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Suppressive effects of coumarins from *Mammea siamensis* on inducible nitric oxide synthase expression in RAW264.7 cells

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ABSTRACT

A methanol extract of the flowers of *Mammea siamensis* (Calophyllaceae) was found to inhibit nitric oxide (NO) production in lipopolysaccharide-activated RAW264.7 cells. From the extract, two new geranylated coumarins, mammeasins A (**1**) and B (**2**), were isolated together with 17 known compounds including 15 coumarins. The structures of **1** and **2** were determined on the basis of their spectroscopic properties as well as of their chemical evidence. Among the isolates, **1** (IC_{50} = 1.8 μM), **2** (6.4 μM), surangins B (**3**, 5.0 μM), C (**4**, 6.8 μM), and D (**5**, 6.2 μM), kayeassamins E (**7**, 6.1 μM), F (**8**, 6.0 μM), and G (**9**, 0.8 μM), mammea A/AD (**11**, 1.3 μM), and mammea E/BB (**16**, 7.9 μM) showed NO production inhibitory activity. Compounds **1**, **9**, and **11** were found to inhibit induction of inducible nitric oxide synthase (iNOS). With regard to mechanism of action of these active constituents (**1**, **9**, and **11**), suppression of STAT1 activation is suggested to be mainly involved in their suppression of iNOS induction.

1. Introduction

Mammea siamensis (Miq.) T. Anders. (Calophyllaceae), known in Thai as "Sarapi", is a small evergreen tree distributed in Thailand, Laos, Cambodia, Vietnam, and Myanmar, etc. The flowers of this plant have been used for a heart tonic in Thai traditional medicine.[1–7] By previous chemical studies on the flowers,[2,3,8,9] seeds,[4,6] twigs,[1,5] and barks[7] of *M. siamensis*, presence of several coumarins,[2,4,5,7–9] xanthones,[1,6] triterpenoids,[3] and steroids[3] have been revealed. In the course of our characterization studies on bioactive constituents in Thai natural medicines,[10–27] the methanol extract of the flowers of *M. siamensis* was found to inhibit nitric oxide (NO) production in lipopolysaccharide (LPS)-activated RAW264.7 cells. By bioassay-guided separation, two new geranylated coumarins, mammeasins A (**1**) and B (**2**), were isolated together with 17 known compounds including 15 coumarins (**3**–**17**). This paper deals with the isolation and structural elucidation of these new geranylated coumarins (**1** and **2**) as well as inhibitory effects of the coumarin constituents on the LPS-activated NO production. Furthermore, to clarify the mechanism of action of the NO production inhibitory activity, effects of three active coumarins (**1**, **9**, and **11**) on protein levels of inducible NO synthase (iNOS), activation of mitogen-activated protein kinases (MAPK) [c-Jun N-terminal kinase (JNK) and p38], and nuclear protein levels of phosphorylated signal transducer and activator of transcription-1 (STAT1) (p-STAT1) as well as nuclear factor κ-B (NF-κB) were examined.

2. Results and discussion

2.1. Effect of methanol extract from the flowers of *M. siamensis* on NO production in LPS-activated RAW264.7 cells

The dried flowers of *M. siamensis* (collected in Nakhonsithammarat Province, Thailand) were extracted with methanol under reflux to yield a methanolic extract (25.66% from the dried flower). The methanol extract was partitioned into an EtOAc–H_2O (1:1, v/v) mixture to furnish an EtOAc-soluble fraction (6.84%) and an aqueous phase. The aqueous phase was subjected to Diaion HP-20 column chromatography ($H_2O \rightarrow$ MeOH) to give H_2O- and MeOH-eluted fractions (13.50% and 4.22%, respectively). As shown in Table 1, the methanol extract was found to inhibit LPS-activated NO production in RAW264.7 cells (IC_{50} = 28.9 μg/mL). By bioassay-guided fractionation, the EtOAc-soluble fraction was found to be the active fraction (IC_{50} = 8.3 μg/mL), although the fraction exhibited cytotoxic effects in MTT assay. On the other hand, the MeOH- and H_2O-eluted fractions showed no activity.

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Table 1
Inhibitory effects of the methanolic extract and its fractions from the flowers of *M. siamensis* on LPS-activated NO production in RAW264.7 cells

	Inhibition (%)[a]				IC_{50} (μg/mL)
	0 μg/mL	10 μg/mL	30 μg/mL	100 μg/mL	
MeOH extract	0.0 ± 1.9	38.1 ± 1.4[b]	55.7 ± 0.8[b]	64.6 ± 0.7[b] (86.7 ± 1.7)	28.9
EtOAc-soluble fraction	0.0 ± 1.4	60.4 ± 0.9[b]	65.0 ± 1.9[b,c] (45.8 ± 1.6)	95.8 ± 0.2[b,c] (27.3 ± 1.1)	8.3
MeOH-eluted fraction	0.0 ± 4.2	3.5 ± 3.7	7.8 ± 1.3	15.2 ± 1.5[b]	>100
H_2O-eluted fraction	0.0 ± 1.4	5.0 ± 0.8	7.6 ± 0.9	−1.7 ± 1.7	>100

[a] Each value represents the mean ± SEM ($N = 4$).
[b] Significantly different from the control, $p < 0.01$.
[c] Cytotoxic effects were observed, and values in parentheses indicate cell viability (%) in MTT assay.

2.2. Chemical constituents from the flowers of *M. siamensis*

The EtOAc-soluble fraction was subjected to silica gel and ODS column chromatography and finally HPLC to furnish mammeasins A (**1**, 0.0293% from the dried flower) and B (**2**, 0.0123%). Additionally, 15 coumarins, surangins B[4,28–30] (**3**, 0.0337%), C[31,32] (**4**, 0.0571%), and D[7] (**5**, 0.0632%), kayeassamins A[33] (**6**, 0.0578%), E[34] (**7**, 0.0113%), F[34] (**8**, 0.0390%), and G[34] (**9**, 0.0171%), mammea A/AC[35,36] (**10**, 0.0555%), mammea A/AD[37] (**11**, 0.0022%), mammea A/AB cyclo D[38] (**12**, 0.0047%), mammea A/AC cyclo D[35] (**13**, 0.0077%), mammea B/AB cyclo D[37,38] (**14**, 0.0016%), mammea B/AC cyclo D[8] (**15**, 0.0055%), mammea E/BB[4,39] (**16**, 0.0194%), and deacetylmammea E/BC cyclo D[9] (**17**, 0.0073%), and β-amyrin[40] (0.0072%) and benzoic acid[40] (0.0043%) were isolated from this plant material (Fig. 1).

