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# Ferulic Acid Dimer Inhibits Lipopolysaccharide-stimulated Cyclooxygenase-2 Expression in Macrophages

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**Abstract.** *Phenylpropanoids may act as nonsteroidal anti-inflammatory drug (NSAID)-like compounds. 4-cis,8-cis-Bis(4-hydroxy-3-methoxyphenyl)-3,7-dioxabicyclo-[3.3.0]octane-2,6-dione (bis-FA, compound* ***2****), a dimer of ferulic acid, was synthesized from ferulic acid (****1****), and its effect on lipopolysaccharide (LPS)-stimulated cyclooxygenase-2 (COX-2) expression in RAW 264.7 cells was compared with those of the parent ferulic acid (****1****) and of iso-ferulic acid (3-hydroxy-4-methoxycinnamic acid) (****3****). LPS-induced gene expression of COX-2 was markedly inhibited by compound* ***2*** *at a concentration of 10 μM and by compound* ***3*** *at 100 μM, but was not inhibited by compound* ***1*** *at 100 μM. This observation suggests that compound* ***2*** *may possess potent anti-inflammatory activity. These ferulic acid-related compounds were able to scavenge the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. The 50% inhibitory concentration for DPPH radicals declined in the order* ***3*** *(40.20 mM) >* ***2*** *(3.16 mM) >* ***1*** *(0.145 mM). Compound* ***1*** *possessed potent anti-radical activity, but no COX-2 inhibitory activity, which may be a result of enhancement of its conjugate properties by abstraction of an H atom from the phenolic OH group, causing loss of phenolic function. In contrast, inhibition of COX-2 expression by compounds* ***2*** *and* ***3*** *could be caused by their increased phenolic function, which is associated with decreased anti-radical activity. Compounds* ***2*** *and* ***3****, particularly* ***2****, may have potential as NSAID-like compounds.*

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*o*-Methoxyphenols, such as ferulic acid (4-hydroxy-3-methoxycinnamic acid) and eugenol (4-allyl-2-methoxyphenol), act as antioxidants, but also act as prooxidants under certain circumstances, such as in light, oxygen-rich and alkaline conditions. The prooxidative activity of o-methoxyphenols can cause adverse effects such as allergic and inflammatory reactions (1). Dimerization of *o*-methoxyphenol monomers can reduce their intrinsic prooxidative properties, because the reactive moieties in the monomer are blocked by the other monomer (2). We have previously synthesized a symmetrical dimer of eugenol, *bis*-EUG (2,2'-dihydroxy-3,3'-dimethoxy-5,5'-di(2-propenyl)-1,1'-biphenyl), by the *ortho-ortho* coupling reaction of eugenols, and an asymmetrical dimer of *iso*-eugenol (4-propenyl-2-methoxyphenol), namely dehydrodiisoeugenol (2-(3-methoxy-4-hydroxyphenyl)-3-methyl-5-(1-propenyl)-7-methoxy-2,3-dihydrobenzofuran), from *iso*-eugenol. These dimers showed inhibitory effects on lipopolysaccharide (LPS)-induced cyclooxygenase-2 (COX-2) expression in the mouse macrophage-like cell line RAW 264.7 (3, 4). Furthermore, ferulic acid derivatives, with a molecular structure in which the two ferulic acid moieties face each other, were reported to suppress COX-2 promoter activity in a concentration-dependent manner (5). These findings strongly suggest that dimers of ferulic acid would be able to inhibit COX-2 expression. With the aim of finding *ortho*-methoxyphenol derivatives with potent anti-inflammatory activity, a dimer of ferulic acid (bis-FA, **2**) and evaluated for its effects on LPS-induced COX-2 expression in comparison with those of ferulic acid (**1**) and iso-ferulic acid (**3**). The activity of these compounds against DPPH radicals was also investigated, since COX-2 inhibitory activity has previously been reported to correlate with 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity (6-8).

