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Biosynthesis of Panaefluoroline B from the Cultured Mycobiont of *Amygdalaria panaeola*

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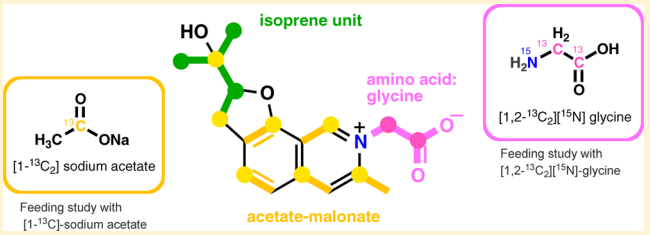
Supporting Information

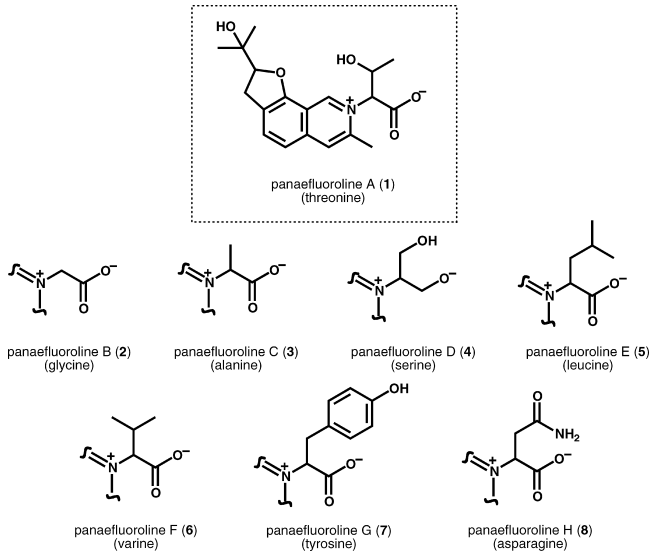
ABSTRACT: Panaefluoroline B (**2**) is a fluorescent yellowish-green pigment produced by the cultured mycobiont of a lichen, *Amygdalaria panaeola*. Panaefluoroline B (**2**) has an isoquinoline skeleton, a C_5 unit, and an amino acid, glycine, in its structure. The biosynthetic pathway of **2** was revealed by feeding experiments using [1-^{13}C]-sodium acetate and [1,2-$^{13}C_2$][^{15}N]-glycine. The analysis of labeling patterns of **2** and its mass spectrum suggested the isoquinoline part is biosynthesized via the acetate−malonate pathway with glycine as the nitrogen source and that the C_5 unit originates from the mevalonate pathway.



Lichens are symbiotic associations of an algal or cyanobacterial photobiont and a fungal mycobiont. They produce many characteristic phenolic compounds, such as depsides, depsidones, and dibenzofurans, which are considered to be biosynthesized by the fungal partners of the association.[1] On the other hand, cultured mycobionts of lichens produce independent substances that were not found from natural lichens.[2−5] Our group has reported previously the structures of various secondary metabolites from cultured lichen mycobionts.[6−8] Fluorescent-colored compounds, named panaefluorolines A−H (**1**−**8**), were isolated from a culture filtrate of the mycobiont of the lichen *Amygdalaria panaeola* (Ach.) Hertel & Brodo (Lecideaceae).[9,10] *A. panaeola* was collected in Finland in 1990, and thallus fragment spores of the lichen were isolated to culture the mycobiont on malt−yeast medium. The biosynthetic pathway of panaefluorolines is of interest because of their structures, consisting of an isoquinoline ring, an amino acid part, and a C_5 unit.

Although tyrosine is normally involved in the formation as the C_6−C_2−N unit in the structure of an isoquinoline ring, by examining the structures of panaefluorolines A−H (**1**−**8**) it was considered that several amino acids might be used as a nitrogen source for its N-linked side chain, while the remaining carbon source of the isoquinoline ring might originate by another biosynthetic route. Metabolic pathways can be studied in lichen mycobionts by in vitro experiments using stable-isotope-labeled precursors followed by NMR-based analysis of labeling patterns. This report describes labeling experiments with [1-^{13}C]-sodium acetate and [1,2-$^{13}C_2$][^{15}N]-glycine to analyze the biosynthetic pathway of panaefluoroline B (**2**).

