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Biosynthetic origin of alkylated decaline-type polyketides and induction of a new metabolite by addition of benzoate in cultured mycobionts of *Pyrenula* sp.

Yukiko Takenaka, Takao Tanahashi*

Kobe Pharmaceutical University, 4-19-1, Motoyamakita-machi, Higashinada-ku, Kobe 658-8558, Japan

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ABSTRACT

The assembly pattern of acetate units and methyl groups in the biosynthesis of a polyketide, pyrenulic acid C (**1**), in cultured mycobionts of a Vietnamese *Pyrenula* sp. was verified by administration of sodium [1-^{13}C]-acetate, sodium [1,2-$^{13}C_2$]-acetate, and L-[methyl-^{13}C]-methionine. The cultured mycobionts were induced to produce a new polyketide, pyrenulic acid I (**2**), by addition of sodium benzoate to malt-yeast extract medium supplemented with 5% sucrose. Its structure was determined by spectroscopic methods.

1. Introduction

Cultures of spore-derived lichen mycobionts were shown to be capable of producing certain lichen substances or novel metabolites in osmotically stressed conditions (Hamada and Ueno, 1990; Miyagawa et al., 1994; Tanahashi et al., 1997; Takenaka et al., 2004, 2010, 2011). In our previous study, pyrenulic acids A–H were isolated from cultured mycobionts of a Vietnamese lichen *Pyrenula* sp. (Le et al., 2014). These alkylated decaline-type polyketides are unusual as lichen substances and have never been detected in lichen thalli. However, these isolated metabolites are structurally related to cladobotric acids from the fermentation broth of a *Cladobotryum* fungus (Mitova et al., 2006). The DNA sequence of the ITS region of the mycobionts indicated 62% sequence similarity with *Cladobotryum* spp., impling that pyrenulic acids A–H could be biosynthesized in a similar manner to cladobotric acids (Le et al., 2014). To support this assumption, feeding experiments with ^{13}C-labelled acetates and methionine were undertaken. During examination of the effect of additives, we detected an uncharacterized metabolite in the cultivated mycobionts on MY5 medium containing sodium benzoate, prompting us to further investigate this metabolite. In this paper, we report the results of feeding experiments with pyrenulic acid C and structural determination of the new metabolite.

2. Results and discussion

2.1. Biosynthetic origin of polyketide 1

The mycobionts of Vietnamese *Pyrenula* sp. cultured on conventional malt-yeast extract medium supplemented with 10% sucrose (MY10) produced pyrenulic acids A-H (Le et al., 2014). Pyrenulic acids are not typical lichen substances but are structurally related to fungal cladobotric acids, which were shown to be biosynthesized from a polyketide chain and *S*-adenosylmethionine (SAM) (Mitova et al., 2006). From our interest in the biosynthetic origin of pyrenulic acids such as pyrenulic acid C (**1**) (Fig. 1), administration experiments with ^{13}C-labeled acetates and methionine were performed. For feeding experiments, the culture conditions were re-examined. The cultured mycobionts were found to produce higher amounts of the metabolites in MY medium supplemented with 5% sucrose (MY5) rather than in MY10, although the growth rate was lower. Thus, feeding experiments with cultured mycobionts were performed on MY5 medium. Feeding with [1-^{13}C]-acetate and [1,2-$^{13}C_2$]-acetate resulted in moderate incorporation into pyrenulic acid C (**1**). The ^{13}C NMR spectrum showed that the C-1 carbon of acetate was incorporated into the carbons at C-1, 3, 5, 7, 9, 11, 13, 15, 17, 19, and 21 in pyrenulic acid C (**1**). The 2D INADEQUATE (incredible natural abundance double quantum transfer experiment) spectrum of pyrenulic acid C isolated from the cultured mycobionts incubated with [1,2-$^{13}C_2$]-acetate showed eleven pairs of ^{13}C-^{13}C couplings, indicating that C-1/C-2, C-3/C-4, C-5/C-6, C-7/C-8, C-9/C-10, C-11/C-12, C-13/C-14, C-15/C-16, C-17/C-18, C-19/C-20,

* Corresponding author.
E-mail address: tanahash@kobepharma-u.ac.jp (T. Tanahashi).

