Antioxidant compounds produced by endolichenic fungus Penicillium sp. induced from Pyxine subcinerea

Hiroko Kawakami
khiroko@akita-pu.ac.jp

Akita Prefectural University
Nao Watabe
Akita Prefectural University
Yuko Matsubuchi
Akita Prefectural University
Kojiro Hara
Akita Prefectural University
Masashi Komine
Akita Prefectural University

Short Report

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Abstract

Endolichenic fungi are expecting for new bioresources of pharmacological compounds. However, the number of investigations targeting antioxidant compounds produced by endolichenic fungi remains limited. To discover new antioxidant compounds, we analyzed the antioxidant activity of 59 methanol extracts derived from isolated lichen mycobionts or endolichenic fungi induced from *Pyxine subcinerea*. We performed this analysis using the oxygen radical absorbance capacity (ORAC) method. As a result, we isolated from an endolichenic fungus identified as *Penicillium* sp. in *Pyxine subcinerea.* This fungus produced a red pigment, and its chemical structure was determined to be sclerotioramine based on the analytical data obtained from NMR, LC-MS/MS, and HPLC-PDA. Sclerotioramine exhibited high antioxidant activity, and the ORAC values (mean ± SD) of sclerotioramine and sclerotiorin were 11.4 ± 0.36 and 4.86 ± 0.70 mmol TE per gram of the respective pure compound. Thus, the antioxidant activity of sclerotioramine was greater than twice that of sclerotiorin. This work represents the first report that the antioxidant activity of sclerotioramine is higher than that of the sclerotiorin.

Introduction

Lichens are symbiotic organisms in which fungi (*e.g.* lichen mycobionts) and photobionts. The lichen mycobionts contain a great number of natural products called “Lichen substances”. Lichen substances are well-known as sources for medicines, dyes, and perfumes. Beside of lichen mycobiont, many kinds of non-obligate microfungi live in lichen thalli and relate with photobionts, constituting a class of organisms referred to as “Endolichenic fungi”. Most isolated endolichenic fungi were classified into subclasses Eurotiomycetes, Dothideomycetes, Leotiomycetes, Pezizomycetes, and Sordariomycetes. Secondary metabolites produced from endolichenic fungi have been expected as new pharmacological bioresources in addition to lichen substances. In fact, some secondary metabolites such as anthraquinones, benzopyranoids, chromanone, furanones, pyrones, quinones, sulfur-containing and xanthones were discovered in endolichenic fungi (Kellogg and Raja, 2016), and Singh et al. (2017) summarized that these metabolites have some useful activities, including anticancer, antifungal, and antioxidant activities.

Antioxidant compounds inhibit the effects of the reactive oxygen species (ROS), avoiding damage to biomolecules related to the factor of some diseases *e.g.* cancer, cardiovascular diseases, and aging of skin (Halliwell and Gutteridge 2015). Many lichen substances have also been reported as antioxidant compounds, such as atranorin, usnic, lecanoric, diffractaic, lobaric, stictic, fumarprotocetraric, salazinic, physodic and orsellinic acid, orcinol, and other orsellinates (White et al., 2014). We previously reported that an endolichenic fungus induced by *Pertusaria laevigata* produced an antioxidant compound identified as norlichexanthone (Kawakami et al. 2019). However, the number of investigations targeting antioxidant compounds produced by endolichenic fungi remains limited.

To find new antioxidant compounds, we performed the antioxidative screening of 59 methanol extracts derived from isolated lichen mycobionts or endolichenic fungi induced from *Pyxine subcinerea*. A methanol extract obtained from endolichenic fungi induced by *P. subcinerea* exhibited relatively high

antioxidant activity. Here, we demonstrate that an endolichenic fungus induced by *P. subcinerea* produces sclerotioramine, a compound that shows antioxidant activity.