2.3. Structures of mammeasins A (1) and B (2)

Mammeasin A (**1**) was obtained as a pale yellow oil with negative optical rotation ($[\alpha]_D^{27}$ −25.4 in $CHCl_3$). Its IR spectrum showed absorption bands at 3503, 1748, 1717, and 1609 cm^{-1} ascribable to hydroxyls, ester carbonyl, α,β-unsaturated γ-lactone, and chelated acyl groups. The UV spectrum exhibited absorption

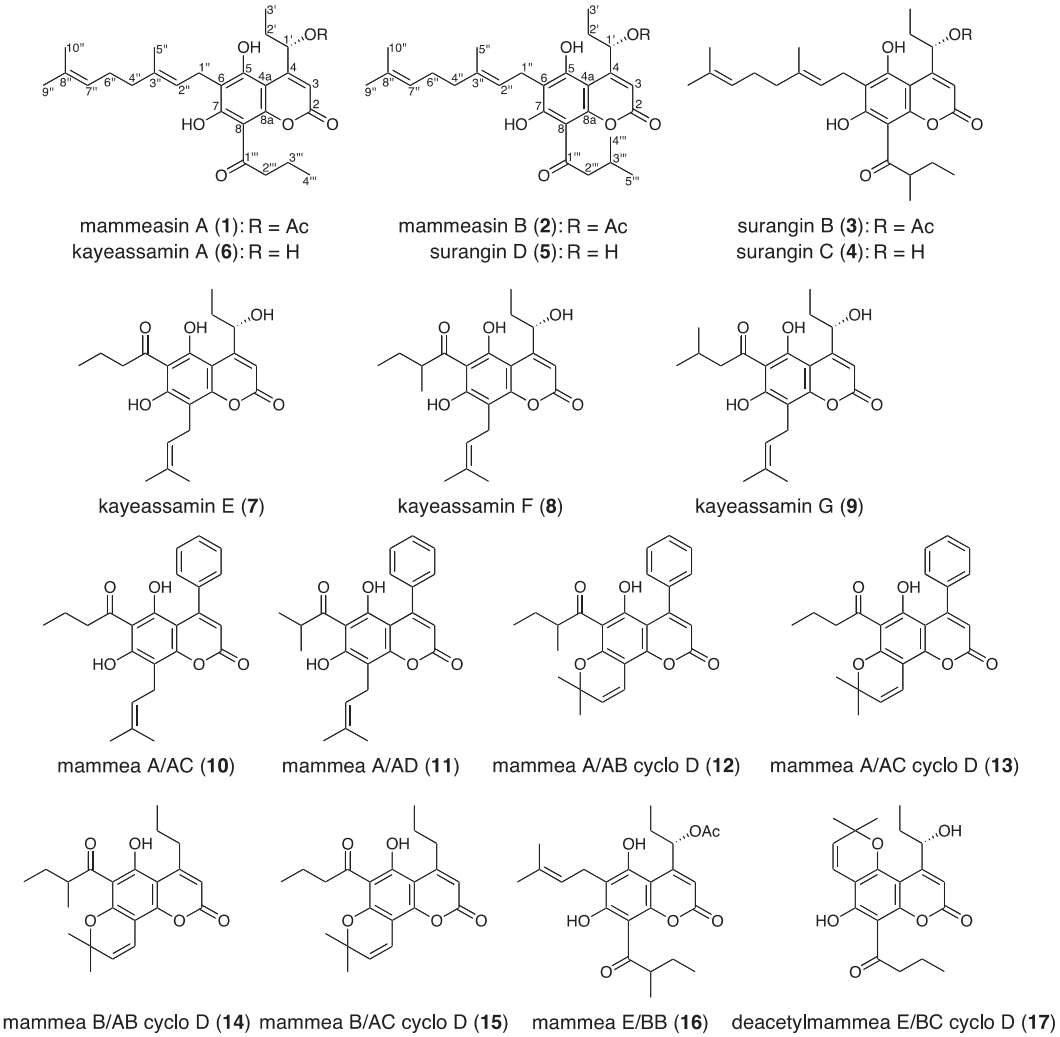

Figure 1. Coumarin constituents (**1**–**17**) from the flowers of *M. siamensis*.

Table 2
^{1}H NMR (700 MHz, $CDCl_3$) data on mammeasins A (**1**) and B (**2**)

Position	**1** δ_H (*J*, Hz)	**2** δ_H (*J*, Hz)
3	6.27 (1H, d, 1.1)	6.27 (d, 1.1)
1′	6.48 (1H, ddd, 1.1, 2.4, 8.0)	6.48 (1H, ddd, 1.1, 2.6, 7.9)
2′	1.68 (1H, m)	1.68 (1H, m)
	1.95 (1H, ddq, 2.4, 14.4, 7.2)	1.95 (1H, m)
3′	1.00 (3H, dd, 7.2, 7.2)	1.00 (3H, dd, 7.2, 7.2)
1″	3.48 (1H, dd, 7.4, 16.5)	3.48 (1H, dd, 7.2, 16.7)
	3.51 (1H, dd, 7.0, 16.5)	3.52 (1H, dd, 7.4, 16.7)
2″	5.24 (1H, ddq, 7.0, 7.4, 1.0)	5.25 (1H, ddq, 7.0, 7.2, 1.0)
4″	2.11 (2H, m)	2.12 (2H, m)
5″	1.86 (3H, d, 1.0)	1.86 (3H, br s)
6″	2.11 (2H, m)	2.12 (2H, m)
7″	5.06 (1H, m)	5.06 (1H, m)
9″	1.68 (3H, s)	1.68 (3H, s)
10″	1.60 (3H, d, 0.8)	1.60 (3H, d, 0.8)
2‴	3.27 (2H, t, 7.2)	3.12 (1H, dd, 6.6, 15.9)
		3.17 (1H, dd, 6.6, 15.9)
3‴	1.78 (2H, tq, 7.2, 7.4)	2.27 (1H, m)
4‴	1.04 (3H, t, 7.4)	1.03 (3H, d, 6.7)
5‴		1.03 (3H, d, 6.7)
1″-*OAc*	2.18 (3H, s)	2.18 (3H, s)
7-O*H*	14.69 (1H, s)	14.67 (1H, s)

maxima at 223 and 330 nm, similar to those of 5,7-dioxygenated coumarins.[5,33,34,37] The EIMS spectrum of **1** showed a molecular ion peak at *m/z* 484 (M^+), and the molecular formula was determined as $C_{28}H_{36}O_7$ by high-resolution EIMS measurement. The ^{1}H and ^{13}C NMR spectra of **1** ($CDCl_3$, Tables 2 and 3), which were assigned by means of various NMR experiments,[41] showed signals assignable to two primary and three vinyl methyls [δ 1.00 (3H, dd, J = 7.2, 7.2 Hz, H_3-3′), 1.04 (3H, t, J = 7.4 Hz, H_3-4‴), 1.60 (3H, d, J = 0.8 Hz, H_3-10″), 1.68 (3H, s, H_3-9″), 1.86 (3H, d, J = 1.0 Hz, H_3-5″)], six methylenes {δ [1.68 (1H, m), 1.95 (1H, ddq, J = 2.4, 14.4, 7.2 Hz), H_2-2′], 1.78 (2H, tq, J = 7.2, 7.4 Hz, H_2-3‴), 2.11 (4H, m, H_2-4″, 6″), 3.27 (2H, t, J = 7.2 Hz, H_2-2‴), [3.48 (1H, dd, J = 7.4, 16.5 Hz), 3.51 (1H, dd, J = 7.0, 16.5 Hz), H_2-1″]}, a methine bearing an oxygen function [δ 6.48 (1H, ddd, J = 1.1, 2.4, 8.0 Hz, H-1′)], and three olefinic protons [δ 5.06 (1H, m, H-7″), 5.24 (1H, ddq, J = 7.0, 7.4, 1.0 Hz, H-2″), 6.27 (1H, d, J = 1.1 Hz, H-3)] together with an acetyl group [δ 2.18 (3H, s); δ_C 21.1, 170.4]. The ^{1}H and ^{13}C NMR spectroscopic properties of **1** were quite similar to those of **6**, except for the signal due to the acetyl group. The ^{1}H–^{1}H COSY experiment on **1** indicated the presence of partial structures shown in bold lines in Figure 2. In the HMBC experiment, long-range correlations were observed between the following proton and carbon pairs (H-3 and C-2, 4a, 1′; H-1′ and C-3, 4a, 1′-$OCOCH_3$; H_2-1″ and C-5–7; H-2″ and C-6, 3″, 5″; H_2-4″ and C-3″; H_2-7″ and C-9″, 10″; H_3-5″ and C-2″–4″; H_3-9″ and C-7″, 8″, 10″; H_3-10″ and C-7″–9″; H_2-2‴ and C-1‴). In order to elucidate the absolute stereostructure, **1** was chemically related to **6**, for which the absolute configuration at the C-1′ was reported.[33] As shown in Figure 3, acetylation of **6** with acetic anhydride (Ac_2O) in pyridine furnished **1**, so that the absolute configuration at the C-1′ was determined to be *S* orientation.

