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Characteristics of Sulfobacin A from a Soil Isolate *Chryseobacterium gleum*

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Abstract A nonmotile, nonspore-forming, Gram-negative, aerobic, small rod-shaped bacterium, isolated from soil, was identified as *Chryseobacterium gleum* on the basis of 16S rRNA gene sequence analysis. It was observed to grow luxuriously at pH 9 and tolerate highly alkaline environment up to pH 12. Orange red color was a peculiar character of these cells which on purification obtained 60–80 mg/l and found to be sphingosine type of sulfonolipid "sulfobacin A" on the basis of infrared, nuclear magnetic resonance, and mass spectral data. Inhibition of sulfobacin A synthesis by incorporation of L-cycloserine in culture growth medium suggested presence of serine palmitoyl transferase which is one of the important enzymes involved in its biosynthesis. Sulfobacin A from *C. gleum* LMG P-22264 exhibited cytotoxicity against four cell lines tested. Maximum activity against human mammary adenocarcinoma cells was indicative of its potential as an anticancer agent.

Keywords *Chryseobacterium gleum* · Sulfonolipid · Sulfobacin A · L-cycloserine · Serine palmitoyl transferase · Cytotoxicity

Introduction

During the reclassification of the members of the genus *Flavobacterium* on the basis of rRNA cistron similarity studies, the genus *Chryseobacterium* was introduced and described first time by Vandamme et al. in 1994 [1]. Chryseobacteria have been isolated from milk and butter [2], fish [3], rice field soil [4], lactic acid beverage [5], raw chicken in a chicken-processing plant [6], etc. Kamiyama et al. had isolated *Chryseobacterium* sp. NR 2993 from soil and observed that it produced sulfobacin A and B, which were sphingolipid (SL) type of compound with long carbon-chain aliphatic amine [7]. The culture under study was isolated from soil and identified as *Chryseobacterium gleum.* It produced a typical orange

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red-colored compound which was structurally characterized as sulfobacin A. Subsequently, its biosynthesis and cytotoxicity were studied.

Characteristics of Sulfonolipids and Its Presence in Microorganisms

Sphingolipid is a long carbon-chain aliphatic amine described for the first time by a German physician Tudichum in 1884 [8]. Carter [9] elucidated the structure of sphingosine. A class of SL comprises a C1 sulfonate group sulfonosphingolipids which are the first known example of this class isolated from *Nitzschia alba* [10]. These compounds are unusual sphingosine derivatives. Similar sulfonolipids, *N*-acyl-2-amino-3-hydroxy-15-methylhexadecane-1-sulfonic acids, were previously found in the cell envelope of gliding bacteria of the genera *Cytophaga*, *Capnocytophaga*, *Sporacytophaga*, and *Flexibacter* [11]. In *Flavobacterium johnsoniae*, sulfonolipids were detected as an unusual component of the cell membrane involved in motility [12]. The role of these sulfonolipids was speculated to be in presenting specific polysaccharides to the outer cell surface in nonmotile organisms [13, 14]. It was observed in *Cytophaga–Flexibacter* group that the biosynthesis of sulfonolipid (Fig. 1) takes place in the outer membrane of the cell [8, 15] where serine palmitoyl transferase (SPT), a pyridoxal phosphate-dependent heterodimeric enzyme, determines the rate of SLs biosynthesis [16].

Although action of SPT might be a major point of regulation, its regulatory mechanisms remain largely unknown to date [17]. L-Cycloserine, β-chloro- and β-fluoro alanine are reported inhibitors of SPT at nanomole concentration [8]. Here, we describe the production and characterization of sulfobacin A from our isolate *C. gleum* LMG P-22264, evidence that proved the involvement in its synthesis and its anticarcinogenic activity.

Materials and Methods

All the chemicals used during experimentation were of analytical research and guaranteed grade, procured from Hi-media, Qualigens, SD Fine (Mumbai, India), and Sigma (USA).

Culture Isolation and Identification

Culture was isolated from the soil of North Maharashtra University campus. This soil is weathered profile of a typical Deccan Basalt. Culture was biochemically characterized and further identified on the basis of 16S rRNA gene sequence analysis.

