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Low molecular weight lignin suppresses activation of NF-κB and HIV-1 promoter

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Abstract—Human immunodeficiency virus type 1 (HIV-1) is a cytopathic retrovirus and the primary etiological agent of acquired immunodeficiency syndrome (AIDS) and related disorders. In cells chronically infected with HIV-1, nuclear factor-κB (NF-κB) activation by external stimuli such as tumor necrosis factor α (TNFα) augments replication of HIV-1. NF-κB involves in many diseases such as inflammation, cancer, and Crohn's disease. In this paper, we exhibit that (i) HIV-1gene expression was inhibited by lignin, (ii) fraction of small molecular mass in HBS lignin (less than 0.5 kDa) had stronger inhibitory effects than large molecular mass (more than 1.3 kDa), (iii) lignin also inhibited activation of NF-κB induced by TNFα, (iv) among six lignin dimer-like compounds, compound **6** containing β-5 bond has more potent inhibitory activity than compounds **1**, **2**, **3**, **4** and **5**, which contain β-1, β-*O*-4, 5-5, or β–β structural units. These results suggested that small molecules of lignin inhibit HIV-1 replication through suppression of HIV-1 transcription from LTR including activation via NF-κB. Low molecular lignin may be a beneficial material or drug leads as a new class for AIDS and NF-κB-related diseases.

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) is a cytopathic retrovirus and the primary etiological agent of acquired immunodeficiency syndrome (AIDS) and related disorders. The recent progress in combination therapy against viral reverse transcriptase and protease has achieved considerable reduction of the viral load in HIV-1-infected individuals and significant improvement in survival, however, chemotherapy could not terminate latent infection, and HIV-1 replicates continuously, even during the prolonged asymptomatic period between primary infection and the development of AIDS.[1–3] To control HIV-1 replication from latently infected cells, it is important to search for new drugs. Among the various steps of viral life cycle, the step of transcription from HIV-1 provirus is conceived to be crucial for viral replication since amplification of the viral genetic information is attainable only through transcription. HIV-1 transcription is directed by the promoter located in the 5′-long terminal repeat (LTR) of the integrated provirus. In cells chronically infected with HIV-1, activation of nuclear factor-κB (NF-κB) by external stimuli such as tumor necrosis factor α (TNF-α) and its binding to LTR triggers the initiation of transcription of viral genes, which results in explosive HIV-1 replication.[4–6] It is expected that inhibitor of HIV-1 transcription could be used for chemotherapy. Lignin is a polyphenolic material arising from oxidative polymerization of three phenylpropanoid monomers, *p*-coumaryl, coniferyl, and sinapyl alcohols (Fig. 1), and are amorphous cell wall polymer is covalently bound to cellulose and hemicelluloses.

The structural elements comprising lignin are linked by various species of carbon-carbon and ether bonds.[7] Lignin extracted from natural products has anti-HIV activities toward cultured cell.[8–11] The synthetic lignin-like polymer (DHP) also has anti-HIV effect in vivo. DHP suppresses the absorption of HIV-1 onto the cultured

Abbreviations: AIDS, acquired immunodeficiency syndrome; DHP, synthetic lignin-like polymer; HBS lignin, High-boiling solvent lignin; HIV-1, human immunodeficiency virus type 1; LTR, 5′-long terminal repeat; NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor-α.
Keywords: HBS; HIV-1; Lignin; LTR; NF-κB; TNF-α.
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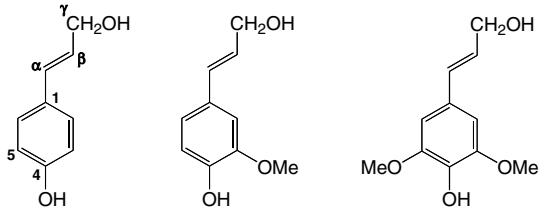

Figure 1. Structures of lignin monomer.

cell surface, and lignin and DHP inhibit activity of HIV-1 protease in vitro. However, mechanism of inhibition of lignin on HIV-1 replication is still obscure. Moreover, there is no information about relationship between structure of lignin and its inhibitory activity.