Table 3
^{13}C NMR (175 MHz, $CDCl_3$) data on mammeasins A (**1**) and B (**2**)

Position	**1** δ_C	**2** δ_C
2	159.5	159.4
3	106.4	106.4
4	157.3	157.1
4a	100.4	100.4
5	158.3	158.3
6	110.2	110.2
7	165.7	165.7
8	104.6	104.6
8a	156.2	156.2
1′	73.7	73.7
2′	28.7	28.7
3′	10.1	10.1
1″	21.6	21.6
2″	120.0	119.7
3″	142.6	142.5
4″	39.7	39.7
5″	16.5	16.5
6″	26.4	26.4
7″	123.3	123.3
8″	132.3	132.3
9″	25.7	25.6
10″	17.7	17.7
1‴	206.5	206.3
2‴	46.8	53.6
3‴	18.1	25.6
4‴	13.8	22.6
5‴		22.6
1′-OAc	170.4	170.3
	21.1	21.0

Mammeasin B (**2**) was also isolated as a pale yellow oil with negative optical rotation ($[\alpha]_D^{26}$ −18.4 in $CHCl_3$). The EIMS of **2** showed a molecular ion peak at *m/z* 498 (M^+), and the molecular formula was determined as $C_{29}H_{38}O_7$, by high-resolution EIMS measurement. The ^{1}H and ^{13}C NMR spectroscopic properties of **2** were similar to those of **1**, and showed signals assignable to a primary, two secondary, and three vinyl methyls [δ 1.00 (3H, dd, J = 7.2, 7.2 Hz, H_3-3′), 1.03 (6H, d, J = 6.7 Hz, H_3-4‴, 5‴), 1.60 (3H, d, J = 0.8 Hz, H_3-10″), 1.68 (3H, s, H_3-9″), 1.86 (3H, br s, H_3-5″)], five methylenes {δ 1.68, 1.95 (1H each, m, H_2-2′), 2.12 (4H, m, H_2-4″, 6″), 3.12, 3.17 (1H each, both dd, J = 6.6, 15.9 Hz, H_2-2‴), [3.48 (1H, dd, J = 7.2, 16.7 Hz), 3.52 (1H, dd, J = 7.4, 16.7 Hz), H_2-1″]}, a methine [δ 2.27 (1H, m, H-3‴)], a methine bearing an oxygen function [δ 6.48 (1H, ddd, J = 1.1, 2.6, 7.9 Hz, H-1′)], and three olefinic protons [δ 5.06 (1H, m, H-7″), 5.25 (1H, ddq, J = 7.0, 7.2, 1.0 Hz, H-2″), 6.27 (1H, d, J = 1.1 Hz, H-3)] together with an acetyl group [δ 2.18 (3H, s); δ_C 21.0, 170.3]. The ^{1}H and ^{13}C NMR spectroscopic properties of **2** were superimposable on those of **5**, except for the singals due to the acetyl group. As shown in Figure 2, the connectivity of the acetyl group in **2** was elucidated on the basis of the ^{1}H−^{1}H COSY and HMBC experiments. In addition, **2** was obtained by acetylation of **5** as shown in Figure 3. Thus, the planar structure of **2** was characterized to be as shown. Since the absolute stereochemistry of **5** has been uncharacterized, the absolute configuration of the C-1′ position in **5** was determined in the present study by the modified Mosher's method.[42] Thus, **5** was derived to the corresponding MTPA esters, 1′-(*R*)-MTPA ester (**5a**) and 1′-(*S*)-MTPA ester (**5b**), by treatment with (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetic chloride [(*S*)-(+)-MTPA-Cl] and its (*R*)-isomer, respectively, in pyridine. As shown in Figure 3, signal due to the proton at C-3 in **5b** was observed at lower field compared with that of **5a** [$\Delta\delta$: positive], while the signals due to protons at C-2′ and C-3′ in **5b** were observed at higher field compared with those of **5a** [$\Delta\delta$: negative]. Thus, the absolute configuration at C-1′ of **5** was determined to be *S* orientation. Consequently, the absolute stereostructures of **2** and **5** were elucidated to be as shown in Figure 3.

2.4. Inhibitory effects on LPS-activated NO production in RAW264.7 cells

Inflammation is a systemic response aimed to decrease the toxicity of harmful agents and repair damaged tissue.[43] A key feature

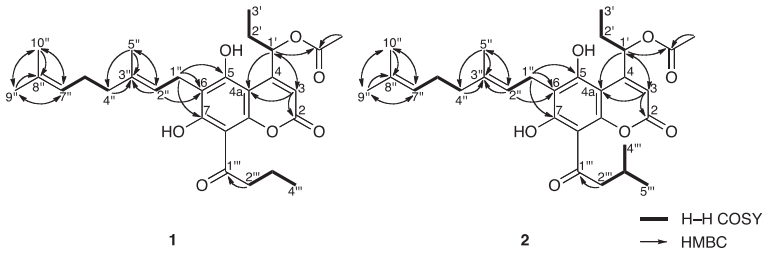

Figure 2. ^{1}H–^{1}H COSY and HMBC correlations of **1** and **2.**

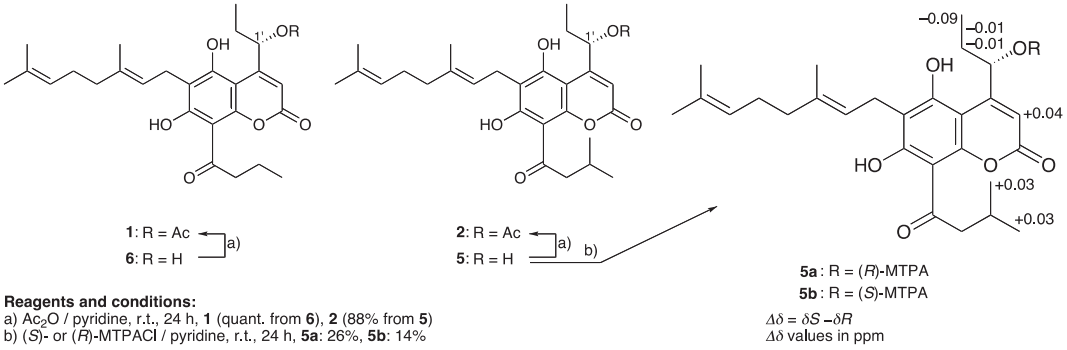

Figure 3. Absolute stereostructures of **1**, **2**, and **5.**

of the inflammatory response is the activation of phagocytic cells involved in host defense, which produce an oxidative burst of reactive oxygen, chlorine, and nitrogen species.[43,44].

