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Isolation and Structure Determination of Streptochlorin, an Antiproliferative Agent from a Marine-derived *Streptomyces* sp. 04DH110

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Abstract An antiproliferative agent, streptochlorin, was isolated from the fermentation broth of a marine actinomycete isolated from marine sediment. Phylogenetic analysis of the 16S rRNA gene sequence indicated that the strain belongs to the genus *Streptomyces*. Bioactivity guided fractionation of the culture extract by solvent partitioning, ODS open flash chromatography, and reversed-phase HPLC gave a pure compound, streptochlorin. Its structure was elucidated by extensive 2D NMR and mass spectral analyses. Streptochlorin exhibited significant antiproliferative activity against human cultured cell lines.

Keywords: *Streptomyces* sp. 04DH110, antiproliferative agent, streptochlorin

Marine microorganisms have been recognized as a rich source of structurally unique bioactive secondary metabolites. Marine bacteria, in particular, have received increased attention as potential sources of bioactive natural products. The large numbers, and diversity, of marine bacteria suggest that this resource will be of significant importance in the discovery of new drugs [2, 3, 6, 8]. Although largely ignored in comparison with their terrestrial counterparts, marine bacteria isolated from both shallow and deep-water sediments have produced many biologically active and/or structurally unique compounds [4, 9, 12].

In the course of our screening program for bioactive marine natural products, we isolated marine actinomycete strains from shallow and deep-sea sediments at various sites off the shores of South Korea and in the western Pacific Ocean. Strain 04DH110 was isolated from shallow water sediment taken at −1 m depth of Ayajin Bay, on the East Sea of Korea. 16S rRNA gene sequence analysis indicated that the strain belongs to the genus *Streptomyces*. The crude extracts of this strain exhibited potent antiproliferative activity against the human leukemia cell line (K-562). Bioassay-guided fractionation by solvent partitioning, ODS vacuum flash chromatography, and purification with a reversed-phase HPLC gave a pure antiproliferative compound, streptochlorin. Streptochlorin was first isolated from *Streptomyces* sp. as a new antibiotic, designated SF2583A [16]. However, the structure of SF2583A was mainly determined by X-ray crystallography, and other biological activities of streptochlorin besides its antimicrobial activity have never been reported. This paper is the first complete structure assignment of streptochlorin by NMR. In this report, we describe the isolation, physico-chemical properties, structure elucidation, and antiproliferative activity of streptochlorin.

The bacterial strain 04DH110 was isolated from marine sediment taken at −1 m depth of Ayajin Bay, on the East Sea of Korea (also known as the Sea of Japan) in August 2004. One g of sediment was incubated for 50 min at 60°C and resuspended in 9 ml of autoclaved seawater. After filtration and serial dilution with autoclaved seawater, 0.1 ml aliquots were spread onto International *Streptomyces* Project (ISP) medium 2, inorganic salt-starch agar (ISP medium 4) [13], and modified Bennett's agar [1]. The plates were incubated for 14–20 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 the significant antiproliferative activity was designated *Streptomyces* sp. 04DH110. The strain 04DH110 is a Gram-positive actinomycete that forms well-developed and branching substrate mycelia and aerial mycelia. 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,

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on which it grew abundantly. The colony morphology of the strain 04DH110 on a Bennett's agar plate at 30°C for 3 days was round, regular, and matt type with pale yellow vegetative structure, having a white mycelium. For taxonomic identification, the strain 04DH110 was analyzed following the 16S rDNA sequence method [5, 11]. Extraction of genomic DNA and 16S rRNA gene amplification were carried out according to Rainey *et al.* [10]. The 16S rRNA gene sequence (1,485 bp) of the strain 04DH110 was aligned using CLUSTAL W software Ver. 1.7 [15] and bootstrap analyses of 1,000 replicates were carried out using MEGA version 2.0 [7]. Furthermore, a BLAST search of 16S rRNA gene sequences available in the DDBJ/EMBL/GenBank database showed the highest similarity of 100% with *Streptomyces fradiae* NBRC 12773T^T (AB184134) and *Streptomyces microsporus* NBRC 13678 (AB184459) (Fig. 3). The strain 04DH110 is currently deposited in the Microbial Culture Collection, KORDI, with the name of *Streptomyces* sp. 04DH110 under the curatorship of H.J.S.