In this paper, we exhibit that (i) HIV-1gene expression was inhibited by lignin, (ii) fraction of small molecular mass in HBS lignin[12] (less than 0.5 kDa) had stronger inhibitory effects than large molecular mass (more than 1.3 kDa), (iii) lignin also inhibited activation of NF-κB induced by TNFα, (iv) among six lignin dimer-like compounds, compound **6** containing β-5 structure has more strong inhibitory activity than the other compounds, which contain β-1, β-*O*-4, 5-5, or β–β structures. These results suggested that small molecules of lignin inhibit HIV-1 replication through suppression of HIV-1 transcription from LTR including activation of NF-κB. This paper is the first report about the mechanism of inhibitory effect of lignin on HIV-1 replication, and demonstrating for the first time about relationship between structure of lignin and its inhibitory activity.

2. Results

2.1. Suppression of HIV-1 gene expression by lignin

There are several methods for extraction of lignin from wood, and properties of those lignins are different, respectively. Therefore, three kinds of lignin, lignin alkali, lignin organosolve, and HBS lignin,[12] were used. At first, to assess whether lignin inhibits HIV-1gene expression, we examined transient luciferase assay using reporter plasmid, CD-12-luc is widely used in studying transcription of HIV-1 gene.[13,14] Transfected cells were cultured in the absence or presence of lignin for 20 h. As shown in Figure 2, lignin alkali did not have effect on HIV-1gene expression. However, 71%, 60%, and 70% decrease in expression were observed with 100 μg/ml lignin organosolve, A-HBS lignin, and B-HBS lignin, respectively. This result suggested that anti-HIV effect of lignin differs with kinds of lignin.

The cells were co-transfected with pCD-12-luc and pCMV-β-galactosidase. Transfected 293-T cells were cultured for 4 h and then incubated for 20 h with vehicle (column 1), 100 μg/ml lignin alkali (column 2), 100 μg/ml lignin organosolve (column 3), 100 μg/ml A-HBS lignin (column 4), and 100 μg/ml B-HBS lignin (column 5). Data are means from three independent experiments.

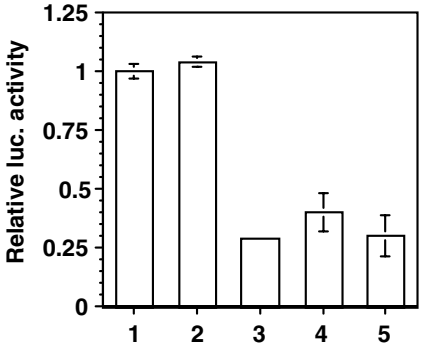

Figure 2. Suppression of HIV-1 gene expression by lignin.

2.2. Distribution of molecular weights of HBS lignin

It is thought that the average molecular weight of lignin considerably differs among extraction methods. To investigate relationship between molecular weight and anti-HIV effect, HBS lignins were fractionated between ether-based Soxhlet extracts and insoluble residue, and their average molecular weights were determined by size exclusion chromatography (Table 1). The average molecular weights of A- and B-HBS lignins were smaller than those of lignin alkali, and those of ether soluble fraction were smaller than those of ether insoluble fraction. The number-average molecular weights of ether soluble fractions of A- and B-HBS lignins were 390 and 470, respectively. Thus, these results suggested that main components of ether soluble fraction are dimer and trimer lignins.

2.3. Relationship between molecular weight and effect on HIV-1 gene expression

As shown in Table 2, the 50% inhibitory concentrations (IC_{50}) of ether soluble fraction of A- and B-HBS lignins for HIV-1gene expression were 25 and 19 μg/ml, respectively. The insoluble fractions of A- and B-HBS lignins showed IC_{50} values of 308 and >400 μg/ml, respectively. From these results, it is thought that low molecular weight lignin (less than 0.7 kDa) has inhibitory effect on transcription of HIV-1 gene rather than high molecular weight lignin (over 1.3 kDa).

