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Streptokordin, a New Cytotoxic Compound of the Methylpyridine Class from a Marine-derived *Streptomyces* sp. KORDI-3238

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Abstract A new cytotoxic compound, streptokordin, and four known compounds, nonactic acid, dilactone, trilactone, and nonactin, were isolated from the fermentation broth of a marine actinomycete strain collected in deep-sea sediments. Biochemical tests and 16S rDNA analysis indicated that the strain belongs to the genus *Streptomyces*. This actinomycete produces various bioactive secondary metabolites. Fractionations by solvent partitioning, silica vacuum flash chromatography, and reversed-phase HPLC gave a pure cytotoxic compound, designated streptokordin. Its structure was elucidated by FAB-MS, ^{1}H, ^{13}C, and 2D NMR spectroscopy. Streptokordin exhibited significant cytotoxicity against seven human cancer cell lines but showed no growth inhibition against various microorganisms including bacteria and fungi.

Keywords *Streptomyces* sp. KORDI-3238, 16S rDNA, cytotoxic, methylpyridine, 4-acetyl-6-methyl-1-*H*-pyridine-2-one

Introduction

The genus *Streptomyces* was first described by Waksman and Henrici [1] and is rich source of bioactive natural products which are extensively used as pharmaceuticals [2]. These bacteria produce about 75% of medically useful agents [3] including a wide array of anti-infective, antibacterial, antifungal, and antitumor agents [4, 5]. The decreasing rate of discovery of novel drugs from established terrestrial sources has motivated the evaluation of new sources of chemically diverse bioactive compounds [6]. The oceans represent an under-explored environment for microbial diversity and novel secondary metabolites. Marine sediments, in particular, have been largely overlooked [7].

In the course of our screening program for bioactive marine natural products, we isolated marine actinomycete strains from deep-sea sediments at various sites in the western Pacific Ocean. Strain KORDI-3238 was isolated from a deep-sea sediment sample collected at Ayu Trough, after applying dry heat to the soil. Biochemical tests including chemotaxonomic methods and partial 16S rDNA analysis indicated that the strain belongs to the genus *Streptomyces*. The crude extracts of this strain exhibited potent cytotoxic activity against the human leukemia cell line (K-562). Bioassay-guided fractionation by solvent partitioning and purification with a reversed-phase HPLC gave a pure cytotoxic compound. Based upon the results of combined spectral analyses, the structure of this compound was determined to be a new methylpyridine derivative named streptokordin (**1**, Fig. 1). In this report, we describe the fermentation and taxonomy of *Streptomyces* sp. KORDI-3238 and the isolation, physico-chemical properties, structure elucidation and biological properties of streptokordin (**1**).

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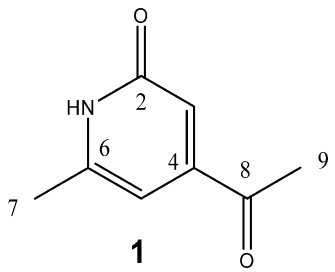

Fig. 1 Structure of streptokordin (**1**) produced by *Streptomyces* sp. KORDI-3238.

Materials and Methods

Microorganism

The producing strain KORDI-3238 was isolated from a deep-sea sediment at Ayu Trough (01°38′355N, 132°43′591E) in the Pacific Ocean. The sediment core sample was collected in 2001 as part of 'Daeyang Program' in the western Pacific Ocean and Philippine Sea by the Korea Ocean Research & Development Institute (KORDI). One gram of sediment was incubated for 50 minutes at 60°C and resuspended in 20 ml of autoclaved seawater. After filtration and dilution with autoclaved seawater (10^{-1}, 10^{-2}, and 10^{-3}, respectively), 0.1 ml aliquots were spread onto starch-casein agar [8], yeast extract - malt agar, International *Streptomyces* Project (ISP) medium 2, inorganic salt - starch agar (ISP medium 4) [9] and modified Bennett's agar [10]. The plates were incubated for 14 days at 30°C, and the resulting colonies were transferred and maintained on the modified Bennett's agar. Among the actinomycete strains isolated, one strain that showed significant cytotoxic activity was designated *Streptomyces* sp. KORDI-3238.