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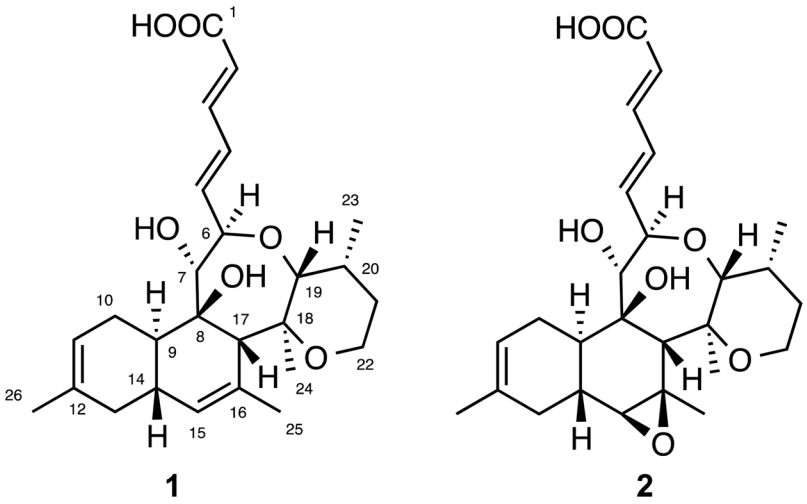

Fig. 1. Structures of pyrenulic acids C (**1**) and I (**2**).

and C-21/C-22 originated from intact acetate units through the acetate-malonate pathway.

In a feeding experiment with L-[methyl-^{13}C]-methionine, C-23, 24, 25, and 26 of pyrenulic acid C (**1**) were extensively enriched. From these findings, pyrenulic acid B (**3**) was assumed to arise from ring-closure of the polyketide chain accompanied by *C*-methylations at C-23, 24, 25, and 26 by SAM in a similar manner to cladobotric acids (Mitova et al., 2006). Subsequent epoxidation of pyrenulic acid B (**3**) could yield a common key intermediate, pyrenulic acid A (**4**), which is hydroxylated to form cladobotric acids A and C in *Cladobotryum* spp. and transformed to pyrenulic acid C (**1**) as well as pyrenulic acids D–H through several steps of oxidation, cleavage of the epoxide ring, and cyclization to the oxepane ring followed by pyran ring formation or dehydration in cultured mycobionts of *Pyrenula* sp. (Fig. 2).

2.2. Induction of new polyketide (2) production in cultured mycobionts supplemented with sodium benzoate

In the biosynthesis of polyketides, the choice of starter unit and extender units is governed by the substrate specificity of polyketide synthases. During the course of feeding experiments, the effects of additives such as sodium acetate, sodium propionate, and sodium benzoate on secondary metabolism were tested. As expected, propionate and benzoate could not be utilized as a starter unit, and pyrenulic acid C (**1**) was the major product. However, it was found that the productivity of pyrenulic acids was enhanced and the profile of metabolites was changed by the addition of benzoate to the medium. The metabolites of mycobionts cultivated on MY5 supplemented with sodium benzoate were fractionated to isolate a new compound **2** along with pyrenulic acid C (**1**).

Compound **2** was isolated as a colorless solid. The molecular formula of **2** was established as $C_{26}H_{36}O_7$ from HR-ESIMS. Its UV spectrum showed strong absorption at 255 nm, and its IR spectrum displayed absorption bands at 3340 (O−H) and 1713 (C=O) cm^{-1}. The ^{1}H NMR spectrum of **2** exhibited the signals of five olefinic protons at δ_H 5.41, 5.90, 6.35, 6.55, and 7.38, seven methine protons, including four oxygenated methines at δ_H 2.94, 3.54, 3.99, and 4.31, four pairs of methylene protons, and four methyl groups at δ_H 1.10, 1.33, 1.45, and 1.69. The ^{13}C NMR, DEPT, and HSQC spectra of **2** showed the presence of a carboxyl carbon, five olefinic methines, and a quaternary olefinic carbon, as well as sixteen sp^3 carbons, which were classified as four CH_3, four CH_2, four oxygenated CH, a non-oxygenated CH, and three oxygenated tertiary carbons. These spectral features were closely similar to those of pyrenulic acid C (**1**), except that **2** showed an oxygenated methine proton signal at δ_H 2.94 and oxygenated sp^3 carbon signals at δ_C 64.7 and 66.1 instead of the olefinic proton signal and two sp^2 carbon signals ascribable to the 15,16-double bond in **1**. All proton and carbon signals were assigned by COSY, HSQC, and HMBC experiments. The signal for the methyl group at δ_H 1.45, which was assignable to H_3-25 from the HMBC interaction with C-17, was further correlated with carbon signals at δ_C 64.7 (C-15) and 66.1 (C-16). The methine proton singlet at δ_H 2.94 showed an HSQC correlation with a carbon signal at δ_C 64.7 (C-15) and HMBC correlations with a methyl at δ_C 26.9 and two CH carbons at δ_C 37.8 and 36.1 (C-9 and C-14). These findings, together with its molecular formula, suggested that the compound **2** was an oxygenated derivative of pyrenulic acid C (**1**), in which an epoxy ring was constructed between the double bond of C-15 and C-16.

