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메조-디하이드로구아레틱산 메틸, 아세틸 치환체의 합성 및 이들 화합물들의 LPS에 의해서 유도된 일산화질소(NO)의 억제 효능에 대한 연구

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Synthesis of Methylated and Acetylated Derivatives of *Meso*-dihydroguaretic Acid and Study of Their Inhibitory Activities on LPS Derived Nitric Oxide (NO) Production

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요 약: 본 연구는 메조-디하이드로구아레틱산(MDGA, 1)과 합성 유도체(2와 3)의 일산화질소(NO)억제 효능에 대한 내용이다. MDGA는 토후박(*Machilus thunbergii* Sieb. et Zucc.)껍질에서 분리된 리그난 화합물이다. 본 연구자들은 디메틸로 치환된 화합물(2)과 디아세틸로 치환된 화합물(3)을 합성하였고, 이 두 화합물의 일산화질소 억제 효능을 MDGA (1)와 비교하여 측정하였다. MDGA (1)와 화합물(3)은 LPS에 의해서 유도된 일산화질소 억제 효능을 나타내었다. RT-PCR 분석을 통해 MDGA (1)와 화합물(3)의 일산화질소 억제 효능은 iNOS의 mRNA 발현의 감소에 기인함을 확인하였다. 이러한 실험 결과로부터 디아세틸로 치환된 화합물(3)은 MDGA의 프로드럭으로 사용될 수 있음을 확인하였다.

Abstract: This study was conducted to examine the inhibitory effects of *meso*-dihydroguaretic acid (MDGA, **1**) and its synthetic derivatives (compound **2** and **3**) against NO production. MDGA is a lignan component isolated from the bark of *Machilus thunbergii* Sieb. et Zucc. We synthesized dimethylated MDGA (**2**), diacetylated MDGA (**3**) and compared NO inhibition of two derivatives with that of MDGA (**1**). MDGA (**1**) and compound **3** showed suppressive effects against the generation of NO in LPS-activated macrophages. RT-PCR analysis suggested that MDGA (**1**) and compound **3** inhibited NO production through the suppression of iNOS mRNA expression. From these results, diacetylated MDGA (**3**) can be used as a pro-drug for MDGA.

Keywords: *meso-dihydroguaretic acid, methylated derivatives, acetylated derivative, nitric oxide, LPS*

1. Introduction

Machilus thunbergii Sieb. et Zucc. (*M. thunbergii*) is a deciduous tree grown in the southern areas of Korea and its bark is commonly used as a folk medicine for the treatment of leg edema, abdominal pain and abdominal distension[1,2]. *Meso*-dihydroguaretic acid (MDGA)[3,4], which is a lignan component in the bark of *M. thunbergii*, has been shown to have broad biological activities such as neuroprotective activity[5], osteoblast differentiation inducing activity[6], inhibitory activity against the expression of MMP-2 and 9[7], antioxidative activity[8] and depigmenting activity[9]. However, no studies of MDGA

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as a nitric oxide (NO) inhibitor have been reported to date. The MeOH extract[10] of this plant exhibited inhibitory activity against NO production in LPS-activated macrophages, but the compounds responsible for this activity have not been identified.

NO[11] is a free radical generated by constitutive and inducible nitric oxide synthase in numerous mammalian cells and tissues. NO constitutively expressed by neuronal NOS (nNOS) and endothelial NOS (eNOS) is a key regulator of homeostasis[12]. However, the synthesis of NO by iNOS is induced by a variety of stimuli, including LPS, bacteria, viruses and postinflammatory cytokines[13]. Excess production of NO can damage DNA, lipids and proteins.

MDGA (**1**) has two aromatic hydroxyl groups which can be considered as a pharmacophore. Although naturally occurring phenols show various biological activities, their uses in cosmetics are limited because of their unfavorable stabilities[14]. Thus, there are strong needs to enhancing chemical stability and biological activities of phenols by minimal modification of aroatic hydroxyl groups[15]. Recently, several aromatic compounds, protected by methyl and acetylgroups, showed potent biological activities. In particular, O-methylated flavonoids exhibited a superior activity than the corresponding hydroxylated derivatives[16,17]. In acetyl derivatives, 3,5,4'-tri-acetyl resveratrol has been studied as a pro-drug for resveratrol[18,19]. In this study, we synthesized di-O-methyl MDGA (**2**) and di-O-acetyl MDGA (**3**) and evaluated their inhibitory effects on NO production.