First examined was which amino acid is incorporated into the biosynthesis of panaefluorolines A−H (**1**−**8**). As an initial cold



experiment, the amino acids alanine, glycine, tyrosine, and leucine were added individually to the culture medium of the lichen *A. panaeola*. As a result, the quantity of panaefluoroline B (**2**) produced was the highest on supplementation with glycine. Since glycine is considered difficult to metabolize in the organism, panaefluoroline B (**2**) may have been mostly produced from the supplemental glycine. Then, [1-^{13}C]-sodium acetate and [1,2-$^{13}C_2$][^{15}N]-glycine were used in labeling experiments to analyze the biosynthetic pathway of compound

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2. ^{13}C-Labeled or ^{13}C- and ^{15}N-labeled panaefluoroline B (**2**) were produced and isolated from cultures grown with added [1,2-$^{13}C_2$][^{15}N]-glycine or [1-^{13}C]-sodium acetate, respectively. The ^{13}C NMR, ^{15}N NMR, and ^{1}H−^{15}N HMBC spectra of ^{13}C- and ^{15}N-labeled panaefluoroline B (**2**) isolated from culture medium containing [1,2-$^{13}C_2$][^{15}N]-glycine by adding culture medium were acquired. The molecular formula, $C_{15}{}^{13}C_2H_{20}{}^{15}NO_4$, was determined from the positive HRFABMS data, *m*/*z* 305.1443, $[M + H]^+$ (calcd for $C_{15}{}^{13}C_2H_{20}{}^{15}NO_4$, 305.1430, $[M + H]^+$). In this way, U−^{13}C- and ^{15}N-labeled panaefluoroline B (**2**) were identified by MS. In the ^{15}N NMR spectrum, a signal at δ 203 was obtained. The ^{13}C NMR spectrum of U−^{13}C- and ^{15}N-labeled panaefluoroline B (**2**) showed strong peaks for the carbonyl carbon (C-1′) at δ 170.4 (d, J = 50.6 Hz, coupled with ^{13}C-2′) and the methylene carbon (C-2′) at δ 62.2 (dd, J = 50.6, 8.3 Hz, coupled with ^{13}C-2′ and ^{15}N). The ^{1}H−^{15}N-HMBC spectrum showed correlations of H-6 (CH), H-9 (CH), H-10 (CH_3), and H-2′ (CH_2) with ^{15}N (Figure 1).

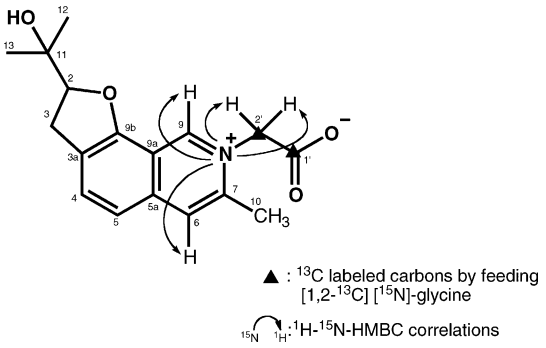

Figure 1. ^{1}H−^{15}N HMBC correlations and ^{13}C-labeled carbons by feeding [1,2-$^{13}C_2$][^{15}N]-glycine.

These results revealed that glycine was involved in the biosynthesis of panaefluoroline B (**2**). Subsequently, in a [1-^{13}C]-sodium acetate feeding experiment, the C-4, C-5a, C-7, C-9, and C-9b carbons were enriched in the ^{13}C NMR data (Figure 2), which helped elucidate that the carbon skeleton of

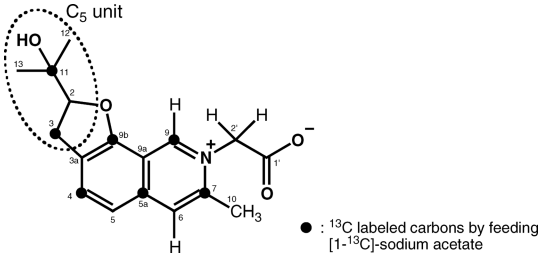

Figure 2. ^{13}C-Labeled carbons by feeding [1-^{13}C]-sodium acetate.

