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Characteristics of Secondary Metabolites from Isolated Lichen Mycobionts

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Abstract

Secondary metabolites, produced by polyspore-derived lichen mycobionts but not by the lichens themselves, were studied. Each of the substances was found in the mycobionts of several rather than one specific species of worldwide distribution. Their appearance as crystals on slant cultures and their nature were similar regardless of the species of lichen mycobiont. Another notable characteristic of these substances was that they were often toxic to photobionts. The biological significance of these metabolites is discussed from the viewpoint of lichen symbiosis.

Keywords: Lichen symbiosis, secondary metabolites, mycobiont

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1. Introduction

Secondary metabolites were often obtained from colonies of spore-derived lichen mycobionts cultured on conventional malt-yeast extract media with 10% added sucrose.

Some of these substances were characteristic lichen substances (e.g. pannarin, atranorin, norstictic acid) (Leuckert et al., 1990; Culberson and Armaleo, 1992; Hamada et al., 1996; Hamada et al., 1997), and were detected in the mycobionts as well as in the lichens under natural conditions.

Other substances not detected in the lichens, were often new, and crystallized on the surface of medium-cultured mycobiont. These substances which differed among the various lichen mycobionts included graphenone, graphisquinone (Miyagawa et al., 1994), dibenzofurans (hypostrepsilic, isostrepsilic, hypostrepsilalic acids) (Miyagawa et al., 1993; Miyagawa et al., 1997; Kon et al., 1997), xanthones (Tanahashi et al., 1999), hybocarpone (Ernst-Russel et al., 1999), isocoumarins (Tanahashi et al., 2000) and other substances found in the mycobionts of fruticose or crustose lichens. These substances were often bioactive or toxic to photobionts, and remained detectable even after multiple transplantation.

In addition to previous work identifying new mycobiont substances, further studies are needed in order to elucidate the biological significance of secondary substances produced by the lichen mycobionts and the origin of lichen symbiosis.

In the present study, we have examined the geographic distribution of four groups of substances and their derivatives, which are produced in large amounts by polyspore-derived mycobiont from many families of crustose lichens. We found that they were distributed worldwide, including Japan, North America and Europe. We have also examined the relationship between these secondary metabolites and the lichen species from which the mycobiont was derived.

2. Materials and Methods

All lichen samples were collected between 1993 and 2000 by N. Hamada (NH) except for the Hawaii sample collected by Prof. V. Ahmadjian, and the Argentina sample collected by Dr. M.T. Alder. The specimens were identified by Prof. M. Nakanishi (Okayama University of Science), Prof. M. Inoue (Akita University), and Dr. H. Miyawaki, and are deposited at the Osaka City Institute of Public Health and Environmental Sciences.

Lichen mycobiont spores were isolated using methods described previously (Hamada et al., 1996). For each species, apothecia from air-dried lichen thalli were attached to the lids of inverted petri dishes containing plain agar, and a number of spores (more than 50) were inoculated on to slants of malt-yeast

extract medium containing 100 g of sucrose (MY10) to which had been added a base medium of 10 g malt extract, 4 g yeast extract, 15 g agar, and one liter of water. The final pH of these solutions was adjusted to 6.8 with KOH. Cultures were kept in the dark at 18±1°C. After about 6 months, the lichen mycobionts that produced secondary substances and formed compact mycelia on the medium were separated from the agar using a needle and used for measurement. The mycelia from slants on which mycobionts grew and produced white or yellow crystals were collected and used for extraction. Some strains of lichen mycobiont were transplanted after approximately 6-months in order to keep them alive.

