Inhibition of Nitric Oxide Production in RAW 264.7 Cells by Azaphilones from Xylariaceous Fungi

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The aim of this study was to discover a novel agent that suppresses nitric oxide (NO) production stimulated by lipopolysaccharide (LPS) in RAW 264.7 cells. We carried out a screening test in fifteen azaphilone compounds, which were isolated from xylariaceous inedible mushrooms. The structure–activity relationship was discussed; accordingly, azaphilones were divided into five groups based on the functional groups attached to the main azaphilone backbone. Rubiginosin A, an azaphilone with an orsellinic acid moiety attached to the bicyclic azaphilone through an ester linkage, showed potential inhibitory activity. To clarify the mechanism involved, total RNA extraction, followed by RT-PCR for inducible nitric oxide synthase (iNOS) and finally electrophoresis on agarose gel were performed. These findings indicated that suppression of the LPS-induced NO production of rubiginosin A is due to the inhibition of iNOS protein synthesis.

Key words azaphilone; nitric oxide; Xylariaceae; RAW cell; inducible nitric oxide synthase (iNOS)

The Xylariaceae (Ascomycete) is a large family and currently comprises more than 36 genera. So far, at least one third of these genera have been chemically investigated.[1] One of these specific metabolites repeatedly isolated from this family is azaphilone, with various substitution groups linked to the main skeleton.[2,3] Although previous publications showed that azaphilones exhibit broad activities in many biological systems, such as cholesteryl ester transfer protein,[4] monoamine oxidase inhibition,[5] endothelin receptor binding,[6] the inhibition of gp120-CD4 binding,[7] telomerase inhibition,[8] the induction of Epstein-Barr virus,[9] and antimicrobial activities,[3,10,11] there is no report about the inhibition of nitric oxide (NO) production by azaphilones.

NO is synthesized from L-arginine by nitric oxide synthase (NOS). Currently, three distinct isoforms of NOS have been identified: Neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS).[12] Of these, iNOS is an important enzyme involved in the regulation of inflammation[13] and in the generation of a large amount of NO.[14] Overproduction of NO by iNOS has been implicated in various pathological processes including septic shock, tissue damage following inflammation, and rheumatoid arthritis.[15—19] Therefore, suppression of nitric oxide (NO) production in macrophages could be a target for potential anti-inflammatory drugs.

In the present study, we screened the inhibitory activity of fifteen azaphilones, which were isolated from mushrooms belonging to the Xylariaceae family, and also elucidated the mechanism of inhibition of NO production stimulated by lipopolysaccharide (LPS) in RAW 264.7 cells.

MATERIALS AND METHODS

Isolation of Azaphilones Fifteen azaphilones, specifically daldinins C, E and F (**1**—**3**) from *Hypoxylon fuscum*,[20,21] multiformin D (**4**) from *H. multiforme*,[11] sassafrins A—C (**5**—**7**) from *Creospharia sassafras*,[10] entonaemin A (**8**) and rubiginosins A—C (**9**—**11**) from *H. rubiginosin*,[22] cohaerins A and B (**12**, **13**) from *H. cohaerens*,[23] rutilins A and B (**14**, **15**) from *H. rutilin*,[24] were isolated from xylariaceous fungi and their structures characterized by a combination of NMR spectrometry and X-ray crystallographic analysis.

Cell Culture RAW 264.7 cells, a mouse macrophage cell line, were grown in RPMI 1640 supplemented with 10% fetal bovine serum, kanamycin (50 μg/ml), and ampicillin (60 μg/ml) at 37 °C in an atmosphere of 5% CO_2 and 95% air.

Inhibition of NO Production of Fifteen Azaphilones One hundred microliters of RAW 264.7 cells (8×10^5 cells/ml) was pipetted into the wells of 96-well culture plates and then cultured. After 24 h incubation, 50 μl of medium containing various concentrations of each azaphilone derivative diluted in DMSO was added to each well. The final DMSO concentration was less than 0.1%. At this concentration, DMSO did not show any NO-induction without stimulation with LPS. Then, 50 μl of vehicle or LPS purified from *Pantoea agglomerans* (4 μg/ml) was added to each well. The cells were further incubated at 37 °C for 24 h. The supernatant (35 μl) was withdrawn, mixed with Griess reagents[25] (35 μl), and then the absorbance at 550 nm measured using a BIO-RAD model 550 Microplate reader. NO concentrations were determined by measuring the amount of nitrite in the cell culture supernatant using Griess reagents and IC_{50} values were calculated as described in Table 1.