16S rRNA Gene Sequence Analysis and Phylogenetic Study

Total DNA was prepared according to the protocol of Niemann et al. [18]. Polymerase chain reaction-based 16S rRNA gene analysis was carried out using primers 16F27 (AGTTTGATC CTGGCTCAG) and 16R1485 (TACCTTGTTACGACTTCACCCCA).

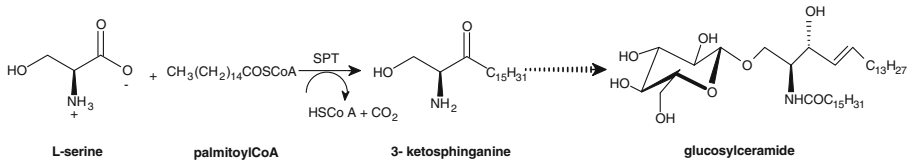

Fig. 1 Key step involving serine palmitoyl transferase (*SPT*) in biosynthesis of sphingolipid

Obtained sequence was subjected to phylogenetic analysis using the software package BioNumerics (Applied Maths, Belgium) and a resulting tree was constructed using the neighbor-joining method.

Culture Conditions for SL Production

Isolate was best grown in a medium containing (g/l) enzymatic hydrolysate of casein 10; $MgSO_4.7H_2O$, 1.18; K_2HPO_4, 0.25; and pH 9.0 and the same was used for production of sulfonolipid. Cells were cultivated on a rotary shaker (120 rpm) at 30 °C for 48 h and harvested by centrifugation at 10,000×*g* for 10 min and biomass was separated from supernatant. Compound was extracted from the biomass as described in the patent [19].

Test for Flexirubin Type Pigment

Orange colonies of *Chryseobacterium* grown on nutrient agar medium were covered with aqueous 20% KOH and observed for the reversible shift to red, purple, or brown color [20].

Purification of the Compound and Characterization

Dried impure compound 0.356 g was dissolved in 5-ml dichloromethane. It was then loaded on silica gel (60–120 mesh, Thomas Baker) column for adsorption and was eluted with 5% petroleum ether in ethyl acetate followed by 20% to get major fraction of orange colored compound which was dried using rotary vacuum evaporator (Buchi R-124, Switzerland). After addition of 0.5 ml $CDCl_3$, it was subjected to nuclear magnetic resonance (NMR) spectroscopy.

Melting Point Determination

Melting point of the SL was determined by using conventional melting point determination method wherein compound was filled in a one side sealed tube and heated in a controlled manner so as to observe its melting.

UV Spectroscopy

It was carried out in the range 325–700 using UV–Visible Spectrophotometer (1601), Shimadzu, Japan.

Elemental Analysis

C, H, N, and S analysis of the compound was carried out by using CHN analyzer (Perkin Elmer Elemental analyzer 2400, USA).

IR, NMR, and Mass Spectroscopy

For structural elucidation of pure sample infrared (IR), NMR, and mass spectroscopy was carried out. In order to confirm the OH group in the structure of the compound, acylation was carried out. To 46.3 mg of purified SL, 7 ml dichloromethane, 0.5 ml acetic anhydride, and 0.5 ml pyridine was added at room temperature and acetate derivative of the compound was obtained. Then, pure water and dichloromethane were added thereby to form two phases: one aqueous and other organic. The organic phase was washed with water and

separated and purified using silica gel column which was eluted with petroleum ether. The fraction obtained by eluting with 100% petroleum ether was subjected to spectroscopy. IR spectra (Perkin-Elmer 833 spectrometer) were recorded in 4,000–450 cm^{-1} range. To the sample, solvent $CDCl_3$ was added and ^1H NMR was recorded with NMR spectrometer (Brucker DPX 200). Later, mass spectroscopy (Autospec mass spectrometer) was performed.

SL Synthesis Inhibition by L-Cycloserine

The culture was grown in the medium stated earlier consisting various concentrations of L-cycloserine [8] at 30 °C for 48 h. Biomass was separated by centrifugation from which the SL was extracted and estimated spectrophotometrically.

