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Effects of Sesquiterpenes and Amino Acid–Sesquiterpene Conjugates from the Roots of *Saussurea lappa* on Inducible Nitric Oxide Synthase and Heat Shock Protein in Lipopolysaccharide-Activated Macrophages

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Abstract—The methanolic extract of the roots of *Saussurea lappa* CLARKE, a Chinese medicinal herb Saussureae Radix, was found to inhibit nitric oxide (NO) production in lipopolysaccharide (LPS)-activated mouse peritoneal macrophages. Among the constituents from the methanolic extract, two sesquiterpene lactones (costunolide and dehydrocostus lactone) and two amino acid-sesquiterpene conjugates (saussureamines A and B) potently inhibited LPS-induced NO production ($IC_{50} = 1.2–2.8$ μM). Saussureamines A and B in addition to costunolide and dehydrocostus lactone did not inhibit iNOS enzyme activity, but they inhibited both induction of inducible NO synthase and activation of nuclear factor-κB in accordance with induction of heat shock protein 72.

Introduction

Among nitric oxide synthase (NOS) family, inducible NOS (iNOS) has been shown to be involved in pathological processes via overproduction of nitric oxide (NO), and is expressed in response to pro-inflammatory agents [interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and lipopolysaccharide (LPS), etc.] in various cells including macrophages, endothelial cells, and smooth muscle cells.[1] Nuclear factor (NF)-κB is a major transcription factor involved in iNOS, TNF-α, IL-1β, and IL-8 genes expression. NF-κB presents as an inactive form due to combination with an inhibitory subunit, IκB, which keeps NF-κB in the cytoplasm, thereby preventing activation of target genes in the nucleus. Cellular signals lead to phosphorylation of IκB following elimination of IκB from NF-κB by proteolytic degradation. Then, the activated-NF-κB is released and translocated into the nucleus to activate transcription of its target genes.[2] Inhibition of iNOS enzyme activity or iNOS induction and inhibition of NF-κB activation may be of therapeutic benefit in various types of inflammation.[1–4]

Saussureae Radix, the roots of *Saussurea* (*S.*) *lappa* CLARKE (Compositae) have been used as a Chinese herbal medicine, which are used as an aromatic stomachic, and also have been used as an important fragrance. With regard to chemical constituents of this plant, several sesquiterpenes, such as costunolide (**1**) and dehydrocostus lactone (**2**), have been isolated from Indian *S. lappa*.[5–7] In the course of our studies on the constituents from the natural medicines with NO production inhibitory activity,[8] the methanolic extract of Chinese *S. lappa* was found to show a potent inhibitory effect on nitrite (NO_2^-) accumulation in LPS-activated mouse macrophages ($IC_{50} = 3.5$ μg/mL).

There have been many pharmacological studies on the activities of extracts or principal constituents, costunolide (**1**) and dehydrocostus lactone (**2**) from the roots of *S. lappa* such as the anti-ulcer,[9] anti-carcinogenesis in rats,[10] the vasorelaxant effect,[11] and the inhibitory effects on killing activity of cytotoxic T lymphocytes.[12] In addition, previous studies of *S. lappa* and other herbal medicines demonstrated that sesquiterpene lactones including costunolide (**1**) and dehydrocostus lactone (**2**) inhibited NF-κB activation thereby preventing iNOS and TNF-α expression.[8a,13] However, the effects of other

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constituents on iNOS or TNF-α expression have not been reported. In the present study, we isolated four active sesquiterpenes [costunolide (**1**), dehydrocostus lactone (**2**), α- and β-costols mixture (**3**), and (−)-elema-1,3,11(13)-trien-12-ol (**4**)] from an ethyl acetate-soluble fraction and five active amino acid-sesquiterpene conjugates [saussureamines A (**8**), B (**9**), C (**10**), D (**11**), and E (**12**)] were isolated from the 1-butanol-soluble fraction. In our previous study on NO production inhibitors from the leaves of *Laurus nobilis*, compounds **1** and **2** were found to show inhibition of iNOS induction in accordance with induction of heat shock protein 72 (HSP 72).[8a]

This study examined the effects of constituents (**1**–**12**) from the roots of *S. lappa* on NO production in LPS-activated macrophages. In addition, we examined the effects of saussureamines A (**8**) and B (**9**) on iNOS enzyme activity, induction of iNOS and activation of NF-κB activated by LPS, and induction of HSP 72 to clarify their mechanisms of action.

