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Exploration of Essential Structure of Malloapelta B for the Inhibitory Activity Against TNF Induced NF-κB Activation

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For the exploration of pharmacophoric moiety of malloapelta B (**1**) possessing the inhibitory activity of NF-κB activation, structural variation of α,β-unsaturated carbonyl motif was attempted. **1** was reduced by catalytic hydrogenation, sodium borohydride, and lithium aluminumhydride. Catalytic hydrogenation with 30 psi or 15 psi of H_2 gas of **1** generated 8-butyl–5,7-dimethoxy-2,2-dimethylchroman (**2**) and 1-(5,7-dimethoxy-2,2-dimethylchroman-8-yl)butan-1-one (**3**), respectively. Reduction with sodium borohydride occurred at the double bond of α,β-unsaturated ketone of **1** to give 1-(5,7-dimethoxy-2,2-dimethyl-2H-chromen-8-yl)butan-1-one (**4**). Reduction of **1** with lithium aluminumhydride and then quenched with methanol and water produced unexpected products, 1-(5,7-dimethoxy-2,2-dimethyl-2H-chromen-8-yl)-3-methoxy-1-butene (**5**) and 1-(5,7-dimethoxy-2,2-dimethyl-2H-chromen-8-yl)-3-hydroxy-1-butene (**6**). These are formed from the isomerization of initial product **9** through the continuous conjugate carbocation intermediate **11**. Addition of ethylmagnesium bromide and dimethyl malonate anion to **1** gave the conjugate adducts **7** and **8**. Ethylmagesium bromide and sodium borohydride reduction unusually gave the conjugate addition due to steric congestion around carbonyl group of **1**. Compound **2** exhibits the reduced inhibitory activity against NF-κB activation and the others do not show the activity. Therefore α,β-unsaturated carbonyl group of **1** should be important for its inhibitory activity.

Key words: Essential structure of malloapelta B, NF-κB activation

INTRODUCTION

Mallotus apelta Muell – Arg. (Euphorbiaceae) is distributed widely in Vietnam and southern area of China. From time immemorial, its leaves have been used as Vietnamese traditional medicine for treatment of chronic hepatitis and enteritis (Chi, 1997). From this plant, many constituents have been isolated including triterpenoid, benzopyran, and coumarino – lignoids (Shan, 1987; Cheng *et al.*, 1999; Cheng and Chen, 2000). Some antibiotic triterpenoids and steroids have also been reported to be isolated from its root (An, 2001, 2003). Since many compounds containing α,β-unsaturated carbonyl moiety (Jia and Turek, 2005) show the inhibitory activity against LPS or TNF induced NF-κB activation which is related to septic shock, autoimmune disorders, and inflammatory diseases (Kim *et al.*, 2005; Shin *et al.*, 2006; Banno *et al.*, 2005), some new benzopyrans from this plant had been investigated their inhibitory activity against TNF induced NF-κB activation (Chau *et al.*, 2005). Among them malloapelta B (**1**) with α,β-unsaturated carbonyl moiety in side chain present a significant NF-κB activity (IC_{50} = 5.0 μM). To explore the possible way of enhancement, the identification of the important structural unit of **1** for the activity was attempted. Thus seven derivatives altered at α,β-unsaturated carbonyl moiety from malloapelta B were synthesized (Scheme 1) and tested the inhibitory activity against NF-κB activation.

