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Flavocristamides A and B, New DNA Polymerase α Inhibitors from a Marine Bacterium *Flavobacterium* sp.

Jun'ichi Kobayashi*, Saika Mikami, Hideyuki Shigemori, Toshifumi Takao[a], Yasutsugu Shimonishi[a], Shunji Izuta[b], and Shonen Yoshida[b]

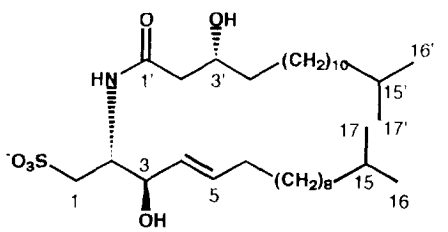
Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan, [a]Institute for Protein Research, Osaka University, Osaka 565, Japan, and [b]Research Institute for Disease Mechanism and Control, Nagoya University School of Medicine, Nagoya 466, Japan

Abstract: Flavocristamides A (**1**) and B (**2**), two new sulfonolipids with inhibitory activity against DNA polymerase α, have been isolated from a marine bacterium *Flavobacterium* sp. and the structures containing absolute stereochemistry were determined by spectroscopic data and chemical means.

Marine microorganisms have proven to be a rich source of compounds which might be useful for the development of new pharmaceutical agents.[1] In our search for bioactive compounds from marine microorganisms,[2] we have examined extracts of marine bacteria and isolated two new 1-deoxyceramide-1-sulfonates possessing inhibitory activitiy against DNA polymerase α, named flavocristamides A (**1**) and B (**2**), from the cultured mycelium of a bacterium *Flavobacterium* sp., which was separated from the marine bivalve *Cristaria plicata*. In this paper we describe the isolation and structure elucidation of **1** and **2**, and its inhibition of DNA polymerase α.

The bacterium *Flavobacterium* sp. was isolated from the marine bivalve *Cristaria plicata* collected at Ishikari Bay, Hokkaido, and grown statically in Zobell broth [peptone (Diffco) 0.1%, yeast extract (Diffco) 0.02% in 90% sea water, pH 7.6] at 25°C for 10 days. The bacterial cells (28 g, wet weight from 14 L of culture) were extracted with $CHCl_3$/MeOH (1:1) and the extract was partitioned between EtOAc and H_2O. The EtOAc soluble portions were subjected to silica gel columns followed by a reversed-phase column to afford flavocristamides A (**1**, 0.006% wet weight) and B (**2**, 0.004%).

Flavocristamide A (**1**) was obtained as a colorless amorphous solid {$[\alpha]^{20}_D$ -17° (*c* 0.27, MeOH)}. HRFABMS analysis revealed the molecular formula to be $C_{34}H_{66}NO_6S$ [*m/z* 616.4594, (M^-), Δ -1.7



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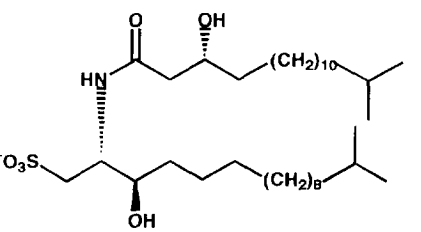

