Antiallergic Agents from Natural Sources 9.[1] Inhibition of Nitric Oxide Production by Novel Chalcone Derivatives from *Mallotus philippinensis* (Euphorbiaceae)

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Three novel chalcone derivatives, mallotophilippens C (1), D (2) and E (3) were isolated from the fruits of *Mallotus philippinensis* MUELL. ARG. These compounds were identified, using chemical and spectral data, as 1-[6-(3,7-dimethyl-octa-2,6-dienyl)-5,7-dihydroxy-2,2-dimethyl-2*H*-chromen-8-yl]-3-(4-hydroxy-phenyl)-propenone, 3-(3,4-dihydroxy-phenyl)-1-[6-(3,7-dimethyl-octa-2,6-dienyl)-5,7-dihydroxy-2,2-dimethyl-2*H*-chromen-8-yl]-propenone and 1-[5,7-dihydroxy-2-methyl-6-(3-methyl-but-2-enyl)-2-(4-methyl-pent-3-enyl)-2*H*-chromen-8-yl]-3-(3,4-dihydroxy-phenyl)-propenone, respectively. They inhibited nitric oxide (NO) production and inducible NO synthase (iNOS) gene expression by a murine macrophage-like cell line (RAW 264.7), which was activated by lipopolysaccharide (LPS) and recombinant mouse interferon-γ (IFN-γ). Furthermore, they downregulated cyclooxygenase-2 (COX-2) gene, interleukin-6 (IL-6) gene and interleukin-1β (IL-1β) gene expression. These results suggest that they have anti-inflammatory and immunoregulatory effects.

Key words *Mallotus philippinensis*; chalcone; Euphorbiaceae; nitric oxide

Mallotus philippinensis MUELL. ARG. (Euphorbiaceae) is widely distributed in the forestes of Kelara. The Granular hairs on the surface of fruits is called Kamala. It has been used as a drug and dye. Earlier investigations on Kamala dye have afforded various flavonoides.[2—5] Kamala powder is used as an anthelmintic and cathartic in traditional medicine.[6]

Macrophages play an important role in non-specific host defense mechanisms.[7] In macrophages, bacterial LPS alone or in combination with cytokines like interferon-γ (IFN-γ) is one of the best-characterized stimuli to induce the transcription of genes encoding pro-inflammatory proteins, resulting in cytokine release and synthesis of enzymes such as cyclooxygenase-2 (COX-2)[8] and inducible nitric oxide synthase (iNOS).[9] NO is a short-lived bioactive molecule that participate in the physiology and pathophysiology of many systems.[10] NO is synthesized *in vivo* from L-arginine by NOS with NADPH and oxygen as cosubstrates.[11] Large amounts of NO may lead to tissue damage. In inflammatory disease such as rheumatoid arthritis, excessive NO production by activated macrophages has been observed. Therefore, it would be valuable to develop potent and selective inhibitors of NO for potential therapeutic use.

Previously, we reported that mallotophilippens A and B, phloroglucinol derivatives from *M. philippinensis*, suppressed the NO production and iNOS gene expression.[3]

In this work, we isolated three novel chalcone derivatives, **1**, **2** and **3** (Fig. 1), from the hexane-soluble fraction and investigated the inhibitory effects of those compounds on NO generation from activated macrophages.

Results and Discussion

Compound **1** had the molecular formula $C_{30}H_{34}O_5$ as found from its high resolution (HR)-EI-MS (*m/z* 474.2405), and its IR spectrum showed hydroxy group (3371 cm^{-1}) absorption. The UV spectrum of **1** showed absorptions at 370, 287, 206 nm. Support for a chalcone structure for **1** was provided by the signals observed in its ^{1}H-NMR (Table 1). In addition to two *trans*-olefinic protons at δ 7.74, and δ 8.01 (J=15.7 Hz), readily assigned to the β and α positions, respectively, the ^{1}H-NMR spectrum displayed signals for four aromatic protons showing the A_2B_2 pattern typical of a 4-substituted A ring. The presence of two *cis*-coupled olefinic protons, together with two methyl group resonances and a quaternary carbon at δ 77.7 in the ^{13}C-NMR spectrum (Table 1), indicated a 2,2-dimethylpyran system.