Materials and methods

Fungal isolation and liquid culture

One initial endolichenic fungus was induced from the thallus of *P. subcinerea* (sampled from Hiraoka Park, Osaka, Japan) applying the lichen tissue culture method (Yamamoto et al. 1985). Its crashed lichen thalli were incubated on solid malt-yeast (MY) medium containing 1.5% agar at 20 °C under light (1042.8 lux), followed by subculture under the same culture conditions. To obtain a larger amount of cultured *Penicillium* sp., 2 g of this fungus was inoculated to 200 mL of liquid MY medium in a 500-mL Erlenmeyer flask and incubated for 3 weeks at 20 °C under light (Fig. 1). This strain was deposited at Akita Prefectural University, Akita, Japan (Registration No. 1322P). The voucher strain of this endolichenic fungus was identified as *Penicillium* sp. based on its sequence of the ribosomal DNA internal transcribed spacer (ITS) region, and the DNA Data Bank of Japan (DDBJ) accession number for the ITS sequence of strain 1322P is LC775762.

Extraction and isolation of antioxidant compounds from cultured fungi

To extract antioxidant compounds, the dried fungus (7.6 g dry weight) of 1322P strain was submerged in methanol and incubated overnight at room temperature, followed by filtration. The filtrate was evaporated *in vacuo* and eluted with water, and then sequentially extracted with solvents that yielded the following fractions: *n*-hexane (170 mg), ethyl acetate (62 mg), *n*-butanol (135 mg), and water (118 mg). The ethyl acetate fraction was separated using thin-layer chromatography (TLC; PLC Silica gel 60 F_{254}, Merck, Tokyo, Japan) with ethyl acetate-chloroform (1:9), yielding Fr. 1 (11 mg), Fr. 2 (4 mg), Fr. 3 (8 mg), Fr. 4 (49 mg), Fr. 5 (6 mg), Fr. 6 (37 mg), Fr. 7 (7 mg), Fr. 8 (7 mg), Fr. 9 (8 mg), Fr. 10 (12 mg), Fr. 11 (7 mg), Fr. 12 (5 mg), Fr. 13 (5 mg), Fr. 14 (6 mg), Fr. 15 (5 mg), Fr. 16 (6 mg), and Fr. 17 (6 mg). Fr. 1 was purified by repeating TLC under the same conditions as above, resulting in the isolation of a red pigment named Compound **1** (1 mg).

Determination of chemical structure by NMR, HPLC-PDA, and LC-MS/MS

The Compound **1** were eluted in methanol-d_4 containing trimethyl silane (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and its ^{1}H- and ^{13}C-NMR spectra were analyzed by a JEOL-ECS 400 spectrometer (^{1}H at 400 MHz and ^{13}C at 100 MHz, JEOL Ltd., Tokyo, Japan). Compound **1** was also analyzed by liquid chromatography with electrospray ionization mass spectrometry (LC-ESI-MS) coupled MS (MS/MS) and high-performance liquid chromatography with photodiode array (HPLC-PDA). LC-ESI-MS analysis was performed using a TSQ Quantum Ultra MS connected with the UltiMate 3000 system (Thermo Fisher

Scientific, Waltham, MA, USA), for which 1 µL of sample was injected onto an InertSustainSwift C18 column (column size, 2.1 × 100 mm; particle size, 3 µm; GL Sciences, Tokyo, Japan) heated at 35°C, and eluted at a flow rate of 200 µL min^{-1}. The mobile phase established with a linear gradient system of A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) as follows: 0–35 min, 50–70% B; 35–40 min, 70–95% B. The collision energy for the MS/MS was set at 15 eV. HPLC-PDA analysis was performed using the SHIMADZU Prominence system (Shimadzu, Kyoto, Japan), for which 10 µL of sample solution was injected onto a YMC-Pack ODS-A C18 column (column size, 4.6 × 150 mm; particle size, 5 µm; YMC Co., Ltd., Kyoto, Japan) heated at 40°C, and eluted at a flow rate of 1 mL min^{-1}. The mobile phase established with a linear gradient system of A (0.1% acetic acid in water) and B (0.1% acetic acid in acetonitrile) as follows: 0–10 min, 10–30% B; 10–20 min, 30–60% B; 20–30 min, 60–80% B; 30–40 min, 80% B. Spectra of absorbance for ultra-violet region were measured at wavelengths of 200–400 nm.