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mmu]. IR absorptions of **1** implied the presence of OH and/or NH (3450 cm^{-1}), amide carbonyl (1640 and 1560 cm^{-1}), and sulfonate (1060 cm^{-1}) groups, respectively. The presence of a sulfonate group was also supported by negative FABMS fragment ions at *m/z* 80 (SO_3^-) and 94 ($CH_2SO_3^-$). The ^1H NMR spectrum of **1** in CD_3OD showed signals due to four secondary methyls (δ_H 0.92), two olefinic protons (δ_H 5.52 and 5.78), and long aliphatic chains (δ_H 1.1 ~ 1.4, 32H). A methylene (δ_H 3.05 and 3.16; δ_C 51.7) was connected to the sulfonate group from comparison of the chemical shifts with those of aliphatic sulfonic acids.[3] The ^{13}C chemical shifts of C-2 (δ_C 52.7) and C-3 (δ_C 75.0) indicated that a nitrogen and an oxygen atoms were attached at C-2 and C-3, respectively. *E*-Geometry of the double bond at C-4 was ascertained by the proton coupling constant ($J_{4,5}$ = 15.5 Hz). The HMBC correlation for H-2 to C-1' indicated that a fatty acid was attached at C-2 through an amide bond. This fatty acid was elucidated to be 3-hydroxy-15-methylhexadecanoic acid from the fragment ions at *m/z* 349 and 419 in the negative-ion FABMS/MS spectrum (Fig. 1). Methanolysis of **1** with 1N HCl/82% MeOH gave methyl ester (**3**) of the fatty acid and an aminosulfonic acid (**4**) (Scheme 1), in which absolute stereochemistry of the former (**3**) at C-3 was identified as *R* by ^1H NMR and EIMS data, and the optical rotation ($[\alpha]_D$ -20°; lit.[4] $[\alpha]_D$ -13°). The relative stereochemistries at C-2 and C-3 in **4** were elucidated to be 2,3-erythro by comparison of proton coupling constants ($J_{2,3}$ and $J_{3,4}$ = 7.1 Hz) with those ($J_{2,3}$ and $J_{3,4}$ = 6.7 Hz) of sphingosine. The cysteic acid generated by $NaIO_4/KMnO_4$ oxidation[5] of **1** was determined to be L-form by chiral HPLC analysis (SUMICHIRAL OA-5000). Thus the absolute stereochemistries of **1** were assigned to be 2*S*, 3*R*, and 3'*R*, respectively.

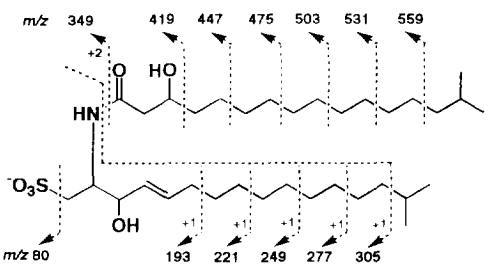
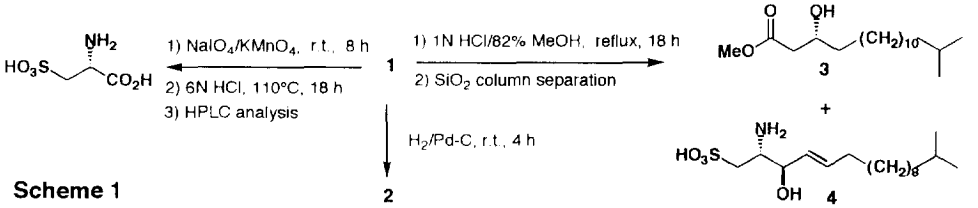


Fig. 1 FABMS/MS Fragment Ions of Flavocristamide A (1) (*m/z* 616, M^-)

Flavocristamide B (**2**) was shown to have the molecular formula, $C_{34}H_{68}NO_6S$, by HRFABMS [*m/z* 618.4750 (M^-), Δ -1.8 mmu]. ^1H and ^{13}C NMR data were very similar to those of flavocristamide A (**1**) except for lack of olefine proton signals observed for **1**. Comparison of FABMS/MS fragment pattern between **1** and **2** implied the presence of 3-hydroxy-15-methylhexadecanoic acid in **2**. Flavocristamide B (**2**) was concluded to be 3,4-dihydro form of flavocristamide A (**1**), since all spectral data ($[\alpha]_D$, ^1H NMR, IR, and FABMS) of hydrogenolysis product of **1** were identical with those of flavocristamide B (**2**).



Scheme 1

Flavocristamides A (**1**) and B (**2**) exhibited inhibitory activity against, an eukaryotic DNA replication enzyme, DNA polymerase α (Fig. 2). The sulfonate group in **1** and **2** seems to be important for the inhibitory activity since ceramide has been reported to have no effect to DNA polymerase α[6], while the double bond at C-4 and C-5 of **1** is not essential for the inhibition. Detailed analysis of the inhibition will appear elsewhere.