the isoquinoline part of panaefluoroline B (**2**) is biosynthesized by the acetate−malonate pathway. Moreover, it was considered that the remaining C_5 unit (Figure 2) was biosynthesized by the mevalonate pathway or methylerythritol phosphate (MEP) pathway. From the [1-^{13}C]-sodium acetate feeding experiment, C-3 and C-11 were also enriched in ^{13}C NMR data for ^{13}C-labeled panaefluoroline B (**2**) (Figure 2). Thus, it was revealed that the C_5 unit is biosynthesized by the mevalonate pathway. It was concluded that the amino acid glycine was incorporated as a nitrogen, its linked side chain and remaining carbons of the isoquinoline moiety were formed via the acetate−malonate route, and the C_5 unit originated from the mevalonate pathway in the biosynthesis of panaefluoroloine B (**2**). Therefore, an interesting and uncommon biosynthetic pathway for the isoquinoline skeleton was elucidated for this compound.

EXPERIMENTAL SECTION

General Experimental Procedures. The ^{1}H and ^{13}C NMR spectra were recorded with a JEOL JNM-AL-400 (^{1}H 400 MHz and ^{13}C 100 MHz) and a JEOL JNM-LA500 (^{1}H 500 MHz and ^{13}C 125 MHz) spectrometer, using CD_3OD with TMS as the internal standard or D_2O solution with DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) as the internal standard. The ^{15}N NMR spectrum was recorded with a JEOL JNM-LA500 spectrometer, and an $HCONH_2$ signal was used as an external standard at δ 265. HRFABMS and LRFABMS were obtained using a JEOL JMS-700 mass spectrometer. Column chromatography was carried out on silica gel (Kanto Chemical silica gel 60N 63-210 μm). HPLC was performed with a JASCO PU 980 unit and a JASCO UV 970 (Gulliver) detector.

Lichen Material and Culturing. *Amygdalaria panaeola* thalli (collection no. f-181) were collected in July 1990 in Prov. Koillismaa, Kuusamo, Finland, by Dr. I. Yoshimura (Hattori Botanical Laboratory, Japan) with Professor T. Ahti (Helsinki University, Finland). One week after being collected, a piece was cut from the thallus. Following the method of Yamamoto et al.,[11] thallus fragments were homogenized in a mortar and pestle with sterilized water, and small segments 150 to 500 μm in size were selected using a two-filter system. Each segment was inoculated onto an agar plate containing 5 mL of malt−yeast extract (MY) medium[12] and cultured at 15 °C in the dark. After 6 months, the mycelia derived from the small segment had grown to a colony that excreted fluorescent yellowish-green material into the medium. Individual agar blocks bearing a mycobiont colony were cut out and transferred to fresh MY medium (5 mL) in a 60 mm diameter Petri dish. The mycobiont (strain no. 0049M) was grown in the dark at 18 °C and subcultured every four months in the culture collection of Akita Prefectural University. About 20 subcultured micobiont colonies (cut to 3 mm to 4 mm square) were cultured on each agar plate [MY medium (5 mL) in a 60 mm diameter Petri dish] in the dark at 18 °C for three months. After incubation, the agar medium was a fluorescent yellowish-green.

A voucher of the collected lichen (*Amygdalaria panaeola*) has been deposited at Hattori Botanical Laboratory, Japan (collection no. f-181).