RNA Isolation and RT-PCR RT-PCR was performed to identify the expression of iNOS mRNA. First, 500 μl of RAW cells (10^6 cells/ml) was added to each well and cultured in 24-well culture plates. After 24 h incubation, 250 μl of samples with three dilutions (12.5, 3.13, 0.78 μM), which were dissolved in DMSO, was added to each well. One hour later, 250 μl LPS (4 μg/ml) or vehicle was added to the wells. The cells were further incubated for 8 h. Total RNA was isolated from RAW cells using TRIzol and the concentration of purified total RNA was determined by absorbance at 260 nm.

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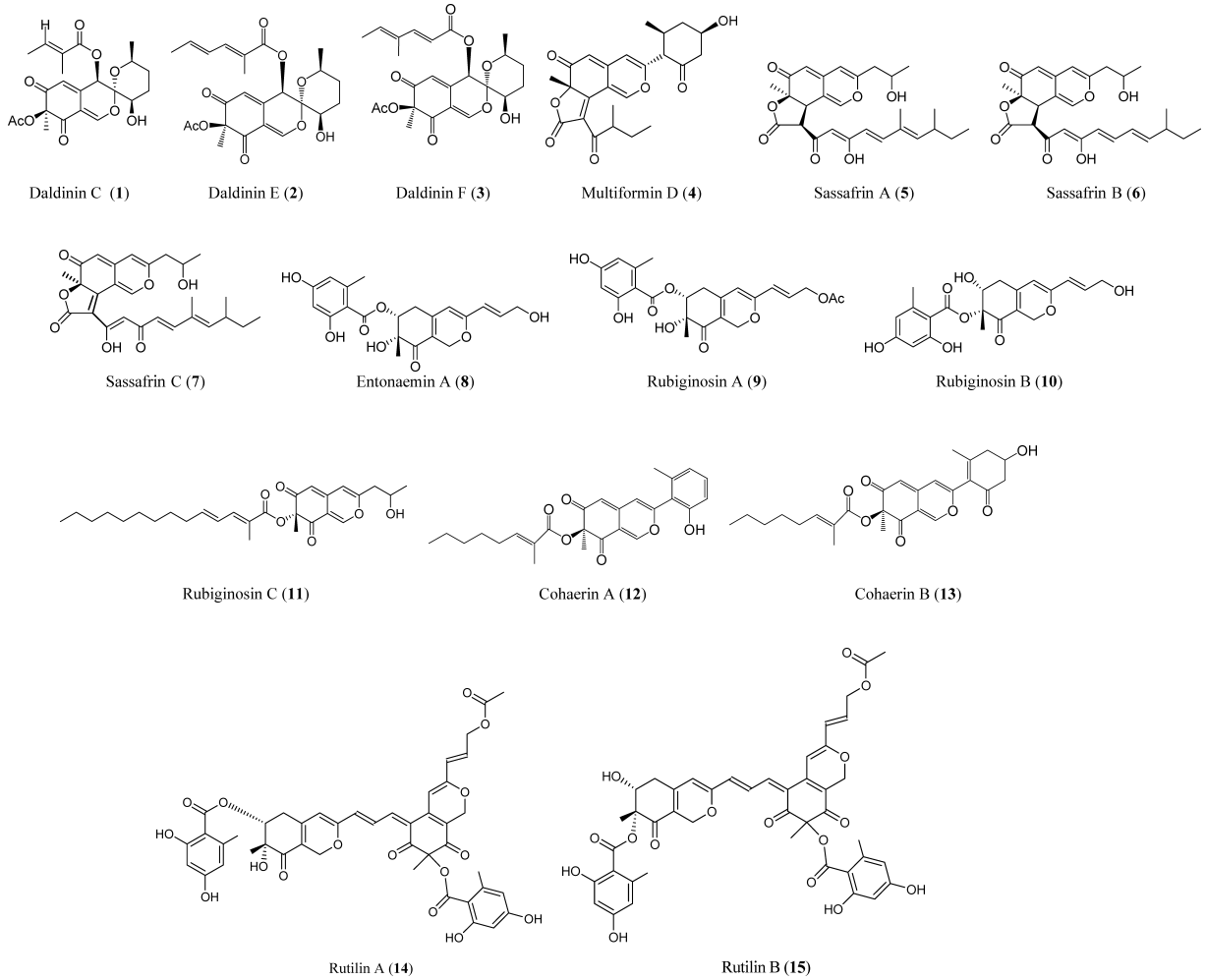

Fig. 1. Structures of Azaphilones (**1**—**15**)

The RNA samples were reverse-transcribed into cDNA using the following method: total RNA (2.5 μg) and oligo (dT) primer (0.5 μl) were combined and the final volume was brought up to 7.5 μl with free RNase water and then the mixture was heated at 70 °C for 10 min. 5×RT buffer (3 μl), 10 M dNTPS (1.5 μl), reverse Ace (MMLV Reverse Transcriptase RNaseH-) (0.75 μl), RNase inhibitor (0.375 μl) and water (1.875 μl) were then added to the mixture, which was incubated at 42 °C for 60 min and then 99 °C for 10 min.