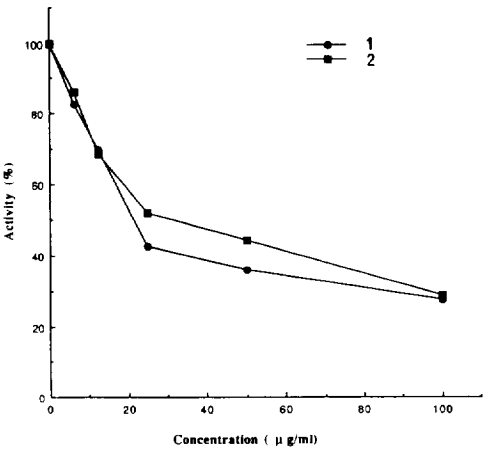


Fig. 2 Inhibition of Calf Thymus DNA Polymerase α by Flavocristamides A (**1**) and B (**2**). Calf thymus DNA polymerase α (0.05 U, 5 μL) was pre-incubated with the various concentrations (5 μL) of compounds **1** and **2** on ice for 30 min, then reaction mixture (15 μL) was added. Incubation was performed for 30 min at 37°C. 100% corresponds to 7610 cpm. Final concentrations of **1** and **2** are shown on horizontal axis.

Experimental Section

General Methods. Optical rotations were determined on a JASCO DIP-370 polarimeter. ^1H and ^{13}C NMR spectra were recorded on JEOL EX-400 and Bruker ARX-500 spectrometers. The 3.35 and 7.26 ppm resonances of residual CD_2HOD and $CHCl_3$, respectively, and 49.0 and 77.0 ppm of CD_3OD and $CDCl_3$, respectively, were used as internal references. EIMS and FABMS spectra were obtained on a JEOL DX-303 spectrometer operating at 70 eV and on a JEOL HX-110 spectrometer, respectively.

Collection and Cultivation. The bacterium *Flavobacterium* sp. was isolated from the bivalve *Cristaria plicata* which was collected at Ishikari Bay, Hokkaido. Cultures of *Flavobacterium* sp. were grown in Zobell broth [peptone (Diffco) 0.1%, yeast extract (Diffco) 0.02% in 90% sea water, pH 7.6]. Cultures were incubated statically at 25°C for 10 days. The cells were harvested by centrifugation (5000 rpm, 10 min).

Extraction and Separation. The bacterial cells (28 g, wet weight) from 14 L of culture were extracted with $CHCl_3$/MeOH (1:1, 100 mL x 3) and evaporated under reduced pressure. The extract was partitioned between EtOAc (100 mL x 3) and H_2O (100 mL). The EtOAc soluble portions (0.17 g) were subjected to a silica gel column (1.0 x 35 cm) eluted with $CHCl_3$/MeOH [95:5 (320 mL) → 85:15 (170 mL)] to afford a fraction (320 ~ 460 mL), which was applied to a silica gel column (2 x 10 cm) with $CHCl_3$/*n*-BuOH/AcOH/H_2O (1.5:6:1:1). The fraction (20 ~ 50 mL) was purified by C_{18} reversed-phase HPLC (DEVELOSIL ODS-HG-5, Nomura Chemical, 1.0 x 25 cm; flow rate 2.5 mL/min; RI detection; eluent MeOH/H_2O, 90:10) to afford flavocristamides A (**1**, 1.6 mg, t_R 11.0 min) and B (**2**, 1.1 mg, t_R 12.0 min).

Flavocristamide A (1). A colorless amorphous solid; $[\alpha]^{20}_D$ -17° (*c* 0.27, MeOH); IR (KBr) ν_{max} 3450, 1640, 1560, 1200, and 1060 cm^{-1}; ^1H NMR (CD_3OD) δ 0.92 (12H, d, *J* = 6.7 Hz, H-16, H-17, H-16', and H-17'), 1.1 ~ 1.4 (32H, m, H-7 ~ H-13 and H-5' ~ H-13'), 1.21 (4H, m, H-14 and H-14'), 1.50 (1H, m, H-4'), 1.55 (2H, m, H-15 and H-15'), 2.09 (2H, m, H-6), 2.35 (2H, m, H-2'), 3.05 (1H, dd, *J* = 14.4 and 8.8 Hz, H-1b), 3.16 (1H, dd, *J* = 14.4 and 3.2 Hz, H-1a), 4.00 (1H, m, H-3'), 4.23 (1H, m, H-3), 4.37 (1H, m, H-2), 5.52 (1H, dt, *J* = 15.5 and 7.1 Hz, H-4), and 5.78 (1H, dd, *J* = 15.5 and 6.8 Hz, H-5); ^{13}C NMR (CD_3OD) δ 23.1 (q, C-16, C-17, C-16', and C-17'), 26.7 (t), 28.6 (t), 29.2 (d, C-15 and C-15'), 30.4 (t), 30.5 (t), 30.7 (t), 30.8 (t), 30.9 (t), 31.1 (t), 33.5 (t), 34.9 (t, C-6), 38.1 (d, C-4'), 40.3 (t, C-14 and C-14'), 45.6 (t, C-2'), 51.7 (t, C-1), 52.7 (d, C-2), 69.8 (d, C-3'), 75.0 (d, C-3), 130.5 (d, C-4), and 134.9 (d, C-5); FABMS (negative, glycerol matrix) *m/z* 616 (M^-), 94 ($CH_2SO_3^-$), and 80 (SO_3^-); HRFABMS *m/z* 616.4594 (M^-), calcd for $C_{34}H_{66}NO_6S$, 616.4611.