Evaluation of the antioxidant activity

Oxygen radical absorbance capacity method (ORAC)

The antioxidant activity of the isolated compound was evaluated by the ORAC method described by our previous report (Kawakami et al. 2019). ORAC values of antioxidant compounds were calculated using the differences of areas under the FL decay reaction curve between a blank and a sample; values were presented in units of mmol of Trolox equivalents (TE) per gram of pure compound.

Results and discussion

Determination of the chemical structure of antioxidant compounds in cultured Penicillium sp.

The chemical structure of compound **1** was determined by NMR (Table 1) comparing with the compound's spectral data of sclerotioramine (Fig. 2) isolated from a strain of *Penicillium citreonigrum* (Wang et al. 2010). LC-ESI-MS analysis of Compound **1** resulted in the detection of a molecular ion peak at 5.8 min with *m/z* 388 ($[M-H]^-$ (100)) and 390 ($[M-H+2]^-$ (33)); these analytical features of the mass spectrum indicate compounds containing one halogen atom. In LC-ESI-MS/MS, Compound **1** produced fragment ions with *m/z* 310 (6), 324 (7), 328 (43, $[M-H-CH_3COOH]^-$), 346 (6), and 388 (100, $[M-H]^-$) (Fig. 3). Compound **1** and authentic sclerotioramine (Sigma-Aldrich, Tokyo, Japan) were eluted at 9.1 min and showed ultraviolet maximum wavelength (UV λ_{max}) absorbance at 219, 351, and 488 nm in HPLC-PDA (Fig. 4). In a previous work, sclerotioramine was isolated from the mycelia of *Penicillium multicolor*, where the compound was thought to be synthesized by replacing the heterocyclic oxygen with amines (Whalley 1963). Authentic sclerotiorin (Sigma-Aldrich, Tokyo, Japan) was eluted at 22.5 min and showed different UV spectra (absorbance at 219, 267, and 366 nm) than sclerotiolamine. These results showed that discrimination between sclerotioramine and sclerotiorin could be clearly determined by HPLC-PDA analysis. We inferred that, in the present study, sclerotioramine was formed via the reaction of sclerotiorin with endogenous ammonia in the fungal culture, although we did not directly detect sclerotiorin in this

fungal methanol extract. For comparisons, NMR, HPLC-PDA, and LC-MS/MS were used to analyze authentic sclerotioramine, providing data consistent with that of Compound **1**. From these results, compound **1** was identified as sclerotioramine.

Some fungal strains that have been classified as members of the genus *Penicillium* have been isolated from various lichen hosts, including two strains of *Penicillium* sp. from *Parmelia* lichens (Padhi and Tayung 2015) and *P. citrinum* from *Parmotrema* lichens (Samanthi et al. 2015; Wickramarachchi et al. 2019)d *simplicissimum* (Oudem.) Thom from *Usnea baileyi* and a *Penicillium* sp. from *U. bismolliuscula* (Santiago et al. 2021). The mycelia of *P. citrinum* isolated from *Parmotrema* lichens have been shown to contain three antioxidant compounds, with two reported on and determined by Samanthi et al. (2015) to be 5'-acetyl-3,5,7'-trimethoxy-3'H-spiro [cyclohexa [2,4]diene-1,1’-isobenzofuran]-3’,6-dione and 4-acetyl-2’-hydroxy-3’,5’,6-trimethoxy biphenyl-2-carboxylic acid, and the other reported on and determined by Wickramarachchi et al. (2019) to be 10-ethylidene-2,4,9-trimethoxy-10,10a-dihydro-7,11-dioxa-benzo[b] heptalene-6,12-dione; sclerotioramine was not found in that fungal strain.

