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Effects of Culture Conditions on Dibenzofuran Production by Cultured Mycobionts of Lichens

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Abstract

Two dibenzofurans, isostrepsilic acid and hypostrepsilic acid, were produced in large quantities by the mycobiont of *Usnea orientalis* cultured on a malt-yeast extract medium containing sugar alcohols. The natural lichen growing in the field produces usnic and salazinic acid but not the above dibenzofurans. Isostrepsilic acid is a new product which has shown to be 3,7-dihydroxy-9-hydroxymethyl-1-methydibenzofuran-2-carboxylic acid. It seems that the production of these compounds is probably due to the combined effects of the high osmotic pressure of the medium and the nutritional conditions. The production of the dibenzofurans was markedly repressed by actively photosynthesizing photobionts.

Keywords: Lichens, lichen mycobiont, dibenzofuran, hypostrepsilic acid, isostrepsilic acid

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1. Introduction

Lichens are well known producers of unique secondary metabolites, typically the depsides, depsidones and dibenzofurans. Apparently these compounds are produced by the mycobiont without the cooperation of the photobiont. Indeed, Culberson and Armaleo (1992) have succeeded in inducing a complete secondary-product pathway in a cultured lichen mycobiont under xeric conditions.

However, many secondary compounds which are not detected in a natural lichen, may be produced by the cultivated mycobiont without an algal partner (Hamada et al., 1987; Miyagawa et al., 1993, 1994).

When the mycobiont of *Usnea orientalis* Asah. was cultured on a malt-yeast extract medium containing sugar alcohols, we found the growth of the mycobiont to be accelerated and thalline like fibrils to be developed (Kon et al., 1993). Simultaneously, a large quantity of extracellular compounds was produced. These compounds were subsequently isolated and their structural elucidation is described in this paper. Furthermore, the concentration of these compounds was measured when the mycobiont was cultured on the medium under various conditions.

2. Material and Methods

Culture conditions

Thalli of *Usnea orientalis* Asah. were collected on Mt. Kuju, Ooita Prefecture in August, 1989. The mycobiont and photobiont were obtained by the following procedures. Growing tissue was obtained from thalli following the technique described by Yamamoto et al. (1985). The tissue was aseptically placed into a sterilized mortar and then homogenized with sterilized distilled water. The homogenate was cultured on a medium composed of 2% malt extract, 0.2% yeast extract and 2% agar. After one month, mycobiont and photobiont colonies were transferred to separate dishes. The mycobiont colonies and photobiont colonies obtained were used for the present study.

For the isolation of dibenzofurans, the mycobiont was inoculated in test tubes containing malt-yeast extract medium with 2% added mannitol and 2% sorbitol at pH 5.8. The mycobionts were cultured at 18°C in the dark for 10 weeks.

Growth was measured by dry weight determinations every 10 days. Sugars or sugar alcohols were added to the medium to attain a final concentration of 0.2 millimolar. pH and temperature were the same as mentioned above.

For comparison, the cultured mycobiont was mixed with different photobionts identified by Dr. Takeshita (personal communication). These

mixed tissue cultures were then cultured on the above medium under light (C. 13 $\mu mol/m^2/s$).

Usnea orientalis photobiont: *Trebouxia galapagensis.*
Usnea confusa subsp. *kitamiensis* photobiont: *Trebouxia aggregata.*
Usnea diffracta photobiont: *Trebouxia* sp.
Ramalina yasudae photobiont: *Trebouxia galapagensis.*
Cladonia vulcani photobiont: *Trebouxia glomelata.*
Caldonia conistea photobiont: *Trebouxia galapagensis.*
Rimelia clavulifera photobiont: *Trebouxia galapagensis.*
Physciella melanchra photobiont: *Trebouxia* sp.

Isolation of the dibenzofurans

The mycelium of the mycobiont was separated from the agar and dried at 50°C for 24 hours. The dried material (20 g) was then extracted overnight with 5 volumes of cold acetone. The supernatant thus obtained was concentrated to dryness under reduced pressure. The residue was dissolved in a small amount of solvent A (methanol:chloroform, 15:35, v/v) and subjected to column chromatography (Wakogel C-200, 2.0 × 20 cm) using the same solvent as eluant. The dibenzofurans obtained were characterized by standardized thin-layer chromatographic methods (Culberson and Elix, 1990).

The TLC and HPLC methods described by Kon et al. (1993) were used for identification and quantification of the compounds.