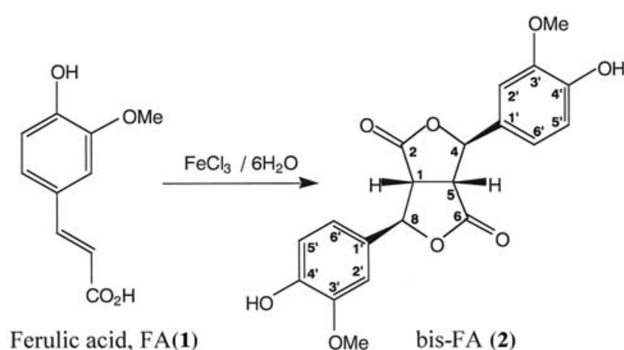


Figure 1. *Synthesis of bis-FA (2) by dimerization of ferulic acid. Ferulic acid (1) was reacted with $FeCl_3/H_2O$.*

## Materials and Methods

*Materials.* The following chemicals and reagents were obtained from the indicated sources: ferulic acid (Tsuno Food Industrial Co. Ltd., Wakayama, Japan); *iso*-ferulic acid (Tokyo Kasei Co. Ltd., Tokyo, Japan); Megaprime DNA labelling system and 5'-[$\alpha$-$^{32}$P]dCTP (Amersham Biosciences Co., Piscataway, NJ, USA); RPMI 1640 (Invitrogen Corp., Carlsbad, CA, USA); FBS (HyClone, Logan, UT, USA); *Escherichia coli* O111 B4-derived LPS (List Biological Laboratories, Inc., Campbell, CA, USA).

*Synthesis of bis-FA.* Compound **2**, bis-FA, was synthesized from ferulic acid by the previously reported procedure (9-11), as shown in Figure 1. Briefly, a solution of ferulic acid (1.94 g, 0.01 mol) in 30 ml of methanol was added slowly to a mixture of $FeCl_3/6H_2O$ in 200 ml of water, and was stirred for 48 h at room temperature in the presence of an air stream. The reaction mixture was extracted five times with $CHCl_3$. The organic layer was washed with $H_2O$ and saturated aqueous NaCl, dried over anhydrous $Na_2SO_4$ and evaporated to give a viscous oily product, which was purified by silica-gel column chromatography to give a colorless solid, **2**, mp 212˚C [213.8-214.7˚C (9); 211-212˚C (10)], in 18% yield. The $^1$H-NMR (500 MHz, DMSO-$d_6$) spectrum was in good agreement with that in the literature (9): δ 3.79 (s, 6H, $OCH_3$ x 2), 4.20 (dd, *J*=1.3 and 1.3Hz, 2H, H-1/5), 5.72 (brs, 2H, H-4/8), 6.79 (d, *J*=8.1Hz, 2H, H-5'), 6.86 (dd, *J*=8.2 and 2.1Hz, 2H, H-6'), 6.99 (d, *J*=2.1Hz, 2H, H-2'), 9.25 (s, 2H, OH x 2). The $^{13}$C-NMR (125 MHz, DMSO-$d_6$) spectrum was similar to reported data in acetone-*d6* (10): δ 48.54 (C1/5), 56.27 ($OCH_3$), 82.54 (C4/8), 111.12 (C2'), 115.92 (C5'), 119.69 (C6'), 129.47 (C1'), 147.84 (C4'), 148.33 (C3'), 175.89 (C2/6). Two-dimensional HMQC (Heteronuclear Multiple Quantum Correlation), HMBC (Heteronuclear Multiple Bond Correlation), and phase-sensitive NOESY (Nuclear Overhauser Enhancement Spectroscopy) experiments proved the structure of **2** proposed previously.

*Cell culture.* Cells of the murine macrophage cell line RAW 264.7, obtained from the Riken Cell Bank, were used. They were cultured to the subconfluent state in RPMI 1640 medium supplemented with 10% FBS at 37˚C under 5% $CO_2$ in air, washed and then incubated overnight in serum-free RPMI 1640. They were then further washed and treated with the test samples.

*DNA hybridization probe.* COX-2 cDNA probes were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). A plasmid containing β-actin cDNA was obtained from the Japanese Cancer Research Bank (Tokyo, Japan). The method used for plasmid preparation was similar to that described previously (12).