The arrangement of the substituents and the placement of the pyran at the 4a′/8a′ positions were established from the results of the heteronuclear multiple bond connectivity (HMBC) experiments (Fig. 2). The chelated hydroxyl group in ring B showed the expected ^2J correlation between its proton and C-7′ (δ 163.8). The HMBC experiment showed a clear ^3J correlation between H-4′ and C-5′ (δ 157.7) and between H-3′ and C-4a′ (δ 102.4); while the chelated

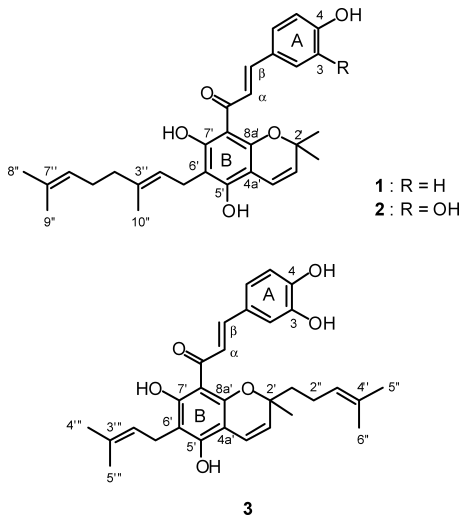

Fig. 1. Chalcone Derivatives from *Mallotus philippinensis*

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Table 1. ^{1}H- and ^{13}C-NMR Spectral Data for Compounds **1** and **2** in $CDCl_3$

Position	**1**		**2**	
	δ_H	δ_C	δ_H	δ_C
1		128.6 s[a)]		129.0 s[a)]
2	7.51 d (8.5)	130.2 d	7.11 d (1.9)	114.9 d
3	6.87 d (8.5)	116.2 d		144.0 s
4		157.5 s		146.2 s
5	6.87 d (8.5)	116.2 d	6.89 d (8.2)	115.6 d
6	7.51 d (8.5)	130.2 d	7.08 dd (1.9, 8.2)	122.3 d
α	8.01 d (15.7)	125.5 d	7.96 d (15.7)	125.6 d
β	7.74 d (15.7)	142.0 d	7.65 d (15.7)	142.4 d
C=O		193.1 s		193.2 s
2′		77.7 s		77.8 s
3′	5.47 d (9.9)	124.8 d	5.46 d (9.9)	124.9 d
4′	6.58 d (9.9)	116.8 d	6.57 d (9.9)	116.7 d
4a′		102.4 s		102.4 s
5′		157.7 s		157.9 s
6′		105.5 s		105.6 s
7′		163.8 s		163.7 s
8′		106.2 s		106.2 s
8a′		154.7 s		154.8 s
1″	3.43 d (7.1)	21.7 t	3.43 d (7.1)	21.7 t
2″	5.31 t (7.1)	121.8 d	5.3 t (7.1)	121.8 d
3″		140.4 s		140.4 s
4″	2.12 t (7.1)	39.7 t	2.12 t (7.1)	39.7 t
5″	2.12 m	26.2 t	2.12 m	26.2 t
6″	5.05 t (7.1)	123.6 d	5.05 t (7.1)	123.6 d
7″		132.4 s		132.4 s
8″	1.69 s	25.7 q	1.69 s	25.7 q
9″	1.61 s	17.7 q	1.61 s	17.7 q
10″	1.83 s	16.2 q	1.82 s	16.2 q
Me-2′	1.54 s	28.0 q	1.53 s	28.0 q
4-OH	5.45 s			
5′-OH	6.43 s			
7′-OH	14.6 s		14.6 s	

Chemical shifts are given in ppm; multiplicities and coupling constant J (parentheses) in Hz. *a*) The multiplicities of carbon signals were determined using the DEPT method, and are indicated as s, d, t and q.

