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ISOLATION OF IKAHONONE, 4-METHYL-2,4-DIHYDROXY-3-PENTANONE FROM *BACILLUS CEREUS* IFM12235

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Abstract – A new ketone, ikahonone (**1**), and five known compounds (**2-6**) were isolated from *Bacillus cereus* IFM12235 collected in Japan. The structure of compound **1** was elucidated using spectral studies, including NMR. The absolute configurations of **1** and **2** were determined by comparing electronic circular dichroism (ECD) spectra with known compounds and calculating the ECD spectra of **1** and **2**. Three compounds (**2-4**) were previously prepared by synthesis, and first isolated as natural products in the present study.

The genus *Bacillus* consists of approximately 300 species, including *B. subtilis*, *B. anthracis*, and *B. cereus*. Numerous species in this genus are known to exist in water and soil, with some species also found in various extreme environments. The genus *Bacillus* produces many bioactive compounds, such as mycobacillin and bacillomycin isolated from *B. subtilis*,^{1,2} and gramicidin S isolated from *B. brevis*.³ *B. cereus* is known to cause food poisoning, while previous studies have reported the isolation of antibiotics cerexin B and thiocillin I from *B. cereus*.^{4,5} Thus, *B. cereus* can also be used as a natural product resource. In the present study, we described the isolation and structural elucidation of a new ketone, ikahonone (**1**), and 5 known compounds, (2*R*)-2-hydroxy-1-(4-hydroxyphenyl)-4-methyl-3-hexanone (**2**),⁶ (3*S*)-3-hydroxy-1-phenyl-2-butanone (**3**),⁷ (2*R*, 3*R*)-4-methyl-1-phenyl-2,3-pentanediol (**4**),⁸ xenocycloin A (**5**),⁹ and xenocycloin B (**6**)⁹ from *B. cereus* IFM12235. Compounds **2-4** were previously synthesized⁶⁻⁸ and first isolated as natural products in the present study.

During our study focused on discovering various bioactive natural products,^{10,11} a screening for antibacterial activity against *B. subtilis* IFM2060 was performed and identified an extract of *B. cereus* IFM12235 as a hit sample (active at 3.1 µg/mL). This strain was cultured in Waksman's liquid medium¹²

(10 L) at 28 °C for 5 days. After centrifugation of the culture extract, the supernatant and methanolic (MeOH) extract of the mycelium cake were combined and partitioned between ethyl acetate (EtOAc) and water. The fractionation of the EtOAc layer was guided by an antibacterial activity test. The EtOAc layer was fractionated by silica gel and reverse-phase HPLC to yield compounds **1-6** (Figure 1).

