-1-ene (**20**, 18 mg, 0.013%). The aliquot (2.5 g) of fraction 3 was subjected to reversed-phase silica gel column chromatography [75 g, MeOH–H_2O (70 : 30→90 : 10, v/v)→MeOH] to give 8 fractions {Fr. 3-1 (78 mg), Fr. 3-2 (968 mg), Fr. 3-3 (111 mg), Fr. 3-4, Fr. 3-5, Fr. 3-6, Fr. 3-7, Fr. 3-8 [=β-sitosterol (167 mg, 0.073%)]}. The fraction 3-1 (78 mg) was purified by HPLC [MeOH–H_2O (50 : 50, v/v)] to give cassumunol C (**3**, 3.6 mg, 0.0016%) and 2,4,5-trimethoxybenzaldehyde (0.5 mg, 0.00022%). The aliquot (484 mg) of fraction 3-2 was purified by HPLC [MeOH–H_2O (70 : 30, v/v)] to give (*Z*)-1-(2,4,5-trimethoxyphenyl)buta-1,3-diene (6.4 mg, 0.0057%). The aliquot (56 mg) of fraction 3-3 was purified by HPLC [MeOH–H_2O (80 : 20, v/v)] to give (±)-*trans*-3-(2,4,5-trimethoxyphenyl)-4-[(*E*)-2,4,5-trimethoxystyryl]cyclohex-1-ene (**21**, 7.3 mg, 0.0064%). The aliquot (14.2 g) of fraction 4 was subjected to reversed-phase silica gel column chromatography [426 g, MeOH–H_2O (50 : 50→70 : 30→80 : 20, v/v)→MeOH] to give 9 fractions [Fr. 4-1 [=vanillic acid (172 mg, 0.071%)], Fr. 4-2, Fr. 4-3, Fr. 4-4, Fr. 4-5 [=(*E*)-4-(3,4-dimethoxyphenyl)but-3-enyl acetate (1.401 g, 0.58%)], Fr. 4-6, Fr. 4-7, Fr. 4-8 (10.0 g), Fr. 4-9]. The aliquot (450 mg) of fraction 4-8 was purified by HPLC [MeOH–H_2O (70 : 30, v/v)] to give (±)-*trans*-3-(2,4,5-trimethoxyphenyl)-4-[(*E*)-2,4,5-trimethoxystyryl]cyclohex-1-ene (**21**, 6.6 mg, 0.061%). The aliquot (5.0 g) of fraction 5 was subjected to reversed-phase silica gel column chromatography [150 g, MeOH–H_2O (60 : 40→70 : 30, v/v)→MeOH] to give 4 fractions [Fr. 5-1 (2.7 g), Fr. 5-2, Fr. 5-3, Fr. 5-4]. The aliquot (700 mg) of fraction 5-1 was purified by HPLC [MeOH–H_2O (47 : 53, v/v)] to give cassumunol D (**4**, 6.4 mg, 0.010%). The aliquot (8.3 g) of fraction 6 was subjected to ordinary-phase silica gel column chromatography [249 g, *n*-hexane–EtOAc (2 : 1→1 : 1→1 : 2→1 : 5, v/v)→EtOAc→MeOH] to give 6 fractions [Fr. 6-1, Fr. 6-2, Fr. 6-3, Fr. 6-4 (647 mg), Fr. 6-5 (4.86 g), Fr. 6-6]. The fraction 6-4 (647 mg) was subjected to reversed-phase silica gel column chromatography [19.4 g, MeOH–H_2O (20 : 80→40 : 60→60 : 40→80 : 20, v/v)→MeOH] to give 9 fractions {Fr. 6-4-1, Fr. 6-4-2, Fr. 6-4-3 (44 mg), Fr. 6-4-4 (44 mg), Fr. 6-4-5 [=cassumunaquinone 2 (51 mg, 0.020%)], Fr. 6-4-6 (288 mg), Fr. 6-4-7, Fr. 6-4-8, Fr. 6-4-9}. The fraction 6-4-3 (44 mg) was further purified by HPLC [MeOH–H_2O (28 : 72, v/v)] to give cassumunol F (**6**, 2.6 mg, 0.0011%). The fraction 6-4-4 (44 mg) was further purified by HPLC [MeOH–H_2O (40 : 60, v/v)] to give cassumunol D (**4**, 8.1 mg, 0.0033%). The fraction 6-4-6 (288 mg) was further purified by HPLC [MeOH–H_2O (68 : 32, v/v)] to give cassumunarin A (19 mg, 0.0080%) and cassumunarin C (10 mg, 0.0043%). The fraction 6-5 (2.63 g) was subjected to reversed-phase silica gel column chromatography [78.9 g, MeOH–H_2O (10 : 90→30 : 70→60 : 40→80 : 20, v/v)→MeOH] to give 7 fractions [Fr. 6-5-1 (131 mg), Fr. 6-5-2 (78 mg), Fr. 6-5-3, Fr. 6-5-4, Fr. 6-5-5, Fr. 6-5-6, Fr. 6-5-7]. The fraction 6-5-1 (131 mg) was further purified by HPLC [MeOH–H_2O (15 : 85, v/v)] to give cassumunol A (**1**, 12 mg, 0.0093%), cassumunol B (**2**, 3.5 mg, 0.0026%), cassumunol E (**5**, 5.8 mg, 0.0044%), and cassumunol F (**6**, 23 mg, 0.017%). The fraction 6-5-2 (78 mg) was further purified by HPLC [MeOH–H_2O (30 : 70, v/v)] to give cassumunol G (**7**, 2.8 mg, 0.0021%), cassumunol H (**8**, 32 mg, 0.024%), and (*E*)-4-(3,4-dimethoxyphenyl)but-3-ene-1,2-diol (**15**, 28 mg, 0.021%). The isolation methods of several known compounds were not shown in "Extraction and Isolation." Those isolation methods were showed in ref. 12.