Materials and Methods

Extraction and isolation

The isolation of the chemical constituents from *S. lappa* was described previously.[9] Briefly, the dried roots of *S. lappa* (cultivated in Yunnan province, China) were extracted with methanol under reflux. The methanolic

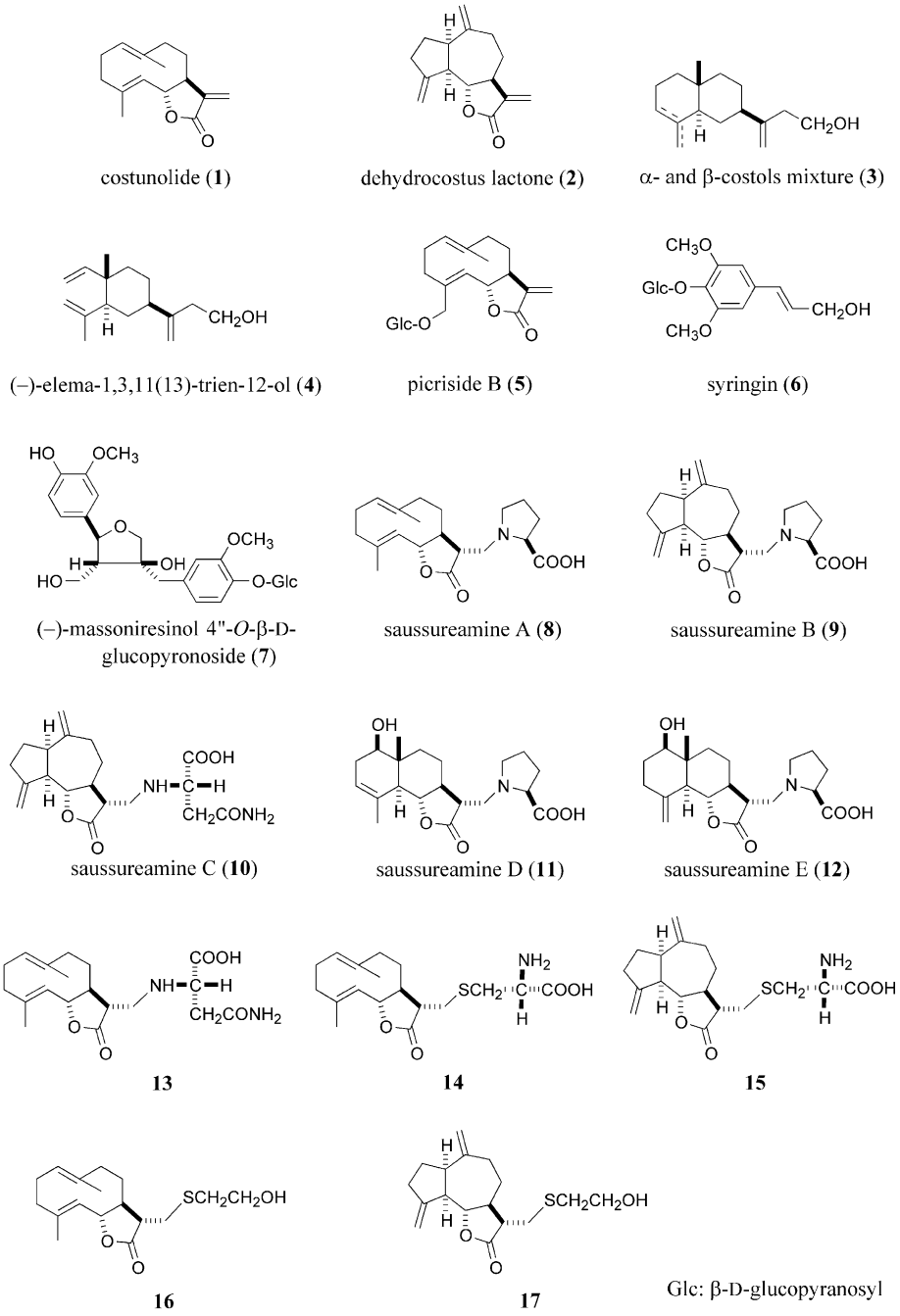

Chart 1.

