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Structural Determination and Proposed Biosynthesis of Alcanivorone, a Novel α-Pyrone Produced by *Alcanivorax jadensis*

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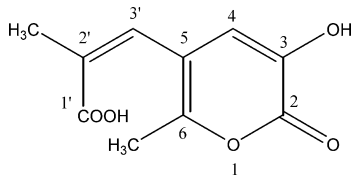
Abstract A novel α-pyrone designated as alcanivorone was found in a culture broth of the marine bacterium, *Alcanivorax jadensis*, and its structure was determined by an analysis of 1D NMR, 2D NMR and MS data. Alcanivorone was produced by *A. jadensis* only when sodium pyruvate was added to the culture medium as a carbon source. Incorporation experiments using stable isotope-labeled pyruvate indicated that alcanivorone was biosynthesized from four molecules of pyruvate.

Keywords marine bacterium, *Alcanivorax jadensis*, α-pyrone, pyruvic acid, biosynthesis

Introduction

Marine microorganisms have gained considerable attention as an important source of novel secondary metabolites [1]. Strains of the *Alcanivorax* group were first isolated from the North Sea as biosurfactant-producing and alkane-degrading marine bacteria, and have also been found in a variety of marine environments including oil spill-contaminated sites [2, 3]. These strains have been shown to be important for the biodegradation of petroleum hydrocarbons in a marine environment, especially under bioremediation conditions [4]. During a series of investigations of new metabolites from marine microorganisms [5, 6], we found a novel α-pyrone, alcanivorone (**1**), from the culture broth of *A. jadensis* (former name: *Fundibacter jadensis* [7]). We describe in this paper the production, structural determination, incorporation experiments with stable isotope-labeled sodium pyruvate, and a proposed biogenesis of **1** based on the incorporation experiments.



Alcanivorone (**1**)

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Materials and Methods

Strains

Alcanivorax jadensis T9^T (DSM 12178^T) was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). Other *Alcanivorax* sp. bacteria, MBIC04549, MBIC04556 and MBIC05570, were isolated from seawater in Heita Bay, Kamaishi, Japan. They were maintained on Marine Agar 2216 (Difco).

Fermentation and Isolation

The NSW medium contained the following components (for 1.0 liter): 1.0 g of ammonium nitrate, 0.2 g of dipotassium hydrogenphosphate, 0.02 g of iron(III) citrate *n*-hydrate, 800 ml of filtered natural seawater, and 200 ml of distilled water at pH 7.6 before autoclaving; an appropriate carbon source, *i.e.*, sodium pyruvate, sodium acetate, glucose or hexadecane, was added according to need. The NSW medium supplemented with 5.0 g/liter of sodium pyruvate (NSW+0.5% sodium pyruvate) was used for the production of **1**. The seed culture was cultured in 50 ml of

1/10 Marine Broth (1/10 MB) at 30°C with rotary shaking at 100 rpm for 5 days. The composition of 1/10 MB (for 1.0 liter) is as follows; 3.74 g of Marine Broth 2216 (Difco), 750 ml of filtered natural seawater and 200 ml of distilled water at pH 7.6 before autoclaving. A 10-ml amount of the seed culture was inoculated into 1.0 liter of NSW+0.5% sodium pyruvate in a 3-liter Erlenmeyer flask. Fermentation was carried out at 30°C with rotary shaking at 100 rpm for 10 days. The fermentation broth (4.0 liters total) was centrifuged at 12,000 *g* for 20 minutes, and the supernatant was extracted twice with an equal volume of EtOAc at pH 2. The precipitate was extracted with $CHCl_3$/MeOH (9 : 1). Both organic extracts were mixed and concentrated *in vacuo*, before being applied to a silica gel column and eluted with $CHCl_3$/MeOH/AcOH (90 : 10 : 0.5). The **1**-containing fractions were gathered and further purified by HPLC under the following conditions: column, COSMOSIL® 5C18-AR (10×250 mm, Nacalai Tesque, Kyoto, Japan); flow rate, 2.0 ml/minute; solvent system, a linear gradient from 50% MeOH - H_2O containing 0.05% phosphoric acid to 100% MeOH containing 0.05% phosphoric acid in 30 minutes; detection, UV λ 250 nm. The HPLC purification was conducted twice to give **1** (7.3 mg) as a white powder. The physico-chemical properties of **1** were as follows: molecular formula, $C_{10}H_{10}O_5$; molecular weight, 210; ESI-MS: positive *m/z* 211 $(M+H)^+$, negative *m/z* 209 $(M-H)^-$; HRFAB-MS: positive *m/z* found 211.0606 $(M+H)^+$, calcd. 211.0607 (for $C_{10}H_{11}O_5$); UV (MeOH) λ_{max} nm (ε), 262 (11,000); IR ν_{max} (KBr) cm^{-1}: 2929, 2608, 2361, 2339, 1697, 1521, 1458, 1419, 1335.