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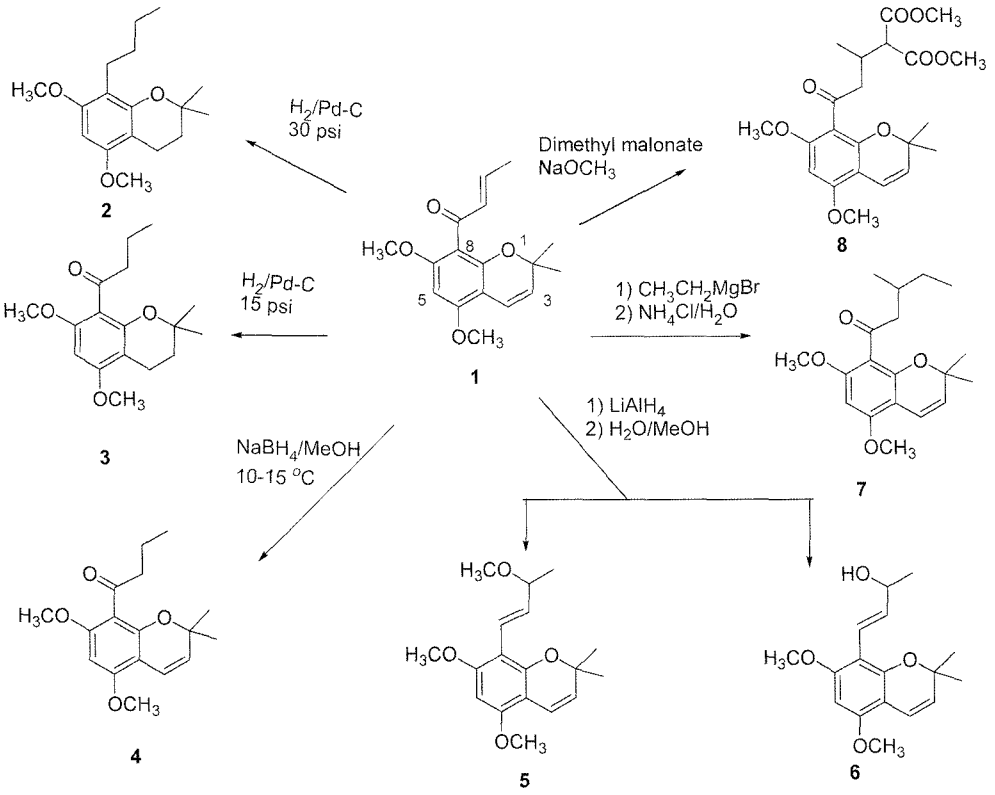
MATERIALS AND METHODS

Melting points (mp) were determined using the electrothermal 1A p100 MKZ apparatus and were uncorrected. All chemicals were purchased from Sigma Aldrich and commercial solvents were purified according to standard procedure prior to use (Perrino and Amarego, 1982). Thin layer chromatography were performed on Merck silica gel



Scheme 1. Modifications of malloapelta B (**1**)

GF-254 pre-coated plates. Flash column chromatography was performed with the. Merck silica gel (230-400 mesh). IR spectra were recorded with the JASCO IR - 100 spectrometer in cm^{-1} and were corrected against the peak at 1601cm^{-1} of polystyrene. NMR spectra were measured against the peak of tetramethylsilane by using Varian Unity 400 spectrometer (400 MHz).

Synthesis of 8-butyl-5,7-dimethoxy-2,2-dimethylchroman (2)

1 (86.4 mg, 0.3 mmol.) was dissolved in 25 mL of methanol and 10% Pd-C (15 mg) was added. After flushing the air by hydrogen gas, the resulting mixture was stirred under hydrogen gas (30 psi) at room temperature for overnight. After removal catalyst by filtration with aid of celite pad, the solvent was removed under vacuum. The crude product was purified by flash chromatography to give **2**.

Yield 80.0%; colorless oil; R_f 0.70 (hexane : acetone = 1.5:1); IR (KBr) : 2950, 1600 cm^{-1}, ^{1}H-NMR (acetone-d_6) δ 0.90 (t, *J* = 7.2 Hz, 3H), 1.27 (s, 6H), 1.31 (m, 2H), 1.41 (m, 2H), 1.71 (t, *J* = 6.8 Hz, 2H), 2.52 (t, *J* = 6.8 Hz, 2H), 2.55 (t, *J* = 6.8 Hz, 2H), 3.78 (s, 3H), 3.79 (s, 3H), 6.19 (s, 1H).

Synthesis of 1-(5,7-dimethoxy-2,2-dimethylchroman-8-yl)butan-1-one (3).