extract was partitioned into an ethyl acetate and water mixture to give an ethyl acetate-soluble fraction and an aqueous phase. The aqueous phase was further extracted with 1-butanol to give a 1-butanol-soluble fraction and a water-soluble fraction. The ethyl acetate-soluble fraction was subjected to silica gel column chromatography to furnish the principal sesquiterpene constituents costunolide (**1**,[5] 1.07% from the natural medicine) and dehydrocostus lactone (**2**,[14] 1.43%). Furthermore, the other fraction was subjected to recycling HPLC to furnish α-and β-costols mixture (**3**,[15,16] 0.005%) and (−)-elema-1,3,11(13)-trien-12-ol (**4**,[16] 0.002%). The 1-butanol-soluble fraction was subjected to Amberlite XAD-2, silica gel, reversed-phase silica gel, and finally Sephadex LH-20 column chromatography to furnish picriside B (**5**,[17] 0.0059%), syringin (**6**,[18] 0.0070%), (−)-massoniresinol 4″-*O*-β-D-glucopyranoside (**7**,[9] 0.0009%), saussureamines A (**8**,[9] 0.0011%), B (**9**,[9] 0.0021%), C (**10**,[9] 0.0006%), D (**11**,[9] 0.0001%), and E (12,[9] 0.0002%). Related amino acid- or 2-mercaptoethanol-sesquiterpene conjugates (**13**–**15**) were synthesized with compounds **1** and **2** using a Michael type addition reaction (Chart 1).[9]

Reagents

Lipopolysaccharide (LPS, from *Salmonella enteritidis*) and N^G-monomethyl-L-arginine (L-NMMA) were purchased from Sigma; 3-(4,5-dimethyl-2-thiazolyl) 2,5-diphenyl tetrazolium bromide (MTT) was from Dojin; RPMI 1640 was from Gibco; protease inhibitor cocktail (Complete Mini) was from Boehringer Mannheim; fetal calf serum (FCS) was from Bio Whittaker; anti-mouse iNOS antibody (monoclonal) was from Transduction Laboratories; anti-mouse IgG antibody conjugated to horseradish peroxidase and the enhanced chemiluminescense (ECL™) kit, L-[U-^{14}C]-arginine, γ-[^{33}P]-ATP were from Amersham; thioglycolate (TGC) medium was from Nissui Seiyaku; iNOS was from OXIS International; NE-PER nuclear and cytoplasmic extraction reagents were from Pierce; NF-κB consensus oligonucleotide and T4 polynucleotide kinase (Gel Shift Assay Kit) were from Promega; Aquasol-2 was from Packard, and all other chemicals were from Wako. Nitrocellulose membranes (0.25 μm) were purchased from Bio Rad; 96-well microplates and culture dishes (6 cm) were from Nunc; and spin columns (UFC30SV00) were from Amicon.

Screening test for NO production

Screening test for NO production was performed as described previously.[8] Briefly, peritoneal exudate cells (5×10^5 cells/well) were collected from the peritoneal cavities of male ddY mice and were suspended in 200 μL of RPMI 1640 supplemented with 5% fetal calf serum (FCS), penicillin (100 units/mL) and streptomycin (100 μg/mL), and pre-cultured in 96-well microplates at 37 °C in 5% CO_2 in air for 1 h. Nonadherent cells were removed by washing with PBS, and the adherent cells were cultured in fresh medium containing 10 μg/mL LPS and various concentrations of test compound for 20 h. NO production in each well was assessed by measuring the accumulation of nitrite (NO_2^-) in the culture medium using Griess reagent. Cytotoxicity was determined by MTT colorimetric assay, after 20-h incubation with test compounds. Each test compound was dissolved in DMSO, and the solution was added to the medium (final DMSO concentration was 0.5%). Inhibition (%) was calculated using the following formula and IC_{50} was determined graphically ($N=4$).

$$\text{Inhibition (\%)} = \frac{A-B}{A-C}\times 100$$

A–*C*: NO_2^- concentration (μM)

[*A*: LPS (+), sample (−); *B*: LPS (+), sample (+);

C: LPS (−), sample (−)]