2. Experimental

2.1. General Methods

All the chemicals were from Sigma. Mass spectra were acquired in the range of (full) *m/z* 125-1,200 (MS-MS ion isolation width, 5 Da; MS-MS collision energy, 45%; and MS-MS scan range, *m/z* of parent ion to ca. 1/3 *m/z* parent ion). ^{1}H, ^{13}C-NMR spectra were recorded on a Varian GEMINI-500BB (500 MHz) spectrometer.

2.2. Synthesis

2.2.1. Synthesis of Compound 2

To a stirred solution of MDGA (**1**, 4.8 g, 30 mmol) and K_2CO_3 (4.0 g, 40 mmol) in acetone (100 mL) under N_2 was added dimethylsulfate (3.6 g, 33 mmol). The reaction mixture was stirred for 2 h at reflux and then the solid was filtered off. The filtrate was evaporated *in vacuo*. The residue was extracted with ethyl acetate (50 mL), washed with water. The solvent was dried over $MgSO_4$ and evaporated to dryness. The crude product was purified by silica gel column chromatography to give desired product **2** in 81% yields.

^{1}H-NMR (500 MHz, $CDCl_3$): d 6.78 (d, 1H, J = 8.1 Hz), 6.70 (d, 1H, J = 8.1 Hz), 6.65 (s, 1H), 3.85 (s, 6H), 2.78 (m, 1H), 2.37 (m, 1H), 1.79 (m, 1H), 0.86 (d, 3H, J = 6.6 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$): d 148.9, 147.3, 134.6, 121.1, 112.5, 111.3, 56.09, 55.98, 39.3, 39.0, 16.3. FAB MS: (m/e) 359 $[M+H]^+$.

2.2.2. Synthesis of Compound 3

To a stirred solution of MDGA (**1**, 4.8 g, 30 mmol) and triethylamine (4.0 g, 40 mmol) in CH_2Cl_2 (100 mL) under N_2 was added acetic anhydride (3.6 g, 33 mmol). The reaction mixture was stirred for 2 h at room temperature and then CH_2Cl_2 was evaporated *in vacuo*. The residue was extracted with ethyl acetate (300 mL), washed with water. The solvent was dried over $MgSO_4$ and evaporated to dryness. The crude product was purified by silica gel column chromatography to give desired product **3** in 78% yields.

^{1}H-NMR (500MHz, $CDCl_3$): d 6.94 (d, 1H, J = 8.1 Hz), 6.73 (d, 1H, J = 8.1 Hz), 6.71 (s, 1H), 3.79 (s, 3H), 2.80 (m, 1H), 2.36 (m, 1H), 2.30 (s, 3H), 1.80 (m, 1H), 0.87 (d, 3H, J = 6.6 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$) d 169.3, 150.9, 140.8, 137.9, 122.4, 121.3, 113.3, 56.0, 39.40, 39.24, 20.8, 16.4. FAB MS: (m/e) 415 $[M+H]^+$.

2.3. Biological Activities

2.3.1 Cell Culture

RAW264.7 cell line was obtained from the Korean Cell

Line Bank (Korea). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 0.1 mg/mL streptomycin at 37 ℃ in a humidified 95% air and 5% CO_2 atmosphere.

2.3.2 Measurements of NO Production

RAW264.7 cells (1×10^6 cells/mL) were preincubated with MDGA and its derivatives for 30 min and continuously activated with LPS (1 μg/mL) for 24 h. Nitrite concentration was measured by adding 100 μL of Griess reagent (1% sulfanilamide and 0.1% N-[1-naphthyl]-ethylenediamine dihydrochloride in 5% phosphoric acid) to 100 μL of culture supernatants for 10 min at room temperature. OD at 570 nm (OD_{570}) was measured using a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, USA). A standard curve of NO was made with sodium nitrite.

2.3.3 Measurements of Cytotoxicity

After the preincubation of RAW264.7 cells (1×10^6 cells/mL) for 18 h, MDGA and its derivatives (0-100 μM) were added to the cells and incubated for 24 h. The cytotoxic effect of MDGA and its derivatives was then evaluated by a conventional MTT assay. At 3 h prior to culture termination, 10 mL of the MTT solution (5 μg/mL in a phosphate buffered-saline, pH 7.4) were added and the cells were continuously cultured until termination. The incubation was halted by the addition of 15% sodium dodecyl sulfate into each well, solubilizing formazan. The absorbance at 570 nm ($OD_{570-630}$) was measured by a Spectramax 250 microplate reader.