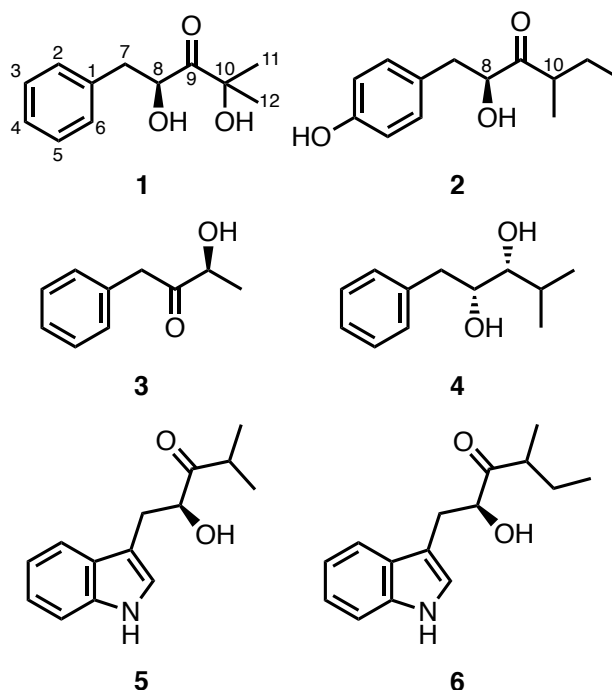


Figure 1. Compounds **1-6** isolated from *Bacillus cereus* IFM12235

The molecular formula of compound **1** was revealed as C₁₂H₁₆O₃ by high-resolution ESI-MS (obsd. *m/z* 231.0960 [M+Na]⁺, calcd. for C₁₂H₁₆O₃Na, 231.0997). The ¹H NMR spectrum of **1** measured in CDCl₃ displayed signals arising from one mono-substituted benzene [δ_{H} 7.24 (3H) and 7.32 (2H)], one oxy-methine [δ_{H} 4.84 (1H)], one methylene [δ_{H} 2.82, and 3.26], and two methyl groups [δ_{H} 1.38 (3H) and 1.43 (3H)]. The ¹³C NMR spectrum of **1**, recorded in CDCl₃, contained 12 peaks. Cross-peaks were observed between H-8 (δ_{H} 4.84) and H₂-7 (δ_{H} 2.82, 3.26) in the COSY data of **1** (Figure 2). The heteronuclear multiple bond correlation (HMBC) spectrum of **1** indicated correlations from the methylene protons [H₂-7 (δ_{H} 2.82, 3.26)] to a quaternary carbon [C-1 (δ_{C} 136.8)] and the sp² carbons [C-2, 6 (δ_{C} 129.4)], suggesting a partial structure **a** (Figure 2). In addition, the HMBC spectrum of **1** revealed correlations between two methyl protons [H₃-11 (δ_{H} 1.43) and H₃-12 (δ_{H} 1.38)] to one sp³ oxygenated carbon [C-10 (δ_{C} 77.4)] and one ketone carbon [C-9 (δ_{C} 214.8)], suggesting another partial structure **b**, as shown in Figure 2. The molecular formula and degree of unsaturation of **1** indicated the existence of two hydroxy groups at C-8 and C-10, with two partial structures, **a** and **b**, connected between C-8 (δ_{C} 74.6) and C-9 (δ_{C} 214.8), leading to the planar structure of compound **1** (Figure 1, Table 1).

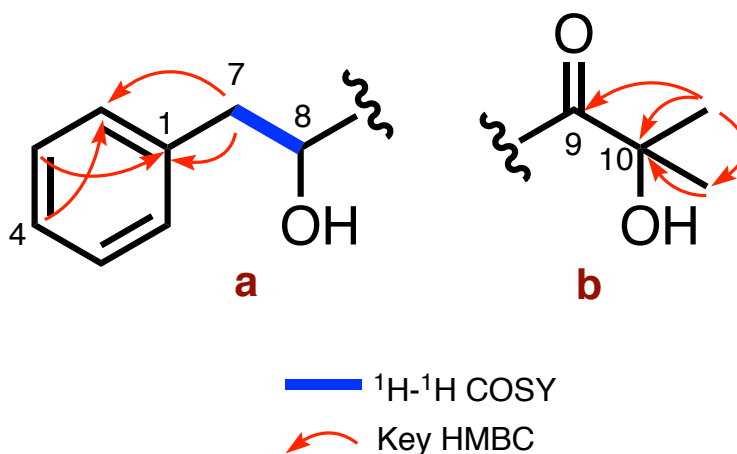


Figure 2. COSY and HMBC correlations for partial structures of compound **1**

Table 1. ^1H and ^{13}C NMR data for ikahonone (**1**)

position	δ_{H}	δ_{C}
1		136.8
2 (6)	7.24 (m, 1H)	129.4
3 (5)	7.32 (m, 1H)	128.6
4	7.24 (m, 1H)	126.9
7	2.82 (dd, 13.8, 8.3, 1H) 3.26 (dd, 13.8, 4.1, 1H)	40.6
8	4.84 (m, 1H)	74.6
9		214.8
10		77.4
11	1.43 (s, 3H)	27.1
12	1.38 (s, 3H)	27.4