Detection of iNOS and HSP 72

Detection of iNOS and HSP 72 was performed as described previously.[8a] TGC-induced peritoneal exudate cells (7.5×10^6 cells/3 mL/dish) were pre-cultured in culture dishes (6 cm i.d.) for 1 h. After washing, culture medium was exchanged for fresh medium containing 5% FCS, 20 μg/mL LPS and test sample for 12 h. Cells were collected in lysis buffer [100 mM NaCl, 10 mM Tris, Complete Mini (1 tab/10 mL), 0.1% Triton X-100, 2 mM ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA)] and sonicated. After determination of the protein concentration of each suspension, the suspensions were boiled in Laemmli buffer.[19] For SDS–PAGE, aliquots of 50 μg of protein of each sample were subjected to electrophoresis in 10% polyacrylamide gels. Following electrophoresis, the proteins were electrophoretically transferred onto nitrocellulose membranes, which were incubated with 5% nonfat dried milk in Tris-buffered saline (T-TBS, 100 mM NaCl, 10 mM Tris, 0.1% Tween 20, pH 7.4) and probed with mouse monoclonal IgG against iNOS or HSP 72 at a dilution of 1:1000. The blots were washed with T-TBS and probed with secondary antibody solution, anti-mouse IgG antibody conjugated to horseradish peroxidase at a dilution of 1:5000. Detection was performed using ECL™ and X-ray film (Hyperfilm-ECL, Amersham).

Electrophoretic mobility shift assay

The electrophoretic mobility shift assay was performed as described previously with some modification.[8b] TGC-induced peritoneal macrophages (7.5×10^6 cells/3 mL/dish) was prepared as described above, and were cultured in RPMI 1640 supplemented with 5% FCS, penicillin (100 units/mL) and streptomycin (100 μg/mL), 20 μg/mL LPS and test compound for 1 h. Cells were collected in ice-cold PBS. Nuclear extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce) according to the manufacturer's instructions. The protein content of each supernatant was determined, and equal amounts of protein (7 μg) and ^{33}P-labeled NF-κB consensus oligonucleotide were added to reaction mixtures. The oligonucleotide-protein

Table 1. Inhibitory effects of the methanolic extract, its fractions and constituents (**1**–**12**) from Saussureae Radix and related compounds (**13**–**17**) on NO production in LPS-activated mouse peritoneal macrophages

	Inhibition %							
	0	0.3	1	3	10	30	100	IC_{50} (μg/mL)
MeOH extract	0.0±6.5	—	16.3±3.5*	44.6±3.6**	85.1±1.4**	104.0±0.2**	107.4±0.4**[a]	3.5
EtOAc-soluble fraction	0.0±6.3	17.7±5.7*	68.3±3.9**	98.9±1.0**	109.4±0.6**	109.3±0.6**[a]	—	0.67
1-BuOH-soluble fraction	0.0±0.7	—	—	−9.0±6.0	6.0±3.2	28.2±2.2**	77.0±1.3**	51
H_2O-soluble fraction	0.0±1.9	—	—	−5.2±1.6	11.3±4.0	11.7±4.0	20.0±4.0*	>100
		0	1	3	10	30	100	IC_{50} (μM)
Constituents from EtOAc-soluble fraction								
Costunolide (**1**)		0.0±2.5	47.6±4.0**	73.8±1.9**	97.3±0.9**	102.0±0.0**[a]	101.1±0.3**[a]	1.2
Dehydrocostus lactone (**2**)		0.0±3.8	45.3±1.5**	74.4±1.6**	92.4±0.3**	100.9±0.3**[a]	100.3±0.3**[a]	1.2
α- and β-Costols mixture (3)		0.0±1.6	−8.5±2.1	−7.3±0.6	3.2±2.9	44.3±2.0**	98.8±1.4**	33
(−)-Elema-1,3,11(13)-trien-12-ol (4)		0.0±5.8	−2.4±4.1	15.9±5.3	42.2±5.1**	89.7±1.2**	101.4±1.1**	12
Constituents from 1-BuOH-soluble fraction								
Picriside B (**5**)		0.0±2.5	1.3±2.5	2.1±2.1	5.6±4.0	26.4±1.8**	68.4±1.4**	55
Syringin (**6**)		0.0±2.0	11.5±0.6	3.8±3.5	−2.8±5.5	−9.4±8.4	2.0±7.7	>100
7		0.0±1.6	−7.6±3.4	−0.7±4.4	−6.1±1.7	4.2±1.1	23.9±2.6**	>100
Saussureamine A (**8**)		0.0±2.8	23.9±1.0**	53.8±2.5**	95.8±0.8**	100.3±0.2**	100.7±0.2**[a]	2.8
Saussureamine B (**9**)		0.0±1.1	11.8±0.8**	52.8±4.9**	88.8±1.6**	100.5±0.2**	100.0±0.2**[a]	2.8
Saussureamine C (**10**)		0.0±9.0	11.7±5.6	20.7±7.5	28.9±8.1*	77.8±3.4**	91.4±2.8**	16
Saussureamine D (**11**)		0.0±4.3	−4.6±1.3	3.2±1.7	3.1±4.0	32.4±4.6**	63.5±2.2**	49
Saussureamine E (**12**)		0.0±5.5	−1.0±6.4	10.6±6.4	16.5±11.1	37.7±5.6**	76.5±2.7**	44
Related compounds								
13		0.0±2.6	18.3±4.8**	12.3±1.6**	37.6±3.3**	73.0±2.0**	97.5±0.5**	16
14		0.0±2.3	2.2±3.6	6.5±3.0	17.9±3.9**	66.6±3.9**	110.5±0.4**	22
15		0.0±2.4	5.7±2.8	−6.9±2.5	12.9±3.5*	39.0±3.6**	95.5±2.6**	36
16		0.0±7.2	−8.5±5.5	−0.8±4.8	6.0±2.3**	51.1±2.5**	93.7±1.6**	29
17		0.0±6.3	−3.0±4.5	11.7±2.2	23.3±8.1*	72.5±2.3**	99.4±0.5**	18

