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Two Neolignans from *Perilla frutescens* and Their Inhibition of Nitric Oxide Synthase and Tumor Necrosis Factor-α Expression in Murine Macrophage Cell Line RAW 264.7

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Abstract—Two neolignans were isolated from leaves of *Perilla frutescens* (Labiatae) as inhibitors of nitric oxide syntheses (IC_{50} 5.9 μM and 53.5 μM, respectively) and tumor necrosis factor-α in lipopolysaccharide-activated RAW 264.7 cells. Their structures were identified as 1β,2α,3β,4α-1,2-dimethyl-3,4-bis-(2,4,5-trimethoxyphenyl)-cyclobutane (magnosalin), and 1α,2β,3β,4α-1,2-dimethyl-3,4-bis-(2,4,5-trimethoxyphenyl)-cyclobutane (andamanicin), and their activities were confirmed as resulted from the suppressed expression of inducible nitric oxide synthase enzyme and from the secretion of tumor necrosis factor-α from activated macrophages.

L-Arginine-derived nitric oxide (NO) is an intracellular mediator produced in mammalian cells by two types of nitric oxide synthase (NOS).[1] A constitutive NOS (c-NOS) is Ca^{2+}-dependent and releases small amounts of NO required for physiological functions[2] whereas the other form, inducible NOS (iNOS) is Ca^{2+}-independent[3] and is induced by lipopolysaccharide (LPS) or proinflammatory cytokines such as IL-1β and IFN-γ.[4] NO produced in large amounts by iNOS and its derivatives, such as peroxynitrite and nitrogen dioxide, play a role in inflammation and also possibly in the multistage process of carcinogenesis.[5] NO is also known to be responsible for the vasodilation and hypotension observed in septic shock.[6] TNF-α, a primary mediator in the septic circulation, is also known to induce iNOS.[7] Therefore, an inhibitor of iNOS may be effective as a therapeutic agent for the treatment of septic shock and inflammation. During our efforts to find new modulators of iNOS[8,9] and TNF-α[10,11] from medicinal plants, leaves of *Perilla frutescens* was selected as one of the target plant sources from the massive activity screening of plant extracts. The extracts of *P. frutescens* were reported as having modulatory activity of NO[12] and TNF-α,[13,14] but the active principle has not been elucidated yet. In this paper, we report the isolation and structural elucidation of two active principles from *P. frutescens* and their inhibitory activity of NO and TNF-α release from LPS-activated macrophages.

For the activity-guided fractionation, NO in the cell culture media was quantitated after incubation with samples during LPS-activation of RAW 264.7 cells as described before.[8] The levels of TNF-α secreted into culture media of RAW 264.7 cells were evaluated as cytotoxicity against L929 cells as previously described[15] with slight modifications.[10] The protein levels of iNOS and TNF-α were assessed by the Western blot analysis by using iNOS specific antibody and TNF-α specific antibody.[11]

Purification and Structural Identification of Active Principles

The dried leaves of *P. frutescens* were purchased from the Kyungdong oriental drug market in Seoul and authenticated by Prof. T. H. Kim at the College of Pharmacy, Sookmyung Women's University. The plant materials (2 kg) were extracted three times with MeOH and the combined extracts (159 g) were concentrated and partitioned between *n*-hexane and H_2O. The *n*-hexane soluble fraction (68 g) exhibited 65% inhibitory activity

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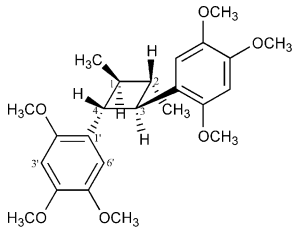
of NO production at 10 μg/mL in LPS-activated RAW 264.7 cells. Activity-guided fractionations of hexane soluble fraction were performed by successive column chromatography on silica gel eluting with *n*-hexane–EtOAc (20:1) and *n*-hexane–acetone (100:1), and finally by reversed-phase HPLC (μ-Bondapak C-18 column, 10×300 mm; 65% MeOH, 2.0 mL/min; UV 254 nm) to yield compound **1** (5 mg) and compound **2** (20 mg).



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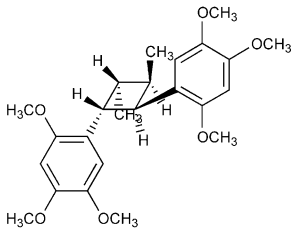

