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Induction of Secondary Products in Isolated Mycobionts from North American Lichens

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Abstract

Isolated, North American lichen mycobionts cultured on 10% sucrose medium produced the same depsides and depsidones as those found in intact lichens, but at low, standard sucrose concentrations the production of these secondary products in the mycobionts was incomplete. Dibenzopyranones, which were the same as those previously reported in *G. scripta* var. *pulverulenta* from Japan, were found in mycobionts of North American *Graphis* sp.

Keywords: Lichen mycobionts, secondary products, depsides, depsidones, dibenzopyranones, *Graphis*

1. Introduction

Previously, we reported increased growth rate of cultured mycobiont colonies and increased production of secondary products in mycobionts when 10 or 20% sucrose was added to a conventional medium (Hamada, 1993; Hamada et al.,

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1996). The addition of sucrose to the medium promoted the production of acetyl-polymalonyl-derived substances, including those produced in many natural lichens as well as a few different ones that have not been reported from lichens. Such typical lichen substances as the depsidones, salazinic acid, norstictic acid and stictic acid, and the depsides, atranorin and gyrophoric acid were found (Hamada and Miyagawa, 1995; Hamada et al., 1996). Other secondary products not yet reported in lichens include the dibenzofuran, hypostrepsilic acid, in mycobionts of *Evernia esorediosa* and the pigment, graphenone, in *Graphis scripta* var. *serpentina*, (Miyagawa et al., 1993, 1994).

In addition to previous work conducted in Japan (Hamada et al., 1996; Tanahashi et al., 1997), further studies are needed in order to understand the full range of secondary metabolites isolated lichen mycobionts can produce and to determine the influence of osmotic conditions on the production of secondary metabolites in lichen mycobionts.

In this study, we examined the secondary metabolites in lichen mycobionts collected in North America and compared the chemistry of these mycobionts to those examined previously.

2. Materials and Methods

All samples were collected in the United States and Canada during 1994 and 1995 by N. Hamada (NH) and T.H. Nash III (TN). The specimens were identified by TN, B. Ryan, H.T. Lumbsch and M.A. Herrera, and are deposited at Osaka City Institute of Public Health and Environmental Sciences. Specimens used were *Lecanora bicincta* Ramond – San Francisco Peaks, AZ, elev. 3,500 m, on rock (TN36936), *Protoparmelia cupreobadia* (Nyl.) Poelt – San Francisco Peaks, AZ, elev. 3,500 m, on rock (TN36945), *Lecidea tessellata* Flörke – Sheep Spring, NM, elev. 2,700 m, on rock (NH9550304), *Rhizocarpon eupetraeum* (Nyl.) Arn. – Mt. Robson, BC (Canada), elev. 2,700 m, on rock (NH9480312), *Usnea mutabilis* Stirton – Ocala, FL, elev. 10 m, on bark (NH9592441), *Graphis* sp. – Fredericton, NB (Canada), elev. 50 m, on bark (NH9572620), *Graphis* sp. – Apalaticola, FL, elev. 10 m, on bark (NH9592553).

Spores were isolated by methods previously described (Hamada et al., 1996). For each species apothecia from air-dried thalli were attached to the lids of inverted petri dishes containing plain agar. Some spores (more than 50) per species were inoculated onto slants with two malt-yeast extract media: one with 4.0 g (conventially MY instead of MY0.4) and one with 100.0 g (MY10) of sucrose added to a basal medium of 10.0 g malt extract, 4.0 g yeast extract, 15.0 g agar, and one liter water. The final pH of these media was adjusted to pH 6.8 with KOH. To maintain constant water content the cultures were kept in the

dark in an incubator at 18±1°C. The mycelia of each species were transplanted to new medium at four months from the start of the experiment. After seven months the lichen mycobionts had produced chemical substances and formed compact mycelia.

These polyspore-derived mycobiont colonies (Table 1) were separated from the agar using a needle, dried in a desiccator for one day and subsequently weighed. Secondary products of each colony were extracted with acetone. The mean concentration of secondary products was expressed as a per cent (i.e. mg substance per 100 mg thallus). The quantity was determined from the relative height of the HPLC chromatographic peaks, using standard curves made with known amounts of authentic substances (Hamada, 1996). The concentration of lichen substances in each lichen specimen was not measured, because it was difficult to separate crustose lichen thalli from the substrate.

Identification of lichen substances: Secondary products from each extract were identified qualitatively by TLC (Culberson, 1972) and quantitatively by HPLC using comparisons with authentic samples (Tanahashi et al., 1997). Four dibenzopyranones in *G. scripta* mycobionts from Japan are designated S1, S2, S3, and S4 (Fig. 1). TLC (Kieselgel GF254) R_f values (×100) for atranorin/norstictic acid/confluentic acid/gyrophoric acid/usnic acid /S1/ S2/S3/S4 were :1) 92/60/69/43/89/70/36/32/28 in benzene-dioxane-acetic acid (180:45:5 v/v/v), 2) 75/27/29/33/65/25/8/4/4 in n-hexane-ethyl ether-formic acid (130:80:20), and 3) 76/28/44/23/65/33/6/7/3 in toluene-acetic acid (20:3). The color of each spot was observed after spraying with 10% H_2SO_4 and heating them at 100°C for 15 min. Analytical HPLC (Waters 600E Solvent Delivery System; Waters 990J PDA Detector) with a 4.6 × 250 mm TSK gel ODS column (Toyo Soda) and water-methanol-acetic acid (20:80:1) flowing at 0.5 ml/min (ca. 1700 psi; t0 = 3.6 min) gave retention times (min) of 22.0 for atranorin, 5.8 for norstictic acid, 12.1 for confluentic acid, 7.9 for gyrophoric acid, and 19.4 for usnic acid.