1 (144 mg, 0.5 mmol.) was dissolved in methanol (50 mL) and 10% Pd-C (15 mg) was added. After absolutely flushing the air by hydrogen gas, the resulting mixture was stirred under hydrogen gas (15 psi) at room temperature for overnight. After removal catalyst by filtration with aid of celite pad, the solvent was removed under vacuum. The crude product was purified by flash column chromatography to give **3**.

Yield 80.5%; white solid; mp. 48.8°C; R_f 0.60 (hexane : acetone = 1.5 : 1); IR (KBr) 2950, 2850, 1600 cm^{-1}. ^{1}H-NMR (acetone-d_6) δ 0.93 (t, *J* = 7.2 Hz, 3H), 1.27 (s, 6H), 1.61 (m, 2H), 1.75 (t, *J* = 7.2 Hz, 2H), 2.56 (t, *J* = 6.8 Hz, 2H), 2.61(t, *J* = 7.2 Hz, 2H), 3.76 (s, 3H), 3.85 (s, 3H), 6.26 (s, 1H).

Synthesis of 1-(5,7-dimethoxy-2,2-dimethyl-2H-chromen-8-yl)butan-1-one (4)

Solution of **1** (288 mg, 1 mmol) in 25 mL of methanol was mixed with 74 mg (2 mmol.) of sodium borohydride in 5 mL of methanol at room temperature (<20°C). After exothermic reaction had ended (about 10 minutes), the mixture was heated at 60°C for 10 minutes. The reaction mixture was then treated with 10 mL of a 3% sodium hydroxide solution and was extracted with ether (3×30 mL). The combined ether layers were dried by anhydrous sodium sulfate and concentrated under vacuum. The obtained residue was purified by flash chromatography to give **4**.

Yield 90.2%; white solid; mp. 62.8°C; R_f 0.50 (hexane : acetone = 1.5:1); IR (KBr) 2950, 1700, 1650, 1600 cm^{-1}; ^{1}H-NMR (acetone-d_6) δ 0.96 (t, J = 7.6 Hz, 3H), 1.39 (s, 6H), 1.69 (m, 2H), 2.73 (t, J = 7.2 Hz, 2H), 3.79 (s, 3H), 3.84 (s, 3H), 5.45 (d, J = 10.0 Hz, 1H), 6.00 (s, 1H), 6.57 (d, J = 10.0 Hz, 1H).

Synthesis of 5 and 6 by reduction of 1 with lithium aluminum hydride

1 (288 mg, 1 mmol.) was dissolved in 40 mL of anhydrous diethyl ether. The resulting solution was slowly added to a stirred solution of 46 mg (1.2 mmol.) of lithium aluminum hydride in 30 mL of anhydrous diethyl ether under nitrogen. After the addition was complete, the reaction was stirred for 15 minutes more at room temperature. The mixture was sequentially treated with 1 mL of methanol-water for 10 minutes, 1 mL of 15% sodium hydroxide for 10 minutes, and 5 mL of water for 10 minutes. After the mixture was filtered, organic layer was separated from filtrate and dried over anhydrous K_2CO_3. The organic layer was concentrated under vacuum and the residue was purified by flash chromatography to give **5** and **6**.

1-(5,7-Dimethoxy-2,2-dimethyl-2H-chromen-8-yl)-3-methoxy-1-butene (5)

Yield 25.2%; colorless oil; R_f 0.6 (hexane : acetone = 1.5 :1); IR (KBr) 2980, 1600 cm^{-1}; ^{1}H-NMR (acetone-d_6) δ 1.23 (d, J = 6.0 Hz, 3H), 1.41 (s, 6H), 3.23 (s, 3H), 3.74 (m, 1H), 3.85 (s, 3H), 3.87 (s, 3H), 5.55 (d, J = 8.8 Hz, H), 6.26 (s, 1H), 6.37 (dd, J = 16.4, 8.0 Hz, 1H), 6.59 (d, J = 8.8 Hz, 1H), 6.70 (d, J = 16,4 Hz, 1H).