Values represent the means±SEM. ($N=4$). Significantly different from controls, *$P<0.05$, **$P<0.01$.
[a]Cytotoxic effect was observed.

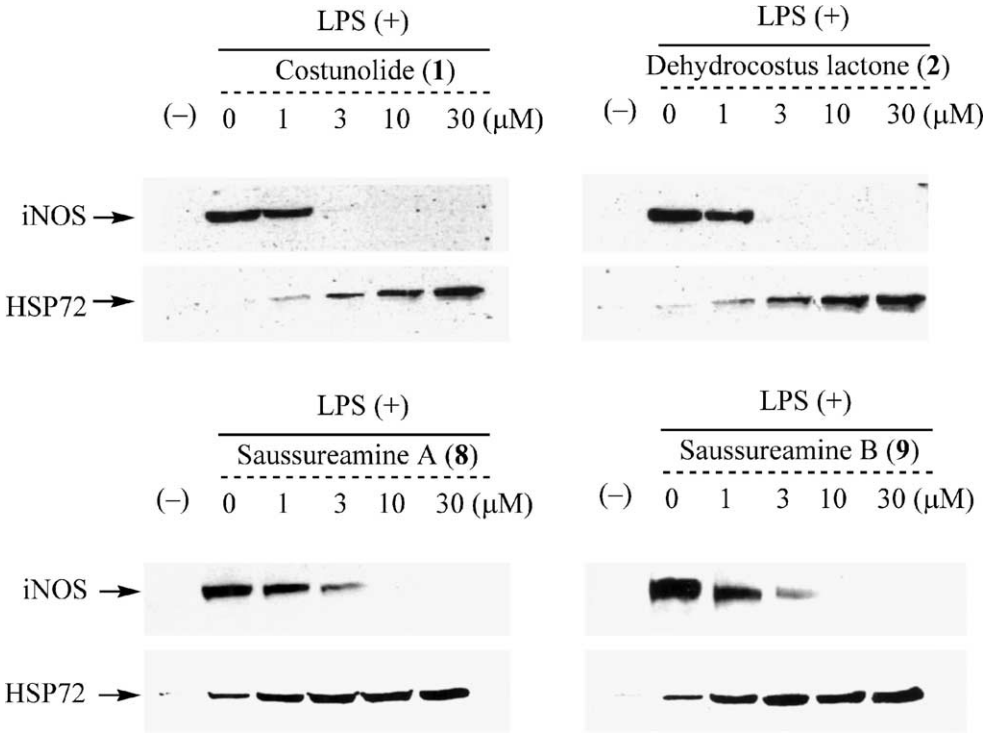

Figure 1. Effects of costunolide (**1**), dehydrocostus lactone (**2**), and saussureamines A (**8**) and B (**9**) on induction of iNOS and HSP 72 in LPS-activated macrophages. The cells were incubated with or without LPS and a test sample for 12 h. Induction of iNOS or HSP 72 was detected by monoclonal antibody against mouse iNOS or HSP 72. Arrows indicate 130 kDa iNOS and 72 kDa HSP 72.

complex was separated by non-denaturing polyacrylamide gel electrophoresis (Gel Shift Assay Kit, Promega), and autoradiography was performed using an imaging analyzer (BAS 5000, Fuji Film). ^{33}P-labeled NF-κB consensus oligonucleotide was labeled using γ-[^{33}P]-ATP (3000 Ci/mmol) and T4 polynucleotide kinase.