Cassumunol A (**1**): Colorless oil; $[\alpha]_D^{26}$ +12.8 (c=0.71, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 280 (3.42) nm; IR (film) ν_{max} 3470, 2962, 1509 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 0.94 (3H, t, J=7.3 Hz, H_3-4), 3.58 (1H, m, H-2), 1.36 (1H, m, H_2-3), 3.90 (3H, s), 4.38 (1H, d, J=6.7 Hz, H-1), 6.81 (1H, dd, J=1.2, 8.0 Hz, H-6′), 6.88 (1H, d, J=8.0 Hz, H-5′), 6.89 (1H, d, J=1.2 Hz, H-2′); ^{13}C-NMR data see Table 2; EI-MS m/z 212 $[M]^+$; HR-EI-MS m/z 212.1042 (Calcd for $C_{11}H_{16}O_4$ $[M]^+$, 212.1048).

Cassumunol B (**2**): Colorless oil; $[\alpha]_D^{26}$ +57.1 (c=0.23, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 280 (3.55) nm; IR (film) ν_{max} 3470, 2944, 1509 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 0.97 (3H, t, J=7.3 Hz, H_3-4), 1.35 (1H, m, H_2-3), 3.91 (3H, s), 3.72 (1H, m, H-2), 4.59 (1H, d, J=6.9 Hz, H-1), 6.83 (1H, dd like, J=1.0, 8.0 Hz, H-6′), 6.90 (1H, d, J=8.0 Hz, H-5′), 6.94 (1H, d like, J=1.0 Hz, H-2′); ^{13}C-NMR data see Table 2; EI-MS m/z 212 $[M]^+$; HR-EI-MS m/z 212.1042 (Calcd for $C_{11}H_{16}O_4$ $[M]^+$, 212.1048).

Cassumunol C (**3**): Pale yellow oil; UV ($CHCl_3$) λ_{max} (log ε) 265 (4.06) nm; IR (film) ν_{max} 3520, 2940, 1509 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 3.62 (dd, J=7.3, 11.0 Hz, H_2-1a), 3.76 (dd, J=3.7, 11.0 Hz, H_2-1b), 3.83, 3.86, 3.90 (all s, CH_3O-2′, 5′, 4′), 4.43 (m, H-2), 6.09 (dd, J=6.7, 15.9 Hz, H-3), 6.50, 6.97 (both s, H-3′, 6′), 6.95 (dd, J=15.9 Hz, H-4); ^{13}C-NMR data see Table 2; EI-MS m/z 254 $[M]^+$; HR-EI-MS m/z 254.1150 (Calcd for $C_{13}H_{18}O_5$ $[M]^+$, 254.1154).