Taxonomy of the Producing Strain KORDI-3238

This strain KORDI-3238 was grown at 27°C for 7 days on ISP-1 (tryptone - yeast extract agar), ISP-2, ISP-4, and ISP-6 (peptone - yeast extract - iron agar) and modified Bennett's agar, and examined visually to determine substrate mycelium pigmentation and spore color. All media used in this study were prepared with natural seawater instead of distilled water and 15 g of agar/liter was added to make agar plates. Morphological characteristics were observed with a scanning electron microscope (Phillips model 515). Standard physiological and biochemical properties of this strain were tested according to the methods of Williams *et al.* [11]. All carbon-sources for carbon-utilization tests were filter-sterilized and tested at the concentrations recommended by Shirling and Gottlieb [9] and Williams *et al.* [11]. The diaminopimelic acid (DAP) isomer [12] and whole-cell sugar pattern [13] were determined by TLC of the whole-organism hydrolysate. Extraction of genomic DNA and 16S rRNA gene amplification were carried out according to Rainey *et al.* [14]. The resulting PCR product was ligated into a pGEM-T easy vector (Promega), sequenced using a Termination Sequencing Ready Reaction kit (Perkin Elmer) and analyzed using an ABI 377 genetic analyzer (Perkin Elmer). The partial 16S rDNA sequence (1461 bp) was aligned using CLUSTAL W software Ver. 1.7 [15]. 16S rDNA sequences used for the phylogenetic analyses were derived and compared with those of other bacterial 16S rDNA sequences available in the DDBJ/EMBL/GenBank database. Kimura's two-parameter model [16] was applied to the calculation of evolutionary distance. A phylogenetic tree was constructed by the neighbor-joining method [17]. Bootstrap analyses of 1000 replicates were carried out using MEGA version 2.0 [18].

Fermentation

A 500-ml Fernbach flask containing 100 ml of the seed medium (ISP-2 medium) was inoculated with a stock culture of the producing strain maintained on a modified Bennett's agar slant. After incubation at 27°C for 4 days on a rotary shaker set at 150 rpm, 20 ml of seed culture was transferred to each of ten 3-liter Fernbach flasks containing 800 ml of the production (modified Bennett's) medium. The fermentation was carried out at 27°C for 7 days on a rotary shaker set at 200 rpm.

Isolation and Physico-chemical Characterization

Streptokordin (**1**) was isolated by the following procedures: After 7 days, the culture broth was centrifuged (2,000×*g* for 15 minutes at 4°C; BECKMAN optima XL-100K ultracentrifuge, USA), and then filtered (0.2 μm pore-size membrane filter) to obtain a cell-free supernatant, followed by extraction with EtOAc. The EtOAc layer was concentrated and the residual suspension (450 mg) was subjected to silica vacuum flash chromatography using gradient mixtures of *n*-hexane and EtOAc as eluents. The fractions eluted with 40% and 60% EtOAc in hexane from silica vacuum flash chromatography were combined (100 mg) and purified by reversed-phase HPLC (YMC ODS-A column, 10×250 mm; 50~80% MeOH; flow rate, 1.5 ml min^{-1}; UV detection at 210 nm) to yield a new cytotoxic compound (**1**, 11.5 mg; Fig. 1) and four known compounds, nonactic acid (5.2 mg), dilactone (8.3 mg), trilactone (1.6 mg), and nonactin (17.4 mg). Their structures were determined by FAB-MS, ^{1}H, ^{13}C, and 2D NMR spectroscopy.