Antioxidant activity of sclerotioramine and sclerotiorin

The ORAC values (mean ± SD) of sclerotioramine and sclerotiorin were 11.4 ± 0.36 and 4.86 ± 0.70 mmol TE per gram of the respective pure compounds. Thus, the antioxidant activity of sclerotioramine was greater than twice that of sclerotiorin. In our previous work describing the antioxidant activities of several known antioxidant compounds, we reported that the ORAC values of sclerotioramine and sclerotiorin were lower than those of norlichexanthone, catechin, ascorbic and gallic acid, and quercetin dihydrate (Kawakami et al. 2019). Santos et al. (2019) reported that sclerotiorin (IC_{50}; 78.12 ± 2.82 μg/mL) showed weak antioxidant activity than gallic acid (IC_{50}; 1.26 ± 0.02 μg/mL), as assessed by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. This work represents the first report that the antioxidant activity of sclerotioramine exceeds that of sclerotiorin.

Sclerotiorin has been shown to exhibit pharmacological activity, including antioxidant and antibacterial activities against *Bacillus subtilis*, *Candida albicans, Escherichia coli, Micrococcus luteus, Salmonella typhimurium*, *Staphylococcus aureus*, and *Streptomyces pyogenes* (Lucas et al. 2007; Santos et al. 2019). In contrast, there have been few reports on the pharmacologic activity of sclerotioramine, although derivatives of this compound have been reported to present potent antifouling activity (Wei et al. 2017). In this study, we describe the potential utility of sclerotioramine as a pharmacologic compound.

Conclusion

This work represents the first report that the antioxidant activity of sclerotioramine exceeds that of sclerotiorin. The cultured *Penicillium* sp. (strain 1322P) isolated from *Pyxine subcinerea* is a valuable endolichenic fungus that produces an antioxidant compound. Our study represents the usefulness of endolichenic fungi as new bioresources for pharmacological compounds.

Declarations

Acknowledgments and Funding

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Author contributions

H.K. cultured fungi, determined the chemical structure of active compounds, and descripted this report. N.W. and Y.M. isolated fungi and purified the active compounds. K.H. identified cultured fungi. M.K. provided financial support.

Availability of data and materials

All data generated or analyzed during this study have been included in this published article.

Code availability Not applicable.

Conflict of interest

We declare that we have no conflicts of interest to declare.

Ethics approval

Not applicable.

Human and animal rights statement

The authors declare that this article does not contain any studies involving human participants or animals.

Consent to participate

Not applicable.

Consent for publication

This manuscript is original and not published elsewhere. All authors have read and approved the final manuscript, and confirm that there are no ethical issues associated with the publication of this manuscript.

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Table

Table 1 is available in the Supplementary Files section.

Figures

Figure 1

Liquid culture of *Penicillium* sp. (strain 1322P) isolated from *Pyxine subcinerea.*