Macrophages play major roles in the immunity and inflammatory responses involved in host defense. Once activated, they initiate the production of cytokines, oxygen and nitrogen species, and eicosanoids. In macrophages, bacterial lipopolysaccharide (LPS) is best able to induce the transcription of genes encoding pro-inflammatory proteins. The stimulation results in the release of cytokines and synthesis of enzymes such as inducible nitric oxide synthase (iNOS). The nitric oxide (NO) radical is known to play a central role in inflammatory and immune reactions.[45,46] It is synthesized through the L-arginine pathway by three types of nitric oxide synthase (NOS): endothelial NOS (eNOS), neural NOS (nNOS) and iNOS.[47,48] eNOS and nNOS are constitutively expressed at low levels. Under normal physiological conditions, iNOS is dormant in resting cells, but under pathological conditions, it produces a large amount of NO leading to a 10-fold higher level of eNOS by interferon-γ (IFNγ) and LPS[49,50] and plays a dual role in chronic infection, inflammation and carcinogenesis.[48,51] As a part of our studies to characterize the bioactive components of natural medicines, we have investigated various NO production inhibitors.[10,52–59]

Table 4
Inhibitory effects of the constituents from the flowers of *M. siamensis* on LPS-activated NO production in RAW264.7 cells

	Inhibition (%)[a]					IC_{50} (μM)
	0 μM	1 μM	3 μM	10 μM	30 μM	
Mammeasin A (**1**)	0.0 ± 1.3	27.5 ± 2.4[b]	72.9 ± 10.5[b,c] (56.4 ± 1.9)	88.1 ± 0.9[b,c] (24.1 ± 0.8)	92.0 ± 1.0[b,c] (10.2 ± 0.1)	1.8
Mammeasin B (**2**)	0.0 ± 2.0	0.6 ± 2.9	31.1 ± 6.7[b,c] (60.3 ± 2.4)	64.7 ± 2.2[b,c] (20.2 ± 0.8)	88.2 ± 1.4[b,c] (13.6 ± 0.4)	6.4
Surangin B (**3**)	0.0 ± 1.5	8.5 ± 0.5	35.7 ± 0.6[b,c] (67.1 ± 4.3)	74.3 ± 0.7[b,c] (42.3 ± 1.2)	91.1 ± 1.1[b,c] (18.1 ± 0.4)	5.0
Surangin C (**4**)	0.0 ± 2.9	18.1 ± 0.7[b]	40.8 ± 1.6[b]	56.7 ± 0.8[b,c] (61.7 ± 0.9)	71.3 ± 0.6[b,c] (42.9 ± 0.8)	6.8
Surangin D (**5**)	0.0 ± 1.4	22.7 ± 0.3[b]	41.1 ± 0.8[b]	55.4 ± 1.0[b]	74.0 ± 0.8[b] (104.5 ± 3.4)	6.2
Kayeassamin A (**6**)	0.0 ± 2.0	5.0 ± 4.7	15.0 ± 1.4[b]	29.7 ± 1.8[b]	56.6 ± 1.2[b] (82.4 ± 1.3)	26.6
Kayeassamin E (**7**)	0.0 ± 0.7	38.7 ± 1.0[b]	38.8 ± 0.2[b]	67.1 ± 0.6[b,c] (42.8 ± 2.3)	63.8 ± 1.6[b,c] (42.2 ± 1.3)	6.1
Kayeassamin F (**8**)	0.0 ± 2.6	31.4 ± 0.6[b]	41.3 ± 1.0[b]	60.7 ± 1.8[b,c] (55.2 ± 2.4)	63.1 ± 1.0[b,c] (31.5 ± 0.9)	6.0
Kayeassamin G (**9**)	0.0 ± 1.9	51.0 ± 0.6[b]	55.9 ± 1.2[b]	81.5 ± 0.6[b,c]	72.3 ± 0.3[b,c] (83.7 ± 1.2)	0.8
Mammea A/AC (**10**)	0.0 ± 14.9	0.6 ± 1.9	14.2 ± 3.0	46.9 ± 5.3[b]	67.6 ± 0.9[b] (96.8 ± 1.7)	13.0
Mammea A/AD (**11**)	0.0 ± 1.5	33.7 ± 2.1[b]	83.4 ± 0.8[b,c] (57.2 ± 0.9)	89.4 ± 0.8[b,c] (27.2 ± 0.9)	102.6 ± 0.4[b,c] (10.1 ± 0.5)	1.3
Mammea A/AB cyclo D (**12**)	0.0 ± 1.3			12.9 ± 1.0[b]	18.3 ± 1.1[b]	>30
Mammea A/AC cyclo D (**13**)	0.0 ± 1.7			9.4 ± 1.4	14.3 ± 0.8[b]	>30
Mammea B/AB cyclo D (**14**)	0.0 ± 0.5			10.6 ± 0.5[b]	13.3 ± 1.5[b]	>30
Mammea B/AC cyclo D (**15**)	0.0 ± 0.7			9.9 ± 0.4[b]	13.8 ± 0.5[b]	>30
Mammea E/BB (**16**)	0.0 ± 1.1	−1.5 ± 1.4	24.2 ± 2.0[b,c] (55.3 ± 3.2)	50.5 ± 1.1[b,c] (29.9 ± 2.3)	89.1 ± 0.3[b,c] (11.0 ± 0.3)	7.9
Deacetylmammea E/BC cyclo D (**17**)	0.0 ± 2.4	15.1 ± 1.9	20.2 ± 2.5[b]	33.1 ± 2.0[b]	62.3 ± 1.1[b] (106.9 ± 4.8)	19.7
SB202190	0.0 ± 2.6			36.8 ± 1.7[b]	70.2 ± 0.3[b] (81.4 ± 1.7)	ca. 16
CAPE[59]	0.0 ± 0.7	5.4 ± 2.0	45.7 ± 3.2[b]	98.4 ± 0.8[b,c] (76.4 ± 6.1)	100.3 ± 0.1[b,c] (15.6 ± 0.7)	3.8

[a] Each value represents the mean ± SEM ($N = 4$).
[b] Significantly different from the control, $p < 0.01$.
[c] Cytotoxic effects were observed, and values in parentheses indicate cell viability (%) in MTT assay.

As a continuation of these studies on bioactive constituents of natural medicines, the effects of the coumarin constituents (**1**–**17**) from the flowers of *M. siamensis* on NO production from LPS-activated RAW264.7 cells were examined, and the results were summarized in Table 4. As the result, compounds **1**–**11**, **16**, and **17** showed NO production inhibitory activities (IC_{50} = 0.8–26.6 μM), although half of them (**1**, **2**, **3**, **4**, **7**, **8**, **11**, and **16**) showed considerable cytotoxic effects even at low concentrations in the MMT assays. Among the active compounds, kayeassamin G (**9**) was found to be the most potent, and strongly inhibited the production of NO (IC_{50} 0.8 μM) without notable cytotoxic effects at the effective concentrations (<10 μM). Whereas mammeasin A (**1**, IC_{50} = 1.8 μM) and mammea A/AD (**11**, 1.3 μM) showed considerable cytotoxic effects although they inhibited the NO production to the same extent as **9**. It is noteworthy that the hydroxyl at C-7 was essential for the strong activity. Once the hydroxyl was masked as the 2,2-dimethylchromene moiety (**12**–**15**), they significantly lost the activity. With respect to the substituents at C-4, C-6, and C-8, no distinct relationships were detected between the structures and the activity in the present study. Regardless of the structure of the substituents, all the tested compounds bearing the C-7 hydroxyl showed a certain degree of activity.

2.5. Inhibitory mechanism of 1, 9, and 11 on iNOS induction

NF-κB is a major transcription factor involved in iNOS and TNF-α gene expression. NF-κB is present as an inactive form due to combination with an inhibitory subunit, IκB, which keeps NF-κB in the cytoplasm, thereby preventing activation of the target gene in the nucleus. Cellular signals lead to phosphorylation of IκB following elimination of IκB from NF-κB by proteolytic degradation. Then, the activated-NF-κB is released and translocated into the nucleus to activate transcription of its target genes.[60] Inhibition of iNOS enzyme activity or iNOS induction and inhibition of NF-κB activation may be of therapeutic benefit in various types of inflammation.[48–51,61]

First, the effects of three coumarins (**1**, **9**, and **11**) on iNOS induction were examined. iNOS was detected at 130 kDa after a 20-h incubation with LPS by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE)–Western blot analysis. As shown in Figure 4, iNOS induction in LPS-activated RAW264.7 cells was suppressed by **1**, **9**, and **11**, and it was closely related to their inhibitions of NO production. These results suggested that the three coumarins (**1**, **9**, and **11**) inhibited NO production due to their inhibitory activities against iNOS induction in LPS-activated RAW264.7 cells. However, the NF-κB levels in a nuclear protein fraction were not reduced by **1**, **9**, and **11** (Fig. 5). Therefore, other mechanisms of action were suggested to exist.