PCR amplification was performed in a reaction volume of 10 μl containing 3 μl of the appropriate cDNA, 1 μl of iNOS or β-actin primer (5 pmol/ml), 5 μl of master mix, and 1 μl of water. The sense primer for iNOS was 5′-GTA-GAAAGTCCAGCCGCAC-3′ and the anti-sense primer was 5′-GTAGCTGCCGCTCTCATCCAG-3′. The sense primer for β-actin was 5′-CCAACCGTGAAAAGATGACC-3′ and the anti-sense was 5′-CAGGAGGAGCAATGATCTTG-3′. The primer sequence were determined for iNOS cDNA (Accession No: BC062378) and β-actin cDNA (Accession No: AK078935).

For iNOS and β-actin, the PCR procedures were carried out using a DNA Engine Opticon™ System Continuous for Fluorescence Detector (MJ Researcher Incorporated, U.S.A.). The PCR was carried out with 20 cycles for β-actin and 25 cycles for iNOS to obtain the results within the exponential range. The PCR amplification was performed after 10 min incubation at 95 °C under the following conditions: 20 to 25 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s, and extension at 72 °C for 20 s, using a thermal cycler. Final amounts of RT-PCR products for iNOS mRNA (203 bp) and β-actin (660 bp) were calculated and the ratios of iNOS/β-actin are described in Fig. 3.

Electrophoresis RT-PCR products for iNOS mRNA and β-actin were analyzed by electrophoresis on 2.5% agarose gel. Electrophoresis was run for 15 min using a Mupid apparatus and the gel was stained by ethidium bromide and then read under UV light. The electrophoresis results for iNOS and β-actin are presented in Fig. 3.

DPPH Free-Radical Scavenging Activity The free-radical scavenging activity of azaphilones was measured using the DPPH method as described previously.[21)]

Statistical Analysis The results represent three independent experiments and are expressed as the mean±S.E.M. The data were analyzed by the *t*-test using SPSS software (12.0 version). The differences were considered to be statistically significant at a p value less than 0.05.

RESULTS

Effects of Azaphilones (1—15) on LPS-Induced Production of NO RAW 264.7 cells were preincubated for 1 h at 37 °C in medium containing six different concentrations of

Table 1. Inhibition of NO Production and Antioxidant Activity of (**1**—**15**)

Samples	Inhibition of NO production IC_{50} (μM)	Antioxidant activity IC_{50} (μM)
Group A		
Daldinin C (**1**)	55.66	412.0[21]
Daldinin E (**2**)	43.68	178.9[21]
Daldinin F (**3**)	30.62	212.3[21]
Group B		
Multiformin D (**4**)	13.81	418.3
Sassafrin A (**5**)	14.68	104.8
Sassafrin B (**6**)	15.66	54.4
Sassafrin C (**7**)	10.02	62.4
Group C		
Entonaemin A (**8**)	14.24	>500
Rubiginosin A (**9**)	2.56	>500
Rubiginosin B (**10**)	15.66	>500
Group D		
Rubiginosin C (**11**)	39.16	42.2
Cohaerin A (**12**)	50.76	77.6
Cohaerin B (**13**)	54.55	63.8
Group E		
Rutilin A (**14**)	1.76	
Rutilin B (**15**)	1.80	
N^G-mehtyl-L-arginine	88.4[26]	
L-Ascorbic acid		16.5[21]

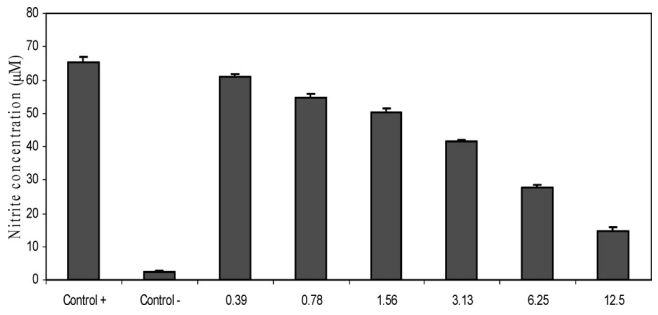

Fig. 2. Effect of Rubiginosin A on LPS-Induced NO Production

RAW cells were incubated in the presence of the indicated concentrations from 12.5 to 0.39 μM of rubiginosin A for 24 h in the presence (+) or absence (−) of LPS. From the supernatant, NO was detected and analyzed using Griess reagent. Data were derived from the three independent experiments.

each compound (**1**—**15**) and then LPS (1 μg/ml) was added and the incubation continued for 24 h. Nitrite concentrations in the conditioned medium were determined. The production of nitrite was increased by treatment with LPS. All azaphilones (**1**—**15**) inhibited the LPS-induced production of nitrite and their IC_{50} values are shown in Table 1. The inhibition of NO production by rubiginosin A (**9**), chosen as a representative azaphilone, at six concentration is shown in Fig. 2. Higher concentrations (more than 30 μM) of each azaphilone were tested first, however, cell viability was decreased due to cytotoxicity (data not shown). Thus, all samples were tested at concentrations less than 12.5 μM to confirm that the inhibition of NO production was not due to cytotoxicity. In the following experiment, we analyzed the effect of rubiginosin A (**9**), which showed potent inhibitory activity among the fifteen azaphilones.