Air-dried mycelia were extracted more than two times with ether or acetone. The extracts were then filtered and condensed under reduced pressure. The residues were identified by TLC (Culberson and Johnson, 1982) and HPLC through comparison with authentic samples identified previously. Rf values (x100) on TLC (Kieselgel GF_{254}) using three solvent systems were measured, and the color of each spot observed by visualization with 10% H_2SO_4 was recorded. TLC (Kieselgel GF_{254}) Rf values (x100) for atranorin/norstictic acid/S1/S2/S3/S4/graphenone/XT5/XT6/XT7/hybocarpone were: 1) 81/34/32/6/10/4/31/41/51/74/51 in hexane-methyl tert. butyl ether-formic acid (140:72:18), and 2) 76/26/37/13/7/4/45/38/43/61/28 in toluene-acetic acid (20:3). Analytical HPLC (Waters 600E Solvent Delivery System; Waters 990J PDA Detector) with a 4.6 x 250 mm TSK gel ODS-120T column and water-methanol-acetic acid (20:80:1) flowing at 1.0 ml/min (ca. 1060 psi; t0=4.3 min) gave retention times (min) of 37.0 for atranorin, 7.6 for norstictic acid, 9.9 for S1, 6.0 for S2, 6.6 for S3, 5.1 for S4, 6.4 for graphenone, 6.6 for XT5, 9.8 for XT6, 18.2 for XT7, and 19.4 for hybocarpone.

3. Results

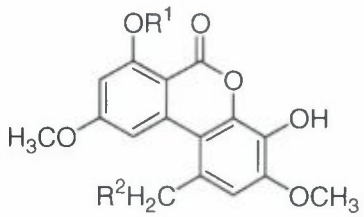
Graphislactones

Four graphislactones, 6*H*-dibenzo[*b*,*d*]pyran-6-one derivatives, were detected in mycobiont colonies of the samples as indicated in Table 1. S1, S2 and S3 (Fig. 1) were major substances in *Graphis scripta* from Japan (Wakayama) (Tanahashi et al., 1997), while S1 and S2 were common in all colonies. S3 and S4 were not detected in colonies of some samples. In all mycobionts producing graphislactones, crystallization appeared similar with numerous fine white graphislactone crystals, highly insoluble even in acetone, found on the surface and interior of the agar surrounding the colonies. Moreover, the color of the media turned dark with the growth of the colonies. The quantity of crystals produced by the mycobiont of *G. prunicola* seemed to increase with repeated transplantation.

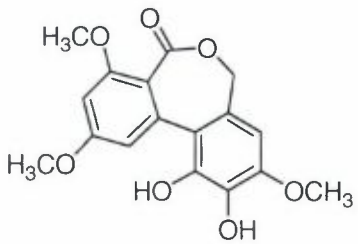
Table 1. Distribution of graphislactones in the lichen mycobionts examined

Lichen species	Country	Sample site	Lichen substance	Mycobiont substances S1	S2	S3	S4	SX
Graphis scripta	Japan	Wakayama	–	+	+	+	+	
G. scripta	Canada	New Brunswick	–	+	+	–	–	
G. prunicola	USA	Florida	–	+	+	+	–	
G. scripta	Slovenia	Slovenia	–	+	+	–	–	+
G. cognata	Japan	Wakayama	–	+	+	+	+	+
G. cognata	Japan	Nagano	–	+	+	–	+	
G. scripta	Japan	Nagano	–	+	+	–	+	
G. scripta	Japan	Nagano	–	+	+	–	–	
G. cognata	Japan	Iwate	–	+	+	+	+	
G. cognata	Japan	Kyoto	–	+	+	+	+	
G. cognata	Japan	Kochi	–	+	+	+	+	
G. cognata	Japan	Hyogo	–	+	+	+	–	
G. cognata	Japan	Kyoto	–	+	+	+	–	
G. cognata	Japan	Nara	–	+	+	–	+	
G. cognata	Japan	Tochigi	–	+	+	+	+	
G. cognata	Japan	Nagasaki	–	+	+	+	+	

Structures of S1, S2, S3, and S4 are indicated in Fig. 1, and XS is of unknown structure. No depsides or depsidones were found in any mycobionts.



S1: $R^1 = R^2 = H$
S2: $R^1 = CH_3$, $R^2 = H$
S3: $R^1 = H$, $R^2 = OH$



S4

Figure 1. Structure of four graphislactones found in lichen mycobionts. These substances are respectively designated S1, S2, S3 and S4 within the text.