Instrumentations

^{1}H, ^{13}C and two-dimensional nuclear magnetic resonance (2D NMR) spectra were measured on a Varian Unity 500 NMR spectrometers in CD_3OD-d_4 at 300 K. ^{1}H and ^{13}C NMR spectra were measured at 500 and 125 MHz, respectively. The resonances of residual CD_3OD-d_4 at δ_H 3.30 and δ_C 49.0 were used as internal references for ^{1}H and ^{13}C NMR spectra, respectively. Mass spectra, including high resolution mass measurement, were measured by using a JEOL JMS-HX 110 mass spectrometer and provided by the Korea Basic Science Institute, Taejeon, Korea. Ultraviolet spectrum was measured in methanol using a Milton-Roy spectrophotometer. IR spectrum was measured on a Mattson Galaxy FT-IR spectrophotometer. All solvents used were spectral grade or were distilled from glass prior to use.

Biological Assays

For cytotoxicity test, a sulforhodamine B (SRB) assay was performed against human cancer cell lines as described previously [19]. Cell suspensions were adjusted to cell densities of 2×10^4 cells/ml in order to assure exponential growth throughout drug exposure. Aliquots of 200 μl/well of these suspensions were used to seed microcultures in a 96-well mircoplate. After incubation for 24 hours, cells were exposed to solutions of compounds. For this purpose, the compounds were dissolved in MeOH and then serially diluted. After incubation for 48 hours in the presence of the compounds, anchorage-dependent cells were directly fixed by slow addition of 50 ml of 50% trichloroacetic acid (TCA) solution per well. Anchorage-independent cells were fixed by pre-centrifugation (150×*g*, 1 minute at 20°C) and the drop-wise addition of 50% TCA. Fixation proceeded for 1 hour at 4°C. After fixation, plates were washed five times with tap water, and air-dried. One hundred microliters of SRB solution (0.4% in 0.1% acetic acid) was added to each well of the 96-well microplates. Staining was done at room temperature for 30 minutes. Residual dye was washed out with 0.1% acetic acid and air-dried. To each well, 100 ml of Tris solution (10 mM, pH 10.5) was added. Optical density (O.D.) was measured in a microtiter plate reader at 540 nm. Each sample concentration was tested in triplicate at least three times.

An agar overlay assay was used to determine the antibacterial and antifungal spectrum of the cytotoxic compound, streptokordin produced by strain KORDI-3238. Activity was tested against various Gram-positive, Gram-negative bacteria, fungi, and yeasts. Cultures of test microorganisms (100 μl) were suspended in 10 ml of soft agar (0.6%) of their specific media. The cells in the soft agar were poured on a solid agar plate of the same medium (1.5% agar). Streptokordin was dissolved in MeOH and 10 μl was applied on a 6-mm-diameter disc (Advantec Inc., Tokyo, Japan) and air-dried to remove MeOH. The discs were directly placed on the lawns of tested microorganisms and then the plates were incubated for 3 days at a temperature that permitted optimal growth of the test microorganisms. The growth inhibition was assessed by the size of clear inhibitory zone around the paper disc.

Results and Discussion

Taxonomy of the Producing Strain

The strain KORDI-3238 is a Gram-positive actinomycete which formed well-developed and branching substrate mycelium and aerial mycelium, but fragmentation of the substrate mycelium was not observed. Good growth was observed on ISP-1, ISP-2, ISP-4, and modified Bennett's agar. The best medium for the culture of this strain was modified Bennett's agar, on which it grew abundantly. This strain grew moderately well on ISP-6. Substrate and aerial mycelium color were medium-dependent. This strain formed pale yellow or light gray aerial mycelium on ISP-1, ISP-2, ISP-4, and modified Bennett's agar (Table 1). Melanin was not produced on ISP-6. The long spore chains were of the *Spirales*-type with smooth surface. Mature spores were oval with a diameter of (0.5~0.7)×(0.7~0.9) μm (Fig. 2). Special morphology such as sclerotia and sporangia were not observed. Physiological characteristics and carbon utilization of strain KORDI-3238 are summarized in Table 2. Diaminopimelic acid (DAP)