The relative configuration of **2** was determined on the basis of the coupling constants and significant NOESY cross-peaks. The coupling constant $J_{6,7}$ (10.0 Hz) suggested that H-6 and H-7 were oriented in an anti-arrangement. Moreover, the coupling constant $J_{19,20}$ (6.0 Hz) and the NOESY correlations of H-6/H-10eq (δ_H 2.28), H-7/H-17, H-7/H-19, 8-OH/H-14, H-17/H-19, H-9/H_3-24, H-22ax (δ_H 3.65)/H_3-23, H-22ax/H_3-24, H-15/H_3-24, and H-15/H_3-25 indicated that H-7, H-17, and H-19 were oriented axially on the upward face. On the other hand, H-9, H-15, H_3-23, H_3-24, and H_3-25 were directed downward (Fig. 3). Accordingly, new compound **2** was determined as shown and designated as pyrenulic acid I.

In conclusion, the feeding experiments with ^{13}C-labelled compounds defined that mycobionts of *Pyrenula* sp. had biosynthetic pathways similar to *Cladobotryum* fungus. Mycobionts of lichen species are genetically related to non-symbiotic fungi, and expected to harbor diverse metabolic genes as in fungi (Stocker-Wörgötter, 2008). However, lichen thalli generally produce no fungal metabolites but instead form characteristic lichen substances. We have already found that the metabolic pathways normally suppressed in lichenized conditions were expressed by isolation and cultivation of mycobionts from lichen thalli. Recent studies showed that chemical epigenetic manipulation techniques could promote the generation of a variety of new compounds from attenuated or silent biosynthetic pathways in fungi (Cichewicz, 2010). The present study showed that the treatment of cultivated mycobionts of *Pyrenula* sp. with benzoate could induce a silent metabolic ability to produce a new metabolite, although the function of benzoate was not clarified.