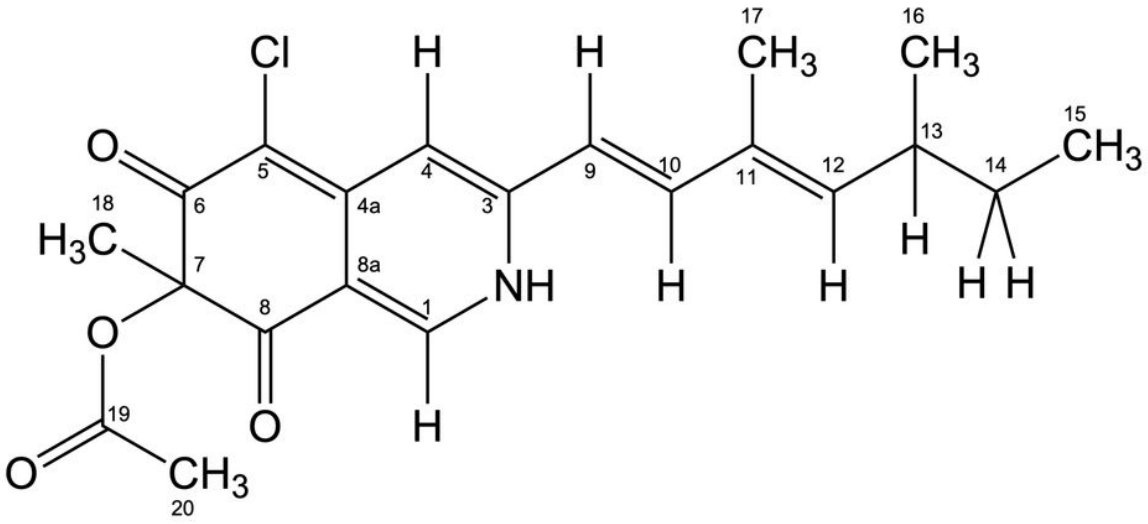

Figure 2

Chemical structure of Compound **1,** determined as sclerotioramine.

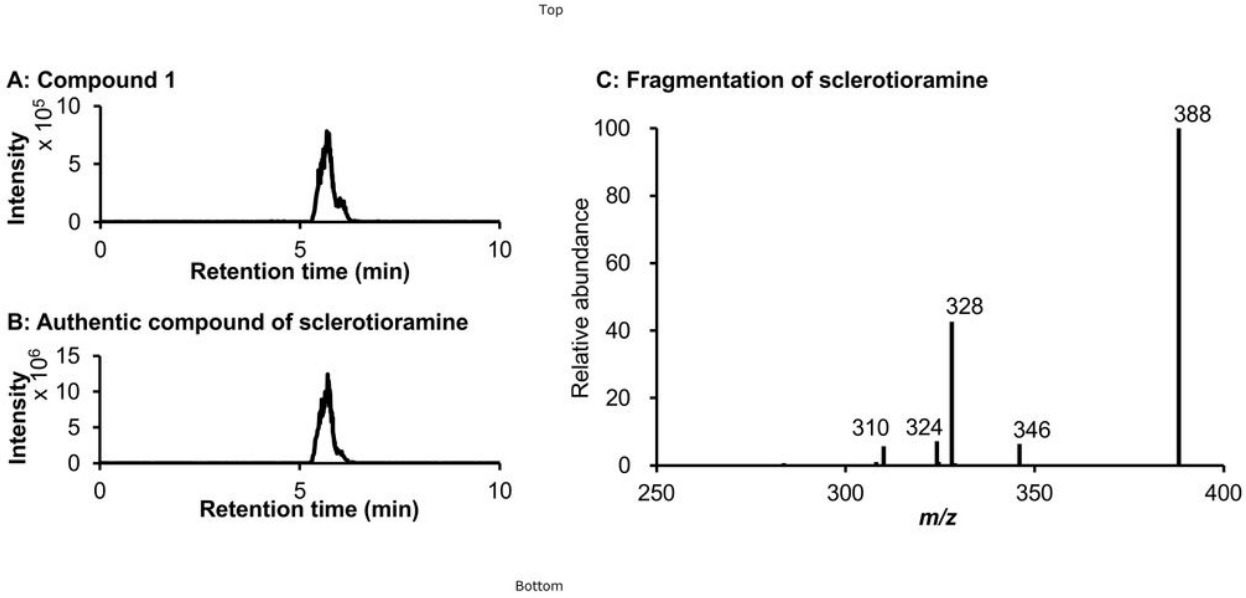

Figure 3

LC-ESI-MS chromatograms of Compound **1**, isolated from *Penicillium* sp. (**A**), and authentic sclerotioramine (**B**). Both were detected at *m/z* 388 in negative ionization mode. Fragmentation of sclerotioramine (**C**).