Table 1 Cultural characteristics of *Streptomyces* sp. KORDI-3238

Medium	Growth	Aerial mycelium	Substrate mycelium	Reverse color	Soluble pigment
Modified Bennett's agar	Very good	Abundant, pale yellow	Purplish red	Purplish red	Purplish red
Tryptone - yeast extract agar (ISP-1)	Good	Abundant, light gray	Deep yellow	Deep yellow	Pale yellow
Yeast extract - malt agar (ISP-2)	Good	Abundant, light gray	Light brown	Dark brown	Pale yellow
Inorganic salt - starch agar (ISP-4)	Good	Thick, light gray	Purplish red	Purplish red	None
Peptone - yeast extract - iron agar (ISP-6)	Moderate	Moderate, light gray	Pale yellow	Pale yellow	None

Fig. 2 Scanning electron micrograph of *Streptomyces* sp. KORDI-3238 grown on modified Bennett's agar at 27°C for 3 days. The bar represents 10 μm.

analysis showed the presence of LL-DAP in the peptidoglycan [20]. Glucose and ribose were detected in the whole-cell hydrolysates. Liquefaction of gelatin and hydrolysis of starch were positive at 7 days. Production of H_2S and reduction of nitrate (weak reaction after 14 days) were observed. This strain utilized D-Glucose, D-Xylose, L-Ramnose, D-mannitol, D-Sucrose, and D-Galactose as sole carbon sources, but not D-fructose and L-Arabinose.

The above results support the identification of this strain as a member of the genus *Streptomyces*. Furthermore, a BLAST search of 16S rDNA sequences available in the DDBJ/EMBL/GenBank database showed the highest similarity of 97% with *Streptomyces lavendulae* IFO 12789^T (D85116) (Fig. 3). The strain KORDI-3238 was deposited in the Korean Culture Center of Microorganisms (KCCM), with the name of *Streptomyces* sp. KORDI-3238 under the accession No. KCCM-10664P in accordance with Budapest treaty.

Fermentation and Isolation

Bioassay-guided purification of cytotoxic compound was conducted by determining the cytotoxic activity against the human leukemia cell line (K-562). *Streptomyces* sp. KORDI-3238 was grown at 27°C with shaking in a 3-liter glass flask containing modified Bennett's medium. After 7 days, the culture broth was centrifuged and then filtrated to obtain a cell-free supernatant. The cytotoxic compound was found in the culture supernatant, not in the cells. Fractionations by solvent partitioning, silica vacuum flash chromatography, and reversed-phase HPLC gave a new cytotoxic compound (**1**, 11.5 mg) as a colorless amorphous powder, and four known compounds, nonactic acid (5.2 mg), dilactone (8.3 mg), trilactone (1.6 mg), and nonactin (17.4 mg). The identification of four known compounds was confirmed by comparison with the chemical shift data from the literature [21, 22].

Table 2 Morphological and physiological characteristics and carbon utilization of *Streptomyces* sp. KORDI-3238

Spore chain morphology	Spirales
Spore surface	Smooth
Spore dimensions (μm)	(0.5~0.7)×(0.7~0.9)
Temperature range for growth (°C)	15~40
Optimal temperature for growth (°C)	25~30
Formation of melanoid pigment on ISP-6	−
Production of H_2S	+
Liquefaction of gelatin	+
Degradation of casein	−
Hydrolysis of starch	+
Reduction of nitrate	+
Carbon utilization	
D-Glucose	+
D-Xylose	+
L-Arabinose	−
L-Ramnose	+
D-Fructose	−
D-Mannitol	+
D-Sucrose	+
D-Galactose	+