Flavocristamide B (2). A colorless amorphous solid; $[\alpha]^{20}_D$ -7.9° (*c* 0.18, MeOH); IR (KBr) ν_{max} 3450, 1640, 1560, 1200, and 1060 cm^{-1}; ^1H NMR (CD_3OD) δ 0.92 (12H, d, *J* = 6.7 Hz, H-16, H-17, H-16',

and H-17'), 1.1 ~ 1.4 (40H, m, H-4 ~ H-13 and H-4' ~ H-13'), 1.23 (4H, m, H-14 and H-14'), 1.57 (2H, m, H-15 and H-15'), 2.37 (2H, m, H-2'), 3.05 (1H, dd, J = 14.4 and 10.0 Hz, H-1b), 3.17 (1H, dd, J = 14.4 and 3.1 Hz, H-1a), 3.73 (1H, m, H-3'), 4.01 (1H, m, H-3), and 4.28 (1H, m, H-2); ^{13}C NMR (CD_3OD) δ 22.8 (q, C-16, C-17, C-16', and C-17'), 26.5 (t), 26.8 (t), 28.4 (t), 29.0 (d, C-15 and C-15'), 30.3 (t), 30.6 (t), 30.9 (t), 34.4 (t), 37.9 (t), 40.1 (t, C-14 and C-14'), 45.3 (t, C-2'), 51.3 (t, C-1), 52.7 (d, C-2), 69.6 (d, C-3'), and 73.8 (d, C-3); FABMS (negative, glycerol matrix) *m/z* 618 (M^-), 94 ($CH_2SO_3^-$), and 80 (SO_3^-); HRFABMS *m/z* 618.4750 (M^-), calcd for $C_{34}H_{68}NO_6S$, 618.4768.

Methanolysis of 1. Compound **1** (1.0 mg) was refluxed with 1N HCl in 82% MeOH (1 mL) for 18 h. The reaction mixture was extracted with *n*-hexane (1 mL x 2), the *n*-hexane layer was concentrated under reduced pressure, and this residue was chromatographed on a silica gel column [*n*-hexane/acetone (3.5:1)] to yield compound **3** (0.4 mg). The aqueous MeOH layer was diluted with $CHCl_3$ (1 mL) and H_2O (1 mL), the $CHCl_3$ layer was evaporated under reduced pressure. The residue was purified by a silica gel column [$CHCl_3$/MeOH/H_2O (65:25:4)] to give compound **4** (0.3 mg). **Compound 3**: A colorless oil; $[\alpha]^{20}_D$ -20° (*c* 0.07, *n*-hexane); IR (film) ν_{max} 3550, 1720, and 1175 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.86 (6H, d, J = 6.6 Hz), 1.00 ~ 1.50 (23H, m), 2.41 (1H, dd, J = 16.4 and 9.0 Hz), 2.52 (1H, dd, J = 16.4 and 3.0 Hz), 2.80 (1H, br), 3.71 (3H, s), and 4.00 (1H, m); EIMS *m/z* 300 (M^+); HREIMS *m/z* 300.2661 (M^+), calcd for $C_{18}H_{36}O_3$, 300.2664. **Compound 4**: A colorless amorphous solid; $[\alpha]^{20}_D$ -9.4° (*c* 0.05, MeOH); IR (film) ν_{max} 3400, 1500, and 1200 cm^{-1}; ^{1}H NMR (CD_3OD) δ 0.92 (6H, d, J = 6.7 Hz, H-16 and H-17), 1.20 ~ 1.50 (16H, m, H-7 ~ H-14), 1.56 (1H, m, H-15), 2.16 (2H, m, H-6), 2.80 (1H, m, H-2), 2.97 (1H, dd, J = 14.8 and 3.0 Hz, H-1b), 3.13 (1H, dd, J = 14.8 and 6.8 Hz, H-1a), 4.16 (1H, t, J = 7.1 Hz, H-3), 5.51 (1H, dd, J = 15.4 and 7.1 Hz, H-4), and 5.92 (1H, dt, J = 15.4 and 6.5 Hz, H-5); FABMS (negative, glycerol matrix) *m/z* 348 $(M-H)^-$; HRFABMS *m/z* 348.2186 $(M-H)^-$, calcd for $C_{17}H_{34}NO_4S$, 348.2209.