The MAPK superfamily of serine/threonine kinases is an important component of cellular signal transduction and also appears to play important roles in inflammatory processes. At least, three MAPK cascades; extracellular signal-regulated kinase (ERK), JNK, and p38 are involved in inflammation.[62–64] Recently, inhibitors of the phosphorylation of JNK, but not of ERK, were reported to reduce LPS-stimulated NO production.[65] In contrast, Hwang et al. reported that the inhibitors of phosphorylation of ERK and p38, but not of JNK, reduced LPS-stimulated NO production.[66] In our previous study,[58] a MAPK-ERK kinase 1 (MEK1) inhibitor (PD98059) acting on the phosphorylation of ERK and an ERK inhibitor (FR180204) showed less inhibition against the production of NO;

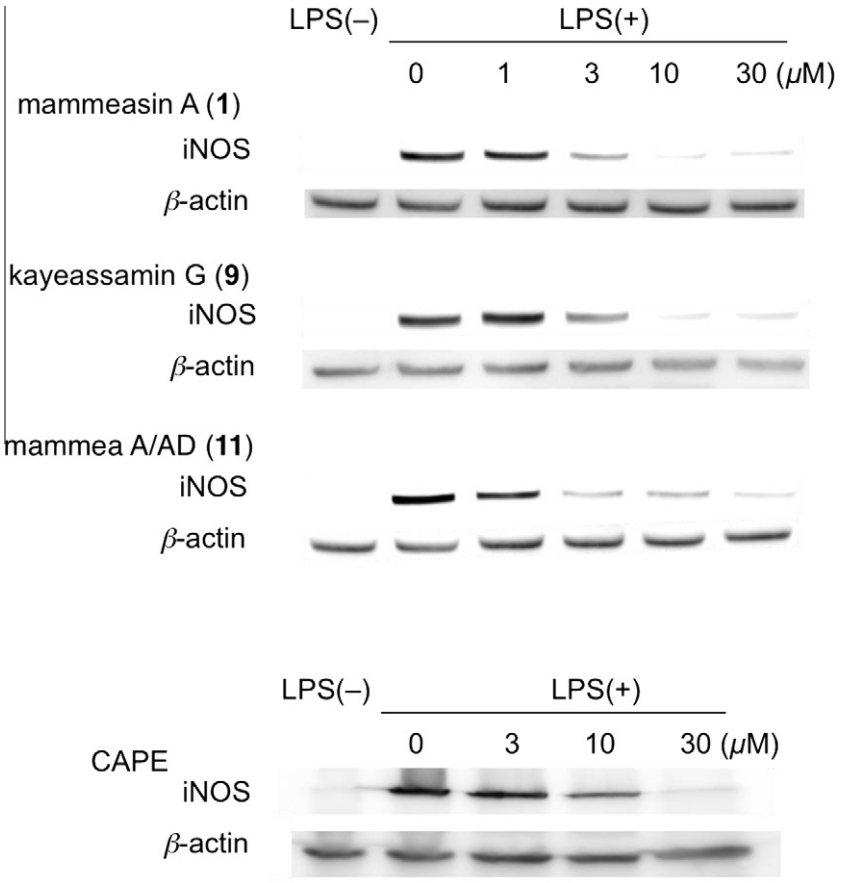

Figure 4. Effects of **1**, **9**, **11**, and CAPE on iNOS protein levels.

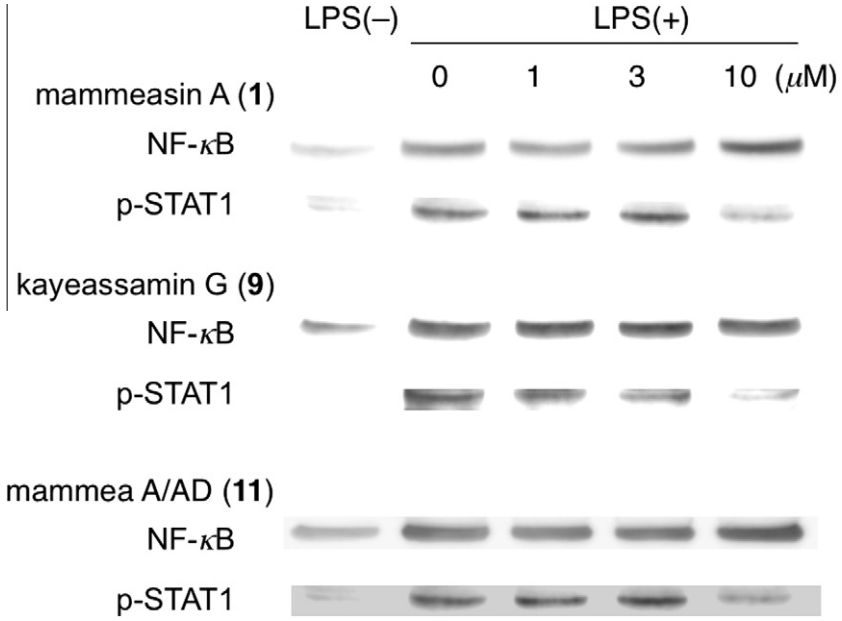

Figure 5. Effects of **1**, **9**, and **11** on nuclear protein levels of NF-κB and p-STAT1.

−2.4 ± 1.1% inhibition at 100 μM and 7.2 ± 2.2% inhibition at 10 μM, respectively. While a JNK inhibitor (SP600125) significantly inhibited the production of NO (74.6 ± 0.6% inhibition at 30 μM, IC_{50} = 17 μM) consistent with the previous report by Lin et al.[65] Furthermore, an inhibitor of phosphorylation of p38, SB202190, showed significant inhibition (IC_{50} = ca. 16 μM) (Table 4). These findings suggested that phosphorylations of JNK and p38 are important steps for expression of iNOS under our experimental conditions. However, in the present study, the active coumarins (**1**, **9**, and **11**) did not inhibit the phosphorylations of these MAP kinases (Fig. 6).

Signal transducer and activator of transcription-1 (STAT1) as well as NF-κB are also important nuclear factor for iNOS expression, and STAT1 is known to activate by IFNβ or IFNγ. Transcription of IFNβ by LPS via Toll-like receptor-4 (TLR4) is reported to be independent of activation of NF-κB, but LPS induce the IFNβ via activation of IFN-regulatory factor-3 (IRF3) in macrophages. The STAT1 is phosphorylated and translocated into the nucleus to activate transcription of its target genes including iNOS.[67–69] Therefore, in the present study, effects of **1**, **9**, and **11**, on phosphorylated STAT1 (p-STAT1) in the nuclear protein fraction were examined. As a result, the protein levels of p-STAT1 in nuclear protein fraction were reduced by **1**, **9**, and **11** in a concentration-dependent manner (Fig. 5). These findings suggest that inhibition of STAT1 activation is mainly involved in the inhibitory effects on iNOS expression by **1**, **9**, and **11**. The detailed mechanism of action including the difference in cytotoxic effects should be studied further.