2.3.4 mRNA Detection

Total RNA from LPS-treated-RAW264.7 cells (5×10^6 cells/mL) was prepared by adding TRIzol Reagent (Gibco BRL) according to the manufacturer's protocol. The total RNA solution was stored at −70 ℃ until used. Semi-quantitative RT reactions were conducted using MuLV reverse transcriptase. Total RNA (1 μg) was incubated with oligo-dT15 for 5 min at −70 ℃ and mixed with a 5 × first-strand buffer, 10 mM dNTPs and 0.1 M DTT. The reaction mixture was further incubated for 5 min at 37 ℃ and for 60 min after the addition of MuLV reverse transcriptase (2 U). Reactions were terminated after 10 min at 70 ℃, and total RNA was depleted by adding RNase H. The PCR reaction was conducted with the incubation mixture (2 mL cDNA, 4 μM 5' and 3' primers, a 10 × buffer [10 μM Tris-HCl, pH 8.3, 50 μM KCl, 0.1% Triton X-100], 250 μM of dNTP, 25 μM of $MgCl_2$, and 1 unit of *Taq* polymerase [Promega, USA]). The following incubation conditions were used: a 30 s denaturation time at 94 ℃, an annealing time of 30 s between 55 and 60 ℃, an extension time of 45 s at 72 ℃, and a final extension of 5 min at 72 ℃. For real-time PCR analysis, 1 μg of RNA was submitted to reverse transcription with the Molony murine leukemia virus reverse transcriptase (Invitrogen). Two microliters of cDNA obtained for each sample were submitted to a qPCR using the SYBR green Master mix method (Applied Biosystems, USA) in the ABO sequence detection system. The results were normalized with the 18S transcript. The primers (Bioneer, Korea) used in this experiment are indicated as follows: iNOS F-5'-GGAGCCTTTAGAC CTCAACAGA-3' and R-5'-TGAACGAGGAGGGTGGT G-3'; GAPDH F-5'-CAATGAATACGGCTACAGCAAC-3' and 5'-AGGGAGATGCTCAGTGTTGG-3'.

3. Results and Discussion

3.1. Sythesis of MDGA Derivatives

MDGA (1) was prepared from *M. thunbergii* as previously described[5]. MDGA was reacted with dimethylsulfate in refluxing acetone to give dimethyl protected MDGA (**2**). Alternatively, MDGA was reacted with acetic anhydride and triethylamine in methylene chloride to afford diacetyl protected MDGA (**3**). The synthetic pathways are shown in Scheme 1.

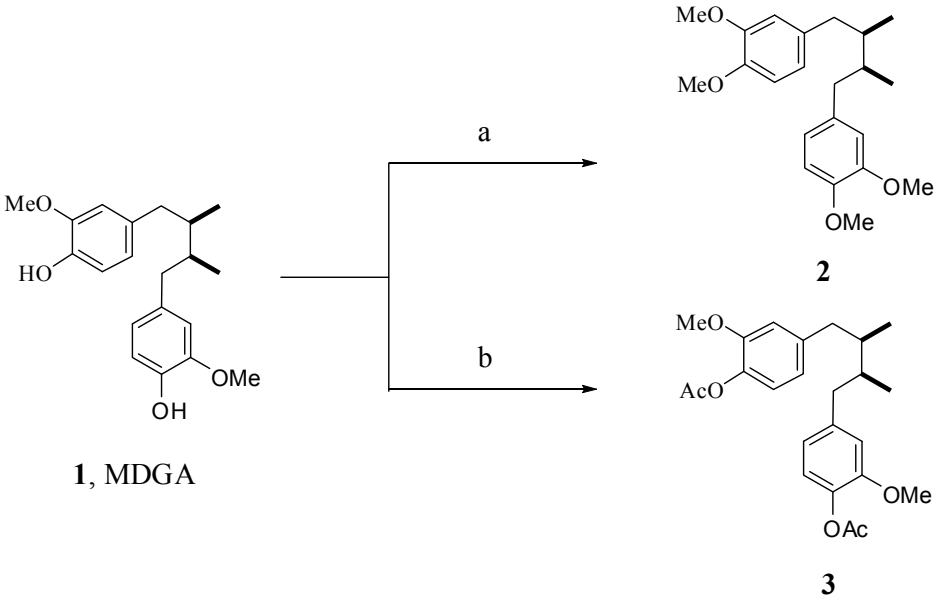
3.2. Inhibition of Nitric Oxide

The inhibitory activities of MDGA and its synthetic derivatives against nitric oxide induced by LPS were

Table 1. NO Inhibitory Activities of *Meso*-dihydroguaretic Acid and Its Derivatives

Compounds	Inhibitory activity [IC_{50}[a], (μM)]	
	NO	Cytotoxicity
1 (MDGA)	42.09	> 200
2	> 200	> 200
3	38.72	> 200
Pentoxifyllin	446	> 1000

[a] Values were determined from logarithmic concentration-inhibition curves and are given as the means of three experiments.