hydroxyl proton showed the expected ^{3}J correlation with C-6′ (δ 105.5) and C-8′ (δ 106.2). The ^{1}H-NMR spectrum of **1** showed signals for a geranyl group: three vinyl methyl signals (see Table 1), two benzylic proton signals (δ 3.43), a broad singlet of four protons at δ 2.12 and a pair of triplet [δ 5.31 (1H) and δ 5.05 (1H)]. From these spectral data, the structure of **1** was determined to be 1-[6-(3,7-dimethyl-octa-2,6-dienyl)-5,7-dihydroxy-2,2-dimethyl-2*H*-chromen-8-yl]-3-(4-hydroxy-phenyl)-propenone and named mallotophilippen C (**1**).

Compound **2** had the molecular formula $C_{30}H_{34}O_6$ as found from its HR-EI-MS (*m*/*z* 490.2347), and its IR spectrum showed hydroxy group (3367 cm^{-1}) absorption. The UV spectrum showed maxima at 382, 266, 208 nm. The ^{1}H-NMR spectrum of **2** was very similar to that of **1** (Table 1). The spectrum displayed signals for three aromatic protons showing the ABX pattern typieal of 3,4-substitueted A ring. HMBC correlation is presented in Fig. 2. From these spectral data, the structure of **2** was determined to be 3-(3,4-dihydroxy-phenyl)-1-[6-(3,7-dimethyl-octa-2,6-dienyl)-5,7-dihydroxy-2,2-dimethyl-2*H*-chromen-8-yl]-propenone and named mallotophilippen D (**2**).

Compound **3** had the molecular formula $C_{30}H_{34}O_6$ as found from its HR-EI-MS (*m*/*z* 490.2350), and its IR spectrum showed hydroxy group (3362 cm^{-1}) absorption. The UV spectrum showed maxima at 382, 269, 207 nm.

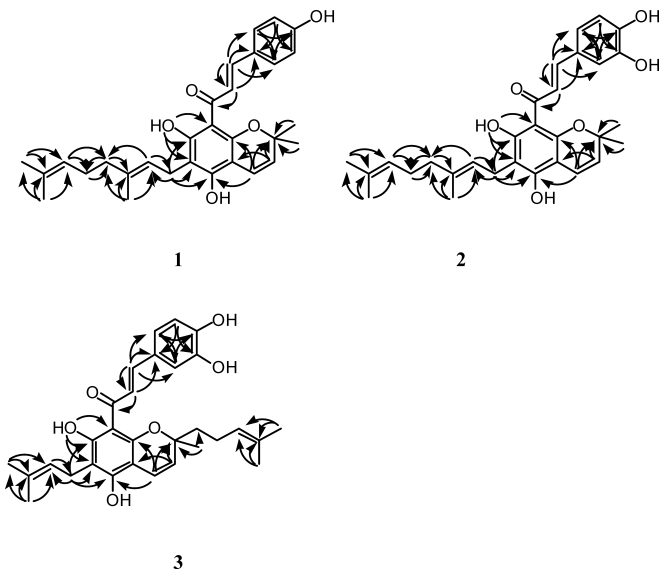

Fig. 2. HMBC Correlation of Compounds **1**—**3**

Compound **3** had a chiral carbon. But, the optical rotation of **3** showed ±0°. Therefore, **3** was indicated racemic mixture. The ^{1}H- and ^{13}C-NMR of spectrum of **3** showed also a saturated chromenochalcone. The ^{1}H-NMR spectrum of **3** showed signals for a isoprenyl group: two vinyl methyl