3. Results and Discussions

Structure of dibenzofurans

Under natural conditions, *U. orientalis* produces both usnic acid (1.32%, cortex) and salazinic acid (2.16%, medulla). As reported previously (Kon at al., 1993), thalli of *U. orientalis* can routinely be induced from their growing tissue *in vitro*. The secondary products present in regenerated fibrils were detected by TLC and HPLC methods, and as in nature, usnic acid and salazinic acid were shown to be produced.

When the mycobiont was cultured on a malt-yeast extract medium containing added sugar alcohols it produced a large quantity of extracellular dibenzofurans. These compounds were not detected in natural *U. orientalis* or in the mycobiont when it was cultured, with or without photobiont, on the normal malt-yeast extract medium.

On TLC plates the spots due to these compounds were pinkish gray in color after spraying with 10% sulfuric acid and heating at 110°C for 10 min. (Rf in

solvent B (hexane:methyl tert.butyl ether:formic acid = 140:72:18) : compound I = 0.41; compound II = 0.11).

The molecular formulae of the compounds I and II were established as $C_{15}H_{12}O_5$ and $C_{15}H_{12}O_6$ respectively, based on the corresponding high resolution mass spectral and/or microanalytical data. The respective chemical structures were deduced from the corresponding ^{1}H NMR and UV spectral data.

Compound I was shown to be 3,7-dihydroxy-1,9-dimethyldibenzofuran-2-carboxylic acid (Fig. 1A), a compound previously called hypostrepsilic acid when it was isolated from the cultured mycobiont of *Evernia esorediosa* (Müll. Arg.) Du Rietz under osmotically stressed conditions (Miyagawa et al., 1993), or norascomatic acid when it was isolated from the lichen *Bunodophoron patagonicum* (Dodge) Wedin (Elix et al., 1994). With both the *Usnea* and *Evernia* mycobionts, hypostrepsilic acid was produced when the mycobionts were cultured on a malt-yeast extract medium containing a sugar or sugar alcohol.

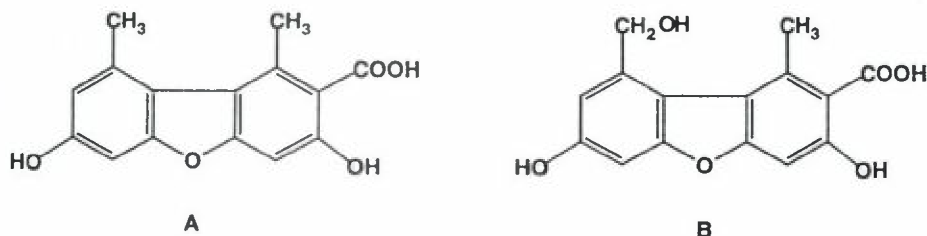

Figure 1. A. Chemical structure of hypostrepsilic acid: 3,7-dihydroxy-1-9-dimethyl-dibenzofuran-2-carboxylic acid. B. Chemical structure of isostrepsilic acid: 3,7-dihydroxy-9-hydroxymethyl-l-methyldibenzofuran-2-carboxylic acid.

The spectroscopic and microanalytical evidence established that isostrepsilic acid was the 9-hydroxymethyl derivative of compound I , i.e. 3,7-dihydroxy-9-hydroxymethyl-l-methyldibenzofuran-2-carboxylic acid (Fig. 1B). This compound has not previously been reported from lichens or their cultured myobionts.

Effects of culture conditions on the production of dibenzofurans

The growth rate of the mycobiont, after an initial lag phase of about 10 days, then accelerated on the medium. In particular, growth on the medium containing 2% added mannitol was faster than that on the medium without the mannitol (Fig. 2). Fig. 3 illustrates the concentration of hypostrepsilic and