Measured in CDCl_3 . 600MHz (^1H). 150 MHz (^{13}C) δ in ppm J in Hz

The absolute configuration of **1** was determined using specific rotation and the ECD spectrum of **1**. The specific rotation of **1** ($[\alpha]_{\text{D}}^{24} +10$ (c 1.0, CHCl_3)) was the same in sign as that of (+)-sattabacin ($[\alpha]_{\text{D}} +35$ (c 0.7, CHCl_3)),¹³ a compound similar to **1**. The stereocenter of (+)-sattabacin, which possessed a hydroxy group, was determined to be the (*S*)-configuration by total synthesis.¹⁴ Reportedly, (-)-sattabacin, an enantiomer of (+)-sattabacin, demonstrated a negative Cotton effect at 290 nm.¹⁵ The ECD spectrum of **1** showed a positive Cotton effect at 292 nm (*Figure S6*), which corresponded well with that of **6**, presenting an *S*-configuration.^{9b} In addition, the experimental ECD spectrum of **1** was compared with the computational ECD spectra of *R*- and *S*- configuration of **1**, calculated by a method similar to that used to

determine the absolute configuration of arungifangin with a free-rotating chain structure,¹⁶ suggesting that compound **1** had an *S*-configuration (Figure S6).

Structures **2-6** were identified by comparing the spectral data with those available in the literature. However, spectral data for **2** have not been previously reported in patents.⁹ Therefore, the structure of **2** was determined based on the analysis of the spectral data obtained in the present study. Additionally, the ECD spectra of **2** were measured and calculated to determine the absolute configuration of **2** (Figure S7). Accordingly, the ECD spectrum of **2** indicates that the C-8 of **2** demonstrates the *S*-configuration. However, the stereochemistry of C-10 of **2** was not determined in this study.

Although the EtOAc extract of *B. cereus* exhibited antibacterial activity against *B. subtilis* IFM2060, the six isolated compounds demonstrated no significant antibiotic effect against *B. subtilis* (minimum inhibitory concentration [MIC], 1 mg/mL or >1 mg/mL). Then, the six compounds were tested in two other assay systems. For pathogenic microorganisms, escaping cellular immunity mediated by macrophages is critical for attacking the host. No compound showed cytotoxicity at 200 μ M when assayed using the mouse macrophage cell line J774.1. In addition, the inflammatory response of the host against bacterial inroads is an important mechanism of innate immune defense; therefore, anti-inflammatory action is a target for pathogenic microorganisms. Nitric oxide (NO) produced by microorganism stimulation, for example, by lipopolysaccharide (LPS), contributes to inflammation. Therefore, the ability of isolated compounds to suppress NO production was examined by the Griess reaction¹⁷ using the LPS-stimulated mouse macrophage-like cell line RAW264. The Griess reaction revealed that compounds **1-6** suppressed the production of nitrate ions at 200 μ M (Figure 3), indicating that these compounds may possess anti-inflammatory activities.

In the present study, we described the isolation of ikahonone and known compounds **2-6** from *B. cereus* IFM12235. In addition, the structure of **1** was elucidated by spectral studies and ECD spectral analysis. Notably, the spectral characterization of compound **2** was described for the first time in the present study. Finally, compounds **2-4** were isolated as natural products.

