Tyrosinase Inhibitory Activities of Meso-dihydroguaiaretic Acid from Machilus thunbergii

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Introduction

Machilus thunbergii (Lauraceae) is the evergreen tree grown in areas of Korea and Japan. M. thunbergii has long been used as a traditional medicine in Korea, China, and Japan to treat various diseases, including edema, abdominal pain, and abdominal distension. In this study, dried stem bark of M. thunbergii extracted in methanol and extract was partitioned into n-hexane, CHCl₃, and BuOH. The CHCl₃-soluble extracts chromatographed on silica gel column using a CHCl₃/acetone and n-hexane/EtOAc mixture to afford Compound 1 and 2. Two dibenzylbutane lignans, macelignan (1) and meso-dihydroguaiaretic acid (2), were isolated from the CHCl₃-soluble extract of M. thunbergii stem bark. The structures of 1 and 2 were determined by ¹H- and ¹³C-NMR spectroscopic data analyses and a comparison with literature data. The tyrosinase inhibitory activity of the isolated compounds was evaluated. Among these compounds, Compound 2 strongly inhibited the monophenolase (IC₅₀=10.2 μM) activity of tyrosinase. A kinetic analysis showed that Compound 2 was a competitive inhibitor. The apparent inhibition constant (K_i) for Compound 2 binding to free enzyme was 4.8 μM. Based on these results, it can be concluded that meso-dihydroguaiaretic acid (2) is a potential candidate for the treatment of melanin biosynthesis-related skin diseases.

Key words: Dibenzylbutane lignans, macelignan, Machilus thunbergii, meso-dihydroguaiaretic acid

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ing from the increased use of tyrosinase enzyme in medicin-
al and cosmetic products [17], and their identification and
isolation from natural sources have been also increased [25].
Natural tyrosinase inhibitors are generally considered to be
free of harmful side effects and can be produced at reason-
able low costs. Therefore, the development and utilization
of more effective tyrosinase inhibitors of natural origin are
desired.

In our continuous search for new tyrosinase inhibitors
from M. thunbergii, the MeOH extracts were subsequently
partitioned and isolated. As a result, two dibenzylbutane li-
gnans, macelignan (1) and MDGA (2) were isolated from
M. thunbergii. In this study, the isolation and structural deter-
minations of these two compounds are described. All the
isolated compounds were evaluated for their tyrosinase in-
hibitory activities.

Materials and Methods

Plant material

The stem bark of M. thunbergii was purchased from an
oriental drug store in Pohang, Gyeongbuk, Korea, in July 2009. A voucher specimen (MT2009-01) has been deposited at the Laboratory of Molecular Neurophysiology, POSTECH, Pohang, Korea.

Instruments

NMR experiments were conducted on a Bruker AM 300
or 500 MHz FT-NMR instrument with tetramethylsilane (TMS) as internal standard. EIMS was collected on Jeol JMS-700 spectrometer. Optical rotations were measured on Perkin-Elmer 343 polarimeter. Silica gel (230-400 mesh, Merck), RP-18 (ODS-A, 12 nm, S-150 m, YMC) and Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden) were used for column chromatography. Thin-layer chromatographic (TLC) analysis was performed on Kieselgel 60 F 254 (silica gel, 0.25 mm layer thickness, Merck) and RP-18 F 254S (Merck) plates.

Extraction and isolation

The dried stem bark (500 g) of M. thunbergii were chopped and extracted three times with 100% methanol (1 l × 3) for 7 days at room temperature. The combined methanol extract was concentrated in vacuo to yield a brown gum (11.3 g). The methanol extract was suspended in H 2O (500 ml), then partitioned in turn with n-Hexane, CHCl 3 and BuOH (each 3-500 ml) to afford n-Hexane extract (1.8 g), CHCl 3 extract (3.5 g), and BuOH extract (1.5 g). The CHCl 3-soluble extract was silica gel column chromatography (230-400 mesh) using CHCl 3 /Acetone (100:1→1:1) mixtures to yield seven sub-

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for the separation consisted of solvent A (0.1% trifluoroacetic acid in water) and solvent B (0.1% trifluoroacetic acid in acetonitrile). A gradient elution procedure was used as 0 min 98% A, 3 min 95% A, 20 min 100% B, 23 min 98% A, 30 min 98% A. The injection volume was 3 μl for analysis. All samples were analyzed in triplicate.