Cytotoxicity Based on Antiproliferative Activity of the Sulfonolipid

Human Cells and Medium for its Cultivation

Human lung carcinoma cell lines (Hep-G2 and Hep3B), human mammary adenocarcinoma cells (MCF-7), and human cervical cancer cell line (SiHA) from National Centre for Cell Science, India were used. Cells were maintained in monolayer enriched medium (MEM; HyClone, Logan, UT, USA) supplemented with heat-inactivated fetal bovine serum (10%), penicillin (100 U/ml), and streptomycin (100 μg/ml). The cells were grown at 37 °C in 5% CO_2 and humidified air atmosphere. Stock solutions of sulfonolipid were prepared in dimethyl sulfoxide (DMSO) at a concentration of 10–11.5 mM and afterwards diluted to the required concentration. The 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was dissolved in 1 ng/ml MEM (without phenol red) and filtered through a Millipore filter, 0.22 μm, before use.

Cell Proliferation Assay

Hep-G2, Hep3B, MCF-7, and SiHA cells were plated at a density of 15,000 cells per well in 96-well tissue culture plates. Cells were allowed to adhere at 37 °C for 24 h and then treated with various concentrations (0, 0.01, 0.1, and 1.0 mg/ml) of sulfonolipid dissolved in DMSO for additional 48 h in triplicates. In the control wells, nutrient medium with corresponding concentration of DMSO was added. Thereafter, cell proliferation was assessed by replacing treatment medium with 50 μl medium containing 1 mg/ml MTT and incubated at 37 °C for 4 h. Medium was then aspirated off and formazan crystals were solubilized in 50 μl of isopropanol. The optical density was read at 570 nm using 630 nm as a reference filter against a blank prepared from cell-free wells. Absorbance given by cells treated with the carrier DMSO alone was taken as 100% cell growth. All assays were performed in triplicates.

Results and Discussion

16S rRNA Gene Sequence Analysis

On the basis of 16S rRNA gene analysis of LMG P-22264, a similarity significant for possible species relatedness (97% sequence similarity) was found with several validly

described *Chryseobacterium* species, indicating that its phylogenetic position belonged to one of these species of genus *Chryseobacterium*. Culture (LMG P-22264) was identified as *C. gleum* when its 16S rRNA gene sequence was compared with MIDI database by Belgian Culture Collection of Microorganisms, Ghent, Belgium (Fig. 2).

Characterization of the Orange Red Compound

The physical appearance of earlier reported sulfobacin A was white [7], but the compound purified from *C. gleum* was orange red colored.

Test for Flexirubin

Reversible shift to purple color on addition of 20% aqueous KOH on colonies lead to conclusion that the compound might be a flexirubin type of pigment. The test could detect the differential phylogenetic characteristic of *Chryseobacterium* sp. [21]. How can it be? As all species of *Chryseobacterium* (previously called *Flavobacterium*; family Flavobacteriaceae) show the presence of flexirubin type of pigment. This test gave the specific characteristic to the family Flavobacteriaceae, hence differentiated phylogenetically. Although the compound passed the flexirubin test, some types of sulfonolipids like *Xanthomonas* sulfonolipid and phenolic carotenoid could have similar structural elements and give a misleading positive reaction [20]. Hence, chemical characterization of the pure compound was carried out. However, further spectroscopic studies revealed that the test compound belonged to a sphingolipid.