2.4. Suppression of NF-κB activation by lignin

NF-κB is an inducible cellular transcription factor that regulates viral gene expression via the two NF-κB sites

Table 1. Distribution of molecular weights of HBS lignin

	Mn	Mw	Mw/Mn
Lignin alkali	5000[a]	28,000[a]	5.6[a]
A-HBS lignin	900	3020	3.6
Ether soluble	390	530	1.3
Ether insoluble	1320	3860	2.9
B-HBS lignin	890	2310	2.6
Ether soluble	470	630	1.3
Ether insoluble	1470	3050	2.1

Mn and Mw indicate number–average molecular weight and weight–average molecular weight, respectively.
[a] Data from Sigma–Aldrich catalogue.

Table 2. Effects of lignin on LTR (HIV-1) promoter

Lignin	Yield (%)	IC_{50} (μg/ml) LTR (HIV-1)
Lignin alkali		>400
Lignin organosolve		78
A-HBS lignin	100	91
Ether soluble	14.1	25
Ether insoluble	85.9	308
B-HBS lignin	100	82
Ether soluble	32	19
Ether insoluble	68	>400

Transfected cells were cultured for 4 h and then treated with or without lignin for 20 h.

Table 3. Effects of lignin on NF-κB activity

Lignin	IC_{50} (μg/ml)	
	NF-κB	NF-κB + TNF
Lignin alkali	>400	>400
Lignin organosolve	68	84.5
A-HBS lignin	200	100
Ether soluble	37	37
Ether insoluble	>400	271
B-HBS lignin	71	67.5
Ether soluble	33	21
Ether insoluble	>400	>400

'NF-κB' column: Transfected cells were cultured for 4 h and then treated with or without lignin for 20 h. 'NF-κB + TNF' column: Transfected cells were cultured for 19 h and then treated with or without lignin for 1 h. Treated cells were stimulated with 3 ng/ml TNFα for 5 h.

located in the HIV-1 LTR enhancer region.[4–6] Treatment with compounds that block NF-κB activation inhibits HIV-1 gene expression and viral replication. Hence, we examined whether lignin suppresses NF-κB activation.

As shown in Table 3, lignin organosolve, A-HBS lignin, and B-HBS lignin also inhibited NF-κB activity in the absence and presence of TNFα, whereas lignin alkali did not show significant inhibitory effects. The ether soluble fractions have more inhibitory effect than ether insoluble fraction and whole HBS lignin. These results suggested that lignin supresses HIV-1 gene expression via inhibition of NF-κB activity, and inhibitory effect is attributed to low molecular weight lignin.

2.5. Inhibitory effects on HIV-1 gene expression and NF-κB activation of lignin model compounds

There are various structures in the lignins from plant and DHP. It is important to explore what is the most effective structural unit. Therefore, we synthesized six lignin dimer-like compounds (Fig. 3) and measured their inhibitory activities (Table 4). Compounds **1** and **2** were used as lignin dimer model of β-*O*-4 substructure, and compounds **3**–**6** were dimer models of β-1, 5–5, β–β, and β-5 substructures, respectively. These substructures are representative structural units in lignin. Among them, compound **6** showed most potent inhibitory activity on LTR (HIV-1) and NF-κB, and compound **2** also had significant activities. Values of IC_{50} on LTR and NF-κB activities of compound **6** were 34 and 19 μg/ml, respectively.