Spectroscopic Measurements

UV and IR spectra were respectively recorded by Beckman DU640 and Jasco FT/IR-430 infrared spectrometers. FAB-MS and HRFAB-MS were recorded by a Jeol JMS-SX 120 instrument. ^{1}H-NMR and all 2D NMR spectra were measured by a Varian Unity INOVA 750 spectrometer, and the ^{13}C-NMR spectrum was measured by a Varian Unity INOVA 500 spectrometer. Chemical shifts are referenced to the solvent peaks of δ_H 3.31 and δ_C 49.15 for CD_3OD.

Antimicrobial Activity

The antimicrobial activity of **1** was measured by the paper disk method. A MeOH soln of a sample was dropped on to a paper disk (6 mm in diameter), and the disk was dried for 30 minutes in a clean enclosure. The sample amount was 5.0 mg/disk. The disk was then put on an agar medium that had been inoculated with each test microorganism, using *Escherichia coli* IFO 3301, *Bacillus subtilis* IFO 3134, *Staphylococcus aureus* IFO 12732, *Salinivibrio costicola* ATCC 33508, and *Candida albicans* IFO 1060. Halo formation was observed after 48 hours of incubation at 30°C.

Feeding Experiment

Sodium [2-^{13}C] pyruvate, sodium [1,2-$^{13}C_2$] pyruvate and sodium [2,3-$^{13}C_2$] pyruvate were purchased from Sigma Chemical Co., USA. The seed culture was cultured for 5 days in 20 ml of 1/10 MB at 30°C with rotary shaking at 100 rpm. A 1-ml amount of the seed culture was inoculated into 100 ml of NSW+0.5% sodium pyruvate, 450 mg of sodium pyruvate and 50 mg of sodium [1,2-$^{13}C_2$] pyruvate in a 300-ml Erlenmeyer flask, and then cultured for 10 days at 30°C with rotary shaking at 100 rpm. The cultured broth was extracted and separated by silica gel column chromatography, before being purified by HPLC as already described. A 1.3-mg amount of labeled **1** was obtained from the 100-ml culture.

The sodium [2-^{13}C] pyruvate and sodium [2,3-$^{13}C_2$] pyruvate feeding experiments were performed by the same procedure as that just described for the sodium [1,2-$^{13}C_2$] pyruvate feeding experiment. A 0.9-mg amount of labeled **1** was obtained from the sodium [2-^{13}C] pyruvate feeding experiment, and a 0.6 mg from the sodium [2,3-$^{13}C_2$] pyruvate feeding experiment.

Results and Discussion

Production of 1 by *A. jadensis*

The effect of carbon source, using sodium pyruvate, sodium acetate, sodium propionate, glucose and hexadecane, on the production of **1** was investigated. Each carbon source was added to the NSW medium at a concentration of 0.5% (w/v). The production of **1** was only detected (12.4 mg/liter) when sodium pyruvate was used as the carbon source. The addition of the other carbon sources, sodium acetate, sodium propionate, or hexadecane did not produce **1**, although the growth of the producing bacterium was almost comparable to the growth by pyruvate. *A. jadensis* didn't grow when glucose was added as a carbon source and didn't produce **1**.

As well as *A. jadensis*, the other *Alcanivorax* strains, MBIC04549, MBIC04556 and MBIC05570, also produced **1** with the addition of sodium pyruvate at a similar level of productivity: 10.0, 9.0 and 8.4 mg/liter, respectively.