Tyrosinase inhibitory activity
Mushroom tyrosinase (EC 1.14.18.1) was assayed as described previously with slight modifications [4], using L-tyrosine or L-3,4-dihydroxyphenylalanine (L-DOPA) as substrate. In a spectrophotometric experiment, the enzyme activity was monitored by dopachrome formation at 475 nm with a Spectra MAX plus spectrophotometer (Molecular device, Sunnyvale, CA) at 30℃. All test samples were dissolved in dimethyl sulfoxide (DMSO) and used for the experiment with dilution. The final concentration of DMSO in the test solution was 1.5%. First, 200 μl of a 4.5 mM L-tyrosine or 12 mM L-DOPA aqueous solution was mixed with 2785 μl of 0.25 M phosphate buffer (pH 6.8) and 10 μl of the test sample, incubated at 30℃ for 10 min. Then, 5 μl of tyrosinase solution (130 units) was added to the phosphate buffer and incubated for additional 20 min. DMSO without test samples was used as the control, and kojic acid was used as a positive control. The assay was conducted in triplicate of separate experiments. The data analysis was performed by using Sigma Plot 2000 (SPSS Inc., Chicago, IL). The inhibitory concentration leading to 50% activity loss (IC50) was obtained by fitting experimental data to the logistic curve by the equation as follows:

\[
\text{Activity} (\%) = 100 \left(1/(1+([I]/IC_{50}))\right)
\]

Inhibition mode was analyzed by Enzyme Kinetics Module 1.0 (SPSS Inc.) equipped with Sigma Plot 2000.

Results and Discussion
The MeOH extract of the M. thunbergii was partitioned successively with n-Hexane, CHCl3, and BuOH soluble fractions, respectively. Then, we were evaluated for the inhibitory activity of the partitioned fraction on tyrosinase. Among the partitioned fractions, the CHCl3 soluble fraction showed significant tyrosinase inhibitory activity (IC50=97.9±5.2 μg/ml), whereas n-Hexane and BuOH fractions showed weaker activity. So, CHCl3 soluble fraction was subject to chromatographed on a normal-phase column and a reverse-phase column, to give two dibenzybutane lignans, macelignan (1) and meso-dihydroguaieric acid (2). Subsequently, HPLC was performed as it is more sensitive, selective, and speedy. The use of ELSD detector is convenient method to identify. The chromatogram of the result of purity analysis by HPLC is shown in Fig. 1. The purity of compounds 1 and 2 were more than 95%.

Compound 1 was obtained as colorless prisms. The EIMS of 1 had a molecular ion peak at m/z 328 [M⁺], consistent with the molecular formula of C20H24O4. The 1H-NMR spectrum of 1 showed one hydroxyl group δH 5.45 (1H, s), one methoxy group δH 3.86 (3H, s), two methyl group δH 0.84 (6H, t, J=6.0 Hz, H-9, 9'), two benzylic methylene protons δH 2.31 (2H, m, H-7b, 7'b) and 2.74 (2H, dd, J=4.5, 13.6 Hz,
paring its spectroscopic data with literature data [20, 26].

3-methoxyphenyl)-8,8'-dimethylbutane (macelignan) by comparing its spectral data with literature data [20, 26]. The isolated compounds were evaluated for their inhibitory activity on tyrosinase. Among them, phenylpropanoids, stilbenes, and other derivatives, including 3,4-dihydroxycinnamic acid [13], oxyresveratrol were identified as tyrosinase inhibitors. Among them, phenylpropanoids containing a hydroxyl group possess potent tyrosinase inhibitory activity. In the case of oxyresveratrol the presence of a hydroxyl group in the ring seems to be related to their inhibitory potency [22]. In this study as well, we suggest that the inhibitory effect of 2 was determined by the guaiacyl

Fig. 2. Inhibitory effect of MDGA (2) on tyrosinase. (A) Effect of MDGA (2) on the tyrosinase catalyzed oxidation of L-tyrosine. Inset: Plot of 1/ν vs. concentration of MDGA (2). (B) Dixon plots for the inhibition of the monophenolase activities of tyrosinase by MDGA (2). Concentrations of substrates for curves were 300 (●) and 450 (○) μM, respectively. (C) Time dependent inhibition of tyrosinase in the presence of MDGA (2). Concentrations of MDGA (2) from top to bottom were 0 (0), 3 (1), 10 (2), 25 (3) μM.