Graphislactones were found in the mycobionts of some species of *Graphis* which produce no depsidones under natural conditions. Graphislactones were

produced by the mycobiont of *G. scripta* collected from Europe (Slovenia), in *G. scripta* and *G. prunicola* from North America (New Brunswick and Florida) and in *G. scripta* and *G. cognata* from Japan. S4 was detected only in mycobionts from Japan. Moreover, additional substances of unknown structure were detected in some mycobionts (Table 1). Graphislactones were detected in the mycobionts of two species collected from Japan. However, the anatomical characteristics of the apothecia of these two species, i.e. *G. scripta* and *G. cognata*, were quite different.

Graphislactones were not produced by the mycobiont of all *G. scripta* samples examined. For example, graphislactones were detected in the mycobionts of lichens from only one (Slovenia) of the eight European collection sites.

Graphenone

In all samples listed in Table 2, yellow-orange crystals of graphenone (Fig. 2) covered the surface of the mycobiont colonies and were scattered across the whole surface of the agar, the color of which turned somewhat yellowish. Crystals of graphenone were found even after transplanting the mycobiont. No other graphenone derivatives were detected in the samples examined.

Graphenone was produced by the mycobiont of several species of *Graphis*, including some which are norstictic acid-positive or negative, and from both the Northern Hemisphere and the Southern Hemisphere (Table 2). Thus it was produced by the mycobiont of *G. scripta* and *G. handelii* from 9 collection sites of Japan, by a *Graphis* sp. from Argentina and by *G. tenella* from Hawaii. Norstictic acid-positive lichens whose mycobiont produced graphenone were predominant in Japan. On the other hand, the two lichen samples from Argentina and Hawaii were norstictic acid-negative, although their mycobiont still produced graphenone.

A small amount of norstictic acid was detected with graphenone in the mycobiont of lichens from Okinawa as well as in the lichen samples, although no depsidones were detected in the mycobionts of *G. handelii* derived from the seven other collection sites.

Xanthones

In the mycobiont of all samples producing xanthones (XT5-7, Fig. 3) (Table 3), yellow crystals covered the surface of the colonies and the surrounding agar, the color of which turned red-brown with the growth of the mycobiont. In particular, xanthones produced by the mycobiont of *Pyrenula pseudobufonia* covered the whole surface of the slant within a few months despite repeated transplantation. Xanthones were found in the mycobiont of some samples from

Table 2. Distribution of graphenone in the lichen mycobionts examined

Lichen species	Country	Sample site	Lichen substance	Mycobiont substance Graphenone
Graphis scripta	Japan	Osaka	–	+
G. tenella	USA	Hawaii	–	+
Graphis sp.	Argentina	Buenos Aires	–	+
*G. handelii**	Japan	Okinawa	Norstictic (+)	+
G. handelii	Japan	Okinawa	Norstictic (+)	+
G. handelii	Japan	Hiroshima	Norstictic (+)	+
G. handelii	Japan	Kyoto	Norstictic (+)	+
G. handelii	Japan	Nara	Norstictic (+)	+
G. handelii	Japan	Kochi	Norstictic (+)	+
G. handelii	Japan	Fukushima	Norstictic (+)	+
G. handelii	Japan	Shiga	Norstictic (+)	+

*Norstictic acid was also found in lichen mycobiont.

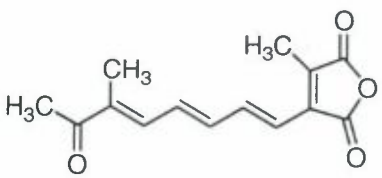


Figure 2. Structure of graphenone.

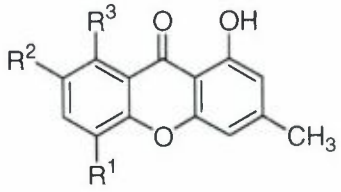
Japan and North America as listed in Table 3. XT6 was detected in all eleven samples examined. XT6 and XT7 were among the major substances detected previously (Tanahashi et al., 1999), although XT7 was not detected in mycobionts of two samples. An additional substance of hitherto unknown structure was detected in one mycobiont (Table 3).