*Northern blot analysis.* Cells ($10^6$/dish) in Falcon 5-cm-diameter dishes (Becton Dickinson Labware, Franklin Lakes, NJ, USA) were treated with test samples, and then total cellular RNA was extracted from them by the AGPC (Acid Guanidine Phenol Chloroform) procedure. As described earlier (13), the RNA was subjected to 1% agarose electrophoresis and blotted onto nylon membranes (Micron Separations, Inc., Westboro, MA, USA). The membranes were then hybridized with cDNA probes that had been labelled with 5'-[$\alpha$-$^{32}$P]dCTP by use of the Megaprime DNA labelling system. After hybridization, the membranes were washed, dried and exposed overnight to Kodak X-ray film (Eastman Kodak Co., Rochester, NY, USA) at –70˚C. β-Actin was used as internal standard for the quantification of total RNA in each lane of the gel.

*Anti-DPPH radical activity.* Radical-scavenging activities were determined with DPPH as a free radical. For each inhibitor, various concentrations were tested in ethanol. The decrease in absorbance was determined at 517 nm for 10 min at room temperature. Antiradical activity was calculated as the concentration (mole/l) of inhibitor necessary to decrease the initial DPPH radical concentration by 50% ($IC_{50}$).

## Results

*COX-2 inhibitory activity.* Northern blot assays were used to investigate the inhibitory effects of compounds, **1**, **2** and **3** on LPS-induced COX-2 gene expression in RAW264.7 cells. Cells that had been pretreated or not with the indicated doses of these compounds were incubated for 1 h, with or without LPS, and then their total RNA was prepared and analyzed by Northern blot assay for COX-2 gene expression. Figure 2 shows that the LPS-induced gene expression of COX-2 was clearly inhibited by *bis*-FA (**2**) at a concentration of 10 μM and was also inhibited by *iso*-ferulic acid (**3**) at the higher concentration of 100 μM. In contrast, the gene expression was not inhibited by ferulic acid (**1**) at a concentration of 100 μM. Compound **2** showed the most potent inhibitory effect on COX-2 expression. None of the compounds tested caused notable cytotoxicity at concentrations up to 500 μM.

*DPPH radical-scavenging activity.* The DPPH radical-scavenging activities of the methoxyphenol derivatives were used to evaluate their antioxidant activity. The 50% inhibitory concentration ($IC_{50}$) was determined (Figure 3). The $IC_{50}$ value declined in the order compound **3** (40.20 mM) > compound **2** (3.16 mM ) > compound **1** (0.24 mM). The DPPH radical-scavenging activities of compounds **3** and **2** were about 200-fold less and 10-fold less, respectively, than that of compound **1**.

