Structure and Biosynthesis of Lecanopyrone, a Naphtho[1,8-*cd*]pyran-3-one Derivative from Cultured Lichen Mycobionts of *Lecanora leprosa*

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From the cultures of spore-derived mycobionts of the lichen *Lecanora leprosa* a novel naphtho[1,8-*cd*]pyran-3-one derivative, lecanopyrone, was isolated. Its structure was determined by spectroscopic methods. The assembly pattern of acetate units in its biosynthesis was verified using sodium [1-^{13}C]-acetate and sodium [1,2-$^{13}C_2$]-acetate.

Key words: Lecanora leprosa, Lichen Mycobiont, Naphtho[1,8-*cd*]pyran-3-one

Introduction

Lichens, a symbiotic association of mycobiont and photobiont partners, produce diverse secondary metabolites, some of which show a wide range of potentially useful biological activities (Huneck, 1999, 2001). Cultures of isolated lichen mycobionts have an ability, under osmotically stressed conditions, to produce substances that have never been detected in the lichenized state but are structurally related to fungal metabolites (Tanahashi *et al.*, 1997). It was pointed out that cultures of lichen mycobionts could be good tools for investigating the secondary metabolism in lichens (Tanahashi *et al.*, 2003). In the course of our studies on cultured lichen mycobionts, we cultivated mycobionts of the lichen *Lecanora leprosa* Fée [*L. cinereocarenea* (Eschw.) Vain.] and isolated five dibenzofurans from their cultures (Tanahashi *et al.*, 2001). Upon examination, we also detected an up to date uncharacterized metabolite in the cultures, prompting us to further investigate this metabolite. In this paper, we report the structural determination and biosynthetic study of this new naphtho[1,8-*cd*]pyran-3-one derivative.

Material and Methods

General experimental procedures

Melting points were measured using a Yanaco micro melting point apparatus and are reported uncorrected. The UV spectra were recorded using a Shimadzu UV-240 spectrophotometer and the IR spectra using a Shimadzu FTIR-8200 infrared spectrophotometer. HR-EI-mass spectra were obtained with a Hitachi M-4100 mass spectrometer. The NMR experiments were performed with Varian VXR-500 spectrometers with tetramethylsilane as internal standard. HPLC was performed using a Waters system (600E Multisolvent Delivery System, 486 Tunable Absorbance Detector). Thin-layer chromatography was performed on precoated Kieselgel 60F_{254} plates (Merck) and spots were visualized under UV light.

Plant material

Specimens of the plant material used are described in a previous publication (Tanahashi *et al.*, 2001). Mycobionts were obtained from the spores discharged from apothecia of a thallus, and were cultivated in test tubes containing modified MY (malt yeast) medium (10 g malt extract, 4 g yeast extract, 100 g sucrose, 15 g agar, 1 l H_2O, pH 7) at 18 °C in the dark.

Chemicals

^{13}C-labeled compounds (99% enriched) were obtained from Sigma-Aldrich Fine Chemicals, St. Louis, MO, USA.

Isolation of compounds

After cultivation for 11–12 months, the harvested colonies (101 test tubes, fresh cell weight

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12.3 g) and slants were extracted continuously with Et_2O and then with acetone. The Et_2O and acetone extracts were repeatedly subjected to preparative TLC (toluene/acetone, 19:1) and preparative HPLC (μBondasphere 5μC18-100Å, MeCN/H_2O, 7:13), giving **1** (1.9 mg from the Et_2O extract, 8.9 mg from the acetone extract) together with previously reported dibenzofurans (Tanahashi *et al.*, 2001).

Lecanopyrone (**1**): Pale yellow crystalline solid, m.p. 225 °C (decomposition) (MeOH). – UV (EtOH): λ_{max} (log ε) = 218 (4.15), 227 (4.17), 267.5 sh (3.92), 277 (4.09), 341 (3.33), 377.5 nm (3.37). – IR (KBr): ν_{max} = 3240, 1769, 1690, 1668, 1647, 1508 cm^{-1}. – ^1H NMR ($CDCl_3$): δ = 3.91 (3H, s, 4-OCH_3), 5.74 (2H, d, J = 1.5 Hz, H_2-10), 5.79 (2H, br s, H_2-3), 6.40 (1H, s, H-5), 6.67 (1H, t, J = 1.5 Hz, H-8), 10.92 (1H, br s, 9-OH). – ^{13}C NMR ($CDCl_3$): δ = 56.2 (4-OCH_3), 71.1 (C-3), 78.7 (C-10), 88.4 (C-5), 96.5 (C-9a), 98.8 (C-3a), 105.4 (C-8), 116.1 (C-6a), 127.1 (C-9b), 148.1 (C-7), 158.1 (C-4), 163.2 (C-6), 164.9 (C-9), 168.0 (C-1). – HR-EIMS: m/z = 258.0544 $[M]^+$ (calcd. for $C_{14}H_{10}O_5$ 258.0529).

