Characterization of Graphislactone A as the Antioxidant and Free Radical-Scavenging Substance from the Culture of *Cephalosporium* sp. IFB-E001, an Endophytic Fungus in *Trachelospermum jasminoides*

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The extracts derived from cultures of 1626 endophytic strains harbored in *Trachelospermum jasminoides* were assayed for more potent antioxidant and/or free radical-scavenging agents. The free radical-scavenging assessment was carried out using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and hydroxyl radical assays, and the antioxidant actions on linoleic acid and human low-density lipoprotein (LDL) models. After extensive spectroscopic analyses, graphislactone A was characterized as the most bioactive secondary metabolite of endophytic *Cephalosporium* sp. IFB-E001 with its free radical-scavenging (in a dose-dependent manner) and antioxidant activities ascertained *in vitro* to be stronger than those of butylated hydroxytoluene (BHT) and ascorbic acid, the two positive references coassayed in the study. From the demonstrated efficacy of graphislactone A in preventing and protecting against oxidative injury, it can be predicted that this metabolite could be a potential agent in the management of oxidative damage-initiated diseases.

Key words endophyte; *Cephalosporium* sp. IFB-E001; *Trachelospermum jasminoides*; graphislactone A; antioxidant; free radical

There is increasing evidence indicating that reactive oxygen species (ROS, e.g., $O_2^{\cdot -}$ and $OH^{\cdot}$) and free radical-mediated reactions are involved in degenerative or pathologic events such as aging, cancer, coronary heart disease, and Alzheimer's disease.¹⁾ Antioxidants are thought to be beneficial and/or curative in the management of ROS-mediated tissue injury.²⁾ However, only a few antioxidants have been approved to date for clinical application due to the low efficacy and/or severe side effects. Therefore there is an urgent need to search for new and more effective antioxidants with negligible or acceptable adverse effects.

Endophytes are bacteria or fungi that spend the whole or part of their lifespan inside the healthy tissues of the host inter- and/or intracellularly without causing discernible symptoms of plant disease.³⁾ In addition to the interest of ecologists in the relationship between endophytes and hosts, chemists and biologists are investigating the chemistry and bioactivity of endophyte metabolites.⁴⁾ The evergreen vine *Trachelospermum jasminoides* (LINDL.) LEM. (Apocynaceae) has been utilized since ancient times in traditional Chinese medical practice for the treatment of rheumatic arthralgia, aching of the loins and knees, and traumatic injuries⁵⁾ and its antiinflammatory fractions have been determined.⁶⁾ However, little is known about endophytes harbored inside the healthy tissues of the climbing plant. As a follow-up to our previous studies aimed at characterizing antioxidant and free radical-scavenging substances from a higher plant⁷⁾ and marine microbe,⁸⁾ we have isolated a total of 1626 endophytic strains from November (2001), April, and June (2002) collections of the *T. jasminoides* growing in the eastern suburbs of Nanjing. The subsequent screening of the $CHCl_3$:MeOH (v/v 1:1) extracts of the corresponding endophyte cultures for antioxidant and free radical-scavenging activity revealed that the most substantially active substance(s) was afforded by the endophytic fungus *Cephalosporium* sp. IFB-E001 residing in the roots of the November collection of *T. jasminoides*. We report here the full characterization of the most bioactive secondary metabolite, graphislactone A, with its free radical-

scavenging and antioxidant activities confirmed *in vitro* to be greater than those of butylated hydroxytoluene (BHT) and ascorbic acid, the two positive references coassayed in the study.

MATERIALS AND METHODS

General EI-MS were recorded on a VG-ZAB-HS mass spectrometer and 1H - and ^{13}C -NMR experiments were performed on a Bruker DRX-500 NMR spectrometer using TMS as an internal standard. Silica gel (200—300 mesh) for column chromatography and silica GF₂₅₄ (10—20 mm) for TLC were products of Qingdao Marine Chemical Factory, China. Sephadex LH-20 was from Pharmacia Biotech, Sweden. All chemicals used in the study were of analytical grade.