1-(5,7-Dimethoxy-2,2-dimethyl-2H-chromen-8-yl)-3-hydroxy-1-butene (6)

Yield 40.0%; white solid; mp: 68.5°C; R_f 0.40 (hexane : acetone = 1.5:1); IR (KBr): 3400, 2960, 1600 cm^{-1}. ^{1}H-NMR (acetone-d_6) δ 1.36 (d, J = 6.4 Hz, 3H), 1.43 (s, 6H), 3.83 (s, 3H), 3.85 (s, 3H), 4.43 (m, 1H), 5.47 (d, J = 10.0 Hz, 1H), 6.03 (s, 1H), 6.59 (d, J = 10.0 Hz, 1H), 6.62 (dd, J = 6.8, 16.0 Hz, 1H), 6.03 (s, 1H), 6.77 (d, J = 16 Hz, 1H).

Synthesis of 1-(5,7-dimethoxy-2,2-dimethyl-2H-chromen-8-yl)-3-methylpentan-1-one (7)

A solution of **1** (288 mg, 1 mmol.) in 30 mL of dry diethyl ether was slowly added to the solution of the ethylmagnesium bromide prepared from 30 mg (1.25 mmol.) of magnesium turnings and excessive ethyl bromide (216 mg, 2 mmol.) in 30 mL of dry diethyl ether with stirring under a nitrogen atmosphere at -10°C. After the mixture had been stirred for 30 minutes, it was washed with a cold and saturated aqueous solution of ammonium chloride (10 mL) and then sufficient aqueous ammonium hydroxide had been added to adjust pH to 7.5-8.0. The ether layer was separated, dried with anhydrous sodium sulfate, and concentrated under vacuum. The residue was purified by flash chromatography to give **7**.

Yield 85.3%; white solid; mp. 62.2°C; R_f 0.70 (hexane : acetone = 1.5:1); IR (KBr): 2950, 1700 cm^{-1}; ^{1}H-NMR (acetone-d_6) δ 0.88 (t, J = 7.6 Hz, 3H), 0.94 (d, J = 6.4 Hz, 3H), 1.22 (m, 1H), 1.38 (s, 6H), 1.42 (m, 1H), 1.96 (m, 1H), 2.50 (dd, J = 7.6, 16.4 Hz, 1H), 2.73 (dd, J = 5.6, 16.4 Hz), 3.81 (s, 3H), 3.85 (s, 3H), 5.54 (d, J = 10.0 Hz, 1H), 6.29 (s, 1H), 6.56 (d, J = 10.0 Hz, 1H).

Preparation of dimethyl 2-(4-(5,7-dimethoxy-2,2-dimethyl-2H-chromen-8-yl)-4-oxobutan-2-yl)malonate (8)

Methanolic solution (10 mL) of sodium methoxide (1 g, 18.5 mmol) was added to the solution of dimethyl malonate (2.44 g, 18.5 mmol.) in methanol (10 mL). The resulting mixture was stirred for 10 minutes and then methanolic solution (30 mL) of **1** (5.86 g, 20.3 mmol.) was added dropwise to keep reaction temperature between 30 and 40°C. After complete addition, the reaction mixture was stirred at room temperature overnight and concentrated to about 20 mL. Water (50 mL) was added and the resulting mixture was extracted with diethyl ether (3×70 mL). Combined diethyl ether extract was neutralized with glacial acetic acid, washed by water, and dried with anhydrous sodium sulfate. After removal of solvent under vacuum, the resulting crude product was purified by flash chromatography to give **8**.

Yield 75.6%; colorless oil; R_f 0.50 (hexane : acetone = 1.5 : 1); IR (KBr), 2890, 1750, 1725, 1700, 1600 cm^{-1}; ^{1}H-NMR (acetone-d_6) δ 1.06 (d, J = 6.4 Hz, 3H), 1.38 (s, 3H), 1.39 (s, 3H), 2.83 (m, 3H), 3.55 (d, J = 6.4 Hz, 1H), 3.70 (s, 6H), 3.81 (s, 3H), 3.85 (s, 3H), 5.54 (d, J = 10.0 Hz, 1H), 6.27 (s, 1H), 6.57 (d, J = 10.0 Hz, 1H).