*Acetylation of **1***

Compound **1** (1.7 mg) was acetylated with Ac_2O/pyridine (each 0.1 ml) and the crude acetate was purified by preparative HPLC (μBondasphere 5μC18-100Å, MeCN/H_2O, 4:1) to yield **1a** (0.9 mg).

1a: Yellow crystalline solid. – UV: λ_{max} (log ε) = 217, 236 sh, 269, 318, 330, 387 nm (photodiode array detector; MeCN/H_2O, 8:2). – ^1H NMR ($CDCl_3$): δ = 2.45 (3H, s, 9-OAc), 3.94 (3H, s, 4-OCH_3), 5.73 (2H, s, H_2-10), 5.81 (2H, s, H_2-3), 6.57 (1H, s, H-5), 6.81 (1H, br s, H-8). – EIMS: m/z (rel. int.) = 300 (17), 258 (100). – HR-EIMS: m/z = 300.0638 $[M]^+$ (calcd. for $C_{16}H_{12}O_6$ 300.0634).

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

Cultured mycobionts of *L. leprosa* were transferred to 11 test tubes containing modified MY medium (10 ml/tube) supplemented with sucrose (250 mg/tube) and sodium [1-^{13}C]-acetate (25 mg/tube). The cultures were grown at 18 °C in the dark for 6 months. The colonies (dry weight 0.76 g) were harvested and extracted with acetone. The acetone extract (240 mg) was purified by preparative TLC with toluene/acetone (4:1) and preparative HPLC (MeCN/H_2O, 7:3) to give **1** (5.2 mg). The previously isolated dibenzofuran derivatives were not obtained under these conditions.

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

Sodium [1,2-$^{13}C_2$]-acetate (25 mg/tube) was administered to the cultures (10 test tubes) grown on modified MY medium (10 ml/tube) supplemented with sucrose (250 mg/tube). After cultivation for 6 months, the colonies (dry weight 0.88 g) were prepared in the same way as for the feeding experiment with sodium [1-^{13}C]-acetate to afford **1** (8.6 mg).

Results and Discussion

The polyspore-derived mycobionts of *Lecanora leprosa* Fée collected in Japan were cultured on conventional malt-yeast extract medium supplemented with 10% sucrose at 18 °C in the dark. After cultivation for 11–12 months, the colonies and agar medium were harvested and extracted with cold Et_2O and acetone. The extracts were separated by a combination of preparative TLC and preparative HPLC to afford the new compound **1**.

Compound **1** was isolated as pale yellow crystals, m.p. 225 °C. Its HR-EI-mass spectrum exhibited a molecular ion peak at m/z 258.0544 $[M]^+$, indicating a molecular formula of $C_{14}H_{10}O_5$. It showed IR bands at 3240, 1769, 1690, 1668, 1647, and 1508 cm^{-1}, suggesting the presence of a hydroxy group, a hydrogen-bound carbonyl group and substituted aromatic system(s). Its ^1H NMR spectrum exhibited signals for a methoxy group at δ_H 3.91 ppm (s), two sets of methylene groups at δ_H 5.74 (2H, d, J = 1.5 Hz) and 5.79 ppm (2H, br s), two aromatic protons at δ_H 6.40 (s) and 6.67 ppm (t, J = 1.5 Hz), and a hydrogen-bound phenolic hydroxy group at δ_H 10.92 ppm (br s). The ^{13}C NMR spectrum of **1** showed a methoxy carbon atom, two oxygenated aliphatic methylene carbon atoms, two aromatic CH carbons, eight quaternary aromatic carbon atoms, three of which were oxygenated, and a carbonyl carbon atom. These spectral features closely resembled those of corymbiferan lactone A (**2**) (Fig. 1), a naphthalene derivative with a lactone bridge isolated from *Penicillium hordei* (Overy and Blunt, 2004). Acetylation of **1** gave a monoacetate, **1a**, suggesting the presence of one hydroxy group in **1**. This