Cassumunol D (**4**): Pale yellow oil; IR (film) ν_{max} 3520, 2936, 1516 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 2.50 (2H, dt, J=6.2, 7.1 Hz, H_2-2), 3.76 (2H, t, J=6.2, H_2-1), 3.83, 3.87, 3.89 (each 3H, all s, CH_3O-2′, 5′, 4′), 6.07 (1H, dt, J=7.1, 15.8 Hz, H-3), 6.50, 6.97 (both s, H-3′, 6′), 6.76 (1H, d, J=15.8 Hz, H-4); ^{13}C-NMR data see Table 2; EI-MS m/z 238 $[M]^+$; HR-EI-MS m/z 238.1213 (Calcd for $C_{13}H_{18}O_4$ $[M]^+$, 238.1205).

Cassumunol E (**5**): Pale yellow oil; UV ($CHCl_3$) λ_{max} (log ε) 281 (3.49) nm; IR (film) ν_{max} 3450, 2962, 1509, 1038 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 2.18 (m, H-3β), 2.27 (m, H-3α), 3.91 (s, CH_3O-3′), 4.02 (ddd like, J=4.3, 8.5, 8.5 Hz, H-4α), 4.26 (br dd like, J=8.5, 15.9 Hz, H-4β), 4.36 (m, H-2), 4.84 (d, J=3.1 Hz, H-1), 6.83 (dd, J=1.9, 8.0 Hz, H-6′), 6.93 (d, J=8.0 Hz, H-5′), 6.95 (br d, J=1.9 Hz, H-2′); ^{13}C-NMR data see Table 2; EI-MS m/z 210 $[M]^+$; HR-EI-MS m/z 210.0899 (Calcd for $C_{11}H_{14}O_4$ $[M]^+$, 210.0892).

Cassumunol F (**6**): Pale yellow oil; UV ($CHCl_3$) λ_{max} (log ε) 281 (3.46) nm; IR (film) ν_{max} 3450, 2962, 1510, 1034 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 1.94 (m, H-3β), 2.20 (m, H-3α), 3.88 (s, CH_3O-3′), 4.11 (br dd like, J=8.6, 15.9 Hz, H-4β), 4.18 (ddd like, J=4.3, 8.6, 8.6, H-4α), 4.24 (ddd like, J=3.7, 6.7, 6.7 Hz, H-2), 4.67 (d, J=3.7 Hz, H-1), 6.82 (dd, J=1.8, 8.0 Hz, H-6′), 6.86 (d, J=1.8 Hz, H-2′), 6.88 (br d, J=8.0 Hz, H-5′); ^{13}C-NMR data see Table 2; EI-MS m/z 210 $[M]^+$; HR-EI-MS m/z 210.0898 (Calcd for $C_{11}H_{14}O_4$ $[M]^+$, 210.0892).

Cassumunol G (**7**): Pale yellow oil; UV ($CHCl_3$) λ_{max} (log ε) 281 (3.43) nm; IR (film) ν_{max} 3450, 2960, 1509, 1025 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 2.17 (m, H-3β), 2.28 (m, H-3α), 3.89, 3.91 (both s, CH_3O-3′, 4′, interchangeable), 4.03 (ddd like, J=4.3, 8.6, 8.6 Hz, H-4α), 4.27 (br dd like, J=8.6, 15.9 Hz, H-4β), 4.38 (m, H-2), 4.86 (d, J=3.1 Hz, H-1), 6.89 (d, J=8.0 Hz, H-5′), 6.91 (br d like, J=8.0 Hz, H-6′), 6.95 (s like, H-2′); ^{13}C-NMR data see Table 2; EI-MS m/z 224 $[M]^+$; HR-EI-MS m/z 224.1044 (Calcd for $C_{12}H_{16}O_4$ $[M]^+$, 224.1048).

Cassumunol H (**8**): Pale yellow oil; UV ($CHCl_3$) λ_{max} (log ε) 281 (3.47) nm; IR (film) ν_{max} 3450, 2962, 1509, 1028 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 1.96 (m, H-3β), 2.21 (m, H-3α), 3.87, 3.89 (both s, CH_3O-3′, 4′, interchangeable), 4.12 (br dd like, J=8.3, 16.5 Hz, H-4β), 4.20 (ddd like, J=4.1, 8.3, 8.3, H-4α), 4.27 (m, H-2), 4.70 (d, J=2.8 Hz, H-1), 6.84 (d, J=8.2 Hz, H-5′), 6.89 (br d like, J=8.2 Hz, H-6′), 6.88 (s like, H-2′); ^{13}C-NMR data see Table 2; EI-MS m/z 224 $[M]^+$; HR-EI-MS m/z 224.1051 (Calcd for $C_{12}H_{16}O_4$ $[M]^+$, 224.1048).

