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NAPHTHAZARIN DERIVATIVES FROM CULTURES OF THE LICHEN *CLADONIA CRISTATELLA**

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Key Word Index—*Cladonia cristatella*; lichen; *Acarospora fuscata*; mycobiont liquid culture; naphthoquinone; naphthazarin; 3-ethyl-2-hydroxy-7-methoxynaphthazarin; 3-ethyl-2-hydroxy-7-methoxy-6-methylnaphthazarin; depside; barbatic acid.

Abstract—High pigment-producing cell aggregates of the cultured spore-derived mycobiont of a lichen, *Cladonia cristatella*, were isolated by clonal selection. The new naphthazarin derivatives, cristazarin (3-ethyl-2-hydroxy-7-methoxynaphthazarin) and 6-methylcristazarin, were identified as red pigments from the liquid culture. A depside, barbatic acid, was also detected in *C. cristatella* and *Acarospora fuscata* mycobiont liquid cultures. Copyright © 1996 Published by Elsevier Science Ltd

INTRODUCTION

Lichens produce many characteristic phenols, such as depsides, depsidones, dibenzofurans, pulvinates, chromones and quinones. Usually, anthraquinones have been reported in lichen mycobiont cultures without the algal partner, e.g. *Cladonia* [1, 2] (bellidiflorin, graciliformin and skyrin), *Caloplaca* [3] (2-chloroemodin, emodin, fragilin and parietin), and *Xanthoria* [3–5] (emodin, erythroglaucin, fallacinal, fallacinol and parietin); the same anthraquinones were usually found in their thalli. We found that cultured *Cladonia cristatella* mycobiont excreted red pigments into the liquid medium. We report herein the isolation and identification of these pigments.

RESULTS AND DISCUSSION

Cell aggregates of spore-derived *C. cristatella* mycobiont (clone no. 72) produced red pigments on an agar plate of malt–yeast extract (MY) medium; however, they were heterogeneous in colour. We selected mycobiont cell aggregates that produced high yields of red pigments by small cell-aggregate cloning [6]. Usually, cultured cells of higher plants are heterogeneous in the production of secondary metabolites, and clonal selection is useful for isolating high-producing cell strains [6]. We confirmed that lichen mycobiont cells behaved similarly to cultured plant cells in this respect and, consequently, that it was very important to select a high metabolite-producing strain among lichen mycobiont cells for the purpose of production of lichen substances.

Selected cell aggregates that produced large amounts of red pigments were transferred and subcultured in MY liquid medium. The culture broth was extracted with ethyl acetate, and this extract yielded two red pigments and a trace of barbatic acid, detected by HPLC analysis. The extract was chromatographed twice to afford two red pigments, cristazarin (**1**) (0.02% w/v) and 6-methylcristazarin (**2**).

To obtain ^{13}C-labelled **1** and **2**, sodium [1-^{13}C]acetate was added to a liquid culture of *C. cristatella* mycobiont in modified Lilly–Barnett (LB) medium. After three weeks incubation, the culture medium was treated with ethyl acetate, and the freeze-dried mycelium was submerged in acetone. Ethyl acetate and acetone solutions were combined and subjected to preparative HPLC to obtain labelled **1** and **2**.

The molecular formula of **1** was established as $C_{13}H_{12}O_6$ based on its HR EI mass spectrum ([M]$^+$, *m/z* 264.0634). The ^{1}H and ^{13}C NMR signals were assigned with the aid of DEPT and ^{13}C–^{1}H COSY spectra and by comparison with the ^{13}C NMR of ^{13}C-labelled **1**. Spectral data suggested that **1** was an ethylmonomethoxytrihydroxynaphthoquinone. ^{13}C-Enriched carbons of **1** were at the 2 (δ 154.8), 4 (180.3), 5 (164.5), 7 (156.8), 9 (110.3) and 11 positions (15.7). NOE was observed between the methoxyl protons

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(δ 3.90) and 6-H (6.71), and ^1H–^{13}C long-range coupling was observed between the methoxyl proton (δ 3.90) and C-7 (156.8); the methoxyl group was attached to C-7. ^1H–^{13}C long-range coupling was observed between the methyl proton (δ 1.04) and C-3 (126.6) or C-11 (15.7); the ethyl group was attached to C-3. ^1H–^{13}C long-range coupling was observed between 6-H (δ 6.71) and C-10 (103.3), C-5 (164.5), C-6 (108.3), C-7 (156.8) or C-8 (156.3). Hence, **1** was identified as 3-ethyl-2-hydroxy-7-methoxynaphthazarin.

The ^1H and ^{13}C NMR spectra of **2** indicated the existence of a methyl group instead of 6-H. ^1H–^{13}C long-range coupling was observed between the aromatic methyl proton (δ 2.10) and C-5 (159.7), C-6 (130.9) or C-7 (153.0). Its UV and IR spectra were almost identical to those of **1**. These data led to the structure of 3-ethyl-2-hydroxy-7-methoxy-6-methylnaphthazarin for **2**.