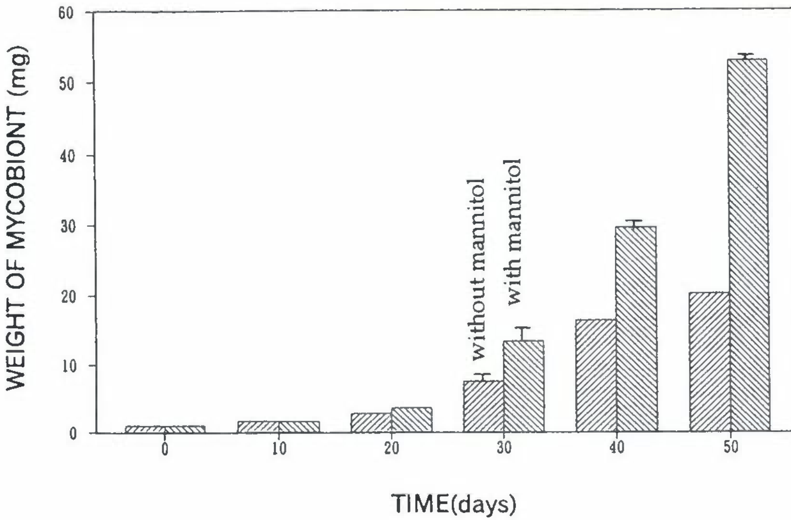

Figure 2. Growth rate of the cultured mycobiont of *U. orientalis* on a malt-yeast extract medium with and without mannitol. Left column (without mannitol) represents the mean ± S.D. of 3 determinations of mycelia. Right column (with mannitol) represents the mean ± S.D. of 3 determinations of mycelia.

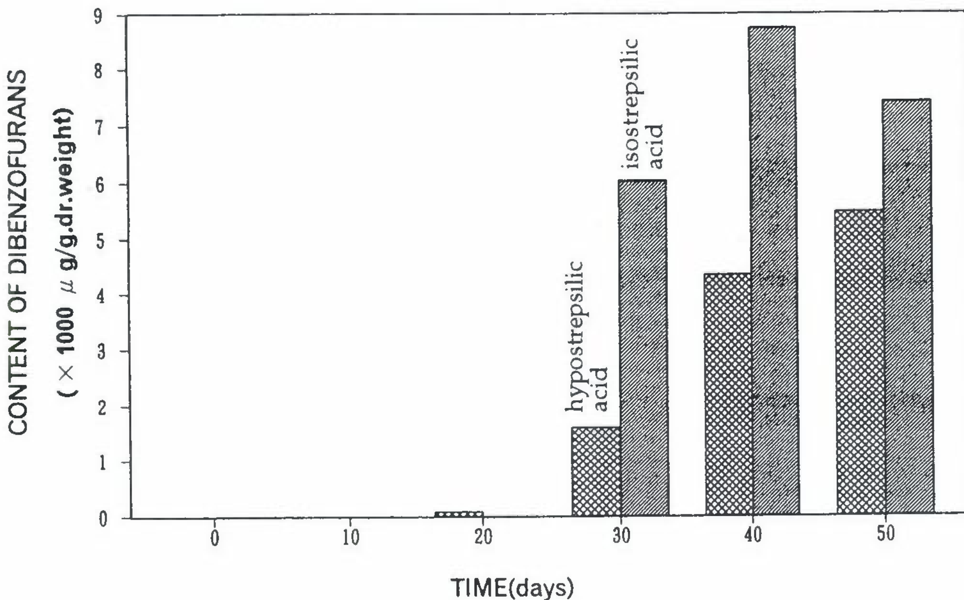

Figure 3. Dibenzofuran content of the isolated mycobiont of *U. orientalis* on a medium containing 2% mannitol. The column indicates the average content of dibenzofuran as determined. From five colonies.

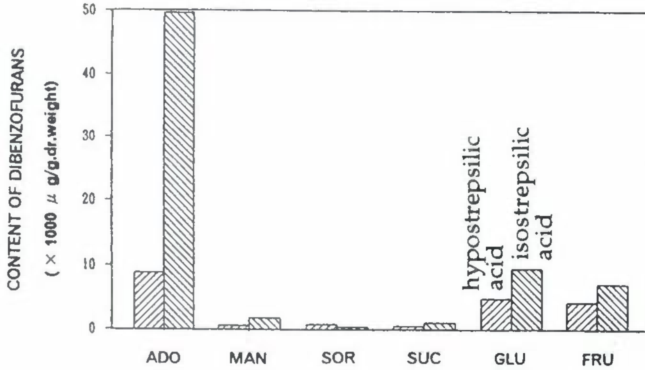

Figure 4. Effects of various sugar alcohols and sugars on the production of dibenzofurans. The polyols and sugars were added so that the final concentration was 0.2 millimolar. ADO: Adonitol, MAN: Mannitol, SOR: Sorbitol, SUC: Sucrose, GLU: Glucose, FRU: Fructose.