Physico-chemical Characterization and Structure Determination

The new cytotoxic compound (**1**, Fig. 1) is soluble in MeOH, EtOH, CH_3CN, PrOH, Me_2CO, CH_2Cl_2, $CHCl_3$, and EtOAc, but is insoluble hexane and Et_2O. The physico-chemical properties of **1** are shown in Table 3. Compound **1** showed UV maxima at 217 nm (ε 10,500) and the positive FAB-MS spectrum of **1** indicated an intense peak at *m/z* 152. The molecular formula of **1** was established as $C_8H_9NO_2$ by HRFAB-MS analysis [*m/z* 152.0716 $(M+H)^+$, (Δ +0.4 mmu)] and ^{13}C NMR spectral data (Table 4). ^1H NMR data (Table 4) of **1** revealed resonances for two olefinic protons (δ 6.15, 6.19) and two methyl groups (δ 2.39, 2.55). Its ^{13}C NMR data also exhibited resonances for four olefinic carbons (δ 101.8, 112.2, 118.4, 142.8), two carbonyl carbons (δ 163.2, 206.5) and two methyl groups (δ 23.4, 33.1). ^1H and ^{13}C correlations were indicated by the gHSQCDEPT spectrum. The small coupling constant (J=2.2 Hz) between H-3 and H-5 suggested that these olefinic protons have a *meta* configuration. The gHSQCDEPT and HMBC analyses of **1** easily gave the structure of a methylpyridine. The carbonyl group (δ 163.2) was assigned at C-2 by its HMBC correlation with H-3, which also showed HMBC cross peaks to C-4 and C-

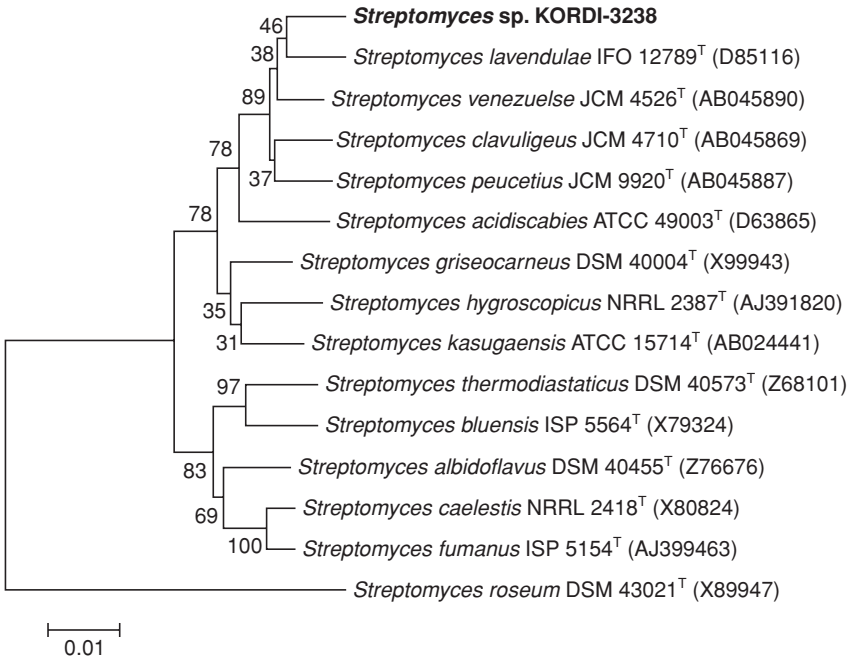

Fig. 3 Phylogenetic tree based on 16S rRNA gene sequence comparison indicating the position of *Streptomyces* sp. KORDI-3238 was Generated using the neighbor-joining method. Bootstrap values, expressed as percentages of 1000 replications, are given at branching points. Bar, 1 nucleotide substitutions per 100 nucleotides.

Table 3 Physico-chemical properties of streptokordin (**1**)

Appearance	Colorless amorphous powder
Molecular formula	$C_8H_9NO_2$
Molecular weight	151
FAB-MS (*m/z*)	
Positive	152 $[M+H]^+$
HRFAB-MS (*m/z*)	
Found	152.0716 $[M+H]^+$
Calcd	152.0712
UV λ_{max} nm in MeOH	217, 280
IR V_{max} cm^{-1} (MeOH)	1550, 1643, 1710

Table 4 ^1H and ^{13}C NMR Data of streptokordin (**1**) in CD_3OD-d_4

No.	^{13}C (mult)	^1H	HMBC
NH			
2	163.2 (s)		
3	101.8 (d)	6.15 (1H, d, *J*=2.2 Hz)	C-2, C-4, C-5
4	118.4 (s)		
5	112.2 (d)	6.19 (1H, d, *J*=2.2 Hz)	C-3, C-4, C-7
6	142.8 (s)		
7	23.4 (q)	2.39 (3H, s)	C-5, C-6
8	206.5 (s)		
9	33.1 (q)	2.55 (3H, s)	C-4, C-8