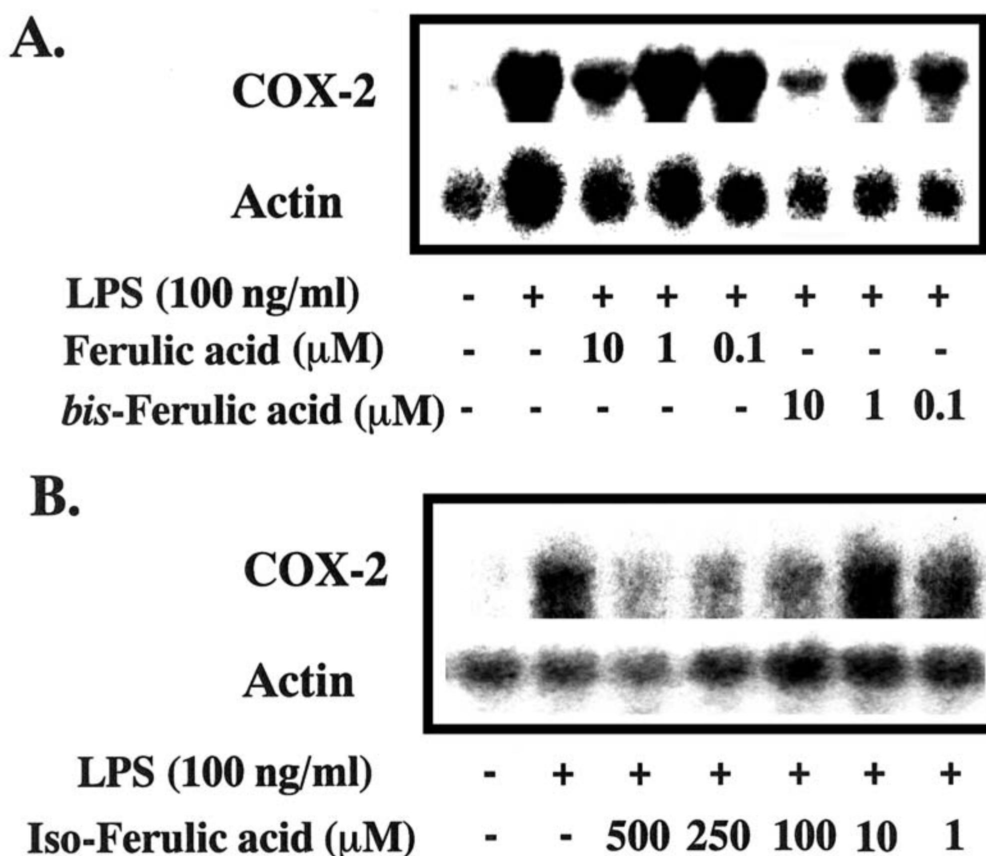


Figure 2. *Regulatory effects of ferulic acid and bis-FA (A) and iso-ferulic acid (B) on LPS-induced expression of the COX-2 gene in RAW 264.7 cells. The cells were pretreated or not for 30 min with the indicated doses of ferulic acid, bis-ferulic acid or iso-ferulic acid. They were then treated or not with LPS at 100 ng/ml. Their total RNA was prepared 3 h after the addition of LPS. Northern blot analysis was performed with COX-2 and beta-actin cDNAs as probes. Three independent experiments were performed and similar results were obtained. To perform the statistical analysis, the data were expressed as relative signal intensity (the ratio of the density of COX-2 to that of actin at each concentration). There was a significant difference between bis-ferulic acid at 10 μM and control ($p<0.01$). In contrast, no significant difference was found between ferulic acid at 10 μM and control. There was a significant difference between iso-ferulic acid at 100 μM, 250 μM and 500 μM and control.*

## Discussion

The rate-limiting step in the conversion of arachidonic acid to prostaglandin $H_2$, the precursor of biologically-active prostaglandins (PGs), is catalyzed by the enzyme COX. COX-2 is induced in a variety of cells by growth factors, cytokines and LPS *via* activation of transcription factors such as NF-kappaB. In addition, COX-2 is closely involved in inflammation, arthritis, Alzheimer's disease, pain and cancer (14-17). In the present study, *bis*-FA (**2**) and *iso*-ferulic acid (**3**) showed potent inhibition of COX-2 expression, whereas ferulic acid (**1**) did not. Correlations between the ability to inhibit LPS-induced COX-2 expression in RAW 264.7 cells and anti-DPPH radical activity have previously been reported for methoxyphenol derivatives (6) and chalcones with phenolic OH groups (7), with these compounds showing both antioxidant activity and COX-2 inhibitory action. Since *o*-methoxyphenols possess potent free radical-scavenging properties, they may suppress LPS-induced gene expression partly by reducing the oxidative stress caused by LPS treatment, possibly *via* inhibition of AP-1 and NF-kappaB DNA-binding activity (18). Nevertheless, in a study of the biological functions of flavanones, which are also phenolic compounds, there was little correlation between the ability to inhibit TPA (12-*O*-tetradecanoylphorbol-13-acetate)-induced COX-2 expression and anti-DPPH radical activity (8). Similarly, in the present study, bis-FA (**2**) and *iso*-ferulic acid (**3**) showed potent COX-2 inhibition without notable cytotoxicity, despite their low anti-DPPH radical activity. We have previously reported that ferulic acid and eugenol preferentially scavenged NO, superoxide ($O_2^-$) and hydroxy ($OH^-$) radicals in addition to