Table 2. ^{1}H- and ^{13}C-NMR Spectral Data for Compound **3** in $CDCl_3$

Position	δ_H	δ_C
1		128.9 s[a)]
2	7.08 br s	114.2 d
3		143.7 s
4		146.2 s
5	6.88 d (7.1)	115.6 d
6	7.06 br d	123.0 d
α	7.94 d (15.7)	125.8 d
β	7.65 d (15.7)	142.2 d
C=O		193.1 s
2′		80.4 s
3′	5.43 d (9.9)	123.6 d
4′	6.62 d (9.9)	117.3 d
4a′		102.0 s
5′		157.5 s
6′		105.3 s
7′		163.7 s
8′		106.1 s
8a′		155.0 s
1″	1.67—1.91 m	41.4 t
2″	2.06—2.23 m	23.2 t
3″	5.14 t (6.6)	124.0 d
4″		132.8 s
5″	1.45 s	25.6 q
6″	1.52 t	17.7 q
1‴	3.41 d (7.1)	21.7 t
2‴	5.29 t (7.1)	121.8 d
3‴		136.6 s
4‴	1.79 s	25.9 q
5‴	1.84 s	17.9 q
Me-2′	1.45 s	26.6 q
7′-OH	14.5 s	

Chemical shifts are given in ppm; multiplicities and coupling constant *J* (parentheses) in Hz. *a*) The multiplicities of carbon signals were determined using the DEPT method, and are indicated as s, d, t and q.

signals, two benzylic proton signals, a triplet proton. And more, a 4-methyl-pent-3-enyl side chain appeareared at two methyl protons, one methine proton and two methylene protons (Table 2). HMBC correlation is presented in Fig. 2. From these spectral data, the structure of **3** was determined to be 1-[5,7-dihydroxy-2-methyl-6-(3-methyl-but-2-enyl)-2-(4-methyl-pent-3-enyl)-2*H*-chromen-8-yl]-3-(3,4-dihydroxyphenyl)-propenone and named mallotophilippen E (**3**).

We have demonstrated that three novel chalcone derivatives inhibit the production of NO induced by LPS and IFN-γ in murine macrophage-like cell line, RAW 264.7. They inhibited NO production dose-dependently with IC_{50} values in the μM range (Table 3). Compounds **1** and **2** exhibited stronger inhibition than quercetin, which was reported previously to inhibit NO production from LPS activated RAW 264.7 cells.[12] Compound **3** showed lower inhibition than quercetin. These compounds up to 30 μg/ml did not affect the cellular viability, as assessed by mitochondrial reduction of MTT after 20 h treatment (data not shown), indicating that they were not cytotoxic. The mechanism for the inhibition on NO production was due to suppression of the expression of iNOS mRNA as shown semiquantitatively by RT-PCR. Compounds **1**, **2** and **3** inhibited dose-dependently the induction of iNOS gene expression (Fig. 3).

The cellular mechanism may be several possibilities. We postulated that these compounds may have effects up-stream of signal transduction such as activation/translocation of transcription factors including nuclear factor kappa B

Table 3. Inhibitory Effect of Compounds **1**—**3** on NO Production by RAW 264.7 Stimulated with LPS/IFN-γ

Compound	IC_{50} μg/ml (μM)
1	3.6 (7.6)
2	4.7 (9.5)
3	18.9 (38.6)
Quercetin[a)]	8.1 (26.8)

a) Positive control.

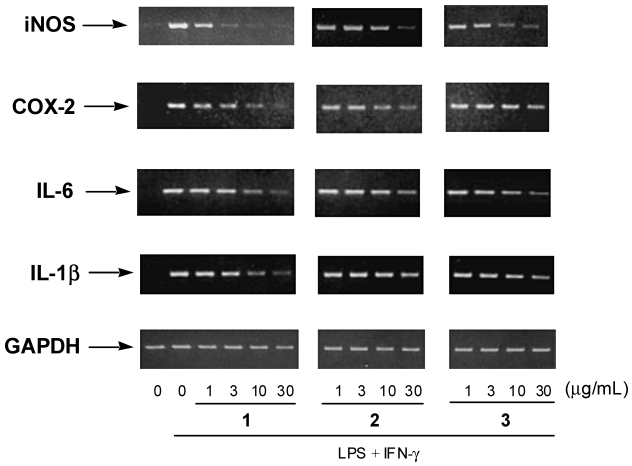

Fig. 3. RT-PCR Analysis of Compounds **1**—**3**

(NF-κB). At the gene level, the promoter of the mouse gene encoding COX-2, IL-6, IL-1 contains consensus sequences that bind several transcription factors including the NF-κB and IFN regulatory factor. We investigated effect of novel chalcone derivatives on COX-2, IL-6 and IL-1β mRNA expression. They inhibited almost dose-dependently COX-2, IL-6 and IL-1β mRNA expression (Fig. 3).