Scheme 1. Reaction conditions: (a) dimethylsulfate, acetone, K_2CO_3, reflux; (b) acetic anhydride, triethylamine, methylene chloride, room temperature.

investigated. The cytotoxicity of these compounds was checked using a MTT assay.

After confirming cell viability, we evaluated NO inhibitory activities at various concentrations. Pentoxifyllin was used as a positive control. The results are shown in Figure 1.

Compound 1 and compound 3 significantly inhibited NO production in a dose dependent manners. The structure of compound **1** (MDGA) comprises two main parts: the C_6 alkyl chain, and two aromatic hydroxyl groups. These two aromatic hydroxyl groups can be considered as a pharmacophore for various biological activities including antioxidative activity. MDGA exhibited potent inhibitory activity against NO production (IC_{50} = 42.09 μM). When two hydroxyl groups of MDGA were methylated, its activity was completely lost (Entry 2 in Table 1). However, diacetylated MDGA (**3**) showed potent inhibitory activity against NO productions. Specifically, the activity of compound **3** (IC_{50} = 38.72 μM) was similar to that of MDGA, even though its two hydroxyl groups are protected by acetyl groups. These results indicated that compound **3** can be used as an alternative of MDGA.

3.3. Expression of iNOS mRNA

To compare the mechanism for the inhibition of NO production by MDGA and compound **3**, we examined the

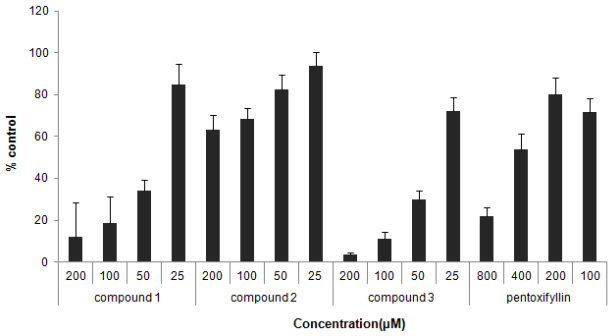


Figure 1. Effects of compound **1**, compound **2** and compound **3** against nitric oxide induced by LPS.

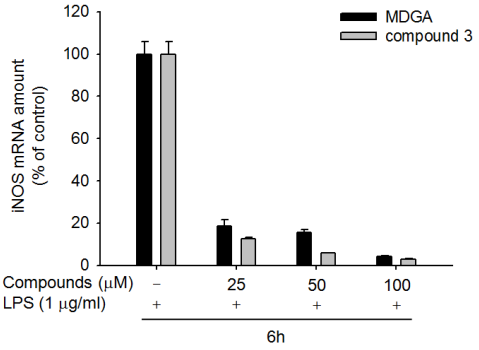


Figure 2. Effects of MDGA (**1**) and compound **3** on the expression of iNOS mRNA in LPS-activated macrophages.

expression of iNOS mRNA in LPS-activated RAW264.7 cells. For RT-PCR analysis, the mRNA of iNOS was induced by treatment of the cells with 1 μg/mL LPS for 6 h (Figure 2). Treatment with MDGA and compound **3** at concentrations of 25, 50 and 100 μM suppressed the expression of iNOS mRNA significantly. In addition, the inhibitory activities of both compounds were found to occur in a dose-dependent manner. These results suggested that the inhibition of NO production by MDGA and compound **3** was due to the decrease of iNOS mRNA. The result in which iNOS mRNA assay showed the similar effect with MDGA corroborates a potential use of compound **3** as an alternative of MDGA.

4. Conclusion

We synthesized MDGA derivatives (**2** and **3**) and compared their inhibitory activities against NO production in LPS-activated macrophages to that of MDGA (**1**). MDGA and diacetylated MDGA (**3**) showed potent inhibitory activities and their activities appeared to be correlated with the suppression of iNOS mRNA. However, dimethylated MDGA (**2**) showed no activity. This study showed that aromatic hydroxyl group can be used as pharmacophore for the inhibition of NO production. Although compound **3** has no aromatic hydroxyl groups, its inhibitory activity was similar to that of MDGA. The activity of diacetylated MDGA (**3**) may be originated from the rapid generation of MDGA by hydrolysis. From these results, we can develop diacetylated MDGA (**3**) as a pro-drug for MDGA. Further studies on the relationship between iNOS mRNA and expression of protein iNOS are underway in our laboratory.

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