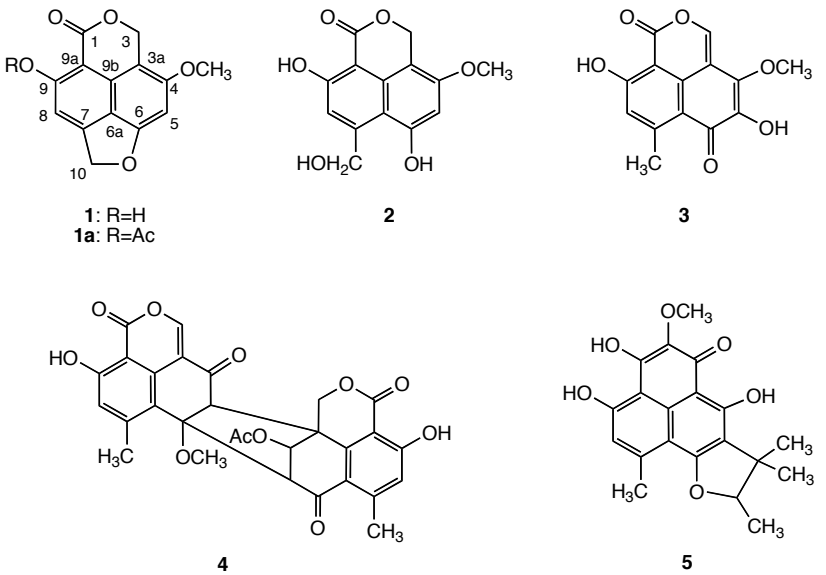

Fig. 1. Chemical structures of lecanopyrone (**1**) and related compounds.

fact, together with its molecular formula, implied that compound **1** was a dehydrated derivative of corymbiferan lactone A (**2**). The location of the functional substituents in the naphthalene ring could be confirmed by HMBC experiments with **1** (Fig. 2). An HMBC correlation between an oxygenated methylene signal at δ_H 5.79 ppm and a carbonyl carbon atom indicated a lactone bridge. A hydrogen-bound hydroxy group, which was adjacent to the carbonyl group, correlated with an aromatic CH carbon atom (C-8) at δ_C 105.4 ppm. The aromatic proton at C-8 had long-range coupling (J = 1.5 Hz) with methylene protons at δ_H 5.74 ppm, and these proton signals showed HMBC interactions with a quaternary aromatic carbon atom at δ_C 148.1 ppm (C-7). Further HMBC correlations were observed from another aromatic proton (H-5) at δ_H 6.40 ppm to two oxygenated aromatic carbon atoms at δ_C 158.1 (C-4) and 163.2 ppm (C-6). A methoxy group was located at C-4 by the HMBC correlation from a methoxy signal at δ_H 3.91 ppm. The HMBC correlation from the methylene protons at δ_H 5.74 ppm to C-6 suggested an ether linkage. Accordingly, the new compound **1** was determined as a dehydrated derivative of corymbiferan lactone A (**2**) in which an ether ring was constructed between the phenolic hydroxy group at C-5 and the hydroxymethyl group at C-7 and designated lecanopyrone (Fig. 1).

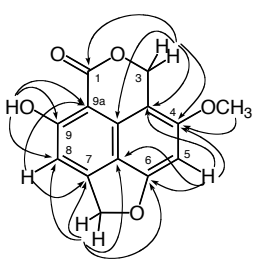

Fig. 2. HMBC correlations of **1**.

Although a naphthopyran derivative, simonyellin (**3**), was isolated from the natural lichen *Simonyella variegata* Steiner (Elix *et al.*, 1995), metabolites with a naphthalene skeleton such as lecanopyrone (**1**) are not typical lichen substances. However, structurally related compounds and their precursor phenalenones are well known as metabolites of non-lichenized fungi. On the basis of the results of a biosynthetic study on the fungal metabolite duclauxin (**4**) (Sankawa *et al.*, 1966), simonyellin (**3**) was postulated to be biosynthesized from a single heptaketide chain *via* an intermediate phenalenone (Elix *et al.*, 1995). On the other hand, a fungal phenalenone, deoxyherqueinone (**5**), was proven to be formed by condensation of a heptaketide folded in a different manner from duclauxin (**4**) (Simpson, 1979). Assuming that lecanopyrone (**1**) is biosynthesized