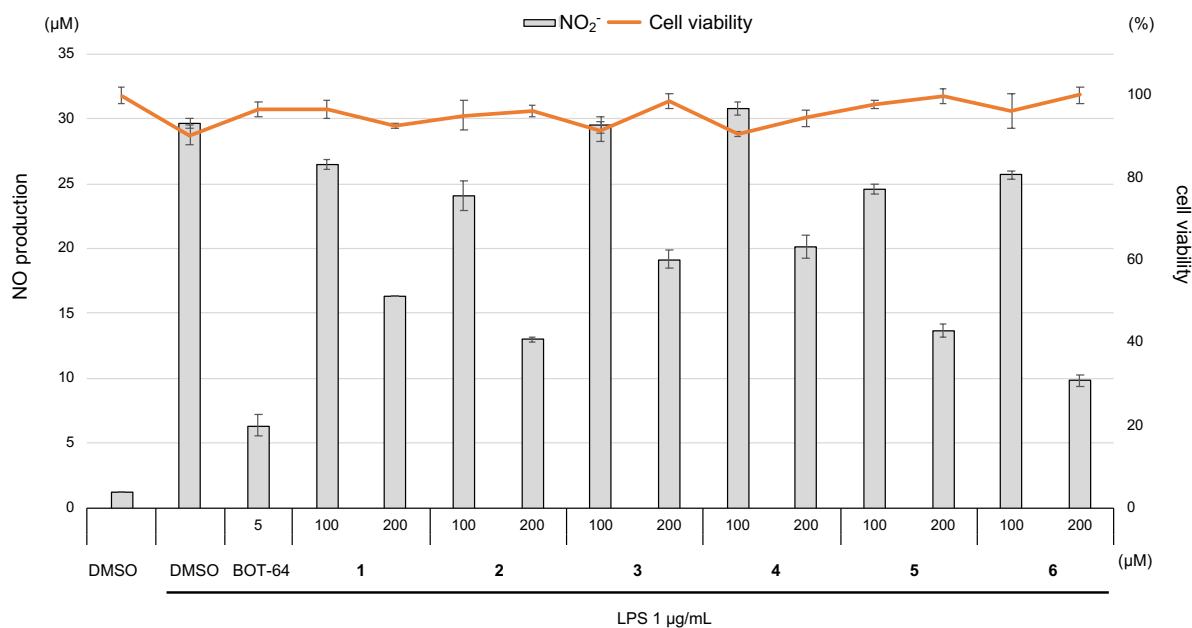


Figure 3. Cell viability and the estimated nitric oxide (NO) production in RAW264 cells for compounds 1-6

EXPERIMENTAL

General experimental procedures

The following instruments were used in the present study: a P-2200 polarimeter (JASCO) for optical rotations; a J-1100 CD spectrometer (JASCO) for circular dichroism; a UV 1280 (Shimadzu) for UV-vis spectra; an ECZ-600 spectrometer (JEOL) for NMR spectroscopy (solvent chemical shifts were used as the internal standard); an AccuTOF LC-plus JMS-T100LP (JEOL) for HR-ESI-MS; two PU 1580 and PU2080 plus (JASCO) pump and UV 970, UV 2075, RI-1530, and RI-2031 plus (JASCO) for HPLC. The following adsorbents were used for purification: silica gel 60 F₂₅₄ (0.25 mm, Merck) and silica gel 60 RP-18 F_{254S} (0.25 mm, Merck) for analytical thin layer chromatography (TLC); Silica gel 60 N (Kanto Chemical Co., Inc.) for column chromatography; COSMOSIL 5C₁₈-AR-II (10.0 × 250 mm, Nacalai Tesque) for preparative HPLC.

Identification of bacterial strain

B. cereus IFM12235 was obtained from soil collected at a mountain road in Ikaho Shrine, Shibukawa City, Japan. *B. cereus* IFM12235 was identified by Dr. Takashi Yaguchi at the Medical Mycology Research Center, Chiba University, and a voucher specimen was deposited with the code IFM12235. This strain was identified by 16S rRNA gene sequencing.

Fermentation and isolation

An aliquot (1 mL each) of the stock strain of *B. cereus* IFM12235 was added to 20 mL of Waksman liquid medium consisting of 2.0% glucose, 0.5% meat extract, 0.5% peptone, 0.5% NaCl, 0.3% dried

yeast, and 0.3% CaCO₃ in a 50-mL Erlenmeyer flask and cultured at 160 rpm for 3 days at 28 °C. The resulting cultures (10 mL each) were transferred to three 500-mL Sakaguchi flasks containing 200 mL of Waksman liquid medium and incubated at 28 °C for 3 days, with shaking at 160 rpm. Each of the resulting 50 mL cultures was added to 10 turnip-shaped flasks (capacity 3 L) containing 1 L Waksman liquid medium and grown with shaking (160 rpm) at 28 °C for 5 days. Subsequently, this culture broth (10 L) was centrifuged at 5000 rpm for 10 min to obtain the supernatant and mycelium cake. The mycelial cake was extracted with MeOH (1 L × 2). The MeOH extract was combined with the supernatant obtained above, and the combined materials were partitioned between EtOAc (10 L × 3) and water to obtain the EtOAc extract (3.2 g). The EtOAc extract showed antibacterial activity against *B. cereus* IFM12235.