Table 1. Tyrosinase inhibitory activity of compounds

<table>
<thead>
<tr>
<th>Compounds</th>
<th>L-Tyrosine IC_{50} (μM)</th>
<th>L-DOPA IC_{50} (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>82.81</td>
<td>&gt;200</td>
</tr>
<tr>
<td>2</td>
<td>10.20</td>
<td>145.32</td>
</tr>
<tr>
<td>Kojic acid</td>
<td>11.55</td>
<td>14.05</td>
</tr>
</tbody>
</table>

μ

L=10^{−6} M (Table 1). In the previous reports, phenylpropanoids, stilbenes, and other derivatives, including 3,4-dihydroxycinnamic acid [13], oxyresveratrol were identified as tyrosinase inhibitors. Among them, phenylpropanoids containing a hydroxyl group possess potent tyrosinase inhibitory activity. In the case of oxyresveratrol the presence of a hydroxyl group in the ring seems to be related to their inhibitory potency [22]. In this study as well, we suggest that the inhibitory effect of 2 was determined by the guaiacyl
The oxidation of monophenol substrates such as L-tyrosine and 2C). As shown in Fig. 2C, the lag time is known for the studied. MDGA (2) showed time dependent inhibition (Fig. 2). o n the tyrosinase catalyzed oxidation of L-tyrosine was that 2 inhibit the hydroxylation of L-tyrosine.

This lag can be shortened. This lag time can be extended from CHCl3-soluble fractions of M. thunbergii. The structures were identified as macelignan (1) and MDGA (2) by the physicochemical and spectroscopic data. The isolated compounds were evaluated for their tyrosinase inhibitory activities. Among them, MDGA (2) inhibited the oxidation of L-tyrosine catalyzed by mushroom tyrosinase. The inhibition mechanism obtained from the Dixon plot show that MDGA (2) is a competitive inhibitor. MDGA (2) only binds the free enzyme to form an EI complex rather than bind the ES complex.

Use of tyrosinase inhibitors is becoming increasingly important in the cosmetic industry due to their skin whitening and preventive effects. Besides being used in the treatment of some dermatological disorders associated with melanin hyperpigmentation, tyrosinase inhibitors have found an important role in the cosmetic and pharmaceutical industries for their skin-whitening effect and depigmentation after sunburn [23]. In this study, it can be concluded that MDGA (2) can be a potential candidate for the treatment of melanin biosynthesis related skin diseases, likely hyper-pigmentation of human as well as animals.

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References

초록: 후박나무에서 분리한 meso-dihydroguaiaretic acid의 tyrosinase 저해활성

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후박나무(녹나무과)는 한국과 일본 등지에 서식하는 상록 교목으로 한국, 중국, 일본에서 부종, 복통, 복부 팽만 등의 질병 치료를 위해 오랫동안 사용되어 오고 있다. 본 연구에서는 후박나무 껍질을 메탄올에 추출하고 메탄올 추출물을 헥산, 클로로포름, 부탄올에 순차적으로 분획하였다. 클로로포름 분획물로부터 2종의 화합물이 분리되었으며 분리된 화합물1과 2의 구조는 1H-, 13C-NMR과 참고 문헌 데이터에 의해 dibenzylbutane lignin 화합물인 macelignan (1)과 meso-dihydroguaiaretic acid (2)로 동정되었다. 분리된 화합물들의 tyrosinase 저해 활성을 측정한 결과, 화합물 2는 tyrosinase 저해 활성 중 monophenolase (IC50 = 10.2 µM)에 대해 높은 저해성을 나타내는 경쟁적 저해제였으며 효소에 결합하는 화합물 2의 저해 상수(Ki 값)는 4.8 µM였다. 따라서 meso-dihydroguaiaretic acid (2)는 멜라닌 생합성과 관련된 피부 질환 치료를 위한 잠재적 후보가 될 수 있을 것으로 판단된다.