3. Experimental

3.1. General experimental procedures

UV spectrum was recorded on a Shimadzu UV-240

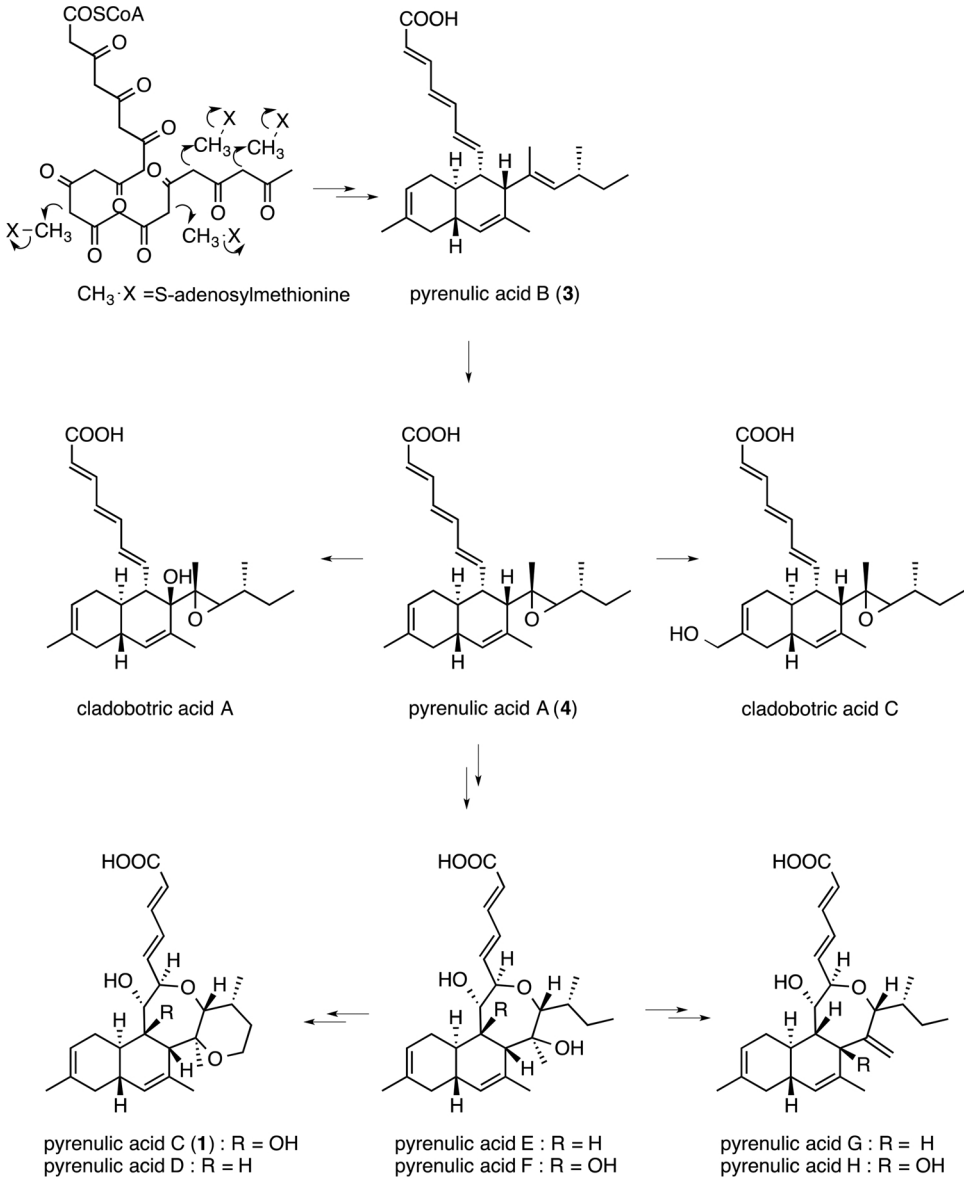

Fig. 2. Biogenetic pathways of pyrenulic acids in *Pyrenula* sp. and cladobotric acids in *Cladobotryum* sp.

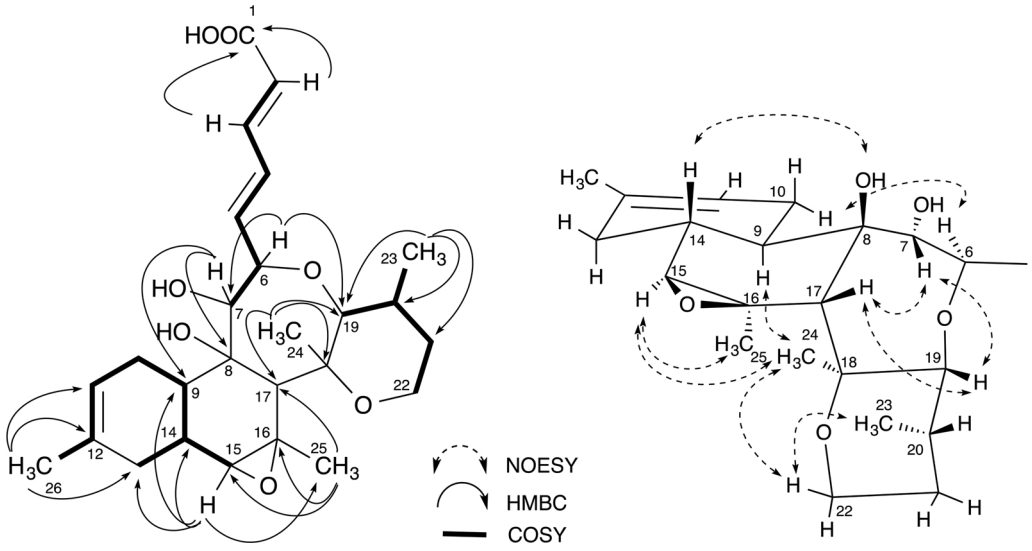

Fig. 3. Key COSY, HMBC and NOESY correlations of pyrenulic acid I (**2**).

spectrophotometer and IR spectrum on a Shimadzu FTIR-8200 infrared spectrophotometer. Optical rotation was measured on a Jasco DIP-370 digital polarimeter. HR-ESIMS was obtained with a Thermo Scientific Q Exactive. NMR experiments were performed with Varian NMR System-500 and Varian UNITY INOVA (500 MHz) spectrometers with tetramethylsilane as an internal standard. Thin-layer chromatography was performed on precoated Kieselgel 60F_{254} plates (Merck) and spots were visualized under UV light. ^{13}C-Labeled compounds (99% enriched) were obtained from Sigma-Aldrich Fine Chemicals, St. Louis, USA.

3.2. Fungal material

The isolation and cultivation of mycobionts of lichen *Pyrenula* sp. and DNA sequence analysis of the culture were as described in our previous report (Le et al., 2014).