The EtOAc extract (3.2 g) was subjected to silica gel column chromatography ($\phi 32 \times 250$ mm, hexane-EtOAc system) to obtain fractions 1A–1I. A portion of fraction 1C (hexane: EtOAc = 2: 1, 161.2 mg) was subjected to reversed-phase HPLC (COSMOSIL 5C₁₈-AR-II [$\phi 10.0 \times 250$ mm]; eluent: 60% MeOH; flow rate: 3.0 mL/min) to extract compounds **3** (4.4 mg, t_R 7.0 min), **1** (2.3 mg, t_R 8.0 min), **2** (3.2 mg, t_R 9.0 min), **5** (6.5 mg, t_R 12.0 min), **4** (3.7 mg, t_R 15.0 min), and **6** (4.9 mg, t_R 19.0 min).

Ikahonone (1)

Colorless solid. $[\alpha]_D^{24} +10$ (c 1.0, CHCl₃). HR-ESI-MS: m/z 231.0960 [M + Na]⁺ (calculated for C₁₂H₁₆NaO₃ 231.0997). UV λ_{max} (MeOH) nm (log ϵ) 204 (5.7) IR (ATR) cm⁻¹: 1750, 1710, 1360, and 1220. CD λ_{max} (MeOH) nm ($\Delta\epsilon$): 217 (-12.5) and 298 (9.7). The ¹H and ¹³C NMR data are presented in Table 1.

(2R)-2-Hydroxy-1-(4-hydroxyphenyl)-4-methyl-3-hexanone (2)

Colorless solid. $[\alpha]_D^{22} +75$ (c 1.0, CHCl₃). HR-ESI-MS: m/z 467.2444 [2M + Na]⁺ (calculated for C₂₆H₃₆NaO₆ 467.2410). UV λ_{max} (MeOH) nm (log ϵ): 225 (5.2) nm and 280 (4.5). IR (ATR) cm⁻¹: 3370, 2970, 1710, 1450, and 1370. CD λ_{max} (MeOH) nm ($\Delta\epsilon$): 208 (-2.3), 233 (-0.2), and 292 (2.6). ¹H-NMR (CDCl₃) δ 7.10 (d, 8.5, H-2, 6), 6.75 (d, 8.5, H-3, 5), 4.46 (m, H-8), 3.47 (br, 8-OH), 3.07 (dd, 14.4, 4.8, H-7), 2.75 (m, H-7), 2.70 (m, H-10), 1.66 (m, H-11), 1.44 (m, H-11), 1.05 (d, 6.9, H-13), and 0.88 (t, 7.5, H-12). ¹³C-NMR (CDCl₃) δ 215.4 (C-9), 154.4 (C-4), 130.3 (C-2, 6), 128.8 (C-1), 115.4 (C-3, 5), 76.8 (C-8), 43.1 (C-10), 39.2 (C-7), 27.1 (C-11), 14.7 (C-13), and 11.4 (C-12).

(3S)-3-Hydroxy-1-phenyl-2-butanone (3)

ESI-MS: m/z 187 [M + Na]⁺. $[\alpha]_D^{24} +12$ (c 1.0, CHCl₃).

(2R, 3R)-4-Methyl-1-phenyl-2,3-pentanediol (4)

ESI-MS: m/z 217 [M + Na]⁺, 389 [2M + H]⁺, 411 [2M + Na]⁺. $[\alpha]_D^{24} +58$ (c 1.0, CHCl₃).

Xenocyloin A (5)

ESI-MS: m/z 232 $[M + H]^+$, 254 $[M + Na]^+$, 485 $[2M + Na]^+$. $[\alpha]_D^{24} +83$ (c 1.0, $CHCl_3$).

Xenocloin B (6)

ESI-MS: m/z 246 $[M + H]^+$, 268 $[M + Na]^+$, 245 $[M - H]^-$. $[\alpha]_D^{24} +131$ (c 1.0, $CHCl_3$).