iNOS enzyme activity

iNOS activity was measured by monitoring the conversion of L-[U-^{14}C]-arginine to L-[U-^{14}C]-citrulline, as described previously.[8b] Briefly, a test sample solution (5 μL) and 40 μL of substrate and coenzyme solution [100 μM L-arginine (containing 50 nCi L-[^{14}C]-arginine), 1 mM NADPH, 3 μM tetrahydrobiopterin (BH_4), 1 μM flavin adenine dinucleotide (FAD), 1 μM flavin mononucleotide (FMN) in 25 mM Tris–HCl buffer, pH 7.4] were pre-incubated at 37 °C for 10 min. iNOS (20 mU/5 μL) was then added to the reaction mixture. After incubation at 37 °C for 30 min, the reaction was terminated by the addition of 400 μL of ice-cold stop solution containing 5 mM EDTA and 50 mM HEPES (pH 5.5). The substrate was adsorbed on AG 50W X-8 ion-exchange resin (Na^+ form, 60–70 mg) packed in spin columns. The L-citrulline, which is ionically neutral at pH 5.5, flowed through the column completely,[20] and was mixed with a scintillation cocktail (Aquasol-2) and radioactivity was determined using a liquid scintillation counter (LS 6500, Beckman). The test compound was dissolved in DMSO and diluted with Tris–HCl buffer (pH 7.4) (final concentration of DMSO: 2%).

Statistics

Values are expressed as means ± SEM. One-way analysis of variance followed by Dunnett's test was used for statistical analysis.

Results

Effects on NO production

First, the effects of the methanolic extract and its fractions on nitrite accumulation from LPS-activated macrophages were examined. Nitrite, a oxidative product of NO, was accumulated in the medium after 20-h incubation with LPS. The nitrite concentration in the medium without inhibitors (control group) was 28.4 ± 1.9 and that in without LPS (unstimulated group) was 1.6 ± 0.3 μM (mean ± SD of 23 experiments). The IC_{50} values of reference compounds, caffeic acid phenethyl ester (CAPE, an inhibitor of NF-κB activation),[4] L-NMMA (a non-selective inhibitor of NOS),[21] and guanidinoethyldisulfide (GED, an inhibitor of iNOS),[22] were 4.0, 28, and 1.4 μM, respectively.[8a]