3. Experimental

3.1. General

The following instruments were used to obtain spectral and physical data: specific rotations, Horiba SEPA-300 digital polarimeter (l = 5 cm); UV spectra, Shimadzu UV-1600 spectrometer; IR spectra, Shimadzu FTIR-8100 spectrometer; ^{1}H NMR spectra, JEOL JNM-ECA700 (700 MHz), JNM-ECA600 (600 MHz), and JNM-ECS400 (400 MHz) spectrometers; ^{13}C NMR spectra, JEOL JNM-ECA700 (175 MHz), JNM-ECA600 (150 MHz), and JNM-ECS400 (100 MHz) spectrometers with tetramethylsilane as an internal standard; EIMS and HREIMS, JEOL JMS-GCMATE mass spectrometer; FABMS and HRFABMS, JEOL JMS-SX 102A mass spectrometer; HPLC detector, Shimadzu SPD-10A UV–VIS detector (230 nm); HPLC column, Cosmosil 5C_{18}-MS-II and πNAP (250 × 4.6 mm i.d. and 250 × 20 mm i.d. for analytical and preparative purposes, respectively).

The following experimental conditions were used for chromatography (CC): highly porous synthetic resin, Diaion HP-20 (Mitsubishi Chemical, Tokyo, Japan); normal-phase silica gel CC, silica gel 60N (Kanto Chemical Co., Ltd., Tokyo, Japan; 63–210 mesh, spherical, neutral); reversed-phase ODS CC, Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd., Aichi, Japan; 100–200 mesh); TLC, pre-coated TLC plates with silica gel 60F_{254} (Merck, 0.25 mm) (normal-phase) and silica gel RP-18 F_{254S} (Merck, 0.25 mm) (reversed-phase); reversed-phase HPTLC, pre-coated TLC plates with silica gel RP-18 WF_{254S} (Merck, 0.25 mm), detection was achieved by spraying with 1% $Ce(SO_4)_2$–10% aqueous H_2SO_4, followed by heating.

3.2. Plant material

The flower of *M. siamensis* was collected in Nakhonsithammarat Province, Thailand on September 2006. The plant material was identified by one of the authors (Y.P.). A voucher specimen (2006.09. Raj-04) of this plant is on file in our laboratory.

3.3. Extraction and isolation

Dried flowers of *M. siamensis* (1.8 kg) were extracted three times with MeOH under reflux for 3 h. Evaporation of the combined extracts under reduced pressure provided a MeOH extract (463.7 g, 25.66%). An aliquot (413.7 g) was partitioned into an EtOAc–H_2O (1:1, v/v) mixture to furnish an EtOAc-soluble fraction (110.34 g, 6.84%) and an aqueous phase. The aqueous phase was subjected to Diaion HP-20 CC (2.4 kg, H_2O → MeOH, twice) to give H_2O-eluted (217.70 g, 13.50%) and MeOH-eluted (68.10 g, 4.22%) fractions, respectively. An aliquot (89.45 g) of the EtOAc-soluble fraction was subjected to normal-phase silica gel CC [3.0 kg, *n*-hexane–EtOAc (10:1 → 7:1 → 5:1, v/v) → EtOAc → MeOH] to give 11 fractions [Fr. 1 (3.05 g), Fr. 2 (2.86 g), Fr. 3 (11.71 g), Fr. 4 (1.62 g), Fr. 5 (4.15 g), Fr. 6 (6.29 g), Fr. 7 (2.21 g), Fr. 8 (2.94 g), Fr. 9 (10.23 g), Fr. 10 (11.17 g), and Fr. 11 (21.35 g)]. The fraction

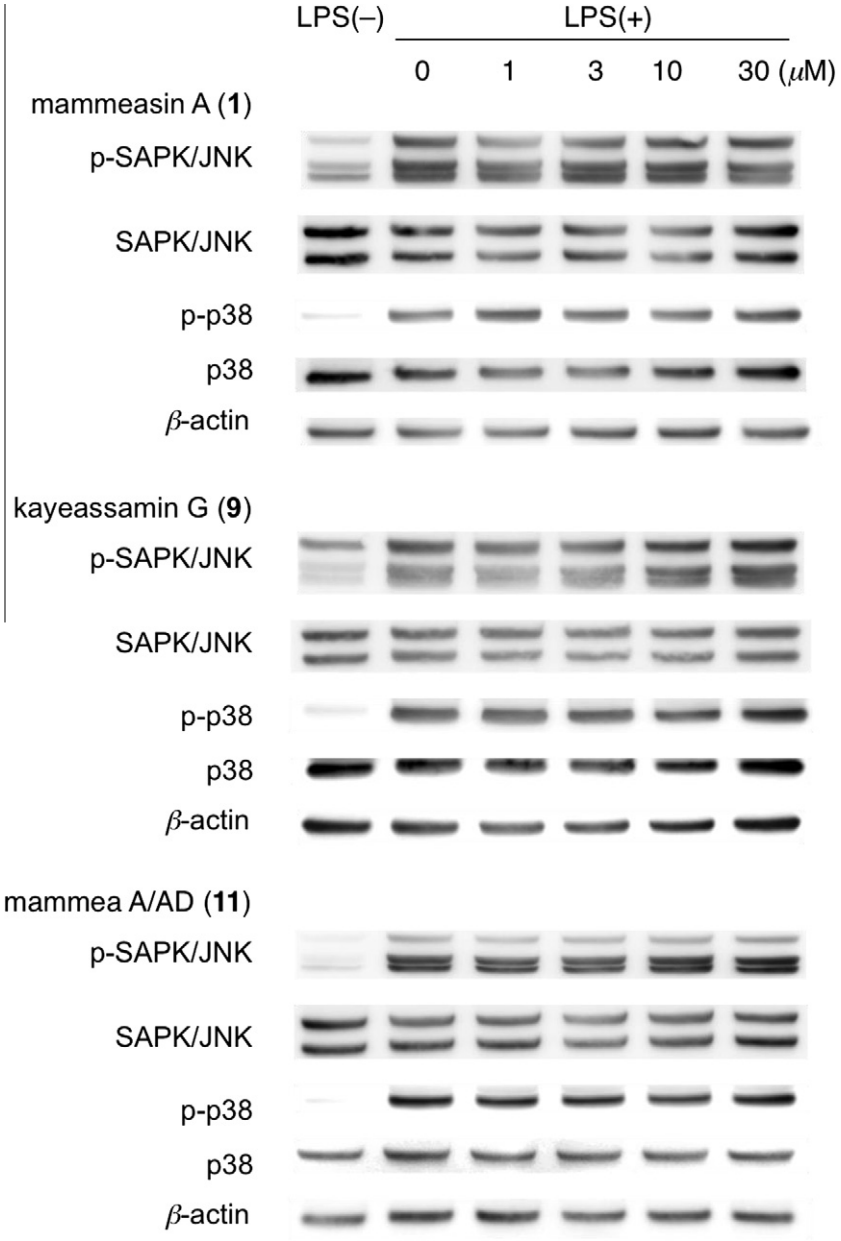

Figure 6. Effects of **1**, **9**, and **11** on SAPK/JNK, p-SPK/JNK, p-p38, and p38 protein levels.