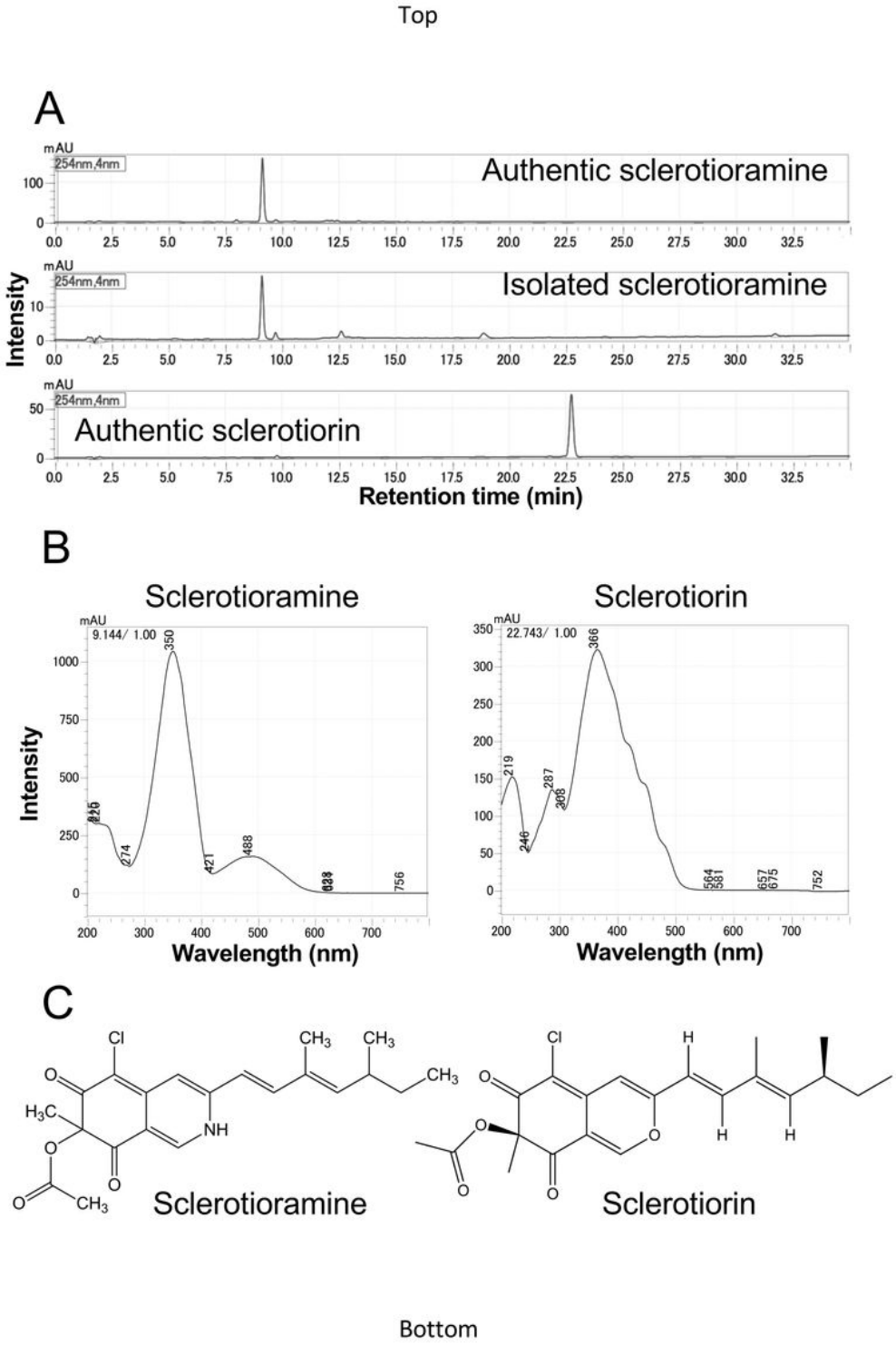

Figure 4

HPLC-PDA chromatograms of compound 1 isolated from *Penicillium* sp. and authentic compound of sclerotioramine and sclerotiorin, which were detected at 254 nm (**A**). UV spectra of sclerotioramine and sclerotiorin (**B**). Chemical structure of sclerotioramine and sclerotiorin (**C**).

Supplementary Files

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- AOL2Table1.jpg