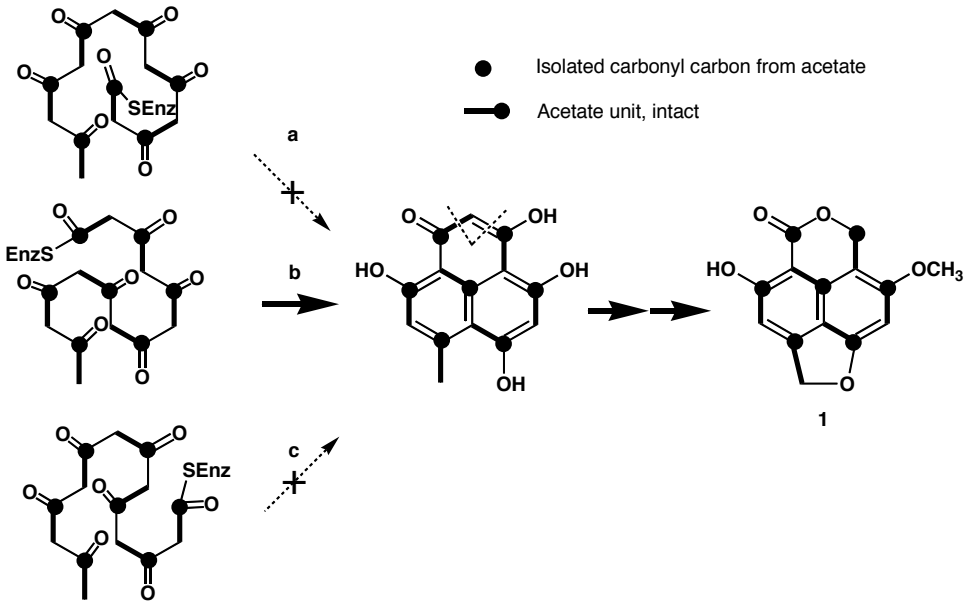

Fig. 3. Folding of the heptaketide chain in the biosynthesis of lecanopyrone (**1**).

via a phenalenone ring system formed by condensation of a heptaketide chain, there are three possible folding patterns, namely, (a) duclauxin-like, (b) deoxyherqueinone-like, and (c) another possible pathway. From our interest in the incorporation pattern of acetate units into lecanopyrone, administration experiments with ^{13}C-labeled acetates were undertaken.

Feeding with sodium [1-^{13}C]-acetate and [1,2-$^{13}C_2$]-acetate resulted in moderate incorporation into lecanopyrone (**1**). The ^{13}C NMR spectrum showed that the C-1 carbon atom of acetate was incorporated into the carbon atoms at C-1, 3, 4, 6, 7, 9, and 9b in lecanopyrone (**1**). The 2D INADEQUATE (incredible natural abundance double quantum transfer experiment) spectrum of lecanopyrone isolated from the cultured mycobionts incubated with [1,2-$^{13}C_2$]-acetate showed five pairs of ^{13}C-^{13}C couplings, indicating that C-3 and C-3a, C-4 and C-5, C-6 and C-6a, C-9a and C-9b, C-8 and C-9, and C-7 and C-10 originated from intact acetate units through the acetate-malonate pathway. Additional weaker contour plots observed between C-5 and C-6 and between C-7 and C-8 could account for a high incorporation of [1,2-$^{13}C_2$]-acetate units. The signal due to C-1 of [1,2-$^{13}C_2$]-acetate-enriched lecanopyrone (**1**) showed a good incorporation of ^{13}C label but no ^{13}C-^{13}C coupling with any other carbon atoms. From these findings, folding patterns (a) and (c) of heptaketide were excluded. Lecanopyrone (**1**) was assumed to arise from folding and cyclization of a heptaketide in a similar manner to that of deoxyherqueinone (b), followed by oxidation of an intermediate phenalenone with elimination of one carbon atom, recyclization to form a lactone ring, and methylation and construction of the ether ring (Fig. 3).

Metabolites with a naphthalene skeleton have not yet been isolated from either lichens or cultured mycobionts of the genus *Lecanora*. The production of lecanopyrone that is structurally and biosynthetically related to fungal metabolites in cultured mycobionts is of great interest. The mycobionts of the lichens seem to have conserved the secondary metabolism of free-living fungi. These metabolic pathways might be normally suppressed in the lichenized condition but expressed in the isolated mycobiont.

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