Structural Elucidation

The molecular formula of **1** was determined to be $C_{10}H_{10}O_5$ by high-resolution FAB-MS [measured, 211.0606; calcd., 211.0607 for $(M+H)^+$]. In the ^{13}C-NMR spectrum, ten

carbon signals, including two carbonyl carbons (δ 172.43 and δ 162.02), six olefinic carbons (δ 157.66, δ 143.84, δ 130.59, δ 125.59, δ 120.24, and δ 120.30) and two methyl carbons (δ 21.99 and δ 12.31) were observed. In the ^{1}H-NMR spectrum, only four kinds of proton signals indicating two methyl protons (δ 2.37 and δ 2.07) and two olefin protons (δ 7.40 and δ 6.47) were observed. All direct connections between the carbons and protons were determined by HSQC. Long-range coupling was observed between the methyl proton (2′-CH_3: δ 2.07; 3H, d, J=1.5 Hz) and the olefinic proton (H-3′: δ 6.47; 1H, d, J=1.5 Hz), and HMBC signals between 2′-CH_3 and C-1′, and H-3′ and C-1′ were observed. A 2-methylacrylic acid moiety was determined by these signals, and the NOE signal between H-3′ and 2′-CH_3 indicating the stereochemistry of the double bond (C-2′=C-3′) to be *Z*. A 3-hydroxy-6-methyl-α-pyrone moiety was determined from HMBC signals from 4-H and from 6-CH_3 as shown in Fig. 1. The connection of the acrylic acid moiety and the α-pyrone moiety was determined by HMBC signals between H-3′ and C-4, and H-3′ and C-6. **1** is a new α-pyrone type metabolite, its NMR data being summarized in Table 1.

Antimicrobial Activity

The antimicrobial activity of **1** was evaluated by the paper disk method (5.0 mg/disk). **1** exhibited mild antimicrobial activity, the halo diameter being 8 mm for *Bacillus subtilis* IFO 3134, 7 mm for *Staphylococcus aureus* IFO 12732, and 22 mm for *Salinivibrio costicola* ATCC 33508, but no inhibitory activity was observed against these three strains in liquid culture assay up to 200 ppm (MIC >200 ppm). No inhibitory activity was apparent toward *Escherichia coli* IFO 3301 and *Candida albicans* IFO 1060 by the paper disk method.

Feeding Experiment and Biosynthetic Pathway of 1

A feeding experiment was carried out with sodium [2-^{13}C] pyruvate. ^{13}C-NMR data for the labeled compound indicated that signal intensities of C-3, C-5, C-6 and C-2′ were clearly higher than those of the non-labeled compound (Table 1). The results indicate that these carbons (C-3, C-5, C-6 and C-2′) originated from the C-2 carbon of pyruvate. A feeding experiment with sodium [1,2-$^{13}C_2$] pyruvate was carried out. The signal intensities of C-2, C-3, C-5, C-6, C-1′ and C-2′ were all higher than those of the non-labeled compound, and ^{13}C-^{13}C coupling of C-2–C-3 (J_{CC}=94 Hz) and C-1′–C-2’ (J_{CC}=69 Hz) was observed (Table 1). These results indicate that C-2 and C-3 originated from a single

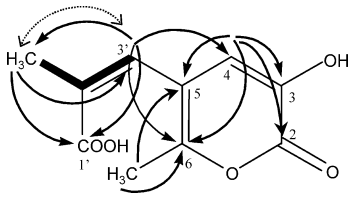

Fig. 1 Structural elucidation of **1** by HMBC.

Bold lines reveal spin systems observed in COSY, and arrows reveal HMBC signals. An HMBC signal between H-4 and C-2 was observed in the measurement used DMSO-d_6 as a solvent. The dotted arrow reveals an NOE signal.

Table 1 NMR data for **1** (750 MHz for ^{1}H, 125 MHz for ^{13}C, CD_3OD) and summary of the feeding experiments

Position	δ ^{1}H (ppm)	δ ^{13}C (ppm)	Specific incorporation (relative intensity to nonlabeled **1**)				
			[2-^{13}C] pyruvate[a]	[1,2-^{13}C] pyruvate[a,c]	J_{CC} (Hz)	[2,3-^{13}C] pyruvate[b,c]	J_{CC} (Hz)
2		162.02	0.8	9.4*	94	1[b]	
3		143.84	12.8*	10.6*	94	6.7*	70
4	7.40 (1H, s)	120.30	1.1	1.1		9.4*	70
5		120.24	11.5*	9.6*		17.7*	63
6		157.66	11.0*	10.6*		6.1*	54
6-CH_3	2.37 (3H, s)	12.31	1[a]	1[a]		19.4*	54
1′		172.43	1.6	11.5*	69	1.1	
2′		130.59	15.2*	11.5*	69	10.5*	44
2′-CH_3	2.07 (3H, d, J=1.5 Hz)	21.99	1.1	0.9		21.1*	44
3′	6.47 (1H, d, J=1.5 Hz)	125.09	0.9	0.9		6.3*	63

[a] Relative enrichment were normalized to the peak intencity of the 6-CH_3 signal.

