Isolation and Identification of A Novel Aporphine Alkaloid SSV, An Antitumor Antibiotic from Fermented Broth of Marine Associated Streptomyces sp. KS1908
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Abstract
A marine actinomycete, Streptomyces sp. KS1908 was isolated from a marine sediment sample collected in the Bay of Bengal, India and identified based on morphological, cultural, physiological, biochemical characteristics, along with the cell wall analysis and 16S rRNA gene sequence analysis. Strain KS1908 has more similarities with Streptomyces albus but some variations were observed. A novel aporphine alkaloid SSV was isolated according to bioactivity guided fractionation of fermented broth through solvent extraction and chromatography. Chemical structure of the aporphine alkaloid SSV was elucidated on the basis of spectroscopic analysis, including two-dimensional (2D) NMR and HR-ESI-MS data. Aporphine alkaloid SSV showed significant antibacterial, antifungal activity and also possesses considerable anticancer activity against human larynx (HEp-2), cervical (HeLa), leukemia HL-60 and MCF-7 breast cancer cell lines.

Keywords: Antitumor antibiotic; Streptomyces sp. KS1908; Aporphine alkaloid SSV; Fermented broth

Introduction
Streptomyces have been shown to possess the ability to synthesize antibacterial, antifungal, insecticidal, antitumor [1-5], anti-inflammatory, anti-parasitic, antiviral, anti-infective, antioxidant and herbicidal compounds [1,2,6-9]. Hence, these are widely recognized as industrially important microorganisms [10]. Moreover, approximately 60% of the antibiotics discovered in the year 1990 and most of the antibiotics are from the genus Streptomyces [11]. These characteristics make this genus an important research area. Earlier literature suggests that many antimicrobial molecules have been isolated from Streptomyces salinus. Salinomycin, a new polyether antibiotic was produced by strain of Streptomyces albus ATCC 21838 [12]. A new macromolecular peptide antibiotic, named NA-1 was isolated from the culture broth of Streptomyces albus AJ 9003 [13]. An antibiotic complex identical to Paulomycins A and B active against multiple resistant strains of staphylococci and other gram-positive bacteria was isolated from cultures of Streptomyces albus G [14]. In present study, bioactive actinomycete was collected from marine sediment and identified as Streptomyces sp. KS 1908 further responsible antibiotic was isolated and spectroscopic assignment of structure was demonstrated here.

Isolation and Taxonomy
Marine sediment samples were collected at Bay of Bengal near Gangavaram Coast, Visakhapatnam, India. Sample serially diluted to isolate as pure culture on a starch casein agar (SCA-soluble starch, 10.0 g; vitamin free casein, 0.3 g; KNO₃, 2.0 g; NaCl, 2.0 g; K₂HPO₄, 2.0 g; MgSO₄•7H₂O, 0.05 g; CaCO₃, 0.02 g; FeSO₄•7H₂O, 0.01 g; agar, 20.0 g; sterilized natural aged sea water, 1.0 L; pH, 7.2; supplemented with rifampicin 25 µg/ml and cycloheximide 75 µg/ml to inhibit bacterial and fungal contamination, respectively) plate, which had been seeded with a sediment sample suspension and incubated at 28°C for 14 days [10]. The isolated actinomycete colonies being filamentous, compact, often leathery giving a conical appearance, and maintained on YEME (Yeast Extract Malt Extract) slants at −20°C [15]. This pure culture was later used for taxonomic and bioactivity studies.

Taxonomic identification was done by physiological conditions, biochemical tests, chemo taxonomic investigations and molecular characterization. Morphological observation through macroscopic based on growth pattern on different media like yeast malt extract agar (ISP-2), Oatmeal agar (ISP-3), Inorganic salts starch agar (ISP-4), Glycerol asparagines agar (ISP-5), tryptone yeast glucose agar, peptone agar, and nutrient agar. The color of aerial mycelium, substrate mycelium and soluble pigment were observed by naked eye. Optical and Scanning electron microscopies (JSM-6610LV, JEOL Ltd.) were used for microscopic observations. Organism growth conditions were studied on SCA at various pH, Temperature and NaCl levels for physiological characterization [16]. Organism was biochemically characterized using the tests viz., Enzymes, H₂S production tests, Carbon and nitrogen utilization tests using different substrates12. Cell wall chemical composition was demonstrated according to the procedures of Lechevalier [17] for chemotaxonomic investigation17. Molecular characterization was done by 16s rRNA gene sequencing [16,18,19].