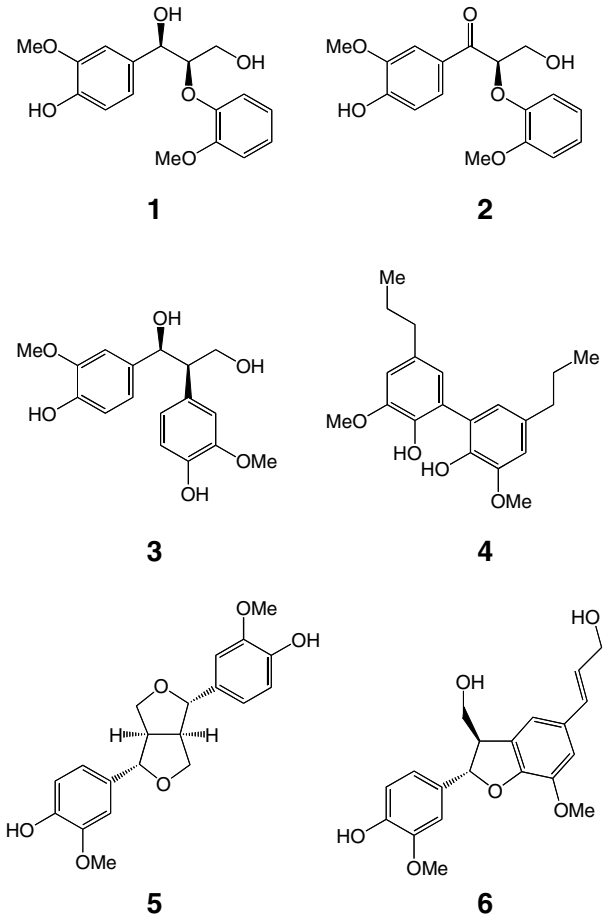

Figure 3. Structures of lignin model compounds.

Table 4. Effects of lignin model compounds on LTR (HIV-1) promoter and NF-κB activity

Compound	IC_{50} (μg/ml)	
	LTR (HIV-1)	NF-κB + TNF
1	>150	126
2	107	43
3	>150	>150
4	>150	>150
5	>150	49
6	34	19

'LTR (HIV-1)' column: Transfected cells were cultured for 4 h and then treated with or without lignin model compound for 21 h.
'NF-κB + TNF' column: Transfected cells were cultured for 20 h and then treated with or without lignin model compound for 1 h. Treated cells were stimulated with 3 ng/ml TNFα for 5 h.

3. Discussion

In addition to transcription factor, several targets such as reverse transcriptase, protease, and integrase have been well studied for chemotherapy of AIDS.[15–20] Many designed drugs were modified from nucleic acid and pep-

tide. However, there are also severe issues accompanying strong side effects and high cost of production. Thus, it is important to explore a new class of anti-HIV agents. In this study, we have addressed the three questions; (i) whether lignin can inhibit HIV-1 gene expression, (ii) what is the mechanism of inhibitory action of lignin, (iii) what is the most important structural unit in decomposed lignin for anti-HIV-1 activity. We observed that lignin olganosolve and HBS lignin inhibit transcription from HIV-1 LTR (Fig. 2 and Table 2). NF-κB activation, which is a critical element for HIV-1 replication, was also inhibited by lignin (Table 3). Moreover, fraction of low molecular weight in lignin and a lignin dimer-like compound containing β-5 structural unit among six model compounds show potent inhibitory activities (Tables 2–4). The most abundant substructure in lignin is a β-*O*-4 structure. However, our previous observations suggested that phenolic ether linkages in lignin were cleaved homolytically via quinone methide intermediates and main recombination structures from the β-*O*-4 structure were phenylcoumaran (β-5) structures under HBS pulping conditions. Pinoresinol (β–β) was identified as a radical coupling product from a phenolic β-aryl ether model compound and β–β substructures were detected in lignin samples by ^{1}H–^{13}C correlation 2D NMR measurements.[12] Compound **6** having representative β-5 structure showed significant effects on both of LTR (HIV-1) promoter and NF-κB activity as shown in Table 4. Compound **5** having representative β–β structure showed selective inhibitory activity against NF-κB mediated transcription of HIV-1. Interestingly, compound **2**, α-keto β-*O*-4 model compound, had moderate inhibitory activities on both LTR (HIV-1) and NF-κB mediated transcription.

Lignin may be a beneficial material or drug leads as a new class of anti-HIV agents possibly effective in the treatment of AIDS. Inhibition of HIV-1 replication by lignin was previously demonstrated. The effective dose, based on 100% inhibition of HIV-1-induced cytopathogenicity in MT-4 cells, of wheat bran lignin was 125 μg/ml.[11] The DHP fractions having a 0.5–1 kDa molecular mass showed stronger anti-HIV-1 activity rather than wheat bran lignin, whereas monomers of *p*-coumaric acid and ferulic acid had no effect on HIV-1 replication.[11] These observations and our results suggest that small decomposed-lignins or lignin model compounds are profitable for suppression of HIV-1. Such small molecules having lignin like structure would have higher cell permeability than high molecular weight lignins.