In conclusion, three novel chalcone derivatives from *M. philippinensis* inhibited NO production from IFN-γ/LPS activated RAW 264.7 cells. Furthermore, they inhibited iNOS, COX-2, IL-6 and IL-1β mRNA expression. On the basis of the current results, we hypothesized that the main inhibitory mechanism of these compounds may be the inactivation of NF-κB. It is known that iNOS participates in the production of pathological changes in adjuvant arthritis and its inhibition would attenuate these changes.[13] There is evidence of an increased NO production in infectious gastroenteritis and rheumatoid arthritis.[14,15] Three novel chalcone derivatives may be a candidate for drugs to treat diseases due to iNOS over-expression.

Experimentalf

General Procedures UV spectra were obtained by a Shimadzu UV-160 spectrophotometer. The NMR spectra were taken on a JOEL JNM GX-400 instrument (400 MHz for ^{1}H-NMR), and chemical shifts were given in ppm relative to internal tetramethylsilane (TMS). Mass spectra were obtained on a Hitachi M-80B spectrometer. Column chromatography was carried out using silica gel (Wako gel C-300, Wako Pure Chemical Ind., Ltd.). TLC was performed on Merck TLC plates (0.25 mm thickness) and compounds were visualized by 5% (v/v) H_2SO_4 in ethanol solution.

Extraction and Isolation Air-dried capsules (500 g) were extracted with acetone at room temperature and yielded 53.4 g of extract. The extract was suspended in water. And the extrct was partitioned with *n*-hexane, ethyl acetate and *n*-butanol, successively. The *n*-hexane fraction was submitted to CC (Sephadex LH-20, $CHCl_3$: MeOH, 1 : 1), gave 4 pooled frs. (A1—A4).

Fraction A3 (29.1 g), submitted to CC (silica-gel) and eluted with *n*-hexane–$CHCl_3$ at increasing polarities to give 6 pooled frs. (B1—B6). Fraction B3 (10.6 g), submitted to CC (silica gel, *n*-hexane : $CHCl_3$), followed by HPLC (SIL-06 column, 100×20 mm, *n*-hexane : EtOAc, 4 : 1) afforded compound **1** (17.3 mg). Fraction B5 (1.3 g) submitted to CC (silica gel, *n*-hexane : EtOAc), followed by HPLC (SIL-06 column, 50×20 mm, $CHCl_3$: MeOH, 49 : 1) afforded compounds **2** (6.7 mg) and **3** (12.9 mg).

Mallotophilippen C (**1**): A reddish-yellow plate, IR ν_{max}^{KBr} cm^{-1}: 3371, 2971, 2919, 2845, 1604. UV λ_{max}^{MeOH} nm (log ε): 370 (4.47), 287 (4.27), 206 (4.48). HR-EI-MS *m/z*: 474.2405 (Calcd for $C_{30}H_{34}O_5$: 474.2406). EI-MS *m/z*: 474 [M^+], 459, 405, 351, 285, 215. ^{1}H- and ^{13}C-NMR presented in Table 1.

Mallotophilippen D (**2**): A reddish-yellow plate, IR ν_{max}^{KBr} cm^{-1}: 3367, 2925, 2853, 1600, 1517. UV λ_{max}^{MeOH} nm (log ε): 382 (4.33), 266 (3.68), 208 (4.62). HR-EI-MS *m/z*: 490.2347 (Calcd for $C_{30}H_{34}O_6$: 490.2355). EI-MS *m/z*: 490 [M^+], 421, 367, 339, 285, 231, 215. ^{1}H- and ^{13}C-NMR presented in Table 1.