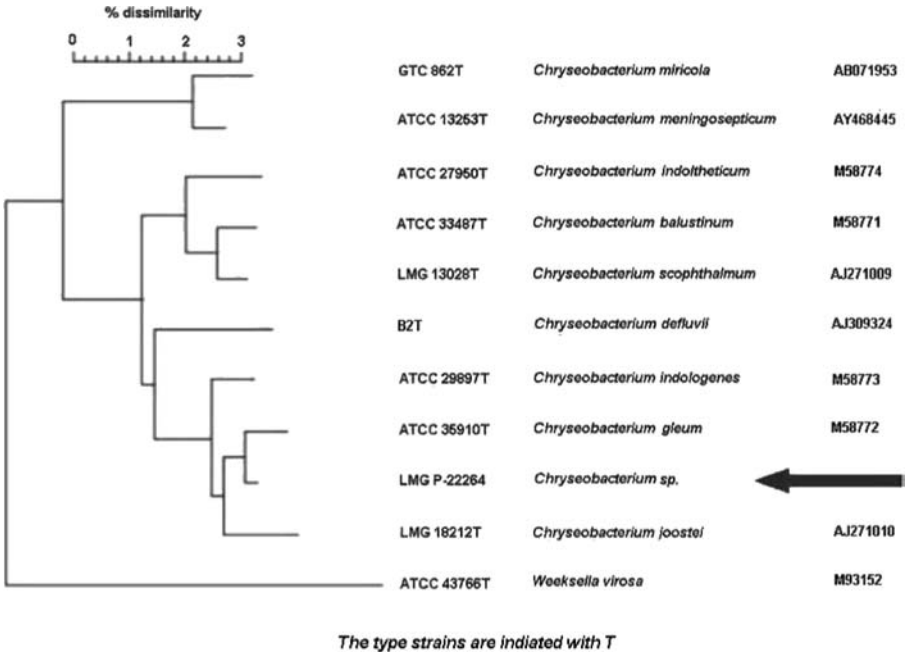

Fig. 2 Phylogenetic position of the isolate LMG P-22264

Melting Point and UV Spectroscopy

Pure compound was found to have MP 285 °C which was subjected to UV spectroscopy that showed two peaks: A_{452} was 1.927 (major peak) and A_{347} was 0.604 (minor peak).

Elemental Analysis

C, H, N, and S analysis of pure compound showed that it contains carbon (57.43%) in majority followed by hydrogen (12.26%) and nitrogen (4.15%). Characteristically, it contained less sulfur (2.28%) concentration.

IR, NMR, and Mass Spectroscopy

The isolated pure compound was characterized by IR, ^{1}H NMR, and mass spectroscopic techniques. The IR spectrum (Fig. 3) confirmed the presence of functional groups $–SO_3H$ (1,215 and 1,103), –CONH (1,646, 1,550, and 1,468), –OH and –NH (3,600–3,200) in addition to C–H (2,925, 2,853, 1,468), and C–O (1,215 and 1,103) groups. ^{1}H NMR (Fig. 4) and ^{1}H NMR of acylated compound (Fig. 5) clearly indicated the presence of 2-NH [7.45 (m/d)], 3-OH [4.41–4.35 (m/d)], and 3′-OH [4.41–4.35 (m)] groups in addition to CH, CH_2, and CH_3 groups.

Furthermore, the mass spectrum (Fig. 6) of compound illustrated the molecular weight 618.6 and fragmentation pattern through $–SO_3$ (80), –CONH (43), $–CH_2$ (14), and $–CH_2$ X n (14 X n), etc. fragment ions having the molecular formula $C_{34}H_{69}NO_6S$.

IR, ^{1}H NMR, and mass spectral data of the pure compound isolated from the *C. gleum* was almost similar to that reported for sulfobacin A [7, 15, 22]. On the basis of spectroscopic characterization, the compound isolated from *C. gleum* was sulfobacin A (Fig. 7) which has not been reported so far.