Materials Following the procedure detailed elsewhere,⁹⁾ a total of 1626 cultivable endophytic fungal isolates were obtained from the surface-sterilized fresh tissues of apparently healthy wild vines of *T. jasminoides* after collection in November (2001), April, and June (2002) from the eastern suburbs of Nanjing, China. The endophytic fungus IFB-E001 affording the most antioxidant and free radical-scavenging culture extract, was then identified as *Cephalosporium* sp. by comparing the morphologic characteristics and the pattern of sporulation, which are nearly identical to those described elsewhere.¹⁰⁾

Samples of a 5-d fresh mycelium of the fungus grown on PDA medium (potato, 200 g; dextrose, 20 g; agar, 20 g; H_2O , 1000 ml) in a Petri dish at 28 °C were inoculated into 1000-ml Erlenmeyer flasks containing 400 ml PD broth medium, followed by shaking (150 rpm) continuously for 4 d at 28 ± 1 °C. The culture liquor (20 ml) of the endophyte *Cephalosporium* sp. IFB-E001 was applied to solid-matrix steady fermentation using millet medium (millet 7.5 g, bran 7.5 g, yeast extract 0.5 g, $FeSO_4$ 0.01 g, tartrate sodium 0.1 g, glutamine sodium 0.1 g and pure corn oil 0.1 ml in tap water 15 ml per bottle). The fermentation, accomplished in a total of 420 bottles, was subsequently accomplished by standing

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for another 30 d at $28 \pm 1^\circ\text{C}$.

Extraction and Isolation The dried powdered mycelia were extracted four times with CHCl_3 :MeOH (v/v, 1:1) at room temperature. Evaporation of the solvent *in vacuo* afforded 750 g of a dark-brownish residue. The crude extract was dissolved in a smaller amount of MeOH at around 45°C and the solution was kept at -20°C overnight, followed by filtration to eliminate waxy substances as the precipitate. Removal of MeOH under reduced pressure gave a brown lump, to which MeOH was added until it dissolved completely. To the MeOH solution, acetone was added drop by drop with acetone 15% (v/v), and the mixture was kept less than -20°C overnight to precipitate salts and saccharides. The filtrate was concentrated *in vacuo* to give a residue (250 g), which was subjected to column chromatography on silica gel (2000 g, 200–300 mesh) eluted with a gradient of CHCl_3 :MeOH (v/v, 1:0→0:1) to give nine fractions (T1, 5.5 g; T2, 21.4 g; T3, 7.3 g; T4, 15.6 g; T5, 18.9 g; T6, 24.7 g; T7, 21.2 g; T8, 14.1 g; T9, 69.8 g) based on TLC monitoring. Fraction T2, the most bioactive fraction, was then chromatographed on silica gel (500 g, 200–300 mesh) using a stepwise gradient of CHCl_3 :MeOH (v/v, 1:0→10:1) and four fractions (T21, 4.9 g; T22, 5.7 g; T23, 7.4 g; T24, 3.8 g) were obtained. T23, the bioactive fraction, was further chromatographed on silica gel (150 g, 200–300 mesh), followed by gel filtration on a Sephadex LH-20 column eluted with CHCl_3 :MeOH (v/v, 1:1) to yield 13 mg of yellowish powdered graphislactone A (Fig. 1) and its spectral data (EI-MS, ^1H - and ^{13}C -NMR, see above) were identical to those reported by Tanahashi *et al.*¹¹⁾

Scavenging Activity against DPPH Radicals The scavenging activity of graphislactone A against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals was measured according to the method of Hou *et al.*¹²⁾ Sample solution 0.3 ml with test amounts (1, 2, 3, 4, 5 μg) of graphislactone A was added to 0.1 ml of 1 M Tris-HCl (pH 7.9), and then mixed with 0.6 ml of DPPH 100 μM in methanol for 20 min under protection from light at room temperature. The absorbance at 517 nm was measured. Deionized water was used as a blank experiment, and BHT as a positive control.

Scavenging Activity against Metal Ion-Dependent Hydroxyl Radicals The hydroxyl radicals were determined using the deoxyribose method described previously.¹²⁾ Sample solution 0.5 ml with one of the test amounts (0.075, 0.165, 0.24, 0.315, 0.405 μg) of graphislactone A was added to 1.0 ml of a solution of potassium phosphate buffer 20 mM (pH 7.4), 2-deoxy-ribose 2.8 mM, EDTA 104 μM , FeCl_3 100 μM , ascorbate 100 μM and hydrogen peroxide 1 mM. The mixtures were incubated at 37°C for 1 h. After incubation, an equal volume of 0.5% thiobarbituric acid in 10% trichloroacetic acid was added and the mixtures were boiled at 100°C for 15 min. Deionized water was used as a blank experiment, and BHT as a positive reference. The absorbance

Fig. 1. Structure of Graphislactone A

at the wavelength of 532 nm was measured.