Mallotophilippen E (**3**): A reddish-yellow plate, $[\alpha]_D^{22}$ ±0° (*c*=0.5, MeOH), IR ν_{max}^{KBr} cm^{-1}: 3362, 2972, 2925, 1598, 1518, 1446. UV λ_{max}^{MeOH} nm (log ε): 382 (4.33), 269 (4.38), 207 (4.62). HR-EI-MS *m/z*: 490.2350 (Calcd for $C_{30}H_{34}O_6$: 490.2355). EI-MS *m/z*: 490 [M^+], 407, 339, 285, 231, 215. ^{1}H- and ^{13}C-NMR presented in Table 2.

Nitrite Assay[16] The cells were seeded at 1.2×10^6 cells/ml onto 96-well flat bottom plate (Sumitomo Bakelite, #8096R, Tokyo) and then incubated at 37 °C for 2 h. Next, the test compound was added to the culture simultaneously with both *Escherichia coli* LPS (100 ng/ml) and recombinant mouse IFN-γ (0.33 ng/ml). Then cells were incubated at 37 °C for approximately 16 h and subsequently chilled on ice. One hundred microliters of the culture supernatant was placed in duplicate in the wells of 96-well flat-bottomed plates. A standard solution of $NaNO_2$ was placed in alternate wells on the same plate. To quantify nitrite, 50 μl of Griess reagent (1% sulfanilamide in 5% H_3PO_4 and 0.1% *N*-1-naphthyletylenediamide dihydrochloride) was added to each well. After 10 min the reaction products were colorimetrically quantified at 550 nm using a Model 3550 MICROPLATE READER (BIO-RAD) and the background absorbance (630 nm) was subtracted. Cytotoxicity was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay method.

Reverse Transcriptase–Polymerase Chain Reaction Analysis The cells were cultured at 1.2×10^6 cells/ml onto 96-well flat bottom plate at 37 °C for 2 h. Then test compound was added to the culture simultaneously with both LPS (100 ng/ml) and IFN-γ (0.33 ng/ml), and the cells were incubated at 37 °C, usually for 8 h. Total RNA was isolated from the cell pellet using RNA isolation kit (QIAGEN, Hilden, Germany). 250 ng of total RNA were reverse-transcribed into cDNA by oligo $(dT)_{12-18}$ primer. The PCR samples contained 30 μl the reaction mixture, comprised of 50 mM KCl, 5 mM $MgCl_2$, 0.2 mM dNTP, 0.6 units of Ampli Taq GOLD (Applied Biosystems, CA, U.S.A.), and 0.4 μmol of sense and antisense primers. The sense primer for iNOS was 5′-ACCTACTTCCTGGACATTACGACCC-3′ and the antisense primer was 5′-AAGGGAGCAATGCCCGTACCAGGCC-3′. The sense primer for COX-2 was 5′-TCAAAAGAAGTGCTGGAAAAGGTT-3′ and the antisense primer was 5′-TCTACCTGAGTGTCTTTGACTGTG-3′. The sense primer for IL-6 was 5′-TGGAGTCACAGAAGGAGTGGC-TAAG-3′ and the antisense primer was 5′-TCTGACCACAGTGAGGAAT-GTCCAC-3′. The sense primer for IL-1β was TGAAGGGCTGCTTC-CAAACCTTTGACC-3′ and the antisense primer was 5′-TGTCCATTGAG-GTGGAGAGCTTTCAGC-3′. The sense primer for glyceraldehydes-3-phosphatedehydrogenase (GAPDH) was 5′-ACCACAGTCCATGCCAT-CAC-3′, and the antisense primer was 5′-TCCACCACCCTGTTGCTG TA-3′. The PCR reaction was performed under the following conditions: denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 1.5 min, using a thermal cycler (GeneAmp PCR Systems 9700; PE Applied Biosystems, U.S.A.). The PCR products were run on a 2% agarose gel and visualized by ethidium bromide staining. The bands in the gel were photographed.

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