Feeding and Isolation of Labeled Panaefluoroline B (2). Alanine, glycine, tyrosine, and leucine were used for the first cold feeding experiment. Each amino acid (3 mg) was added to 6 mL of agar medium in a Petri dish. The mycobiont was cultured on agar medium supplemented with each amino acid at 18 °C in the dark. After incubation for one month, the agar medium was fluorescent yellowish-green. Then, the agar in each dish was collected and freeze-dried. The weights of the freeze-dried agar medium were 318.0 mg (with alanine added), 115.7 mg (glycine), 517.0 mg (tyrosine), and 159.6 mg (leucine). Each dried agar medium was extracted with MeOH (500 mL) three times for 24 h at 4 °C, and the weight of MeOH extract was 48.9 mg (alanine), 42.5 mg (glycine), 226.8 mg (tyrosine), and 33.8 mg (leucine), respectively. Then, each MeOH extract was applied to a column containing HP-20, eluted with H_2O to remove sugars, and eluted with MeOH to give the panaefluorolines. The weights of the MeOH-eluted fractions were 14.8 mg (alanine), 13.5 mg (glycine), 21.1 mg (tyrosine), and 5.4 mg (leucine). The fluorescent compounds in each MeOH-eluted fraction were examined by TLC ($CHCl_3$−MeOH−H_2O, 6:4:1; detected by spraying with 50% H_2SO_4 in MeOH heated at 120 °C for 5 min). Panaefluoroline B (**2**) was obtained as the main component when glycine was added as the amino acid source in the medium for cultivation of the mycobiont. In the other cases, where alanine, tyrosine, or leucine was supplemented to the agar medium, many other panaefluorolines were detected by TLC. It was thus decided to use [1,2-$^{13}C_2$][^{15}N]-labeled glycine for feeding experiments. [1,2-$^{13}C_2$][^{15}N]-Labeled glycine (30 mg) was

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added to malt extract (1.2 g), yeast extract (0.12 g), and agar powder (0.9 g) in 60 mL of purified water and sterilized for 10 Petri dishes {3 mg of [1,2-$^{13}C_2$][^{15}N]-labeled glycine was supplemented in 6 mL of MY agar medium in a Petri dish}. The mycobiont of *A. panaeola* was cultured in agar medium supplemented with [1,2-$^{13}C_2$][^{15}N]-labeled glycine at 18 °C in the dark. After incubation for three months, the agar medium was fluorescent yellowish-green. The agar medium from 10 dishes was collected and lyophilized. The dried powder obtained from the medium (2.28 g) was extracted three times with MeOH (500 mL) for 24 h at 4 °C.

The MeOH extract (1.10 g) was applied to a Diaion HP20 column, the sugars were washed out with H_2O (3 L), and the fluorescent compounds were eluted with MeOH (500 mL). The MeOH eluate (83.6 mg) was chromatographed on a Kanto Chemical silica gel 60N 63-210 μm column with a gradient solvent system of $CHCl_3$–MeOH–H_2O [30:10:0.5 (1350 mL), 20:10:1 (180 mL), 6:4:1 (240 mL), and MeOH (100 mL)] to give Fr. a (18.8 mg), Fr. b (4.5 mg, [U–$^{13}C_2$][^{15}N]-glycine-labeled panaefluoroline B), and Fr. c (22.9 mg). The ^{13}C NMR spectra, ^{15}N NMR spectrum, and ^{1}H–^{15}N-HMBC of [U–$^{13}C_2$][^{15}N]-glycine-labeled panaefluoroline B (**2**) were measured.

Panaefluoroline B (2): yellowish-green, amorphous solid; HRFABMS (positive) *m/z* 302.1376 $[M + H]^+$ (calcd for $C_{17}H_{20}NO_4$, 302.1392, $[M + H]^+$).

[1,2-$^{13}C_2$][^{15}N]-Labeled Panaefluoroline B: yellowish-green, amorphous solid; HRFABMS (positive) *m/z* 305.1443 $[M + H]^+$ (calcd for $C_{15}{}^{13}C_2H_{20}{}^{15}NO_4$, 305.1430, $[M + H]^+$); FABMS (positive) *m/z* 305 ($[M + H]^+$), 185, 93, 75, 57.

■ ASSOCIATED CONTENT

Supporting Information

TLC of the fluorescent compounds in the MeOH-eluted fraction on the first cold feeding experiment, the ^{13}C NMR spectrum of unlabeled panaefluoroline B (**2**) and [1,2-$^{13}C_2$]-[^{15}N]-glycine-labeled panaefluoroline B (**2**), the ^{1}H–^{15}N HMBC spectrum of [1,2-$^{13}C_2$][^{15}N]-glycine-labeled panaefluoroline B, and the ^{13}C NMR spectrum of unlabeled panaefluoroline B (**2**) and [1-^{13}C][^{15}N]-sodium acetate-labeled panaefluoroline B. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.5b00055.

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Notes

The authors declare no competing financial interest.

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