4. Experimental

4.1. Lignin

Lignin Alkali and Lignin Organosolve were purchased from Sigma Chemical Co (St. Louis, MO, USA). High-boiling solvent lignin (HBS lignin) was previously described.[12] A-HBS lignin and B-HBS lignin were prepared from *Acacia mangium* and *Betula platyphylla*, respectively.[21] HBS lignins were fractionated according to solubility of the components in ether by the Soxhlet method.

4.2. Lignin model compounds

Lignin model compounds were obtained by reported methods with slight modifications.[22–25]

4.2.1. Compounds 1 and 2. To a solution of 10 g of ω-(2-methoxyphenoxy)-acetoguaiacone in 100 ml of ethanol were added 5 g of potassium carbonate and 100 ml of formalin. The reaction mixture was stirred for 2 h at 50 °C. Standard workup and recrystallization from a mixed solution of ethyl ether and benzene gave 6.3 g of a desired crystalline compound **2** in 56.7% yield, mp 104 °C (lit.[22] 98 °C).

To a solution of 15 g of compound **2** in 1000 ml of 0.1 N NaOH was added 2 g of sodium borohydride. The resultant mixture was stirred for 12 hours at room temperature. The reaction mixture was adjusted to pH 3 with diluted HCl and extracted with $CHCl_3$. The organic solution was dried over Na_2SO_4 and concentrated in vacuo to give 9.0 g of solid material. Recrystallization of above solidified product from ethyl ether gave 6.1 g of pure compound **1** in 40.9% yield, mp 114.5 °C (lit.[22] 119–120 °C).

4.2.1.1. Compound 1. ^{1}H NMR ($CDCl_3$): δ 2.81 (1H, br t, Cγ-OH), 3.48 (1H, br m, Cγ-Ha), 3.62 (br d, Cγ-Hb), 3.73 (1H, s, Cα-OH), 3.86 (3H, s, OCH_3), 3.90 (3H, s, OCH_3), 4.02 (1H, ddd, Cβ-H), 4.95 (1H, d, Cα-H), 5.77 (1H, s, Ph-OH), 6.00–6.60 (6H, m, aromatics).

^{13}C NMR ($CDCl_3$): d 55.9 (OCH_3), 55.9 (OCH_3), 61.1 (Cγ), 74.0 (Cα), 89.5 (Cβ), 109.5, 112.2, 114.4, 120.3, 121.0, 121.7, 124.2, 131.5, 145.6, 146.7, 147.7, 151.3 (aromatics).

4.2.1.2. Compound 2. ^{1}H NMR ($CDCl_3$): δ 3.04 (1H, br t, Cα-OH), 3.86 (3H, OCH_3), 3.94 (3H, OCH_3), 4.07 (2H, br t, Cγ-H), 5.40 (1H, t, Cβ-H), 6.15 (1H, s, Ph-OH), 6.40–7.00 (5H, m, aromatics), 7.60–7.70 (2H, m, aromatics).

4.2.2. Compound 3. To a solution of 10 ml of LDA (2.0 M) in THF were added dropwise a solution of methyl benzylhomovanillate (4.29 g, 0.015 mol) in 20 ml over 20 min at −70 °C and stirred for 30 min. Benzylvanillin (3.63 g, 0.015 mol) in 20 ml of THF was added dropwise to the resultant greenish yellow solution at the same temperature. The reaction temperature was gradually raised to −58 °C over 1 h and quenched with dry ice. The standard workup followed by recrystallization with EtOH gave 2.74 g of erythro-β-hydroxy ester as first crystals and 2.91 g of threo-β-hydroxy ester as second crystals. Erythro isomer was recrystallized repeatedly from 1% MeOH in $CHCl_3$ and 20% MeOH in $CHCl_3$ to yield a pure erythro isomer.