Biological assay

Cell culture

Human cervical adenocarcinoma HeLa cells (ATCC, Rockville, MD, U.S.A.) were maintained in Dulbecco's modified essential medium (DMEM) supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL) and 10% heat-inactivated fetal bovine serum (FBS). Cells were transfected with the NF-κB reporter construct using lipofectin (Gibco/BRL, Gaithersburg, MD, U.S.A.) and maintained in the same medium except for addition of 500 μg/mL G418.

NF-κB reporter assay

NF-κB activity was evaluated by κB-dependent reporter gene assay (Jin *et al.*, 2002). Briefly, HeLa cells stably transfected with kB element linked to SEAP (secreted alkaline phosphatase) gene were seeded in a 96 wells plate at 5×10^4 cells/well and incubated for 3 h, then cells

were treated with various concentration of test compounds and stimulated by 10 ng/mL TNF-α for 24 h. 100 μL of supernatant were transferred to a new 96 wells plate, heated at 65°C for 10 min then mixed with 100 μL of 2 X SEAP buffer and incubated at 37°C for 10 min. The reaction was initiated by the addition of 20 μL of 31.6 μg/mL p-nitrophenyl phosphatate dissolved in 1X SEAP buffer and incubated at 37°C for 3 h. The optical density of reaction mixture were measured at 405 nm with a microplate reader (Molecular Devices Co., Menlo park, CA, U.S.A.). Celastrol was used as positive control.

Cytotoxicity assay

Cytotoxicity was evaluated by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) uptake method. HeLa cells were seeded in a 96 wells plate at 5×10^4 cells/well and incubated at 37°C for 3 h, and then cells were treated with various concentration of test compounds and incubated at 37°C. After 24 h incubation, 10 μL of 5 mg/mL MTT was added to each well and incubated for another 4 h. After removing of supernatant, formazan crystals were dissolved in 100 μL DMSO and the optical density were measured at 570 nm with a microplate reader.

RESULTS AND DISCUSSION

Since α,β-Unsaturated carbonyl motif frequently appears in NF-κB inhibitors, thus this functional group has been considered as one of the major pharamcophoric moieties. For the confirmation of this fact in Malloapelta B (**1**), structural variation at this position was attempted as shown in Scheme 1. Reduction of double bond or carbonyl group was attempted. Catalytic hydrogenation (Jung *et al.*, 2003) of **1** at 30 psi of H_2 gas in the presence of 10% Pd/C resulted in conversion of α,β-unsaturated carbonyl to butyl and saturation of 3,4-double bond in pyran ring to yield **2**. However the same reaction at 15 psi of H_2 gas only reduced two C=C double bonds to give **3**, which was confirmed by IR and NMR spectra. Peaks for protons on double bonds at δ 5-6 region of NMR of **1** completely disappeared and new methylene groups of **3** appeared at δ 1.61 (m, 2H), 1.75 (t, J = 7.2 Hz, 2H), 2.56 (t, J = 6.8 Hz, 2H), and 2.61 (t, J = 7.2 Hz, 2H). Triplet of $-CH_3$ found at δ 0.93 and peak at 1700 cm^{-1} for carbonyl group also revealed that the hydrogenation only occur on C=C double bonds.

When the reduction of **1** was carried out by sodium borohydride in methanol, the reaction occurs only on C=C double bond in side chain to form compound **4** with butanoyl side chain. Although reaction of α,β-unsaturated ketone with sodium borohydride used to preferentially reduce carbonyl function (Johnson and Rickborn, 1969), steric congestion around carbonyl and conjugation with phenyl exerted 1,4-addition of hydride in this reaction.