3 (11.71 g) was subjected to reversed-phase ODS CC [340 g, MeOH–H_2O (90:10 → 95:5, v/v) → MeOH → acetone] to afford seven fractions [Fr. 3–1 (49.3 mg), Fr. 3–2 (3109.8 mg), Fr. 3–3 (4679.3 mg), Fr. 3–4 (1089.1 mg), Fr. 3–5 (1034.0 mg), Fr. 3–6 (280.8 mg), and Fr. 3–7 (85.6 mg)]. The fraction 3–4 (497.9 mg) was purified by HPLC [Cosmosil 5C_{18}-MS-II, MeOH–1% aqueous AcOH (90:10, v/v)] to give mammea A/AB cyclo D (**12**, 27.8 mg, 0.0047%), mammea A/AC cyclo D (**13**, 46.0 mg, 0.0077%), mammea B/AB cyclo D (**14**, 9.8 mg, 0.0016%), and mammea B/AC cyclo D (**15**, 32.9 mg, 0.0055%). The fraction 3–5 (502.8 mg) was purified by HPLC [Cosmosil 5C_{18}-MS-II, MeOH–1% aqueous AcOH (95:5, v/v)] to give surangins C (**4**, 8.2 mg, 0.0013%) and D (**5**, 24.9 mg, 0.0039%), and β-amyrin (46.0 mg, 0.0072%). The fraction 6 (6.29 g) was subjected to reversed-phase ODS CC [200 g, MeOH–H_2O (80:20 → 90:10 → 95:5, v/v) → MeOH → acetone] to afford 10 fractions [Fr. 6–1 (44.7 mg), Fr. 6–2 (157.2 mg), Fr. 6–3 (928.8 mg), Fr. 6–4 (3117.0 mg), Fr. 6–5 (128.8 mg), Fr. 6–6 (487.1 mg), Fr. 6–7 (230.8 mg), Fr. 6–8 (280.5 mg), Fr. 6–9 (102.9 mg), and Fr. 6–10 (96.5 mg)]. The fraction 6–3 (514.6 mg) was purified by HPLC [Cosmosil 5C_{18}-MS-II, MeOH–1% aqueous AcOH (80:20, v/v)] to give mammea A/AC (**10**, 35.6 mg, 0.0049%), mammea A/AD (**11**, 15.8 mg, 0.0022%), and mammea E/BB (**16**, 140.1 mg, 0.0194%). The fraction 6–4 (536.2 mg) was purified by HPLC [Cosmosil 5C_{18}-MS-II, MeOH–1% aqueous AcOH (90:10, v/v)] to give mammeasins A (**1**, 65.8 mg, 0.0293%) and B (**2**, 21.6 mg, 0.0096%), surangin B (**3**, 58.2 mg, 0.0259%), and **10** (112.6 mg, 0.0501%). The fraction 6–5 (128.8 mg) was purified by HPLC [Cosmosil 5C_{18}-MS-II, MeOH–1% aqueous AcOH (90:10, v/v)] to give **2** (24.1 mg, 0.0019%) and **3** (15.1 mg, 0.0012%). The fraction 6–6 (487.1 mg) was purified by HPLC [Cosmosil 5C_{18}-MS-II, MeOH–1% aqueous AcOH (90:10, v/v)] to give **10** (6.0 mg, 0.00050%). The fraction 9 (10.23 g) was subjected to reversed-phase ODS CC [300 g, MeOH–H_2O (80:20 → 90:10, v/v) → MeOH → acetone] to afford five fractions [Fr. 9–1 (2809.0 mg), Fr. 9–2 (5678.0 mg), Fr. 9–3 (385.9 mg), Fr. 9–4 (422.0 mg), and Fr. 9–5 (51.9 mg)]. The fraction 9–1 (544.5 mg) was purified by HPLC [Cosmosil 5C_{18}-MS-II, MeOH–1% aqueous AcOH (85:15, v/v)] to give kayeassamins E (**7**, 28.6 mg, 0.0113%),

F (**8**, 98.7 mg, 0.0390%), and G (**9**, 43.4 mg, 0.0171%), deacetylmammea E/BC cyclo D (**17**, 18.6 mg, 0.0073%), and benzoic acid (10.9 mg, 0.0043%). The fraction 9–2 (526.2 mg) was purified by HPLC [Cosmosil 5C_{18}-MS-II, MeOH–1% aqueous AcOH (90:10, v/v)] to give **4** (63.6 mg, 0.0525%), **5** (66.7 mg, 0.0551%), and kayeassamin A (**6**, 68.1 mg, 0.0562%). The fraction 9–3 (385.9 mg) was purified by HPLC [Cosmosil 5C_{18}-MS-II, MeOH–1% aqueous AcOH (90:10, v/v)] to give **4** (43.1 mg, 0.0033%), **5** (54.3 mg, 0.0042%), and **6** (20.5 mg, 0.0016%).

3.3.1. Mammeasin A (1)

Pale yellow oil, $[\alpha]_D^{27}$ −29.7 (*c* 1.08, MeOH), $[\alpha]_D^{27}$ −25.4 (*c* 1.08, $CHCl_3$). EIMS (*m/z*, %): 484 (M^+, 7), 301 (100). High-resolution EIMS: Anal. Calcd for $C_{28}H_{36}O_7$ (M^+): 484.2461. Found: 484.2466. UV [MeOH, nm (log ε)]: 223 (4.62), 330 (4.65). IR (film): 3503, 1748, 1717, 1609, 1458, 1399, 1233, 1194, 1136 cm^{-1}. ^1H NMR (700 MHz, $CDCl_3$) δ: given in Table 2. ^{13}C NMR (175 MHz, $CDCl_3$) δ_C: given in Table 3.

3.3.2. Mammeasin B (2)

Pale yellow oil, $[\alpha]_D^{27}$ −19.2 (*c* 0.30, MeOH), $[\alpha]_D^{26}$ −18.4 (*c* 0.30, $CHCl_3$). EIMS (*m/z*, %): 498 (M^+, 8), 315 (100). High-resolution EIMS: Anal. Calcd for $C_{29}H_{38}O_7$ (M^+): 498.2618. Found: 498.2622. UV [MeOH, nm (log ε)]: 223 (4.48), 331 (4.58). IR (film): 3500, 1744, 1719, 1607, 1458, 1404, 1233, 1196, 1132 cm^{-1}. ^1H NMR (700 MHz, $CDCl_3$) δ: given in Table 2. ^{13}C NMR (175 MHz, $CDCl_3$) δ_C: given in Table 3.

3.4. Acetylation of surangin D (5) and kayeassamin A (6)

To a solution of **5** (4.5 mg) in pyridine (1.0 mL) was added Ac_2O (0.8 mL), and the mixture was stirred at room temperature for 24 h. The reaction mixture was poured into water and the resulting mixture was extracted with EtOAc. The extract was washed with 5% aqueous HCl, saturated aqueous $NaHCO_3$, and brine, then dried over anhydrous $MgSO_4$ and filtered. Removal of the solvent under reduced pressure gave a pale yellow oil, which was purified by HPLC [Cosmosil 5C_{18}-MS-II, MeOH–1% aqueous AcOH (90:10, v/v)] to furnish **1** (4.3 mg, 88%). According to the similar procedure, **2** (4.2 mg, quant.) was obtained from **6** (3.6 mg).

3.5. Preparation of (*R*)-MTPA ester (5a) and (*S*)-MTPA ester (5b) from surangin D (5)

A solution of **5** (2.3 mg) in pyridine (1.0 mL) was treated with (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetic chloride [(*S*)-(+)-MTPA-Cl, 7.5 μL] and the mixture was stirred at room temperature for 24 h. The reaction mixture was poured into ice-water and extracted with EtOAc. The extract was successively washed with 5% aqueous HCl, saturated aqueous $NaHCO_3$, and brine, then dried over anhydrous $MgSO_4$ and filtered. Removal of the solvent from the filtrate under reduced pressure furnished a pale yellow oil, which was purified by HPLC [Cosmosil 5C_{18}-MS-II, MeOH–1% aqueous AcOH (90:10, v/v)] to give the (*R*)-MTPA ester derivative (**5a**, 0.9 mg, 26%). According to the similar procedure, **5b** (0.5 mg, 14%) was obtained from **5** (2.5 mg) by using (*R*)-(−)-MTPA-Cl (7.5 μL).