Naphthazarin derivatives have been isolated from the marine invertebrate animal, *Echinoderma* (holothurians, asteroids and ophiuroides) [7] and a plant, *Lomandura hastilis* [8], as major red pigments. 7-*O*-Demethylcristazarin and 2, 7-dihydroxy-3-ethylnaphthazarin were reported in *Echinothrix diadema* [9]. Squamarone, 2,6,8-trihydroxy-3-methyl-7-ethyl-1,4-naphthoquinone, biosynthetically similar to 6-methylcristazarin, has been isolated from a lichen of the Lecanoraceae, *Squamarina cartilaginea*, as an orange pigment (0.0019%) [10]. The difference in the substitution pattern of both naphthoquinones is very interesting and it would be important to elucidate the biosynthesis of the cristazarins.

Cladonia cristatella growing in natural habitats produces usnic, didymic, rhodocladonic and barbatic acids [11]. Castle and Kubsch [2] reported usnic and didymic acids from its mycobiont culture; however, we could not detect them in our strain (no. 72) of *C. cristatella* mycobiont culture.

Cristazarins were found only in strain no. 72, but not in our other spore- and thallus-derived mycobiont cultures under the same conditions. Therefore, this strain is a mutant producing high amounts of cristazarins.

There are several reports on the detection of depsides (atranorin [12], barbatic acid [5], lecanoric acid [13], 4-*O*-demethylbarbatic acid [14, 15], 4-*O*-demethylsphaerophorin [16] and squamatic acid [5, 17]) in solid cultures of lichen mycobionts, but in liquid culture, Leuckert *et al.* [18] reported the production of a depsidone, dechloropannarin, in a lichen mycobiont, *Lecanora dispersa*. In liquid cultures of two lichen mycobionts of *C. cristatella* and *Acarospora fuscata*, we first found a depside, barbatic acid (3) that was detected in the thallus and in the first stage of resynthesis of *C. cristatella* [19]; therefore, some lichen mycobionts also have the capacity for depside production in liquid cultures.

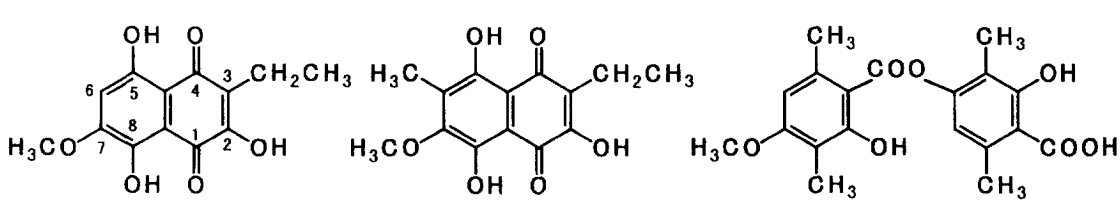
In another paper [20], we reported factors affecting naphthoquinone production in liquid cultures of a *C. cristatella* mycobiont.

EXPERIMENTAL

^1H and ^{13}C NMR spectra were recorded on a JEOL GSX-400 spectrometer in DMSO d_6 with TMS as int. standard. Analyt. HPLC was carried out with a photodiode array detector at 28° under the following conditions: column, 150 × 4.6 mm, packed with Shiseido Capsel Pack C_{18} AG-120; solvent system, MeOH–H_2O–H_3PO_4 (75:25:0.9) at 1 ml min^{-1}. Prep. HPLC was carried out at 25° under the following conditions: column, 150 × 19 mm, packed with μBondasphere 5 μm C_{18}-100 Å; solvent system, a linear gradient of MeOH containing 0.1% HCO_2H–0.1% HCO_2H in H_2O (7:3–100:0) over 30 min at 10 ml min-1; detection, 254 nm.

Material and clonal selection. Strain no. 72, which originated from a single ascospore isolated from a group of spores discharged by an apothecium of *C. cristatella* Tuck. thallus collected on June 4, 1980, in Smoky Mountain National Park, NC, U.S.A., and stored in the herbarium of Clark University, was cultured on an agar plate of MY medium composed of 2% (w/v) Difco malt extract, 0.2% (w/v) Difco yeast extract and 0.2% (w/v) agar. Cultured mycobiont sepd from tissue culture (strain no. ACFU-01) induced from the *A. fuscata* (Nyl.) Arnold thallus (f288), which was collected in Kuusamo, Koillismaa, Finland, and stored in the herbarium of Nippon Paint Co. was also cultured on an agar plate of MY medium.

The colour of cell aggregates of *C. cristatella* and *A. fuscata* mycobiont strains was mottled red and white. Cell aggregates of each strain growing on agar plates were divided into small segments and placed on MY agar medium in a Petri dish 9 cm in diameter. Segments were then cultured at 15° in the dark for 4 weeks. The reddest cell aggregates were selected and divided into



1 2 3

small segments. These were transplanted on to fr. medium and cultured under the conditions described above. The clonal selection procedure was repeated $\times 6$.