A 500-ml Fernbach flask containing 100 ml of the seed medium (modified Bennett's medium) was inoculated with a stock culture of the producing strain 04DH110 maintained on a modified Bennett's agar. After incubation at 30°C for 3 days on a rotary shaker set at 150 rpm, 20 ml of seed culture was transferred to each of twenty 2-l Fernbach flasks containing 600 ml of the production (modified Bennett's) medium. The fermentation was carried out at 30°C for 7 days on a rotary shaker set at 200 rpm. Purification and isolation of streptochlorin was guided by the antiproliferative activity against the human leukemia cell line (K-562). After 7 days, the culture broth was centrifuged (2,000 ×*g* for 15 min at 4°C) and then filtrated (0.2 μm pore-size membrane filter) to obtain a cell-free supernatant, followed by extraction with ethyl acetate (EtOAc). The EtOAc layer was concentrated *in vacuo* and the residual suspension (707 mg) was subjected to ODS open flash chromatography with a stepwise gradient mixture of MeOH/H_2O as eluant. The fraction eluted with 60%MeOH in water was purified by reversed-phase HPLC (YMC ODS-A column, 10×250 mm; 55–70% MeOH; flow rate, 1.5 ml/min; UV detection at 210 nm) to yield a pure antiproliferative compound, streptochlorin (4.5 mg, Fig. 1).

Table 1. ^{1}H and ^{13}C NMR data of streptochlorin in CD_3OD-d_4.

Position	^{1}H (mult, J=Hz)	^{13}C (mult)	HMBC
1	–	–	–
2	7.83 (1H, s)	125.42 (d)	C-3, C-4a, C-5', C-7a
3	–	103.62 (s)	–
4	8.01 (1H, d, 8.3)	121.37 (d)	C-6, C-7a
4a	–	125.93 (s)	–
5	7.16 (1H, t, 8.3)	121.74 (d)	C-4, C-7
6	7.23 (1H, t, 8.3)	123.89 (d)	C-4, C-7a
7	7.47 (1H, d, 8.3)	112.95 (d)	C-4a, C-5
7a	–	137.82 (s)	–
1'	–	–	–
2'	8.19 (1H, s)	150.05 (d)	C-4', C-5'
3'	–	–	–
4'	–	121.61 (s)	–
5'	–	145.35 (s)	–

Streptochlorin: yellowish amorphous solid; UV (MeOH) λ_{max} 215 nm (ε 12,300), 271 nm (ε 4,900), 287 nm (ε 4,800); IR (film) V_{max} 1,626, 1,456, 1,126, 744 cm^{-1}; Positive-HRFAB-MS *m/z* 219.0329 $[M+H]^+$ (calcd for $C_{11}H_8ClN_2O$, 219.0325).

The molecular formula of streptochlorin was established as $C_{11}H_7ClN_2O$ by HRFAB-MS analysis [*m/z* 219.0329 $(M+H)^+$, Δ+0.4 mmu] and ^{13}C NMR spectral data (Table 1). ^{1}H NMR data (Table 1) of streptochlorin revealed resonances for 6 aromatic protons (δ 8.19, δ 8.01, δ 7.83, δ 7.47, δ 7.23, δ 7.16). Among these olefinic protons, two *ortho*-coupled doublets at δ 8.01 and δ 7.47 (both J=8.3 Hz) and

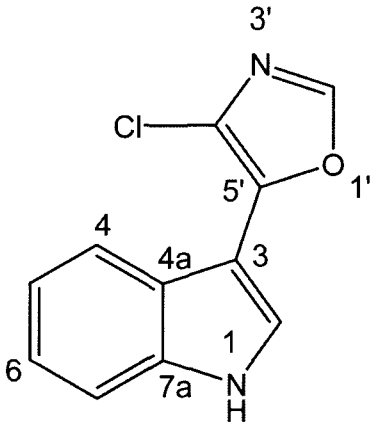

Fig. 1. Structure of streptochlorin.

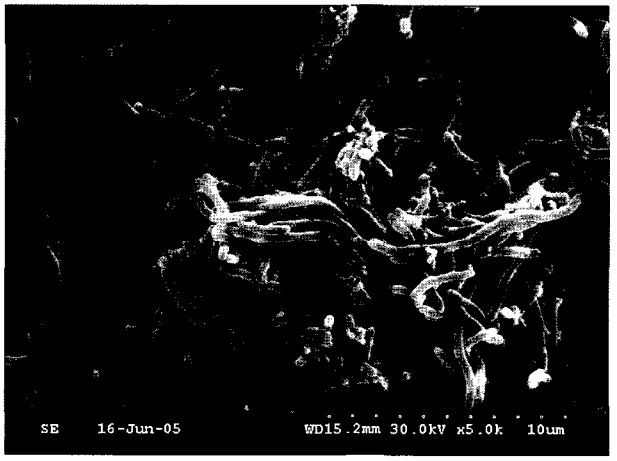

Fig. 2. Scanning electron micrograph of *Streptomyces* sp. 04DH110 grown on modified Bennett's agar at 30°C for 3 days.