In *Pyrenula* species, xanthones were detected in the mycobionts of *P. pseudobufonia* from North America (Florida) and in those of *P. japonica* from 5 collection sites of Japan. Xanthones were also found in the mycobionts of *Lecidella elaeochroma* and of a *Buellia* sp. Atranorin and norstictic acid were detected in the lichenized *Buellia*, unlike the other lichens listed in Table 3. Some species of *Pyrenula* and *Lecidella* are known to contain various xanthones (Leuckert and Knoph, 1993). However, the three xanthones detected in the mycobionts (Table 3) were different from the trace yellow pigments detected in the lichen samples.

Table 3. Distribution of xanthones in the lichen mycobionts examined

Lichen species	Country	Sample site	Lichen substance	Mycobiont substances XT5	XT6	XT7	XTX
Pyrenula japonica	Japan	Osaka	–	+	+	+	+
P. japonica	Japan	Wakayama	–	–	+	+	
P. japonica	Japan	Akita	–	–	+	+	
P. pseudobufonia	USA	Florida	–	–	+	+	
P. japonica	Japan	Shiga	–	+	+	+	+
P. japonica	Japan	Chiba	–	+	+	+	
P. japonica	Japan	Chiba	–	+	+	–	
Buellia sp.	Japan	Okinawa	Norstictic (+) Atranorin (+)	+	+	–	
Pyrenula sp.	Japan	Okinawa	–	+	+	+	
Lecidella elaeochroma	Japan	Tottori	–	+	+	+	
Pyrenula sp.	Japan	Hachijo	–	+	+	+	

Structures of XT5, XT6, and XT7 are indicated in Fig. 3, and XTX is of unknown structure. No depsides or depsidones were found in any mycobionts.



XT5: $R^1 = R^3 = H$, $R^2 = OH$
XT6: $R^1 = R^3 = OH$, $R^2 = H$
XT7: $R^1 = OCH_3$, $R^2 = H$, $R^3 = OH$

Figure 3. Structure of three xanthones found in lichen mycobionts. These substances are respectively designated XT5, XT6 and XT7 within the text.

Hybocarpone

No crystals were detected on the surface of the agar with mycobionts which produced hybocarpone (Fig. 4), but the color of the agar turned wine-red in every sample. Hybocarpone, a dimeric naphthazarin, was detected in the agar, but the quantity was very small. Therefore, only the colonies were used for extraction of hybocarpone. Hybocarpone was common in the mycobionts of all

Table 4. Distribution of hybocarpone in the lichen mycobionts examined

Lichen species	Country	Sample site	Lichen substance	Mycobiont substances Hybocarpone	Other products
Lecanora nipponica	Japan	Akita	Atranorin (+)	+	
L. hybocarpa	USA	Louisiana	Atranorin (+)	+	+
L. cinereofusca	Japan	Fukushima	Atranorin (+)	+	
L. mikuraensis	Japan	Hachijo	Atranorin (+)	+	

No depsides or depsidones were found in mycobionts.

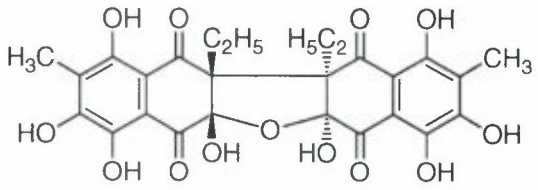


Figure 4. Structure of hybocarpone.

samples from Japan and North America as listed in Table 4. However, arthothelin and 6-O-methylarthothelin were detected as minor substances in the samples from Louisiana. The major substance, hybocarpone, was found without atranorin in the mycobiont of some species of *Lecanora*, although the corresponding lichens contained atranorin (Table 4). Hybocarpone was found in *L. hybocarpa* from Louisiana and in *L. nipponica*, *L. cinereofusca*, and *L. mikuraensis* from Japan.