2

Compound **1** and **2** showed similar spectral patterns in MS, IR, UV, ^{1}H NMR and ^{13}C NMR spectra.[16] In ^{1}H NMR spectra of **1**, we found one methyl peak at δ 1.19, three methoxy peaks at δ 3.68∼δ 3.86, two methine protons at δ 1.75 and δ 3.26 which correlated together in ^{1}H–^{1}H COSY spectra, and two isolated olefinic protons at δ 6.46 and δ 6.94. All protonated carbons of **1** were identified in DEPT spectra, and four non-protonated carbons were also found in ^{13}C NMR spectra. From these data we could induce the probable molecular formula of **1** as $C_{12}H_{16}O_3$ (MW 208). From the FABMS data of **1**, molecular weight was confirmed as 416, and **1** could be considered as a symmetric dimer. From the analogy of ^{1}H and ^{13}C NMR data with reported spectral data,[17] structure of **1** was identified as 1β,2α,3β,4α-1,2-dimethyl-3,4-bis-(2,4,5-trimethoxyphenyl)-cyclobutane (magnosalin). The structure of magnosalin was reported in 1983[18] and corrected by X-ray crystallography afterwards.[17] Those structures of **1** and **2** are very confusing and they can only be distinguished by NMR spectral patterns. The signals of methine and benzylic protons of **2** were observed at lower field (δ 2.72 and δ 3.80, respectively) due to the anisotrophic effects of aromatic rings. These protons of **2** are fall into deshielding zone, while those of **1** are fall into shielding zone. By the comparison with the published spectral data, structure of **2** was identified as 1α,2β,3β,4α-1,2-dimethyl-3,4-bis-(2,4,5-trimethoxyphenyl)-cyclobutane (andamanicin). Magnosalin and andamanicin were reported from *Magnolia salicifolia*[18] and *Piper sumatranum*,[19] respectively, and these structures has not been reported from *P. frutescens* yet.

Biological Evaluation

When compound **1** and **2** were treated into the cell culture media during LPS (1 μg/mL)-activation of RAW 264.7 cells, they showed dose-dependent inhibition of NO syntheses and their IC_{50}'s were 5.9 and 53.5 μM, respectively. When **1** was treated after induction of iNOS by activated cells, the inhibition of NO syntheses by **1** was very weak (17% inhibition at 30 μM comparing with LPS-control), while aminoguanidine (0.1 mM), known as iNOS specific inhibitor, exhibited 79% inhibition of NO production (data not shown). These results implied that **1** might not be an inhibitor of iNOS enzyme activity, but an inhibitor of iNOS expression in LPS-activated cells. This postulation was confirmed by the dose-dependently reduced amounts of iNOS on Western blot by the treatment of **1** and **2** (Fig. 1a). We also examined whether **1** and **2** affect the level of TNF-α, another mediator of septic shock, in LPS-activated RAW 264.7 cells. We measured the LPS-induced TNF-α released into the cell culture media by the method of L929 cell cytotoxicity. The treatment with 10 μM of **1** showed a relative cytotoxicity of 26% compared with LPS control. This means that **1** decreased the release of TNF-α from activated cells which showed cytotoxicity against L929 cells. The inhibitory activity of TNF-α production of **1** is much higher than that of **2** that showed 84% of cytotoxicity at 10 μM (Fig. 2), and this

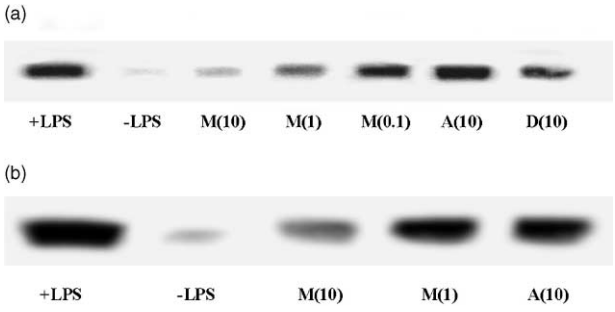

Figure 1. Western blot of iNOS and TNF-α. (a) Effects of magnosalin (M, μM), andamanicin (A, μM) and dexamethasone (D, μM) on the expression of iNOS in LPS-activated RAW 264.7 cells. The cytosolic fractions prepared as described elsewhere[8] were applied on 8% SDS-polyacrylamide gels and transferred to PVDF membrane by standard method. It was incubated with mouse iNOS polyclonal antibody and developed by the method of user's manual of ECL kit (Amersham Pharmacia Biotech, UK). (b) Effects of magnosalin (M, μM), andamanicin (A, μM) on the release of TNF-α into the culture media of LPS-activated RAW 264.7 cells. Cell culture media were concentrated by precipitation with cold acetone and applied on 15% SDS-polyacrylamide gels and transferred to PVDF membrane. It was incubated with mouse TNF-α polyclonal antibody and developed by the method of user's manual of ECL kit (Amersham Pharmacia Biotech, UK).