Antioxidant Activity against Linoleic Acid Peroxidation The antiperoxidation activity of graphislactone A against 20 mM of linoleic acid emulsion was measured at intervals after reactions at 37°C using the thiocyanate method.¹³⁾ During incubation, a 0.1-ml aliquot of the reaction mixture containing graphislactone A 50 μg was mixed with 4.7 ml of 75% ethanol, 0.1 ml of 30% ammonium thiocyanate, and 0.1 ml of 20 mM ferrous chloride in 3.5% hydrochloric acid. Precisely 3 min after the addition of ferrous chloride to the reaction mixture, the absorbance at the wavelength of 500 nm was determined. Deionized water was used as a blank experiment, and ascorbic acid 0.5 mg as a positive control. Increased absorbance at 500 nm of the reaction mixture indicates a decrease in antioxidant activity against linoleic acid peroxidation.

Protection against Cu^{2+} -Induced Low-Density Lipoprotein Peroxidation EDTA in the purchased low-density lipoprotein (LDL) was removed prior to the peroxidation experiment, as described elsewhere.¹⁴⁾ The commercial LDL solution was dialyzed three times against a 500-fold volume of phosphate buffer 10 mM (pH 7.4, containing NaCl 0.16 mM). The capacity of graphislactone A to protect against Cu^{2+} -induced human LDL oxidation in a total 1.1-ml sample volume was measured in the thiobarbituric acid-reactive substance (TBARS) assay at 532 nm.¹⁵⁾ LDL (0.5 mg protein ml^{-1}) was incubated at 37°C under air in phosphate buffer 10 mM (pH 7.4) containing CuSO_4 10 μM for 24 h with or without graphislactone A. The peroxidation reaction was stopped by adding EDTA 100 μM . The TBARS value of 24-h LDL peroxidation was assumed to be 100%. BHT was utilized as a positive control.

Statistical Analysis Experimental values are expressed as mean $\pm$ S.D. of triplicate experiments, as indicated in the legends. Student's *t*-test was used for comparison between two treatments, and a *p* value <0.01 was considered statistically significant.

RESULTS

Scavenging Activities of Graphislactone A against DPPH Radicals The effects of graphislactone A at the test concentrations on the scavenging of DPPH radicals was determined spectrophotometrically. As demonstrated in Fig. 2, the more graphislactone A added, the higher the DPPH-scavenging activities. The IC_{50} value of graphislactone A for DPPH radicals was 2.9 $\mu\text{g ml}^{-1}$ (*viz.*, 9.6 μM), which was more potent than that of BHT (IC_{50} , 3.2 $\mu\text{g ml}^{-1}$; *viz.*, 19.5 μM), the positive control employed in this experiment.

Scavenging Activities of Graphislactone A against Metal Ion-Dependent Hydroxyl Radicals The effects of graphislactone A on the scavenging of hydroxyl radicals are shown in Fig. 3. Graphislactone A exhibited dose-dependent hydroxyl radical-scavenging activities at the test concentrations used. The effects of BHT against hydroxyl radicals were less than those of graphislactone A. The scavenging activity of graphislactone A against hydroxyl radicals was 70% and 91% at 0.05 and 0.27 $\mu\text{g ml}^{-1}$, respectively.

Antioxidant Activity of Graphislactone A against Linoleic Acid Peroxidation As illustrated in Fig. 4, graphislactone A retarded linoleic acid peroxidation during the inter-

Fig. 2. Scavenging Activities of Graphislactone A (■) at 1, 2, 3, 4, and 5 µg ml⁻¹ against DPPH Radicals

BHT (□) served as positive control. The DPPH radical-scavenging activity (%) was calculated using the following equation: $[(A_{517} \text{ of blank} - A_{517} \text{ of sample}) / A_{517} \text{ of blank}] \times 100$.

Fig. 3. Hydroxyl Radical-Scavenging Activities of Graphislactone A (■) at Different Concentrations

BHT (□) served as a positive control. The hydroxyl radical-scavenging activity (%) was calculated using the following equation: $[(A_{532} \text{ of blank} - A_{532} \text{ of sample}) / A_{532} \text{ of blank}] \times 100$. A difference was considered statistically significant when $p < 0.01$ (**) vs. positive control.