To a suspension of the erythro isomer in 30 ml of THF were added 3.19 ml (22.7 mmol) of triethylamine and

1.4 ml (11.4 mmol) of TMSCl at 0 °C. The reaction temperature was raised to 70 °C and stirred for 2.5 hours. The above mixture was transferred to a dropping funnel and $LiAlH_4$ (0.863 g, 22.7 mmol) in 25 ml of anhydrous THF to give 1.78 g (94% yield) of pure 1,2-bis-(4-benzyloxy-3-methoxyphenyl)-propane-1,3-diole after a standard workup and recrystallization from EtOAc-hexane (1:2). Hydrogenolysis of the resultant compound (1.66 g, 3.32 mmol) with 1 g of Pd–C in 200 ml of MeOH under hydrogen atmosphere gave 0.9 g (yield 18.7%) of crystalline compound (**3**) after standard workup followed by recrystallization from EtOAc-hexane, mp 158 °C (lit.[26] 151–154 °C; lit.[27] 158–159 °C).

4.2.2.1. Compound 3. ^{1}H NMR(DMSO-d_6): δ 2.72 (br q, 1H, Cβ-H), 3.43–3.49 (m, 1H, Cγ-Ha), 3.60, 3.64 (s, 6H, $OCH_3 \times 2$), 3.62–3.68 (m, 1H, Cγ-Hb), 4.38 (br t, 1H, Cγ-OH), 4.80 (br t, 1H, Cα-H), 4.88 (br d, 1H, Cα-OH), 6.46–6.62 (m, 6H, aromatics), 8.54, 8.64 (s, 2H, Ph-OH × 2); ^{13}C NMR (DMSO-d_6): δ 55.4 (Cβ), 55.6, 55.7 ($OCH_3 \times 2$), 62.9 (Cγ), 72.6 (Cα), 110.9, 114.0, 114.6, 118.7, 121.9, 131.5, 136.0. 144.7, 144.9, 146.6, 146.8 (aromatics).

4.2.3. Compound 4. To a solution of above 4-propylguaiacol (1.0 g) in EtOH (20 ml) was added a solution of horseradish peroxidase (3 mg, 100 U/mg) in 20 ml of water. To the reaction mixture was added dropwise 3% H_2O_2 (3.5 ml) over 30 min with vigorous stirring. The resultant crystals were collected and washed with 50% aqueous EtOH to give crude compound. Recrystallization from EtOH gave 0.81 g of 4,4′-dipropyl-6,6′-biguaiacol (**4**) (yield 43.5%), mp 149.5 °C (lit.[28] 150–152 °C; lit.[29] 152 °C).

^{1}H NMR ($CDCl_3$): δ 0.96 (6H, t, J = 7.3, Cγ-H), 1.65 (4H, sextet, Cβ-H), 2.56 (4H. t. J = 7.7, Cα-H), 3.92 (6H, s, OCH_3), 6.02 (2H, Ph-OH), 6.72 (2H, s, C6-H), 6.74 (2H, s, C2-H).

4.2.4. Compounds 5 and 6. To distilled water was added a solution of coniferyl alcohol (18.0 g) in acetone (33 ml). To the resultant solution was added a solution of horseradish peroxidase (6.8 mg) in distilled water (150 ml), and then dropwise 0.5% H_2O_2 (1000 ml) over 60 min with stirring. The reaction mixture was stirred for 1 h and added NaCl (250 g). The resultant solution was extracted with EtOAc and organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was chromatographed on silica gel (MeOH: $CHCl_3$, 5:95 to 10:90) to give 716 mg (yield 4.0%) of pinoresinol (**5**), mp 157.1 °C (lit.[28] 156.5–158 °C) and 3.87 g (yield 21.6%) of dehydrodiconiferylalcohol (**6**), mp 118.2 °C (lit.[29] 120–121 °C) after recrystallization from acetone-hexane (1:1) and acetone, respectively.