Preparation of the Acetonide Derivatives (1a) from 1 A solution of **1** (2.5 mg, 0.012 mmol) in CH_2Cl_2 (0.5 ml) was treated with 2,2-dimethoxypropane (0.1 ml) and Amberlyst 15 ion-exchange resin [1 mg, Sigma-Aldrich (Japan)], and the mixture was stirred at room temperature for 12 h. The resin was removed by filtration. Removal of the solvent from the filtrate under reduced pressure gave a residue that was purified by silica gel column chromatography (*n*-hexane–AcOEt=2 : 1, v/v) to furnish **1a** (2.4 mg, 80%).

1a: Colorless oil; ^{1}H-NMR (CD_3OD, 600 MHz) δ: 0.93 (3H, t, J=7.6 Hz, H-4), 1.44, 1.52, 3.91 (3H each, both s, H_3-2″, 3″, C$\underline{H}_3$O-3′), 3.69 (1H, td, J=4.1, 8.3 Hz, H-2), 4.47 (1H, d, J=8.3 Hz, H-1), 6.82 (1H, dd, J=2.0, 8.2 Hz, H-6′), 6.89 (1H, d, J=8.2 Hz, H-5′), 6.94 (1H, d, J=2.0 Hz, H-2′); EI-MS m/z 252 $[M]^+$; HR-EI-MS m/z 252.1367 (Calcd for $C_{14}H_{20}O_4$ $[M]^+$, 252.1361).

Pivaloyl Protection of 15 A solution of **15** (4.1 mg, 0.018 mmol) in dry pyridine (1.0 ml) was treated with pivaloyl chloride (10 ml, 0.09 mmol) at 0 °C for 2 h. The reaction mixture was poured into ice-water and extracted with EtOAc. The EtOAc extract was successively washed with H_2O and brine, then dried over Na_2SO_4 powder and filtered. After removal of the solvent under reduced pressure, the residue was purified by normal-phase silica gel column chromatography [*n*-hexane–EtOAc (5 : 1, v/v)] to furnish a pivaloyl derivative of **15** (4.8 mg, 85%).

Pivaloyl Derivative of **15**: Pale yellow oil; ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 1.22 (9H, s, ($C\underline{H}_3$)$_3$CCOO), 3.89, 3.90 [3H each, both s, C$\underline{H}_3$O-3′, 4′ (interchangeable)], 4.14 (1H, dd, J=7.1, 11.4 Hz, H-1a), 4.25 (1H, dd, J=3.8, 11.4 Hz, H-1b), 4.55 (1H, m, H-2), 6.04 (1H, dd, J=6.3, 15.9 Hz, H-3), 6.64 (1H, d, J=15.9 Hz, H-4), 6.82 (1H, d, J=8.7 Hz, H-5′), 6.92 (1H, dd like, J=1.9, 8.7 Hz, H-6′), 6.94 (1H, d like, J=1.9, H-2′); ^{13}C-NMR ($CDCl_3$, 67 MHz) δ_C 67.9 (C-1), 71.2 (C-2), 125.2 (C-3), 132.1 (C-4), 129.4 (C-1′), 108.8 (C-2′), 149.0, 149.1 [C-3′, 4′ (interchangeable)], 111.1 (C-5′), 119.9 (C-6′), 55.8, 55.9 [$\underline{C}H_3$O-3′, 4′ (interchangeable)], 27.2 [($\underline{C}H_3$)$_3$CCOO], 38.9 [(CH$_3$)$_3\underline{C}$COO]; EI-MS m/z 308 $[M]^+$; HR-EI-MS m/z 308.1626 (Calcd

for $C_{17}H_{24}O_5$ $[M]^+$, 308.1624).