Table 1. Comparison of the yields (+ = <0.1%; ++ = 0.1 to 1.0%) of secondary products identified from mycobionts of North American lichens cultured on media with 0.4% sucrose (MY) or with 1.0% sucrose (MY10).

Species	Site	Secondary product in intact lichen	MY Yield	MY10 Yield
Lecanora bicincta	AZ	Atranorin	+	+
Protoparmelia cupreobadia	AZ	Norstictic acid	–	+
Lecidea tessellata	NM	Confluentic acid	+	+
Rhizocarpon eupetraeum	BC	Gyrophoric acid	+	++
Usnea mutabilis	FL	Usnic acid	–	+

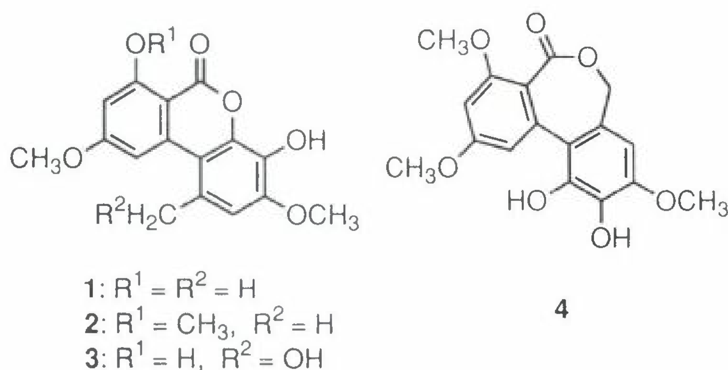

Figure 1. The structure of four dibenzopyranones found in *Graphis scripta* var. *pulverulenta* from Japan. The structures designated 1, 2, 3 and 4 in the figures are respectively designed S1, S2, S3 and S4 within the text.

3. Results

On MY10 the depsidone, norstictic acid, and the depsides, atranorin, confluentic acid and gyrophoric acid, were detected in concentrations greater than 0.01% in mycobionts from Arizona and other North American localities (Table 1). Similarly, usnic acid was detected in mycobionts of *Usnea mutabilis*. These are the same secondary products as those found in the corresponding intact lichens.

In contrast, on MY norstictic acid in mycobionts of *Protoparmelia cupreobadia* and usnic acid in the mycobiont of *Usnea mutabilis* were not detected (Table 1). Furthermore, in the mycobiont of *Rhizocarpon eupetraeum*, gyrophoric acid was found in lower concentration on MY than on MY10.

For mycobionts of *Graphis* sp. from New Brunswick and Florida, numerous white crystals were found on the surface of MY10 slants, but not on MY slants. These crystals were mixtures of dibenzopyranone derivatives (Fig. 1), and corresponded to those (S1 and S2 as major secondary products and S3 and S4 as minor secondary products) found in mycobionts of *G. scripta* var. *pulverulenta* from Japan. The dibenzopyranones designated S1 and S2 were found in both mycobionts from North American collections, but S3 was detected only in the mycobiont from the Florida collection. Lastly, S4 was not detected in either North American mycobiont. None of the dibenzopyranones were detected in the lichenized condition.

4. Discussion

In this study we found the same depsides and depsidones in isolated mycobionts from North American specimens as were previously detected in Japanese specimens (Hamada et al., 1996). Because the specimens were collected from a wide variety of habitats and geographic locations, we infer that most lichen mycobionts are able to produce the same secondary substances as occur in natural lichens regardless of their origin.

In our preliminary experiments, these mycobionts were also cultured on 20% sucrose medium. However, none of these mycobionts produced lichen substances. Thus, the production of secondary metabolites in mycobionts may be optimally induced on media containing 10% sucrose.

The identification of the two *Graphis* sp., whose mycobionts produce dibenzopyranones, is uncertain, although their morphological characters are very close to *Graphis scripta*. The two collection sites, Florida and New Brunswick, are separated by 18° of latitude along the east coast of North America, and the collection site of Tanahashi et al. (1997) in Japan is approximately on the opposite site of the earth. Thus, although dibenzopyranones have not yet been found in the lichenized condition, they appear to be commonly produced in mycobionts isolated from highly unrelated locations. Especially large amounts of dibenzopyranones were produced in the Florida mycobionts, although they were transplanted many times to new slants.

The biological significance in the production of dibenzopyranones is unknown. However, a dibenzopyranone was also isolated from *Alternaria* spp., and was considered a mycotoxin (Raistrick et al., 1953). Thus, they may serve as antibiotics.

In Japanese samples, all mycobionts (sample # NH931038, NH931043, and NH931045) of *G. scripta*, including var. *pulverulenta*, produced dibenzopyranones, as were found in our North American mycobionts. Thus, the production of these substances may be a significant taxonomic character at the species level. In contrast, the occurrence of large yellow crystals of the pigment graphenone, as previously reported in the mycobionts of *G. scripta* var. *serpentina* from Japan (Miyagawa et al., 1994), may only be taxonomically significant at the variety level. Because this same variety is also reported from North America (Fink, 1935), it would be interesting to determine if it also produces graphenone. Because *Graphis scripta* has a world-wide distribution, further collections of *G. scripta* should be done in order to evaluate varietal differences in secondary products of this species.

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