Fermentation
Sporulated isolate prepared with sterile water according to 0.5Mc Farland standard. The resulted spore suspension at 10% level was transferred aseptically into a 250 ml Erlenmeyer flask containing 45 ml of the inoculation medium [8]. The flasks were inoculated and incubated at 28°C for 48 hrs at 120 rpm. Thoroughly washed pellet containing cell
mass was suspended in sterile 0.9% sodium chloride solution and used as inoculum was then transferred to the modified production medium [8] and incubated at 30°C for 96 hrs at 160 rpm on a rotary shaker. The fermented broth was used for extraction of the active principle.

**Bioactivity guided fractionation and purification**

The fermented broth was aseptically collected in a sterile centrifuge tube and centrifuged at 4000 rpm for 15 min at 4°C. The culture filtrate (supernatant) and mycelial pellet obtained were extracted separately for identification of the active principle source. Antibiotics from the cell mass were isolated usually by extraction with polar and non-polar solvents while that from the fermented medium were extracted by solvent extraction only when the antibiotic has a reasonably high degree of solubility in non-polar organic solvents. Bioautography was performed to identify bioactive fraction. The other alternative technique for the separation of bioactive principle from the culture filtrate is the adsorption of the compound on some inert material like silica [20].

**Compound identification and Structure elucidation**

Thin Layer Chromatography (TLC) was analyzed on the glass percolated silica gel plates GF254, and spots were checked by UV light, Iodine and spraying with 10% sulfuric acid in methanol followed with heating. The melting point was determined on Fisher-Johns melting point apparatus. FT-IR spectra were recorded on a Perkin-Elmer spectrophotometer with KBr pellet. The sample was scanned between 400 and 4000 cm⁻¹ wave number. High Resolution Mass Spectrum (HRMS) was recorded on QSTAR XL HYBRID MS System and EI MS was recorded on VG 7070H (70 eV). The NMR data of purified compound was acquired using an AMX-400 spectrometer (Bruker, Rheinstetten, Germany). 1H NMR spectra were obtained at 400.13 MHz and 13C NMR spectra were obtained at 100.6 MHz. All NMR spectra were recorded in DMSO-d6. The chemical shifts were expressed in δ (ppm) using DMSO-d6 and TMS as internal reference. Dragendorff’s reagent test was used for the identification of alkaloid [21].

**Antimicrobial assay**

Anti-microbial studies were carried out on clinical isolates of human pathogenic bacteria and dermatophytic fungi, *Salmonella typhi*, *Vibrio cholerae*, *Shigella dysenteriae*, *Enterococcus faecalis* are gastrointestinal pathogens, which were collected at King George Hospital, Visakhapatnam, India. *Proteus vulgaris* NCIM 2813, *Pseudomonas aeruginosa* NCIM 5031 cultures were collected from NCL, Pune, India. *Staphylococcus aureus* MTCC 7443, *Bacillus subtilis* MTCC 8141, *Aspergillus niger* MTCC 6484, *Aspergillus awamori* MTCC 7711, *Candida albicans* MTCC 1346, *Trichophyton rubrum*. *MTCC* 3272 cultures were collected from Indian microbial technology, India. Clinical isolate of *Candida albicans* was collected from skin lesions. Zone of inhibitions were determined using agar well diffusion method ad Minimum Inhibitory Concentration (MIC) was done by broth dilution assay. Microbial broth cultures (Mueller Hinton broth for bacteria, Sabouraud Dextrose broth for fungus) were adjusted the absorbance to 0.6 (Optical Density at 620 nm) in Spectrophotometer for bacteria, Sabouraud Dextrose broth for fungi) were adjusted the absorbance to 0.6 (Optical Density at 620 nm) in Spectrophotometer for bacteria, Sabouraud Dextrose broth for fungi). The other alternative technique for the separation of bioactive principle from the culture filtrate is the adsorption of the compound on some inert material like silica [20].

**Molecular docking**

Topoisomerase I (PDB ID 1T8I) and IIA (PDB ID 2XCT) crystal structures of proteins were obtained from Protein Data Bank. Co-crystallized ligands and water molecules are removed from target protein using Argus lab. Ligands are prepared using Chemoffice (Cambridge). Energy minimization was done using molecular mechanics. The minimized was executed until root mean square value reached smaller than 0.001 Kcal/mol. Such energy minimized ligands and receptor used for docking studies using Molegro Virtual Docker [29].