Fig. 4. Determination of the Antioxidant Activity of Graphislactone A (□) against Linoleic Acid Peroxidation Reactions during the Interval of 30 h at 37 °C Using the Thiocyanate Method

Deionized water (◇) was used as a blank. Ascorbic acid (△) (0.5 mg) served as a positive control.

val of 30 h at 37 °C compared with the blank. Within the first 12 h of reaction, the positive control, ascorbic acid, showed antioxidant activity against linoleic acid peroxidation similar to that of graphislactone A. However, in the case of ascorbic

Fig. 5. Protective Effects of Graphislactone A (■) against Cu²⁺-Induced Human LDL Peroxidation Using the TBARS Assay with BHT (□) as a Positive Control

The TBARS formed after 24-h LDL peroxidation was considered to be 100%. Student's *t*-test was used for the comparison of the oxidized LDL with the other treatments. A difference was considered statistically significant when $p < 0.01$ (**) or $p < 0.001$ (***) vs. blank control.

acid, a drastic increase in linoleic acid peroxidation was found, similar to that of the blank, 30 h after the reaction, indicating that ascorbic acid 0.5 mg was nearly depleted after 30-h reaction, whereas graphislactone A retained the ability to delay lipid peroxidation. There was a 4.4-fold retardation of linoleic acid peroxidation in samples containing graphislactone A compared with those containing the blank at the end of the 30-h reaction.

Protection against Cu²⁺-Induced LDL Peroxidation

Relative to the blank control, an approximately 17-fold increase in LDL lipid peroxidation was induced by the presence of Cu²⁺. The presence of graphislactone A significantly inhibited lipid peroxidation in human LDL in a dose-dependent manner (Fig. 5). At 4 µg ml⁻¹, the protective activity of graphislactone A was up to 62%. The IC₅₀ of graphislactone A for the protection against LDL peroxidation was 2.2 µg ml⁻¹ (7.3 µM), whereas the IC₅₀ of BHT was 3.4 µg ml⁻¹ (20.7 µM). Therefore graphislactone A is more active than BHT.

DISCUSSION

As a relatively untapped reservoir of bioresources, some endophytic microorganisms have been demonstrated to be excellent producers of biologically active and/or structurally novel metabolites.¹⁶ In this study, we found that the phenolic fungal metabolite graphislactone A exhibited strong free radical-scavenging and antioxidant activities using *in vitro* assays. This compound was previously reported as a metabolite of the lichen mycobiont *Graphis* spp.^{11,17}

The DPPH radical model system is widely used to investigate the scavenging activities of natural compounds.^{8,14} In the present study, graphislactone A exhibited stronger radical-scavenging activity against DPPH radicals than did the positive control BHT (Fig. 2). In addition to DPPH radicals, hydroxyl radicals are considered to be potent oxidants, which can react with all biomacromolecules functioning in living cells.¹⁸ In the present study, the deoxyribose assay was employed to evaluate the hydroxyl radical-scavenging activity of graphislactone A. Graphislactone A showed a pronounced activity in eliminating hydroxyl radicals in a dose-dependent manner, which was greater than that of BHT (Fig. 3). These

results suggest that graphislactone A may attenuate the process of metal ion-dependent hydroxyl radical formation and thus reduce hydroxyl radical-induced cell injury.

The products of lipid peroxidation (such as malondialdehyde) can damage proteins and DNA.¹⁹⁾ In the antioxidant assay performed using the linoleic acid peroxidation model, graphislactone A was found to be more antioxidant than ascorbic acid (Fig. 4). The oxidation of LDL has been found to be associated with foam cell formation and atherosclerosis development,²⁰⁾ and more recent studies have revealed that the ultimate atherogenic agents are modified (mainly oxidized) forms of LDL.²¹⁾ We therefore performed human LDL oxidation using Cu²⁺ as the oxidative initiator to confirm the antioxidant activity of graphislactone A. Under our experimental conditions, graphislactone A significantly suppressed TBARS formation in LDL oxidation induced by Cu²⁺ (Fig. 5). Therefore the phenolic fungal metabolite graphislactone A may be a potential agent to prevent LDL oxidation *in vivo* and in the management of atherosclerosis.

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