Determination of the Absolute Stereochemistry at C-2 in 1. A stock oxidant solution of $NaIO_4$ (2.09 g) and $KMnO_4$ (0.04 g) in H_2O (100 mL) was prepared. This solution (40 μL) together with K_2CO_3 solution (40 μL; 2.5 g/L) was added to **1** (0.2 mg) in H_2O (0.5 mL) and the mixture was stirred at room temperature for 8 h. After the solution was acidified with 1N HCl aq. and excess oxidant was destroyed with KI. The solution was extracted with *n*-BuOH (0.5 mL x 2). The organic layer was washed by $Na_2S_2O_3$ aq. and evaporated under reduced pressure. The residue was hydrolyzed with 6N HCl aq. at 110°C for 18 h. The residue was dissolved in H_2O for chiral HPLC analysis. The chiral HPLC analysis was carried out using a SUMICHIRAL OA-5000 column [Sumitomo Chemical Industry, 4 x 150 mm; flow rate: 0.5 mL/min; eluent: H_2O containing 0.5 mmol $CuSO_4$; detection: UV at 254 nm]. Retention times of standard L- and D-cysteic acid were 21.4 and 25.7 min, respectively, and that of cysteic acid contained in oxidative products of **1** was found to be 21.4 min.

Hydogenation of 1. A solution of compound **1** (0.4 mg) in MeOH (0.5 mL) was stirred in the presence of 5% Pd-C (0.4 mg) under H_2 for 4h at room temperature. The reaction mixture was filtered through celite and the filtrate was evaporated under reduced pressure. The residue was purified by a silica gel column (0.4 x 5 cm) with $CHCl_3$/MeOH/H_2O (65:25:4) to give a reduction product (0.4 mg): $[\alpha]^{23}_D$ -7.1° (*c* 0.05, MeOH); ^{1}H NMR (CD_3OD) δ 0.92 (12H, d, J = 6.7 Hz, H-16, H-17, H-16', and H-17'), 1.1 ~ 1.4 (40H, m, H-4 ~ H-13 and H-4' ~ H-13'), 1.23 (4H, m, H-14 and H-14'), 1.57 (2H, m, H-15 and H-15'), 2.37 (2H, m, H-2'), 3.05 (1H, dd, J = 14.4 and 10.0 Hz, H-1b), 3.17 (1H, dd, J = 14.4 and 3.1 Hz, H-1a), 3.73 (1H, m, H-3'), 4.01 (1H, m, H-3), and 4.28 (1H, m, H-2); FABMS (negative, glycerol matrix) *m/z* 618 (M^-), 94 ($CH_2SO_3^-$), and 80 (SO_3^-).

Assay of DNA Polymerase α. The standard reaction mixture for DNA polymerase α (25 μL) contained 40 mM potassium phosphate (pH 7.2), 4 mM dithiothreitol, 40 μM each of dATP, dGTP, and dCTP, and [^{3}H]dTTP (200 cpm/pmol), 8 mM $MgCl_2$, 200 μg/mL of activated calf thymus DNA, and 0.3 units calf thymus DNA polymerase α.[6] DNA polymerase α and the inhibitor was pre-incubated on ice for 30 min, then reaction mixture was added. Incubation was performed for 30 min at 37°C. The activity without an inhibitor was taken at 100%, and the remaining activity at the several concentration of inhibitor was determined as percent.

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