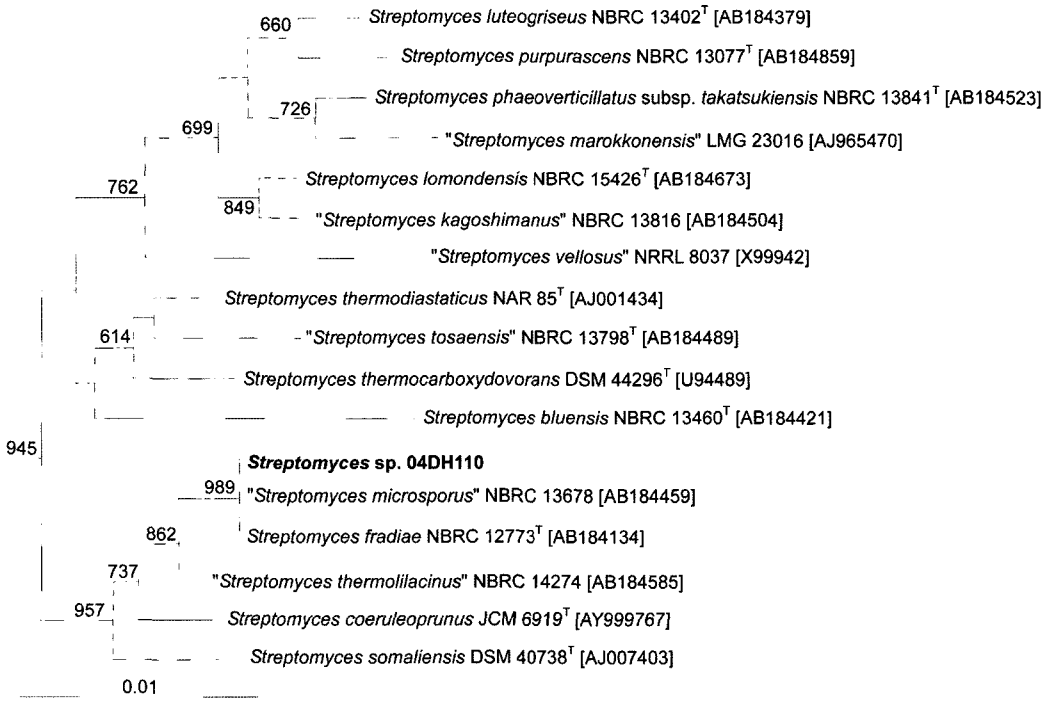

Fig. 3. Phylogenetic tree based on nearly complete 16S rRNA gene sequence (*E. coli* equivalent positions 51 to 1477) showing the relationship between strain 04DH110 and closely related members in the genus *Streptomyces*.
The tree is based on the Jukes-Cantor distance model and the neighbor-joining method. Bootstrap values >50% of 1,000 resampled are shown. Bar, 0.01 substitutions per nucleotide position.

two triplets at δ 7.23 and δ 7.16 (both J=8.3 Hz) indicated the presence of a 1,2-disubstituted benzene ring. One singlet proton in the aromatic region at δ 7.83 together with the positive Ehrlich reaction on TLC indicated a 3-substituted indole ring. Its ^{13}C NMR data (Table 1) also exhibited resonances for 11 olefinic carbons, of which 6 (δ 125.42, 121.37, 121.74, 123.89, 112.95, 150.05) were sp^2 methine and the remaining 5 (δ 103.62, 125.93, 137.82, 121.61, 145.35) were sp^2 quaternary carbons. ^1H and ^{13}C correlations were indicated by the gHSQC. Long-range couplings from the aromatic proton at δ 7.83 (s, H-2) to quaternary aromatic carbons at δ 103.62 (C-3), 125.93 (C-4a), and 137.82 (C-7a) also proved the presence of the indole ring. The presence of chloride in streptochlorin was supported by 3:1 isotope ion peaks at *m/z* 219/221 in the positive FAB-MS. The remaining ^1H and ^{13}C NMR signals were assigned to a chlorinated oxazole ring. The assignment was confirmed by interpretation of HMBC data. The proton signal at δ 8.19 (s, H-2') showed correlations to C-4' and C-5'. HMBC correlation from H-2 (δ 7.83) to C-5' (δ 145.35) established the link between the indole and chlorinated oxazole rings. Thus, the gross structure of streptochlorin was elucidated to be 4-chloro-5-(1*H*-indol-3-yl)oxazole (Fig. 1).

For the antiproliferative activity assay, a sulforhodamine B (SRB) assay was performed as described previously [14]. Streptochlorin exhibited significant *in vitro* growth inhibitory activity against human cultured cell lines such as human leukemia cells (IC_{50}=1.05 μg/ml) and immortalized hepatocytes (CHANG) derived from normal human liver (IC_{50}=>10.9 μg/ml). Streptochlorin showed slightly weaker antiproliferative activity than doxorubicin against human leukemia cell line (K-562). Streptochlorin was first isolated from the lipophilic extract of the mycelium of *Streptomyces* sp. SF2583 as a new antibiotic, named SF2583A [16]. SF2583A was reported to have antimicrobial activity but antiproliferative activity to human cultured cells has never been reported. The finding of the present study indicates that streptochlorin exhibits the growth inhibitory activity against human cultured cell lines. The strain *Streptomyces* sp. 04DH110 seems to be an interesting candidate for studying bioactive compounds. Further pharmacological studies and an investigation of the mode of action of streptochlorin are in progress.

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