In order to selective reduction of carbonyl, compound **1** was treated with lithium aluminum hydride. The results are much complicated with the formation of isomerizied products **5** and **6** as shown in Scheme 2. Peaks in NMR of **5** at δ 1.23 (d, J = 6.0 Hz, 3H), 3.74 (m, 1H), 6.37 (dd, J = 16.4, 8.0 Hz, 1H *trans*), 6.70 (d, J = 16,4 Hz, 1H *trans*) indicate the presence of 3-methoxybutene motif. This typical pattern in NMR of **6** [δ 1.36 (d, J = 6.4 Hz, 3H), 4.43 (m, 1H), 6.62 (dd, J = 6.8, 16.0 Hz, 1H *trans*), 6.77 (d, J = 16 Hz, 1H *trans*)] also confirms 3-hydroxybutene system. It is believed that these compounds are formed from the intermediate **9** during the treatment of water and methanol after reaction completed. This isomerization occurs due to the formation of more stable carbocation intermediate **11**, which has continuous conjugation system of phenyl and vinyl to cation unlike **10**.

Reaction of **1** with ethylmagesium bromide generated conjugate addition product **7**. In IR spectrum, the band for carbonyl group appears at 1700 cm^{-1}. In ^{1}H-NMR spectrum, ethylenic protons (δ 5-7) on side chain of **1** disappeared and the signals at δ 0.88 (t,J=7.2Hz, CH_3), δ 1.22 and δ 1.376 (m, $-CH_2$), δ 1.99 (m, -CH-), and δ 2.50 (dd, J=7.6 Hz, J=16 Hz) prove the presence of 3-methylpentanoyl group of **7**. Considering that reaction of organomagnessium halide to α,β-unsaturated carbonyl compound usually produce 1,2-addition product, this result is unusual. Presumably, steric congestion around carbonyl function might cause the conjugate addition.

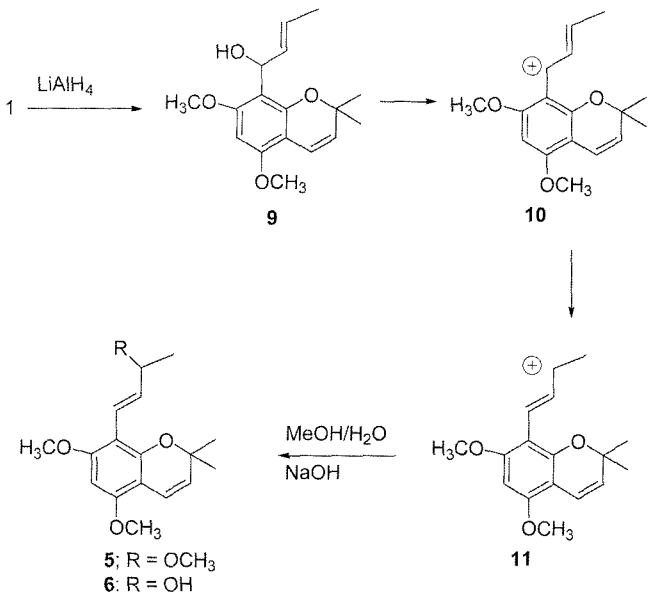
Reaction of **1** with dimethyl malonate in the presence of sodium methoxide produced conjugate addition product **8**.

Compounds synthesized were tested for their inhibitory activity on TNF-induced NF-κB activity using transfected Hela cell and the results are shown in Table I. Only compound **2** show the slightly decreased inhibitory activity. The rest of compounds show the inhibitory activity at the nearly same concentration of cell cytotoxicity. Thus these

Table I. IC50 values (μM)[a] of malloapelta B and its derivatives on NF-κB inhibition and cytotoxicity

Compound	NF-κB	Cytotoxicity
1	3.50 ± 0.68	5.31 ± 0.92
2	8.92 ± 1.33	14.74 ± 0.97
3	34.58 ± 1.62	30.0 ± 2.49
4	29.86 ± 1.15	36.0 ± 0.87
7	12.17 ± 0.94	12.29 ± 1.37
5	12.53 ± 2.04	11.11 ± 1.36
6	> 50	> 50
8	> 50	> 50
Celastrol[b]	0.08 ± 0.85	0.23 ± 1.14

[a]Data are mean ± SD obtained from triplicate experiments
[b]Positive control (Jin *et al.*, 2002).



Scheme 2. Formation of **5** and **6**

compounds are considered to be inactive. The results revealed that the α,β-unsaturated carbonyl moiety and the C=C double bond contribute an important role to the activity of malloapelta B (**1**).

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