Effects of Three Concentrations of Rubiginosin A (9) on LPS-Induced iNOS mRNA Expression Incubation with LPS (1 μg/ml) for 8 h markedly induced iNOS expression in RAW cells (Fig. 3). The LPS-induced iNOS expression was suppressed by rubiginosin A (**9**) at three different

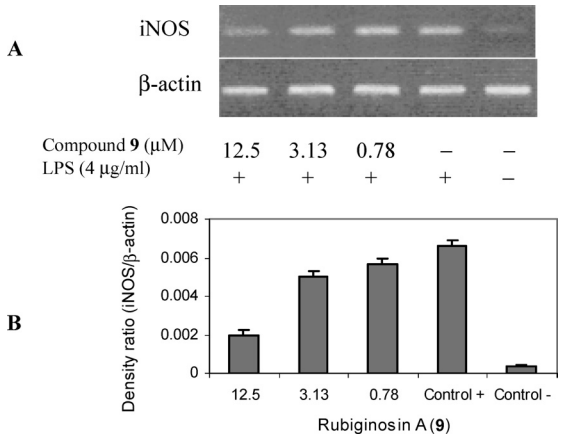

Fig. 3. Results of RT-PCR Analysis of the iNOS mRNA Level of **9**

RAW cells were incubated in the presence of the indicated concentration (μM) of rubiginosin A for 8 h in the presence (+) or absence (−) of LPS. RNA was extracted and subjected to RT-PCR analysis of iNOS. Data were derived from the three independent experiments.

concentrations compared to the positive control. This finding indicated that **9** inhibited the LPS-induced production of nitrite through the inhibition of iNOS expression.

DISCUSSION

Azaphilones can be divided into five groups based on their constitutional formulas.

Group A contains daldinins C, E and F (**1**—**3**) constituting *spiro*-tricyclic derivatives, which only weakly suppressed NO production (Table 1). Group B consists of multiformin D (**4**) and sassafrins A—C (**5**—**7**), which are azaphilones with a lactone ring in their molecule. Their inhibitory activities are stronger than those of group A. Group C [entonaemin A (**8**) and rubiginosin A and B (**9**, **10**)] are azaphilones with an orsellinic acid moiety attached to the bicyclic azaphilone backbone though an ester linkage. In this group, rubiginosin A (**9**) showed the strongest inhibition of NO production. The location of the orsellinic acid moiety did not affect the activity of the compounds. However, the presence of an acetyl group increased the activity. The fourth group, group D [rubiginosin C (**11**), cohaerins A and B (**12**, **13**)], which are azaphilones with a fatty acid side chain linked with bicyclic azaphilone by an ester bond, exhibited weak activity. The greatest inhibitors of NO production is group E consisting of the dimeric azaphilones rutilins A and B (**14**, **15**) with IC_{50} values of 1.76 and 1.80 μM, respectively. Therefore, the number of orsellinic acid moieties in the molecule and conjugated double bonds in dimeric compounds dramatically increased their inhibitory activities. These results again confirmed that the acetyl group was necessary for inhibition, whereas, the location of orsellinic acid did not change their activities. The inhibition of NO synthesis by these azaphilones is strong compared to the IC_{50} of 88.4 μM of N^G-mehtyl-L-arginine (L-NMMA), a known inhibitor of nitric oxide synthase.[26]

RT-PCR was used to examine how mRNA expression was affected by treatment with **9**. The results also showed a significant correlation with mRNA expression level as detected by electrophoresis on agarose gel (Fig. 3). Taking the results from RT-PCR and electrophoresis together, the inhibitory activity of the active azaphilone rubiginosin A on LPS stimu-

lated-NO production in RAW cells is most likely due to a decrease in iNOS mRNA expression. The active compound affected inducible iNOS mRNA expression, but did not affect constitutive gene expression, such as β-actin. All azaphilones showed weak to very weak antioxidant activities (Table 1) compared to the known standard, ascorbic acid. In particular, compounds (**8**—**10**) in group C were void of any activity, so the inhibition of NO production in RAW cells was not due to their free-radical scavenging activities.

In conclusion, we have identified a series of azaphilone compounds as novel potential inhibitors of NO production using a cell-based system, and found that dimeric azaphilones exhibited the strongest inhibitory activities.

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