3.5.1. Compound 5a

^1H NMR (400 MHz, $CDCl_3$) δ: 1.03 (6H, d, *J* = 6.8 Hz, H_3-4‴, 5‴), 1.05 (3H, t, *J* = 6.8 Hz, H_3-1″), 1.61 (3H, br s, H_3-10″), 1.68 (3H, br s, H_3-9″), 1.77, 2.01 (1H each, both m, H_2-2′), 1.88 (3H, br s, H_3-5″), 3.49 (3H, s, –OCH_3), 5.07 (1H, br t-like, H-7″), 5.27 (1H, br dd-like, H-2″), 5.63 (1H, dd-like, H-1′), 5.83 (1H, br s, H-3) [7.45 (3H, m), 7.52 (2H, m), Ph-H].

3.5.2. Compound 5b

^1H NMR (400 MHz, $CDCl_3$) δ: 1.04 (3H, t, *J* = 6.6 Hz, H_3-1″), 1.06 (6H, d, *J* = 6.6 Hz, H_3-4‴, 5‴), 1.60 (3H, br s, H_3-10″), 1.64 (3H, br s, H_3-9″), 1.76 (3H, br s, H_3-5″), 1.76, 2.00 (1H each, both m, H_2-2′), 3.49 (3H, s, -OCH_3), 5.06 (1H, br t-like, H-7″), 5.27 (1H, br dd-like, H-2″), 5.64 (1H, dd-like, H-1′), 5.88 (1H, br s, H-3) [7.46 (3H, m), 7.54 (2H, m), Ph-H].

3.6. Bioassay

3.6.1. Cell culture

The murine macrophage cells (RAW264.7, ATCC No. TIB-71) were obtained from Dainippon Pharmaceutical, Osaka, Japan and cultured in Dulbecco's modified Eagle's medium (DMEM, containing 4500 mg/L glucose) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL) (Sigma Chemical Co., St. Louis, MO, USA). The cells were incubated at 37 °C in 5% CO_2/air.

3.6.2. Effects on production of NO in LPS-stimulated macrophage RAW264.7 cells

The total amount of nitrite in a medium is used as an indicator of NO synthesis.[59] The screening test for NO production using RAW264.7 cells was described previously.[58,59] Briefly, RAW264.7 cells were cultured in DMEM, and the suspension of the cells were seeded into a 96-well microplate at 2.5×10^5 cells/100 μL/well. After 6 h, nonadherent cells were removed by washing with PBS, and the adherent cells were cultured in 100 μL of fresh medium containing the test compounds for 10 min, and then 100 μL of the medium containing LPS (from *Escherichia coli*, 055: B5, Sigma) was added to stimulate the cells for 18 h (final concentration of LPS was 10 μg/mL). The nitrite concentration was measured from the supernatant by Griess reaction. Inhibition (%) was calculated using the following formula and the IC_{50} was determined graphically (*N* = 4). Caffeic acid phenethyl ester (CAPE), an inhibitor of NF-κB activation, was used as a reference compound.[59,60].

$$\text{Inhibition } (\%) = (A - B)/(A - C) \times 100$$

A−*C*: nitrite concentration (μM);
A: LPS (+), Sample (−); *B*: LPS (+), Sample (+); *C*: LPS (−), Sample (−).

3.6.3. Determination of cytotoxic effects

Cytotoxicity was evaluated by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*- tetrazolium bromide (MTT) colorimetric assay according to the previous reported conditions.[59] Briefly, RAW264.7 cells were cultured in DMEM, and the suspension of the cells were seeded into a 96-well microplate at 1.0×10^5 cells/200 μL/well. After 6 h, nonadherent cells were removed by washing with PBS, and the adherent cells were cultured in 100 μL of fresh medium containing the test compounds for 18 h. An aliquot of the medium (100 μL) was removed and MTT (10 μL, 5 mg/mL in PBS) solution was added. After a 2-h incubation at 37 °C, the medium was removed, and isopropanol containing 0.04 M HCl was added to dissolve the formazan produced in the cells. The optical density (OD) of the formazan solution was measured with a microplate reader at 570 nm (reference: 655 nm).

3.6.4. SDS–PAGE and Western blot analysis

RAW264.7 cells (5.0×10^6 cells/2 mL/well) were seeded into a 6-well multiplate and allowed to adhere for 6 h at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were then washed with PBS, 1 mL of DMEM containing various concentrations of the

samples was added to each well, and after incubation for 10 min, 1 mL of DMEM containing LPS was added to stimulate the cells for 30 min or 12 h (final concentration of LPS, 10 μg/mL). The adhered cells were collected using a cell scraper in a lysis buffer (15 mM NaCl, 1 mM Tris, 1% Triton-X, 0.2 mM EGTA, 2.8 mM β-glycerophosphate) containing protease inhibitor cocktail (Thermo Scientific) and phosphatase inhibitor cocktail (PhosSTOP, Roche). Then, cells were disrupted three times (Microson™ ultrasonic cell disruptor, USA) for 30 s, and centrifuged at 10,000 rpm for 10 min. Protein concentrations of cell lysates were determined using the BCA™ protein assay kit. For protein sample preparation; 100 μL of supernatant was transferred to 50 μL of a dissolving agent (0.9 mM EGTA, 200 mM SDS, 2.8 mM Tris, 8% glycerol, 0.03% bromophenol blue, 6% mercaptoethanol). Then, the samples were heated in boiling water for 5 min. After cooling down, the samples were kept at −80 °C until used.[58,59]

Nuclear protein fraction was extracted 30 min after the stimulation with LPS using Nuclear and Cytoplasmic Extraction Reagent (Thermo Scientific) according to the manufacturer's instructions. The nuclear protein solution was concentrated using a Centrifugal Filter Units (Millipore Co., Ltd.) and applied for the electrophoresis.

Equivalent amounts of protein (50 μg of protein/lane for iNOS and β-actin, 25 μg of protein/lane for others) were electrophoresed in 10% SDS–polyacrylamide gels (Bio-Rad ready gel J) and transferred onto a polyvinylidene difluoride (PVDF) membranes (Bio-Rad, HC, USA). The membrane was then soaked in Tris-buffered saline containing 0.1% Tween 20 (T-TBS) with gentle shaking for 10 min, three times. For the blocking of the nonspecific sites, the membrane was soaked in Blocking One-P (for phosphorylated proteins: p-ERK1/2, p-JNK, p-p38, p-STAT1; Nacalai Tesque, Japan) or Blocking One (for others: iNOS, ERK1/2, JNK, p38, NF-κB, β-actin) by shaking for 0.5 h. The membrane was rinsed with T-TBS and incubated with specific primary antibodies: p-ERK1/2, ERK1/2, JNK1/2, p38, p-p38, NF-κB p65, STAT1, p-STAT1 (Ser 727), iNOS and β-actin (1:1000, Cell Signaling Technology). After incubation for 1 h at rt, the membrane was rinsed in T-TBS, and incubated in secondary antibodies (HRP-conjugated goat anti-mouse and anti-rabbit IgG, 1:5000) in an immunoreaction enhancer solution (Can Get Signal, Toyobo, Japan) for 1 h. Next, the membrane was shaken in T-TBS for 10 min, three times. The proteins were detected using an enhanced chemiluminescence (ECL) plus Western blotting detection system (Amersham™ GE Healthcare, Biosciences). The images of membranes were recorded using a luminescent image analyzer LAS-4000 mini (Fuji film, Japan). In our preliminary experiments, the amount of iNOS protein markedly increased 6 h after the treatment with LPS and remained higher after 24 h, and levels of p-ERK, p-JNK, p-p38, and p-STAT1 increased after 10 or 30 min and remained high for several hours (data not shown). Therefore, the effects of test compounds on iNOS protein levels were determined 12 h after the stimulation, and on other protein levels were determined 0.5 h after the stimulation.

3.7. Statistical analysis

All data are expressed as means ± SEM. The data analysis was performed with an one-way analysis of variance (1-ANOVA), followed by Dunnett's test. Probability (p) value of less than 0.05 was considered to be significant.

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