[b] Relative enrichment were normalized to the peak intencity of the C-2 signal.

[c] For the splitted signals, a sum of intencities of splitted peaks was used.

* Enriched signal.

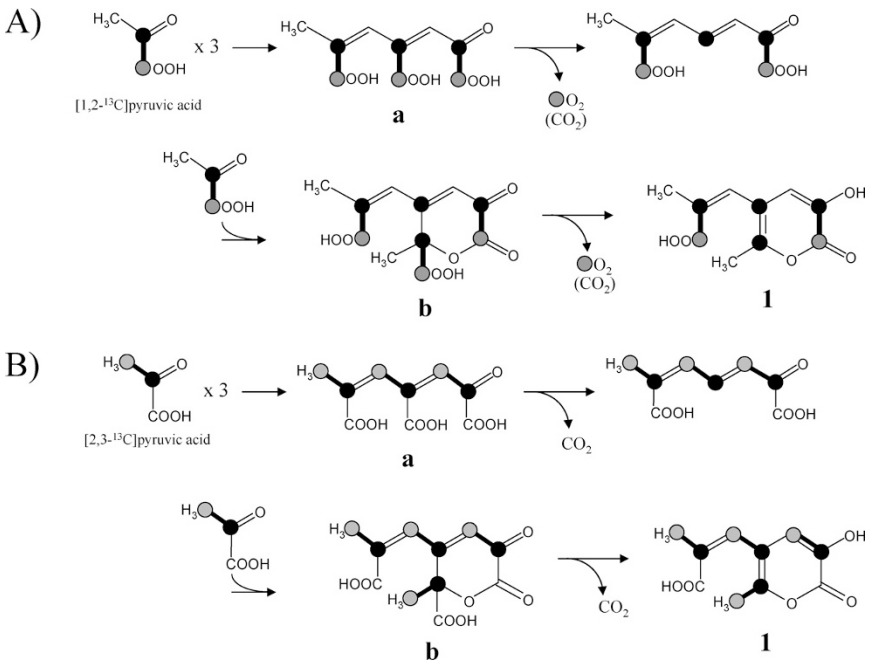

Fig. 2 Hypothetical biosynthetic pathway for **1**.
●, ● and ○: C-1, C-2 and C-3 in pyruvic acid, respectively.

molecule of pyruvate, and that C-1′ and C-2′ were also from a single molecule. The signal intensities of C-5 and C-6 were clearly increased, but no vicinal C-C coupling was apparent. These results indicate that the incorporated two moles of pyruvate were decarboxylated during biosynthesis. We therefore propose the biosynthetic pathway shown in Fig. 2A. The first step may be the condensation of three moles of pyruvate to form **a**, and then decarboxylation and condensation with another pyruvate, and successive lactone formation to afford **b**. The second decarboxylation from **b** gave natural compound **1**. The incorporation pattern of [1,2-$^{13}C_2$] pyruvate into **1** is shown in Fig. 2A.

To clarify the origin of 2′-CH_3 and 6-CH_3 and confirm the proposed biosynthesis shown in Fig. 2A, the feeding experiment with sodium [2,3-$^{13}C_2$] pyruvate was also carried out. The signal intensities of C-3, C-4, C-5, C-6, 6-CH_3, C-2′ 2′-CH_3, and C-3′ were higher than those of the non-labeled compound, and ^{13}C-^{13}C coupling of C-3–C-4 (J_{CC}=70 Hz), C-5–C-3′ (J_{CC}=63 Hz), C-6–6-CH_3 (J_{CC}=54 Hz), and C-2′–2′-CH_3 (J_{CC}=44 Hz) was observed (Table 1). These results indicated that 6-CH_3 and 2′-CH_3 were also from pyruvate, and the incorporation pattern of [2,3-$^{13}C_2$] pyruvate into **1** is shown in Fig. 2B, supporting the proposed biosynthesis of **1**. The other carbon sources including acetate, propionate, hexadecane and glucose did not produce **1**. We also investigated the incorporation of a methyl-^{13}C-methionine, but no incorporation was observed (data not shown), suggesting that pyruvate was the sole carbon source to produce **1**.

It is very interesting that marine bacteria, *Alcanivorax* species, biosynthesized novel α-pyrone, **1** using only pyruvate *via* proposed pathway shown in Fig. 2, being completely different from polyketide biosynthesis. Molecular cloning and biochemical approaches would clarify this unique biosynthetic mechanism in *Alcanivorax* species.

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