**Results**

**Isolation and Taxonomy of isolated marine actinomycete**

The isolated bioactive actinomycete colonies being filamentous, compact, often leathery giving a conical appearance, dry surface on SCA, which can easily be distinguished from fungi and non filamentous bacteria. Morphological and cultural observations of the isolate grown on different ISP media given in Table 1, revealed that vegetative mycelium showed yellow-brown color, aerial hyphae were abundant, well-developed with white color on different test media and substrate mycelium with pale yellow color. It didn't produce any pigments but faint yellow color pigmentation on Yeast-malt extract agar (ISP-2). The scanning electron micrograph of the strain KS1908 revealed that aerial mycelia were monopodially branched with compact spirals of sporophore terminating in long open coils. Each spore chain consisted...
of 8-20 white, oblong to cylindrical shaped spores, 0.6 ~ 0.7 x 0.8 ~ 0.9 µm in size, having smooth surface (Figure 1). The chemotaxonomic investigations revealed that the cell wall peptidoglycan of isolate contained L-diaminopimelic acid and glycine. This indicates that isolate belongs to cell wall type I which is characteristic of the genus Streptomyces.

16S rRNA gene sequence analysis (Genbank accession no. KC556777) and other cultural, biochemical physiological, chemotaxonomic characteristics revealed that Strain KS1908 has close similarities with Streptomyces albicus [12] (Figure 2 and Tables 1 and 2) but some variations were observed so named as Streptomyces SP. KS 1908.

Bioactivity guided fractionation and purification

Fermented broth of sterptomyces sp. KS1908 was extracted using various solvents but only ethyl acetate extract showed bioactivity. Then ethyl acetate extract was run by chromatography using silica gel to obtain bioactive fraction II (255 mg), which was further fractionated with Sephadex LH-20 column and separated into five major fractions that included with fraction IIc. Preparative reverse phase HPLC was used to get light brown colored pure bioactive compound SSV in FIIc2 fraction. Schematic representation of detailed fractionation and purification was given in Figure 3.

Structural elucidation of aporphine alkaloid SSV

Compound SSV was obtained as light brown needles having melting point 68-72°C; [a] 25° ~ -28.0 (c = 1.5, MeOH). Dragendorff’s reagent test showed positive [16]. The molecular formula of SSV was assigned as C_{19}H_{19}NO_{4} from its elemental and mass spectral analyses (HRMS: m/z 326.1386 [M+H]⁺, 348.1206 [M+Na]⁺). This was corroborated by the decoupled [13] C-NMR spectrum which showed signals for all the nineteen carbons of the molecule (Table 3). The mass fragmentation pattern of compound SSV showed typical of aporphine alkaloid [17]. The IR absorption bands at 3430 (OH), 945 (-O-CH2-O-) cm⁻¹ indicated the presence of hydroxyl and methylenedioxy groups.

The proton NMR spectrum of compound SSV showed three, one proton singlet peaks at δ 7.55, 6.75 and 6.57 corresponding to H-11, H-8 and H-3 respectively of an aporphine alkaloid (Table 3). The two, one proton singlet peaks at δ 6.11 and 5.97 indicated the presence of one methylenedioxy group on C1-C2 and two, three proton singlet peaks at δ 3.77 and 2.42 attributed to one methoxyl group and one N-methyl group, respectively. The proton NMR spectrum also showed a one proton singlet peak at δ 9.11 attributed to hydroxyl group. Further the correlations observed in HMOC, HMBGC and HSCGC confirmed that methylenedioxy group was present at C-1 and C-2 carbons, methoxyl group was present on C-9 and free hydroxyl group was present on C-10 (Table 3). Thus from the foregoing spectral studies, the structure of compound SSV was established as 10-hydroxy-9-methoxy-1,2-methylenedioxy-6-methyl-4,5,6,6a-tetrahydro-7H,6a-azabenzanthrene (Figure 4).

Antimicrobial and anticancer activities of aporphine alkaloid SSV

Aporphine alkaloid SSV showed good antimicrobial activity especially on multi drug resistant clinical isolates including bacteria and fungi. Aporphine alkaloid SSV was more effective against bacteria than fungi.