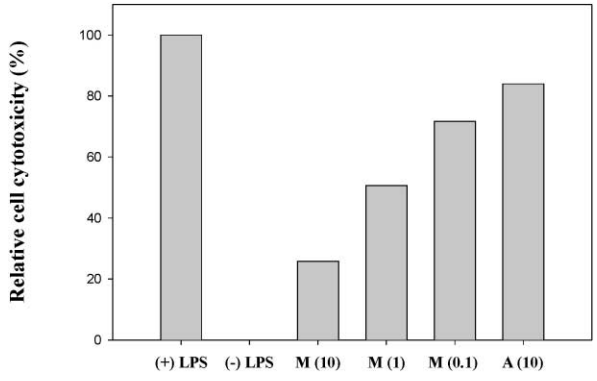

Figure 2. The effect of magnosalin (M, μM) and andamanicin (A, μM) on cell cytotoxicity of TNF-α from LPS-activated RAW 264.7 cells against L929 cells. Bioassay samples were prepared from RAW 264.7 cells that were activated by LPS (1 μg/mL) for 5 h. L929 cells (3×10^6/mL) were seeded in a 100 μL of RPMI-1640 media into 96 well plates. Following a 18 h incubation at 37 °C in 5% CO_2 in air, spent media were removed and replaced with media with 10% FBS and actinomycin D (1 μg/mL). Bioassay samples were added into the plates and incubated for 18 h followed by the MTT-based colorimetric assay. Results represent the mean of three experiments.

activity order of **1** and **2** is parallel to the NO inhibition. Westernblot analyses (Fig. 1b) showed that RAW 264.7 cells activated by LPS in the presence of **1** and **2** contained reduced amounts of TNF-α protein compared with LPS control groups. Regarding the inhibitory activity of NO and TNF-α production by **1** and **2** in activated RAW 264.7 cells, **1** (magnosalin) is about 10 times potent than those of **2** (andamanicin). It is very interesting that these activity differences just came from the difference of relative position of methyl groups at C-1 and C-2 in their stereostructures. These findings are valuable for the understanding the further mechanism of their biological activities and for the structural optimization of these compounds essential for the study for drug development from these lead compounds. Because NF-kB is important in mediating transcriptional control of iNOS and TNF-α, these active compounds from *P. frutescens* might demonstrate their activity through the effect on the LPS-induced NF-kB signaling cascade. The exact activity mechanism of these compounds should be further studied.

In conclusion, magnosalin (**1**) and andamanicin (**2**) purified from the leaves of *P. frutescens* reduced the induction of iNOS and TNF-α in LPS-activated RAW 264.7 cell culture system. Owing to these biological properties of magnosalin and andamanicin, they may thus have potential in the treatment of endotoxemia and inflammation accompanied by the overproduction of NO and TNF-α.

Acknowledgement

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16. Physical and spectroscopic data: 1α,2α,3β,4α-1,2-dimethyl-3,4-bis-(2,4,5-trimethoxyphenyl)-cyclobutane (**1**, magnosalin): colorless needles ($CHCl_3$); mp 97–98 °C; $[\alpha]_D^{23}$ −4.64° (*c* 0.25, $CHCl_3$); UV (EtOH) λ_{max} (log ε) 204 (4.86), 232 (4.23), 292 (4.01) nm; IR (KBr) λ_{max} 2956, 2834, 1515, 1399, 1038 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.19 (6H, d, J = 5.5 Hz, 2×CH_3), 1.75 (2H, brs, H-1 and H-2), 3.26 (2H, diffused d, J = 9.03 Hz, H-3 and H-4), 3.68 (6H, s, 2×OCH_3), 3.84 (6H, s, 2×OCH_3), 3.86 (6H, s, 2×OCH_3), 6.46 (2H, s, H-3′), 6.94 (2H, s, H-6′); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 19.5 (q, CH_3), 43.9 (d, C-1 and C-2), 45.8 (d, C-3 and C-4), 56.6 (q, OCH_3), 57.1 (q, OCH_3), 98.3 (d, C-3′), 112.7 (d, C-6′), 124.3 (s, C-1′), 143.5 (s, C-5′), 147.9 (s, C-4′), 152.l (s, C-2′); FABMS m/z 417 $[M+1]^+$ (calcd for $C_{24}H_{33}O_6$).1α,2β,3β,4α-1,2-dimethyl-3,4-bis-(2,4,5-rimethoxyphenyl)-cyclobutane (**2**, andamanicin): colorless powder ($CHCl_3$); mp 109–110 °C; $[\alpha]_D^{20}$ +2.84° (*c* 0.5, $CHCl_3$); UV (EtOH) λ_{max} (log ε) 204 (4.84), 231 (4.27), 291 (4.03) nm; IR (NaCl, $CHCl_3$ solution) 2952, 2848, 1510, 1398, 1038 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.17 (6H, dd, J = 4.56, 2.04 Hz, 2×CH_3), 2.72 (2H, brs, H-1 and H-2), 3.51 (6H, s, 2×OCH_3), 3.64 (6H, s, 2×OCH_3), 3.79 (6H, s, 2×OCH_3), 3.80 (2H, brs, H-3 and H-4), 6.30 (2H, s, H-3′), 6.55 (2H, s, H-6′); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 15.5 (q, CH_3), 34.4 (d, C-1, C-2), 42.9 (d, C-3, C-4), 56.5 (q, OCH_3), 56.9 (q, OCH_3), 57.1 (q, OCH_3), 98.0 (d, C-3′), 112.7 (d, C-6′), 122.5 (s, C-1′), 142.8 (s, C-5′), 147.7 (s, C-4′), 152.3 (s, C-2′); EI–MS m/z 416 $[M]^+$ (calcd for $C_{24}H_{32}O_6$).
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