3.3. Feeding experiment with sodium [1-^{13}C]-acetate

Cultured mycobionts of *Pyrenula* sp. were transferred to 10 test tubes containing modified MY5 medium (10 ml/tube). After cultivation for 1 month, an aqueous solution of sodium [1-^{13}C]-acetate (10 mg/tube) was added to the cultures. The cultures were grown at 18 °C in the dark for 9 months. The colonies (dry weight 1.83 g) were harvested and extracted with MeOH. The extract (337 mg) was purified by preparative TLC with $CHCl_3$-MeOH (9:1) to give **1** (161 mg).

3.4. Feeding experiment with sodium [1,2-$^{13}C_2$]-acetate

Sodium [1,2-$^{13}C_2$]-acetate (10 mg/tube) was administered to the cultures (10 test tubes) grown on the modified MY5 medium (10 ml/tube). After cultivation for 9 months, the colonies (dry weight 1.88 g) were worked up in the same way as for the feeding experiment with sodium [1-^{13}C]-acetate to afford **1** (156 mg).

3.5. Feeding experiment with L-[methyl-^{13}C]-methionine

L-[methyl-^{13}C]-methionine (10 mg/tube) was administered to the cultures (20 test tubes) grown on the modified MY5 medium (10 ml/tube) for 1 month. After cultivation for 8 months, the colonies (dry weight 5.12 g) were extracted with Et_2O. The extract (649 mg) was purified by preparative TLC with toluene-acetone (4:1) and toluene-AcOH (4:1) to give **1** (120 mg).

3.6. Induction and isolation of pyrenulic acid I (2)

The mycobiont cultures were grown on MY5 medium for 3 weeks and then an aqueous solution of sodium benzoate (25 mg/tube) was added to the cultures, which were cultivated for a further 3 months. The harvested colonies (80 test tubes, dry weight 11.2 g) were extracted with MeOH. The MeOH extract was partitioned between H_2O and *n*-BuOH. The *n*-BuOH (972 mg) was subjected to preparative TLC ($CHCl_3$-MeOH, 9:1), giving fatty compounds (151.4 mg), pyrenulic acid C (**1**) (70.9 mg), and pyrenulic acid I (**2**) (36.3 mg).

3.7. Pyrenulic acid I (2)

Colorless solid; $[\alpha]_D^{29}$–18 (c 0.89, $CHCl_3$); UV (EtOH) λ_{max} (log ε): 255 (4.16) nm. IR (KBr) ν_{max}: 3340, 1713, 1692, 1642 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.10 (3H, d, J = 7.5 Hz, H_3-23), 1.33 (3H, s, H_3-24), 1.42 (1H, br t, J = 13.0 Hz, H-21), 1.45 (3H, s, H_3-25), 1.69 (3H, br s, H_3-26), 1.86 (1H, m, H-21), 1.90 (1H, m, H-9), 2.08–2.17 (4H, m, H-10, H_2-13, H-20), 2.13 (1H, s, H-17), 2.20 (1H, m, H-14), 2.28 (1H, br d, J = 15.0 Hz, H-10), 2.94 (1H, s, H-15), 3.47 (1H, dd, J = 13.0, 5.5 Hz, H-22), 3.54 (1H, d, J = 6.0 Hz, H-19), 3.65 (1H, td, J = 13.0, 2.0 Hz, H-22), 3.99 (1H, d, J = 10.0 Hz, H-7), 4.31 (1H, br dd, J = 10.0, 4.5 Hz, H-6), 5.41 (1H, br d, J = 5.0 Hz, H-11), 5.90 (1H, d, J = 15.0 Hz, H-2), 6.21 (1H, 8-OH), 6.35 (1H, dd, J = 15.0, 4.5 Hz, H-5), 6.55 (1H, ddd, J = 15.0, 11.0, 1.0 Hz, H-4), 7.38 (1H, dd, J = 15.0, 11.0 Hz, H-3); ^{13}C NMR ($CDCl_3$): δ 13.7 (C-23), 14.2 (C-24), 23.0 (C-26), 26.9 (C-25), 29.8 (C-10), 31.3 (C-21), 32.3 (C-20), 35.7 (C-13), 36.1 (C-14), 37.8 (C-9), 54.4 (C-22), 58.7 (C-17), 64.7 (C-15), 66.1 (C-16), 74.1 (C-19), 75.6 (C-6), 76.1 (C-8), 77.7 (C-7), 78.4 (C-18), 120.8 (C-2), 122.8 (C-11), 129.4 (C-4), 132.6 (C-12), 142.9 (C-5), 146.2 (C-3), 171.6 (C-1). HRESIMS: m/z: 459.23950 $[M\text{-}H]^-$ (calcd. for $C_{26}H_{35}O_7$ 459.23842).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.phytol.2018.05.020.

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