5. The methyl group (δ 2.39) was connected to C-6 by the HMBC correlations (H-7/C-6 and H-7/C-5). An acetyl unit was assigned by HMBC cross peak from a singlet methyl signal of H-9 (δ 2.55) to the ketone carbon signal of C-8 (δ 206.5). Moreover, this acetyl unit was connected to C-4 of the methylpyridine by the HMBC cross peak from H-9 to C-4. Thus, the gross structure of **1** was elucidated to be 4-acetyl-6-methyl-1-*H*-pyridin-2-one (Fig. 1).

Biological Activities

Streptokordin (**1**) exhibited significant *in vitro* cytotoxicity against several human cancer cell lines (Table 5) with IC_{50} values of less than 10 μg/ml. Furthermore, antibacterial and antifungal activities of streptokordin were also investigated to know anti-microbial effects on other microorganisms. Streptokordin did not show any obvious inhibitory effects on the growth of the following species at the concentration of 1.0 mg/ml: Gram-positive bacteria (*Bacillus cereus* KCTC-1012, *B. subtilis* KCTC-1021, *Listeria monocytogenes* KCTC-3569, and *Staphylococcus aureus* KCTC-1916), Gram-negative bacteria (*Escherichia coli* KCTC-1923, *Klebsiella pneumoniae* subsp. *pneumoniae* KCTC-2690, *Pseudomonas aeruginosa* KCTC-2592, and *Salmonella typhimurium* KCTC-1926), and fungi

Table 5 Cytotoxicity (IC_{50}=μg/ml) of streptokordin (**1**) against human cancer cell lines[a]

Cancer cells	**1**	Doxorubicin
MDA-MB-231	7.5	0.8
HCT 15	7.8	0.9
PC-3	3.2	0.7
NCI-H23	3.5	0.5
ACHN	4.7	0.6
LOX-IMVI	7.4	0.5
K-562	8.6	0.3

[a] All cancer cell lines were obtained from ATCC. MDA-MB-231, human breast cancer; HCT 15, human colon cancer; PC-3, human prostate cancer; NCI-H23, human lung cancer; ACHN, human renal cancer; LOX-IMVI, human skin cancer; K-562, human leukemia.

(*Aspergillus niger* KCTC-6196, *Candida albicans* KCTC-7728, and *Saccharomyces cerevisiae* KCTC-7913). It is very interesting that **1** possesses cytotoxic activity without any effects on other microorganisams. Therefore, **1** might become a useful cytotoxic anticancer agent. Further pharmacological studies and an investigation of the mechanism of action are in progress.

In addition to **1**, four known compounds, nonactin, nonactic acid, dilactone, and trilactone, were isolated from *Streptomyces* sp. KORDI-3238. Nonactin is an ionophore antibiotic macrolide [21] produced by various *Streptomyces*, including *Streptomyces griseus* subsp. *griseus* ETH A7796. Nonactin has been shown to possess antitumor activity and to be an effective inhibitor against the multidrug-resistant erythroleukemia cell line K-562 [24]. It is assumed that the biological activity of nonactin is mediated by chelation of alkali metal ions [25]. Nonactic acid is a precursor in the biosynthesis of nonactin. Nonactic acid has activity as a plant growth stimulator and exhibits specific insecticidal effects [23]. Recently, a dilactone and a trilactone were isolated and described that exhibited a significant activity against both Gram-negative and Gram-positive bacteria and also against fungi [22]. In conclusion, *Streptomyces* sp. KORDI-3238 produces a new cytotoxic compound (**1**) as well as the nonactin-type of useful macrolide antibiotics. Thus, *Streptomyces* sp. KORDI-3238 seems to be an interesting candidate for studying bioactive compounds. This strain may become a valuable tool for genetically-based combinatorial methods for the biosynthesis of bioactive compounds.

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