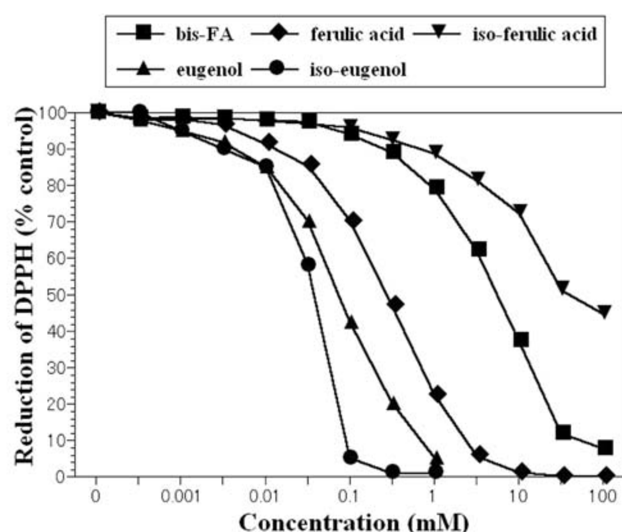


Figure 3. *Reduction of DPPH (% of control) as a function of concentrations of antioxidants. The procedures are described in Materials and Methods.*

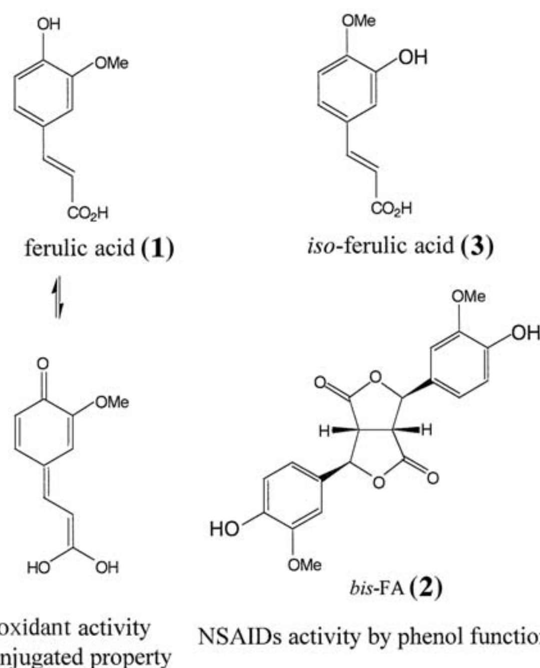


Figure 4. *Ferulic acid (**1**) shows an enhanced antioxidant effect because of conjugation, and its phenolic function is lost. In contrast, iso-ferulic acid (**3**) and bis-FA (**2**) act as NSAID-like compounds because they show increased phenolic function.*

DPPH radicals and, on the basis of a stoichiometric study, suggested the occurrence of dimerization for these compounds (19). As shown in Figure 4, in biological systems both ferulic acid and eugenol are easily oxidized by scavenging various radical species, possibly associated with dimerization during the oxidation process as a result of their high resonance stability. Thus, our observation of the lack of inhibitory effect of ferulic acid (**1**) on COX-2 expression may be explained by high resonance stabilization after reduction of the phenolic function, *i.e.* enhancement of its conjugate properties after abstraction of the H atom from the phenolic OH group. Similarly, eugenol may show enhanced conjugate properties after radical oxidation. As expected, eugenol shows little anti-inflammatory activity, as characterized by NFkappaB inhibition and COX-2 inhibition (3). In contrast, *iso*-ferulic acid (**3**) predominantly retains its phenolic function, resulting in COX-2 inhibition (Figure 4).

In the present study, the dimer of ferulic acid, compound **2**, showed potent COX-2 inhibition. We previously reported that dehydrodiisoeugenol, an asymmetric dimer of *iso*-eugenol, showed potent COX-2 inhibition and NFkappaB inhibition at the low concentration of 0.1 μM, whereas *iso*-eugenol, the parent monomer, did not (4). The DPPH radical-scavenging activity of dehydrodiisoeugenol ($ID_{50}$ 1.30 mM) was markedly less than that of *iso*-eugenol ($ID_{50}$ 0.055 mM) and similar to that of *bis*-FA ($ID_{50}$ 3.16 mM). Overall, these findings indicate that the ability of derivatives of phenylpropanoids, such as ferulic acid and eugenol, to inhibit COX-2 gene expression is dependent on their retention of a phenolic function. Dimerization of *o*-methoxyphenols, leading to high levels of phenolic function, may be a useful tool for the development of NSAID-like compounds.

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