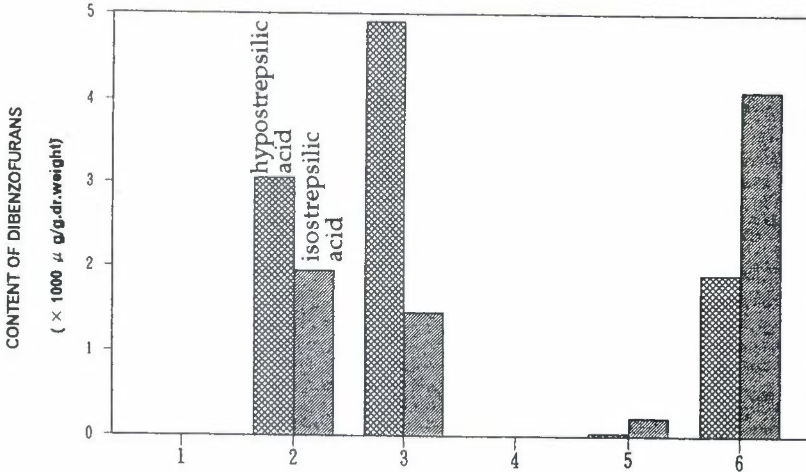

Figure 5. Effect of photobiont on the production of dibenzofurans. 1: Mycobiont was cultured on the medium without mannitol in the dark. 2: Mycobiont was cultured on the medium containing 2% mannitol in the dark. 3: Mycobiont was cultured on the medium containing 2% mannitol and 2% sorbitol in the dark. 4: Mycobiont and its natural photobiont were cultured on the medium containing 2% mannitol in light. 5: Mycobiont and its natural photobiont were cultured on the medium containing 2% mannitol and 2% sorbitol in light 6: Mycobiont and its natural photobiont were cultured on the medium containing 2% mannitol in the dark.

isostrepsilic acids measured when the mycobiont was cultured on a medium containing 2% mannitol. Ten days after inoculation, neither compound could be detected in the mycobiont. However, 30 days after inoculation, the concentration of both compounds rapidly increased parallel with the growth of the mycobiont. The relationship between the rate of growth and dibenzofurans production resembled a similar increase in the production of secondary products observed during a growth spurt of *Cladonia grayi* (Culberson et al., 1992).

The effects of various sugar alcohols and sugars on the production of dibenzofurans under identical osmotic conditions are shown in Fig. 4. The highest production of hypostrepsilic and isostrepsilic acids was observed on a medium containing added adonitol. The addition of mannitol, sorbitol and sucrose to the medium had less effect than the addition of adonitol.

In experiments on the mycobiont of *Evernia esorediosa,* Miyagawa (1993) reported that hypostrepsilic acid was not formed when the mycobiont was lichenized, but that it was produced when the isolated mycobiont was subjected to the osmotically stressed conditions. Similarly, graphenone and graphisquinone were produced by isolated mycobionts cultured on osmotic slants (Miyagawa 1994). Kinoshita et al. (1993) reported that usnic acid production was enhanced by increasing the agar concentration. These authors suggested that the increasing usnic acid content may be correlated with water availability rather than the nutritional conditions.

However, in our experiments the production of these dibenzofurans may be due to the combined effects of the high osmotic pressure of the medium as well as the nutritional conditions.

The effects of various photobionts on the production of the two dibenzofurans is shown in Fig. 5. Hypostrepsilic and isostrepsilic acids were detected when the mycobiont (alone) was cultured on a malt-yeast extract medium containing 2% mannitol. The hypostrepsilic acid content increased when the mycobiont was cultured on the same medium containing 2% mannitol and 2% sorbitol. However, when the mycobiont was cultured with the *U. orientalis* photobiont on a malt-yeast extract medium containing 2% mannitol, it did not produced any dibenzofurans. Further, when this mixed tissue was cultured on a malt-yeast extract medium containing 2% mannitol and 2% sorbitol, it did produce small quantities of hypostrepsilic acid. However, when such mixed tissue was cultured in the dark on a similar medium containing 2% mannitol, the dibenzofuran production was not repressed by the photobiont. These results indicate that the actively photosynthesizing photobionts have a repressive effect on dibenzofuran production by the mycobiont.