CD λ_{max} (MeOH) nm ($\Delta\epsilon$): 225 (-13.9), 240 (-6.8), and 292 (11.9).

Antibacterial activity

An aliquot (1 mL each) of the stock strain of *B. subtilis* IFM2060 was added to 5 mL of nutrient broth (NB) liquid medium consisting of 1.6% NB in a 10-mL Erlenmeyer flask and cultured at 160 rpm for 24 h at 28 °C. After *B. subtilis* was inoculated on a 96-well clear plate at a density of 1.0×10^4 colony-forming unit (CFU)/well, 1 μ L of sample solutions prepared at an appropriate concentration was immediately added to each well and cultured for 24 h at 28 °C. After culturing *B. subtilis* with the samples, 10 μ L of a 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to each well. Following cultivation for 12–24 h, the absorbance of each well was measured at 570 nm to determine the viability of *B. subtilis*.

Cell cytotoxicity assay

Cell viability was measured using the fluorometric microculture cytotoxicity assay (FMCA) method.¹⁸ Mouse macrophage cell line, J774.1 cells were seeded at a density of 4×10^3 cells/well onto a 96-well black microplate and pre-cultured in a CO₂ incubator at 37 °C. After incubation for 24 h, the medium was removed, and 200 μ L of the sample containing Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), adjusted to an appropriate concentration, was added to each well. Next, the cells were cultured for 72 h. After removing the medium, the cells were washed once with 200 μ L of phosphate-buffered saline (PBS); then, 200 μ L of the fluorescein diacetate solution (3.5 μ g/mL) was added to each well. After incubation for 1 h, fluorescein fluorescence was measured (excitation, 485 nm; emission, 538 nm). Data are presented as the mean \pm standard deviation (SD) of three independent experiments. Dimethyl sulfoxide (DMSO) was used as a negative control.

NO production assay

The mouse macrophage cell line, RAW264 cells, were seeded at a density of 3×10^4 cells/well onto 96-well black microplates and then pre-cultured in a CO₂ incubator at 37 °C. After incubation for 24 h, the medium was removed, and 90 μ L of the sample containing DMEM with 10% FBS, adjusted to an appropriate concentration, was added to each well. After incubation for 2 h, 10 μ L of LPS (from *E. coli* O55, Wako, 127-05141) was added to afford cell stimulation for 24 h (final concentration of LPS, 1 μ g/mL). The nitrite concentration was measured in the supernatant using the Griess reaction. The culture supernatant (50 μ L) was transferred into a 96-well clear plate, with 50 μ L of the Griess solution, consisting of 2.5% phosphoric acid, 1.0% sulfanilamide, and 0.1% N-1-naphthylethylenediamine dihydrochloride. After reacting for 30 min in the dark, the absorbance of each well was measured at 570

nm. The amount of nitrite produced was calculated from the absorbance of the calibration curve prepared using NaNO₃. The remaining cells and solutions in the 96-well black plate were used to measure the viability of RAW264 cells by the FMCA method. Data are presented as the mean \pm SD of three independent experiments. BOT-64 cells were used as the positive control.

Conformational analyses and ECD calculations for 1 and 2

Conformational searches were performed with the Spartan18 software (Wavefunction Inc.) using a PC (Windows 10 Home; Intel Core i7-10770; 2.90 GHz; RAM 16 GB). Stable conformers were initially searched using the Merck molecular force field (MMFF) method. Stable conformers suggested by MMFF were optimized using the Hartree-Fock (HF)/3-21G model, and stable conformers up to 10 kJ/mol were further optimized using the B3LYP/6-31G model. ECD calculations for compounds were performed using Gaussian R16W (Gaussian) on a PC (Windows 10 Education; Intel Xeon E31245; 3.30 GHz; RAM 16 GB). The dominant conformers of compounds covered >80% of the population, according to Boltzmann's law. For these conformers, time-dependent density functional theory (TDDFT) calculations were conducted at the B3LYP/def2-TZVP level. The resulting rotational strength data were converted to Gaussian curves to obtain ECD spectra of the different conformers. The spectra were corrected to obtain the theoretical ECD spectrum.

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