Subculture method. High pigment-producing cell aggregates of *C. cristatella* mycobiont were transferred into 100 ml liquid MY medium in a 300-ml Erlenmeyer flask and subcultured on a rotatory shaker at 130 rev min^{-1} at 15° in the dark for 3 weeks. High pigment-production cell aggregates of the *A. fuscata* mycobiont were subcultured in MY liquid medium under the same conditions.

Isolation and identification of metabolites. The culture broth (6 l) of *C. cristatella* mycobiont after 3 weeks of culture was passed through a 150 μm nylon mesh to remove mycelium. EtOAc (12 l) was added to the red filtrate, and the red pigments were transferred to the organic layer. The solvent was evapd to yield the red extract (2.6 g), which was analysed by HPLC. Compound **1** had a R_t of 3.12 min, and **2** of 9.12 min. Compound **3** was detected at R_t 19.14 min; its R_t and UV spectrum were identical to those of the authentic compound [21]. The extract was dissolved and recrystallized from Me_2CO to afford **1** (1763 mg) as red needles, mp 154–157° (uncorr.). The mother liquor was evapd to dryness and subjected to CC (Kieselgel 60) with a stepwise gradient from hexane to EtOAc to obtain 3 frs. Fr. 2 was evapd to dryness and recrystallized from MeOH to afford **2** (61.6 mg) as deep red needles, mp 148–151° (uncorr.).

Cristazarin (**1**): EI MS *m/z* (rel. int.) : 264.0634 $[M]^+$, calc. for $C_{13}H_{12}O_6$; 264.0634 (100), 249 $[M-Me]^+$ (33), 221 (17). UV λ_{max}^{MeOH} nm (log ε): 316 (3.86), 506 (3.83). IR ν_{max}^{KBr} cm^{-1}: 3270, 2970, 1600, 1480, 1400. ^1H NMR (400 MHz, DMSO-d_6): δ 1.04 (3H, *t*, $J = 7.3$ Hz, -Me), 2.49 (2H, *q*, $J = 7.3$ Hz, $-CH_2-$), 3.90 (3H, *s*, -OMe), 6.71 (1H, *s*, 6-H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 12.5 (-Me), 15.7 ($-CH_2-$), 56.7 (-OMe), 103.3 (C-10), 108.3 (C-6), 110.3 (C-9), 126.6 (C-3), 154.8 (C-2), 156.3 (C-8), 156.8 (C-7), 164.5 (C-5), 175.5 (C-1), 180.3 (C-4).

6-Methylcristazarin (**2**). UV λ_{max}^{MeOH} nm (log ε): 318 (3.87), 498 (3.77). IR ν_{max}^{KBr} cm^{-1}: 3300, 2950, 1600, 1460, 1400, 1320. ^1H NMR (400 MHz, DMSO-d_6): δ 1.03 (3H, *t*, $J = 7.3$ Hz, -Me), 2.10 (3H, *s*, Ar-Me), 2.47 (2H, *q*, $J = 7.3$ Hz, -CH2-), 3.92 (3H, *s*, -OMe). ^{13}C NMR (100 MHz, DMSO-d_6): δ 8.9 (-Me), 12.3 (-Me), 15.6 ($-CH_2-$), 60.4 (-OMe), 105.6 (C-10), 109.4 (C-9), 125.9 (C-3), 130.9 (C-6), 153.0 (C-7), 155.0 (C-8), 155.3 (C-2), 159.7 (C-5), 178.7 (C-1), 184.1 (C-4).

Incorporation of [1-^{13}C]*acetate.* Na [1-^{13}C]OAc was purchased from Cambridge Isotope Laboratories. A 10% soln (1 ml) of the precursor was added aseptically to the liquid culture of *C. cristatella* mycobiont (initial fr. wt 2 g) in 100 ml modified LB medium [22] on days 0, 1, 2 and 3. Modified LB medium consisted of 4% (w/v) sucrose, 0.2% (w/v) -L-asparagine, 0.1% (w/v) KH_2PO_4, 0.05% (w/v) $MgSO_4.7H_2O$, pH 6. After 3 weeks incubation in the dark at 15° on a rotatory shaker (130 rev min^{-1}), the culture broth was filtered through nylon mesh (150 μm), and the medium extracted $\times 3$ with EtOAc (200 ml); the mycelium was freeze-dried and submerged in Me_2CO (20 ml) at 4° overnight. EtOAc and Me_2CO solns were combined and evapd to yield a reddish residue. This residue was subjected to prep. HPLC. The red eluates at R_t *ca* 10 min and at *ca* 15 min were evapd to dryness and recrystallized from MeOH to afford labelled **1** and **2**, respectively.

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