As shown in Table 4, zone of inhibition found to be between 9-14 mm at 30 µg of compound. MIC range found to be between 1-100 µg/ml. 14 mm was the inhibitory zone showed by SSV on S. typhi and with lowest MIC of 1 µg/ml. Aporphine alkaloid SSV showed potent antibacterial activity against both gram positive and gram negative bacteria. Compound SSV showed very effective activity against gastrointestinal pathogenic bacteria (S. typhi, V. cholerae, E. faecalis and E. coli). Dermatophytic fungi (T. rubrum and C. albicans) showed slight resistance. Aporphine alkaloid SSV showed comparable antimicrobial potency with ciprofloxacin and fluconazole (antibiotics).

Anticancer activity of aporphine alkaloid SSV

Anticancer activity of compound SSV performed by MTT assay and found significant cytotoxic activity on human larynx (HEp-2), cervical (HeLa), human leukemia HL-60 and MCF-7 breast cancer cell lines with the IC₅₀ values of 1.10, 1.13, 2.85 and 4.44 µg/ml, respectively. Aporphine alkaloid SSV showed comparable potency with camptothecin (Table 5).

Docking of aporphine alkaloid SSV

Molecular docking studies of aporphine alkaloid SSV were on topoisomerases using Molegro Virtual Docker. Docked energy or binding energy was inversely proportional to affinity of compounds towards enzyme. Lower binding energy indicated higher binding affinity. -98.4 kcal was the binding energy of aporphine alkaloid SSV on topo II, which was less than docked score of cipropiloxacin (~79.7 kcal/mol). Aporphine alkaloid SSV showed comparable binding energy (-78.6 kcal/mol) with camptothecin (~77.1 kcal/mol) on topoisomerase I. Figure 4, demonstrated that Compound SSV binding interactions

| Medium                        | Characteristics |
|-------------------------------|-----------------|
|                               | Growth          | Vegetative mycelia | Aerial mycelia | Spore mass | Soluble pigment |
| Nutrient agar                 | Good            | Moderate, pale yellow | Moderate, white | Poor, white | Nil            |
| Yeast – malt extract agar (ISP-2) | Abundant        | Moderate, yellow | Abundant, white | Moderate, white | Faint yellow |
| Oatmeal agar (ISP-3)          | Abundant        | Moderate, yellow | Abundant, white | Moderate, white | Nil            |
| norginic salts starch agar (ISP-4) | Good            | Moderate, yellow | Good, white | Poor, white | Nil            |
| Glycerol asparagines agar (ISP-5) | Good            | Moderate, yellow | Good, white | Moderate, white | Nil            |
| Tyrosine agar (ISP-7)         | Moderate        | Moderate, pale-yellow | Good, white | Poor, white | Nil            |
| Yeast – starch aga            | Moderate        | Good, pale yellow | Good, white | Moderate, white | Nil            |
| Sucrose – nitrate agar        | Moderate        | Moderate, pale yellow | Moderate, white | Poor, white | Nil            |

Table 1: Cultural characteristics of the isolated Streptomyces sp. KS1908.

Figure 1: Scanning electron micrograph of Streptomyces sp. KS1908.
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Figure 2: Phylogenetic tree of obtained by distance matrix analysis of 16s rRNA gene sequence of closely related streptomyces sps. obtained from BLAST results and constructed using neighbor-joining method, showing phylogenetic position of Streptomyces sps. KS1908 with yellow marking.

Figure 3: Isolation and purification procedure of compound SSV.
isolated from bioactive fraction of fermented broth and chemically novel aporphine alkaloid SSV is an antitumor antibiotic which was inhibition [30]. Pimprinine, an extracellular alkaloid has been special class of antitumor antibiotics which act through topoisomerase II inhibition [30]. Pimprinine, an extracellular alkaloid has been isolated from the culture filtrate of Streptomyces CDRIL-312 [31]. Alkaloid group of aporphine alkaloids are topoisomerase I inhibitors from Streptomyces sp [32].

In conclusion, bioactive streptomyces sp. KS1908 was isolated and characterized from marine associated actinomycetes further novel aporphine alkaloid SSV is an antitumor antibiotic which was isolated from bioactive fraction of fermented broth and chemically characterized through advanced spectroscopic data. Therefore, the isolated aporphine alkaloid SSV can be promising agent for treatment of cancer and microbial infections.

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