4.2.4.1. Compound 5. ^{1}H NMR ($CDCl_3$): δ 3.10 (2H, m, Cβ-H), 3.86 (2H, dd, J = 3.5, Cγ-H_1), 3.91 (6H, s, OCH_3), 4.25 (2H, dd, J = 6.8, 8.9, Cγ-H_2), 4.74 (2H, d, J = 4.1, Cα-H), 5.59 (2H, s, C_4-OH), 6.82 (2H, d, J = 8.1, C_5-H), 6.87 (2H, s, C_2-H), 6.89 (2H, dd, J = 1.4, 8.1, C_6-H); ^{13}C NMR ($CDCl_3$): δ 54.2 (Cβ), 56.0 (OCH_3), 71.7 (Cγ), 85.8 (Cα), 108.5 (C_2), 114.2 (C_5), 118.9 (C_6), 132.9 (C_1), 145.1 (C_4), 146.6 (C_3).

4.2.4.2. Compound 6. ^{1}H NMR (acetone-d_6): δ 3.53 (1H, br q, J = 6.4, Cβ-H), 3.78–3.86 (3H, m, γ-OH, Cγ-H), 3.82 (3H, s, OCH_3), 3.86 (3H, s, OCH_3), 4.15–4.22 (3H, m, γ′-OH, Cγ-H), 5.56 (1H, d, J = 6.4, Cα-H), 6.25 (1H, dt, J = 15.8, 5.4, Cβ-H), 6.53 (1H, d, J = 15.8, Cα-H), 6.79–7.04 (5H, m, aromatics), 7.65 (1H, s, C_4-OH); ^{13}C NMR (acetone-d_6) δ 54.7 (Cβ), 56.2 (OCH_3), 56.3 (OCH_3), 63.3 (Cγ′), 64.5 (Cγ), 88.4 (Cα), 110.3 (C_2), 111.5 ($C_{2'}$), 115.5 (C_5), 115.9 ($C_{6'}$), 119.4 (C_6), 128.1 (Cβ'), 130.2 ($C_{5'}$), 130.3 (Cα'), 131.7 ($C_{1'}$), 134.2 (C_1), 144.9 ($C_{3'}$), 147.1 (C_4), 148.1 (C_3), 148.7 ($C_{4'}$).

4.3. Plasmids

Construction of HIV-1 LTR luciferase expression plasmid: CD-12-luc (containing the HIV-1 LTR U3 and R) was previously described.[6] The pNF-κB-luc reporter plasmid was purchased from Stratagene (Garden Grove, CA, USA). The expression vector, pCMV-β-galactosidase, was previously described.[30]

4.4. Luciferase assay

293-T cells were maintained in RPMI-1640 medium (WAKO, Osaka, Japan) containing 10% fetal bovine serum, 100 μg/ml streptomycin, and 20 U/ml penicillin G at 37 °C under 5% CO_2. For transient transfections cells were transfected using Fugene-6 (Roche Diagnostics Inc., Mannheim, Germany) according to the manufacturer's recommendation.

The cells in 10-cm dishes were co-transfected with 5 μg reporter plasmid and 0.008 μg pCMV-β-galactosidase. Two hours later, cells were harvested into new 12-well plates and cultured for 3–19 h, treated with or without lignin, and then stimulated with or without 5 ng/ml TNFα (PEPROTECH EC Ltd, London, UK). Luciferase activity was measured with the Luciferase Assay System (Promega, Madison, WI, USA). β-Galactosidase activity was measured with the β-Glo™ Assay System (Promega). Chemiluminescence was determined by a microplate luminometer, Veritas™ (Promega). β-Galactosidase activities were used to normalize transfection efficiency and cell number.

4.5. Size exclusion chromatography

Acetylation of lignin samples was done with acetic anhydride and pyridine at room temperature. The obtained acetylated lignin samples were purified according to the method of G. Gellerstedt,[31] and then samples were dissolved in tetrahydrofuran and were analyzed for average molecular weights by Size exclusion chromatography. A HITACHI Liquid Chromatograph L-6200 with a UV detector, L-4000 (280 nm) was used. Shodex GPC packed column KF-802 and KF-803 L were connected in a series and molecular weight was calibrated with standard polystyrene (tetrahydrofuran, flow-rate: 0.5 ml/min, 40 °C).

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