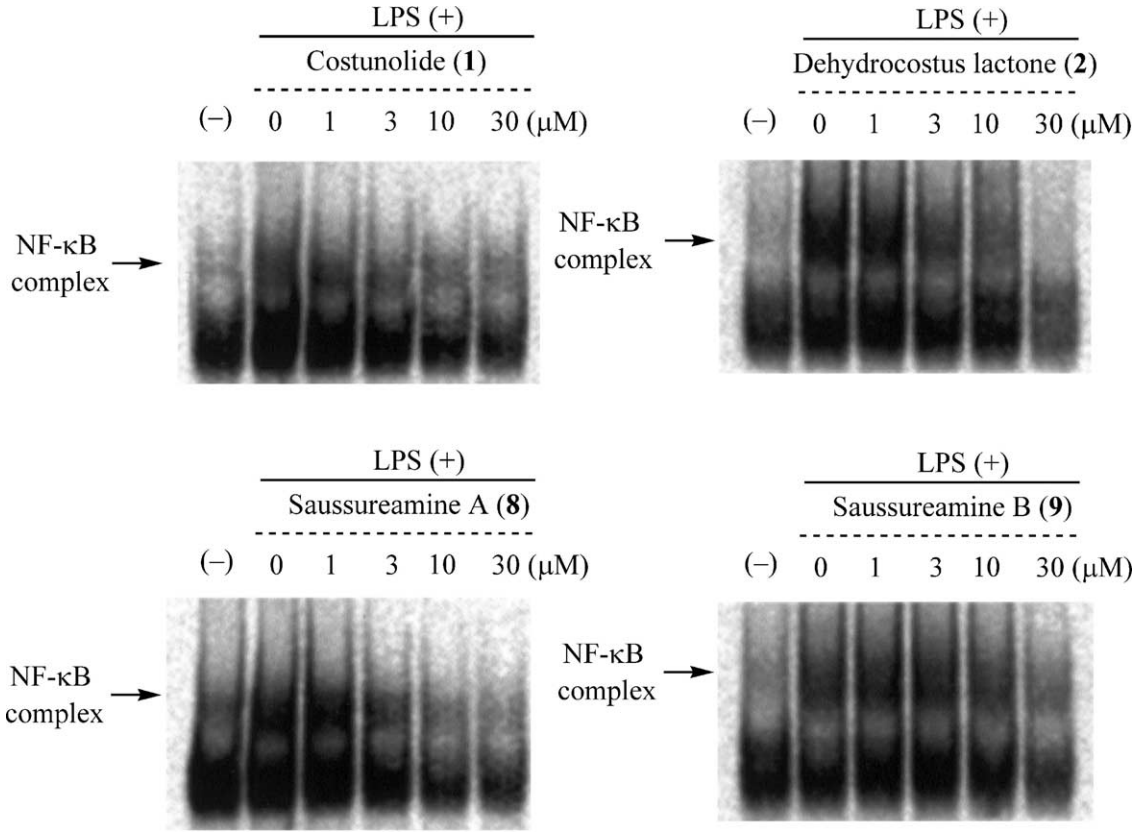

Figure 2. Effects of costunolide (**1**), dehydrocostus lactone (**2**), and saussureamines A (**8**) and B (**9**) on LPS-induced activation of NF-κB in macrophages. The cells were incubated with or without LPS and a test sample for 1 h, and the proteins of cell lysate were added to the reaction mixture containing NF-κB consensus oligonucleotide labelled with ^{33}P-ATP. The oligonucleotide-protein complex was separated electrophoretically and an autoradiograph was obtained.

As shown in Table 1, the methanolic extract and its fractions inhibited nitrite accumulation. In particular, the ethyl acetate-soluble fraction showed potent inhibitory activity ($IC_{50}=0.67$ μg/mL). Two principal sesquiterpenes [costunolide (**1**), dehydrocostus lactone (**2**)] with an α-methylene-γ-butyrolactone moiety showed the most potent activity ($IC_{50}=1.2$ and 1.2 μM). On the other hand, α- and β-costols mixture (**3**), (−)-elema-1,3,11(13)-trien-12-ol (**4**), a sesquiterpene glucoside [picriside B (**5**)] and three amino acid-sesquiterpene conjugates [saussureamines C (**10**), D (**11**), and E (**12**)] showed weak inhibitory effects ($IC_{50}=12$–55 μM), but saussureamines A (**8**) and B (**9**) exhibited relatively potent activity ($IC_{50}=2.8$ and 2.8 μM). A phenylpropanoid glucoside [syringin (**6**)] and a lignan glucoside [(−)-massoniresinol 4″-*O*-β-D-glucopyranoside (**7**)] showed no or weak activity ($IC_{50}>100$ μM). Compounds **1**, **2** at 30 μM and **8**, **9** at 100 μM showed cytotoxicity in the MTT assay.

Effects of costunolide (1), dehydrocostus lactone (2), and saussureamines A (8) and B (9) on iNOS enzyme activity

Next, the effects of costunolide (**1**), dehydrocostus lactone (**2**), and saussureamines A (**8**) and B (**9**) on iNOS enzyme activity were examined. A reference compound, L-NMMA, inhibited iNOS enzyme activity with an IC_{50} of 13 μM, but compounds **1**, **2**, **8**, and **9** did not show any effects; the inhibitions of **1**, **2**, **8**, and **9** at 100 μM were 12, 13, 0, and 3%, respectively.

Effects of costunolide (1), dehydrocostus lactone (2), and saussureamines A (8) and B (9) on induction of iNOS and HSP 72 proteins

iNOS and HSP 72 were detected at 130 kDa and 72 kDa after 12-h incubation with LPS by SDS-PAGE-Western blotting analyses as shown in Figure 1. Costunolide (**1**), dehydrocostus lactone (**2**), and saussureamines A (**8**) and B (**9**) inhibited the induction of iNOS with an increase in the HSP 72 level in a concentration-dependent manner.

Effects of costunolide (1), dehydrocostus lactone (2), and saussureamines A (8) and B (9) on NF-κB activation

Finally, the effects of costunolide (**1**), dehydrocostus lactone (**2**), and saussureamines A (**8**) and B (**9**) on activation of NF-κB were examined by electrophoretic mobility shift assay. Cells were incubated with or without LPS and a test sample for 1 h, and proteins of the cell lysate were added to reaction mixtures containing NF-κB consensus oligonucleotide labeled with ^{33}P-ATP. The oligonucleotide-protein complex was separated electrophoretically. As shown in Figure 2, detection of oligonucleotide-NF-κB (NF-κB complex) was prevented by **1**, **2**, **8**, and **9** in a concentration-dependent manner.