We also mixed the photobionts isolated from *Usnea confusa* subsp. *kitamiensis, Usnea diffracta, Ramalina yasudae, Cladonia vulcani, Cladonia conistea, Rimelia clavulifera* and *Physciella melanchra* with the *U. orientalis*

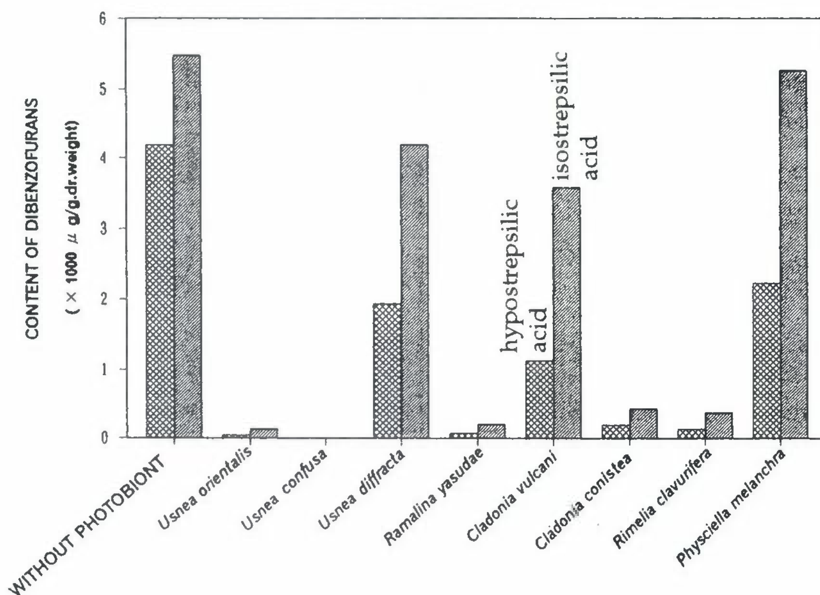

Figure 6. Effect of natural and non-natural photobionts on the production of dibenzofurans.

mycobiont. The repressive effects of the various photobionts on dibenzofurans production is shown in Fig. 6. In contrast, the mycobiont alone produced both dibenzofurans.

Trebouxia galapagensis isolated from *U. orientalis, Ramalina yasudae* and *Rimelia clavulifera* and *Trebouxia excentrica* isolated from *Cladonia conistea* all had repressive effects. However, other photobionts isolated from *Usnea diffracta, Cladonia vulcani* and *Physciella melanchra* did not completely repress the production of the dibenzofurans. Thus the production of dibenzofurans was not completely blocked when the mycobiont was cultured with an unnatural photobiont.

Hypostrepsilic acid

The crude hypostrepsilic acid crystallized from acetone-light petroleum to form cream colored microcrystals, m.p. 228–230° (227–229°), alone or admixed with a synthetic sample (Found: C, 66.0; H, 4.6; mol. wt. 272.0684. Calc. for $C_{15}H_{12}O_5$; C, 66.2; H, 4.4%; mol. wt. 272.0685). UV λ max (EtOH) nm (log ε): 309

(4.2), 257 (4.5), 241 (4.65). ^1H NMR (CD_3SOCD_3) δ 2.72 (3H, s, 9-Me); 3.03 (3H, s, 1-Me); 6.55, 6.75 (each 1H, d, J = 2.2 Hz, H-6, H-8); 6.60 (1H, s, H-4); 9.74 (1H, bs, OH). EIMS (70 eV) *m/z* 273 (5%), 272 (M, 25), 255 (18), 254 (100), 229 (7), 228 (60), 227 (23), 199 (5), 198 (22).

Isostrepsilic acid

The crude isostrepsilic acid crystallized from 10% aqueous acetone to form cream colored microcrystals, m.p. 210–220° dec. (Found: C, 62.2; H, 3.2%. $C_{15}H_{12}O_6$ requires C, 62.5; H, 3.2%). UV λ max (EtOH) nm (log ε): 309 (4.3), 259 (4.6), 241 (4.7). ^1H NMR (CD_3SOCD_3) δ 3.01 (3H, s, 1-Me), 4.88 (2H, d, J = 7 Hz, CH_2); 5.80 (1H, t, J = 7Hz, 9-OH); 6.60 (1H, 8, H-4); 6.77, 6.89 (each 1H, d, J = 2.2 Hz, H-6, H-8); 9.65 (1H, bs, OH). EIMS (70 eV) *m/z* 270 (12%, M-H_2O), 241 (22), 228 (23), 227 (26), 213 (62), 199 (35), 185 (100).

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