4. Discussion

The main collection sites were Japan, with very few samples from other Asian countries, followed by North America, Europe and Oceania. More lichens with mycobionts which produce these four groups of substances would perhaps be found if further sampling were conducted in other Asian countries. These mycobiont substances often had worldwide distributions and were found in mycobionts derived from one or more genera. The production of these metabolites must therefore have some common significance for many lichen mycobionts.

In the mycobionts examined, the production of these substances was stable after transplantation of the mycobiont. Moreover, the substances examined were sometimes produced in large quantity, often accounting for more than 1% of the dry weight of mycobiont colonies. The metabolic pathway to these substances is therefore thought to be an inherent characteristic of the lichen mycobiont.

Under the mycobiont culture conditions used, the normal metabolic pathway observed in the lichen association seems to be inoperative, but the dormant fungal metabolism was induced in the isolated mycobiont. The dormant metabolic pathway is thought to have biological significance only for the mycobiont, and is conserved in the mycobionts but is not expressed under lichenized conditions.

Derivatives of these mycobiont substances were also found in free-living fungi, and may serve as toxins. For example, isocoumarins are known phytotoxic metabolites (Tanahashi et al., 2000). Similarly, dibenzofurans, e.g. hypostrepsilic, isostrepsilic and hypostrepsilalic acids, detected in lichen mycobionts, seem to be toxic to photobionts. Hybocarpone is also bioactive, and a potent cytotoxin (Ernst-Russel et al., 1999). Graphislactones possessed the same basic skeleton as the mycotoxin, alternariol, observed from free-living fungi (Tanahashi et al., 1997), although a small amount of alternariol was also isolated from lichens (Archer and Elix, 1998).

The substances examined in this study were secreted outside the mycobiont colonies, and the mycobionts examined were derived from crustose lichens, e.g. *Graphis*, *Lecanora*, and *Pyrenula*. Dibenzofurans however were accumulated inside the mycobiont colonies of the fruticose lichens *Evernia*, *Usnea*, and *Stereocaulon*.

In a previous paper, usnic acid was reported from inside mycelia of *Ramalina siliquosa*, and *R. pertusa* (Hamada, 1991), but usnic acid has also been found on media surrounding colonies of the mycobiont of *Lecanora pulverulenta*, as has 2-chloroemodin and derivatives in mycobiont colonies of *Caloplaca flavorubescens* (Hamada et al., 1996). Thus, mycobionts of fruticose lichens (with a more complex growth form than crustose lichen) seem to have a storage system for secondary metabolites inside colonies, although the shapes of the cultured colonies are similar for both crustose and fruticose lichens.

The pathway to the secondary metabolites, hybocarpone and dibenzofurans, in lichen mycobionts occurs beyond the boundary of the lichens, such as the lichen substance salazinic acid. Moreover, the relationship between the secondary substances produced by lichen mycobionts and the taxonomic characteristics of lichens appears complicated. For example, *G. handelii* and *G. scripta* are quite different in chemical characteristics (Table 2), whereas *G. scripta* and *G. cognata* are quite different in anatomical characteristics (Table 1).

We postulate that these mycobionts preserve a secondary metabolic pathway of free-living fungi from the pre-symbiosis age. Namely, isolated mycobionts may have the same origin as free-living fungi, e.g. *Alternaria*, but have developed different life styles and separated in the evolutionary process. Free-living fungi evolved to produce antibiotic compounds, while mycobionts evolved the symbiotic system with photobionts, preserving the metabolic pathway of ancient mycobiont. Studies on the relationship of the secondary metabolites of lichen mycobionts to those of free-living fungi may be effective in elucidating the origin of lichens.

The metabolic pathways leading to mycobiont substances could be expressed for the survival of the mycobiont in the pre-lichenized age, but suppressed in the lichenized condition because of their toxicity to photobionts. The photobiont may thus secrete substances which cause the metabolism of mycobiont to produce characteristic lichen substances, so that the production of lichen substances is stimulated by symbiosis (Culberson and Ahmadjian, 1980). In the case of *G. handelii* from Japan, this switching from graphenone to the depsidone norstictic acid is complete.

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