Preparation of the (*S*)- and (*R*)-α-Methoxy-α-(trifluoromethyl)phenylacetate (MTPA) Esters (15a, 15b) Solution of the pivaloyl derivative of **15** (1.5 mg, 0.005 mmol) in pyridine (1.0 ml) was treated with (−)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (9 μl, 0.05 mmol), and the mixture was stirred at rt for 2 h. The reaction mixture was poured into ice-water and extracted with EtOAc. The EtOAc extract was successively washed with H_2O and brine, then dried over Na_2SO_4 powder and filtered. After removal of the solvent under reduced pressure, the residue was purified by reversed-phase silica gel column chromatography [MeOH–H_2O (50 : 50, v/v)] to give (*S*)-MTPA ester derivative (**15a**, 1.2 mg). Using a similar procedure, (*R*)-MTPA ester derivative (**15b**, 1.6 mg) was obtained from the pivaloyl derivative of **15** (1.4 mg, 0.005 mmol). The NMR data of **15a** was completely same as that of **15b**.

(*S*)- and (*R*)-MTPA Ester Derivatives (**15a**, **15b**): Pale yellow oil; ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 1.15, 1.19 (9H each, both s, $(C\underline{H}_3)_3CCOO\times2$), 3.53, 3.62, 3.88, 3.89, 3.90, 3.90 [3H each, both s, $C\underline{H}_3O\times6$], 4.17 (1H, dd like, J=7.6, 12.4 Hz, H-2a), 4.18 (1H, dd like, J=6.8, 12.4 Hz, H-2a*), 4.37 (1H, dd, J=3.5, 12.4 Hz, H-2b), 4.45 (1H, dd, J=2.8, 12.4 Hz, H-2b*), 5.88 (1H each, m, H-2, 2*), 5.90 (1H, m, H-3), 6.01 (1H, dd, J=7.6, 15.8 Hz, H-3*), 6.57 (1H, d-like, J=15.8 Hz, H-4), 6.71 (1H, d, J=15.8 Hz, H-4*), 6.81—6.94 (6H, m, H-2′, 2′*, 5′, 5′*, 6′, 6′*), 7.35—7.41 (6H, m, Ph-H), 7.54 (4H, br d like, J=7.5 Hz, Ph-H).

Cell Culture Human fibrosarcoma HT1080 cells (Cell No. JCRB9113) were obtained from Health Science Research Resources Bank (Osaka, Japan). These cells were maintained in MEM and RPMI1640 supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Invasion Assay The invasion assay of HT1080 cells was performed using Cell Culture Insert™ and 24-well multiplates as reported previously. The upper side of each filter of Cell Culture Insert™ was pre-coated with Matrigel (25 μg/filter). Briefly, 100 μl of 0.25 mg/ml Matrigel in phosphate buffered saline (PBS) solution was added onto each filter (pore size 8 μm), incubated for 4 h at 37 °C, and dried at room temperature. Cell Culture Insert™ with Matrigel-coated filters was inserted into the 24-well multiplates with 700 μl/well MEM supplemented with FCS [FCS (+)]. A mixture of HT1080 cells (1×10^6 cells/ml) suspended in 100 μl MEM without FCS [FCS (−)] and test compound solution in 100 μl MEM [FCS (−)] was then added onto the filters and incubated for 24 h. After incubation, the cells crossing the filters were collected after treatment of trypsin solution (0.25% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) in PBS), and the invaded cells were resuspended in RPMI1640 [FCS (−), phenol red (−)] and seeded onto 96-well black microplates. After incubation for 4 h at 37 °C in 5% CO atmosphere, Cell Counting Kit-F™ was used for counting of the invaded cells according to the manufacturer's instruction. The test compound was dissolved in dimethylsulfoxide (DMSO) and final concentration of DMSO in the medium was 0.1%.

Cytotoxicity After 24, 48, or 72 h incubation of HT1080 cells (1×10^5 cells/100 μl/well) with test compounds in MEM [FCS (+)] in 96-well black microplates, the medium was exchanged for RPMI1640 [FCS (−), phenol red (−)], and then 10 μl of calcein-AM in PBS solution (Cell Counting Kit-F™) was added to each well. After 30 min at rt, the fluorescence intensity of each well was measured with a microplate reader (ex: 485 nm, em: 520 nm, FLUOstar OPTIMA, BMG Labtechnologies, Offenburg, Germany).

Statistics Values are expressed as mean±S.E.M. One-way analysis of variance followed by Dunnett's test was used for statistical analysis.

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