Discussion

Recent studies demonstrated that sesquiterpenes with an α-methylene-γ-butyrolactone moiety showed inhibitory effects on NF-κB activation, thereby iNOS induction following inhibition of NO production.[8a,13] In agreement with the previous findings, costunolide (**1**) and dehydrocostus lactone (**2**) with an α-methylene-γ-butyrolactone moiety showed potent inhibition of NO_2^- accumulation in the medium, and were reported to inhibit iNOS induction or NF-κB activation in LPS-activated mouse or RAW 264.7 macrophages.[8a,13d] On the other hand, a sesquiterpene glucoside, picriside B (**5**), showed less activity than compound **1**, although it has an α-methylene-γ-butyrolactone moiety. In our previous studies, glycosides, such as stilbene glucosides (e.g. rhaponticin), showed less activity than their corresponding aglycons.[8b,8c] It is suggested that glycosides including compound **5** are hardly permeable to cell membranes and could not reach the active sites, and therefore, showed less activity.

Saussureamines A (**8**) and B (**9**), which were conjugated with L-proline at the α-methylene part, also relatively retained the activity, and they inhibited both NF-κB activation and iNOS induction in a concentration-dependent manner. These findings suggest that compounds **8** and **9** inhibit NF-κB activation, thereby suppressing iNOS induction similar to sesquiterpene lactones.

Several mechanisms of inhibition of NF-κB activation by the sesquiterpene lactones have been proposed, (1) inhibition of degradation of IκB due to inhibition of IκB posphorylation;[23] (2) conjugation with sulfhydryl (SH) groups of target protein (e.g., p65 subunit of NF-κB).[13a,24] An earlier study demonstrated that sesquiterpene lactones (e.g., cynaropicrin, reynosin and santamarin) combined with SH groups of proteins, and protection of SH groups from sesquiterpene lactones by L-cysteine, dithiothreitol and 2-mercaptoethanol abolished the inhibition of pro-inflammatory cytokine production in LPS-stimulated macrophages.[13b]

Recent studies demonstrated that heat shock responses or HSP induction by thermal and non-thermal stimuli (e.g., ischemia, heavy metals) inhibited signal transduction pathways including NF-κB, mitogen-activated protein kinases and stress-activated kinases in the stimulation of pro-inflammatory cytokines and agents.[25,26] Induction of HSPs, especially HSP 72, inhibits translocation of NF-κB to nucleus thereby inhibiting iNOS induction.[26] In our previous study, we reported that sesquiterpene lactones (**1**, **2**) induced HSP 72 in macrophages possibly due to a combination with SH groups of proteins.[8a] We also reported that compounds **8** and **9** chemically liberated the sesquiterpene lactones (**1**, **2**) and L-proline, and that treatment of **1** and **2** with L-cysteine gave compounds **14** and **15**, which are adducts between the SH group and the α-methylene-γ-butyrolactone part.[9b] These findings in the previous and present studies suggested that the amino acid moiety of **8** and **9** could be replaced with SH groups of proteins, and the HSP induction might be involved in the inhibition of iNOS induction by compounds **1**, **2**, **8**, and **9**. On the other hand, saussureamines C (**10**), D (**11**), E (**12**), and related compounds **13**–**17** showed less activity than compounds **8** and **9**. The α-methylene-γ-butyrolactone

part of sesquiterpene lactones prominently reacted with the SH group,[9b] so that compounds **14**–**17** showed less activity than compounds **8** and **9**. The weak effects of other amino acid-sesquiterpenes (**10**–**13**) may depend on their reactivity to the SH groups of target proteins.

In conclusion, not only sesquiterpene with an α-methylene-γ-butyrolactone moiety [costunolide (**1**) and dehydrocostus lactone (**2**)], but also amino acid-sesquiterpene conjugates [saussureamines A (**8**) and B (**9**)] isolated from Saussureae Radix showed potent inhibitory effects on NO production in LPS-activated macrophages. With regard to the mode of action of compounds **8** and **9**, they inhibited NF-κB activation and iNOS induction in accordance with induction of HSP 72 similar to sesquiterpene lactones, **1** and **2**.

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