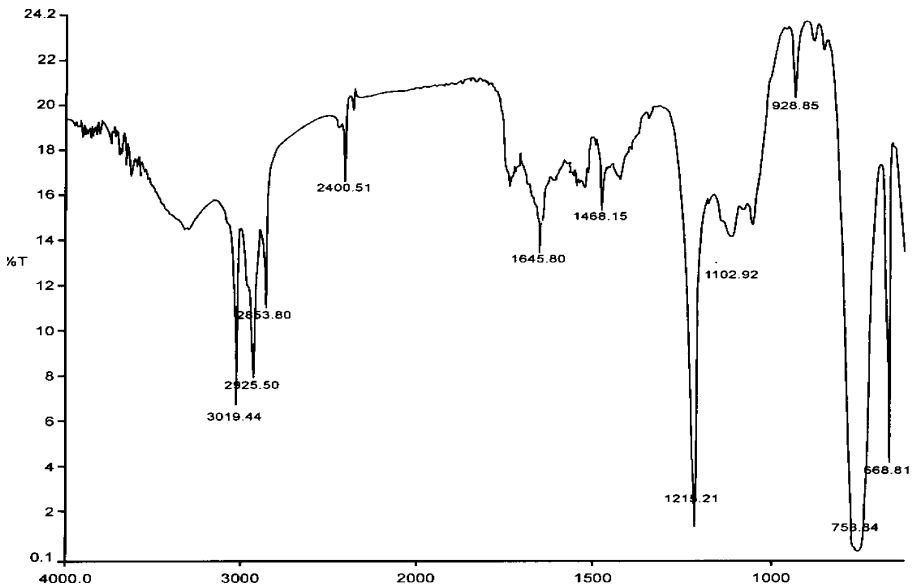

Fig. 3 IR spectrum of sulfobacin A from *C. gleum* LMG P-22264

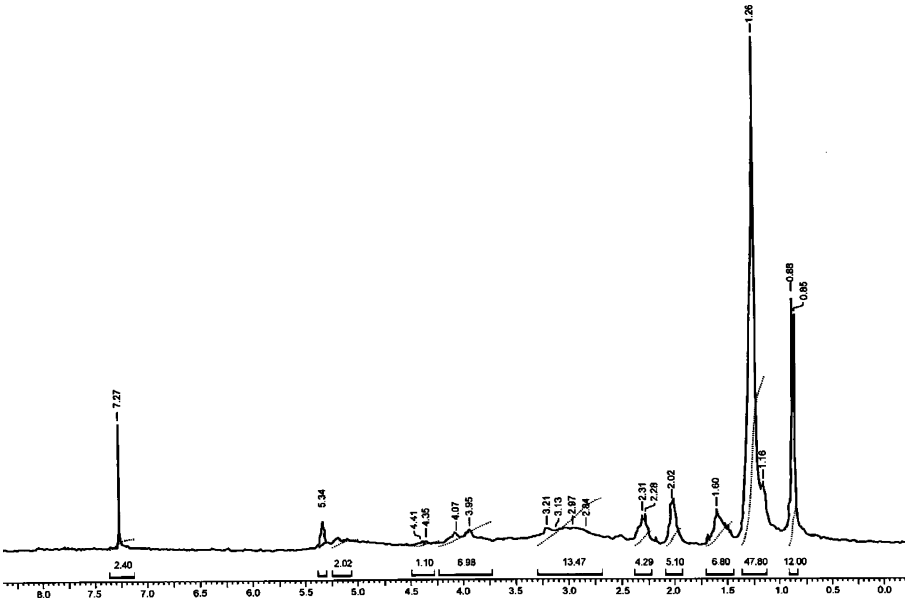

Fig. 4 [1]H NMR of sulfobacin A from *C. gleum* LMG P-22264

The isolation and characterization of sulfonolipids, unusual sphingosine derivatives such as sulfobacin A and sulfobacin B, has been reported by Kamiyama et al. [7] who discovered sulfobacins A and B as the first von Willebrand factor (vWF) receptor antagonists of microbial origin while screening for novel vWF in the culture broth of *Chryseobacterium* sp. NR 2993. These compounds do possess potent inhibitory activity against the binding of vWF to glycoprotein Ib/IX receptors. vWF is a large multimeric glycoprotein present in blood plasma and its primary function is binding to other proteins, particularly factor VIII

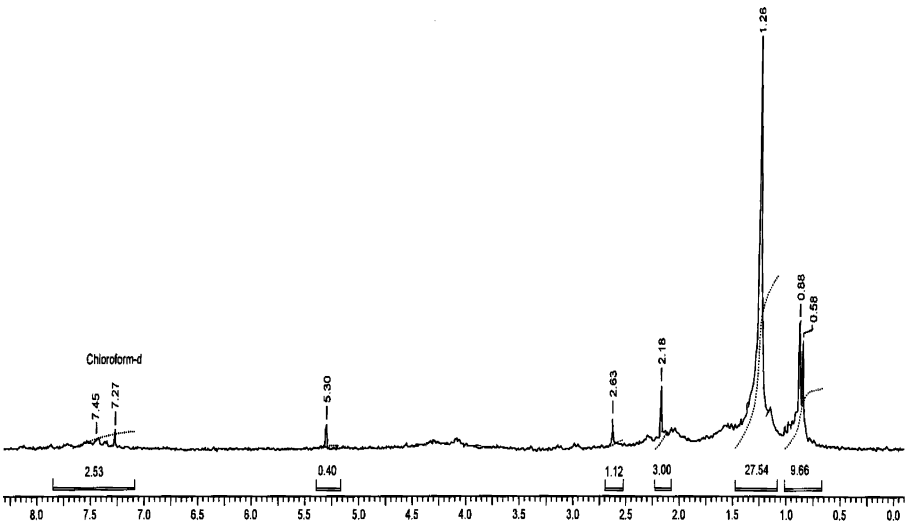

Fig. 5 [1]H NMR of acylated sulfobacin A from *C. gleum* LMG P-22264

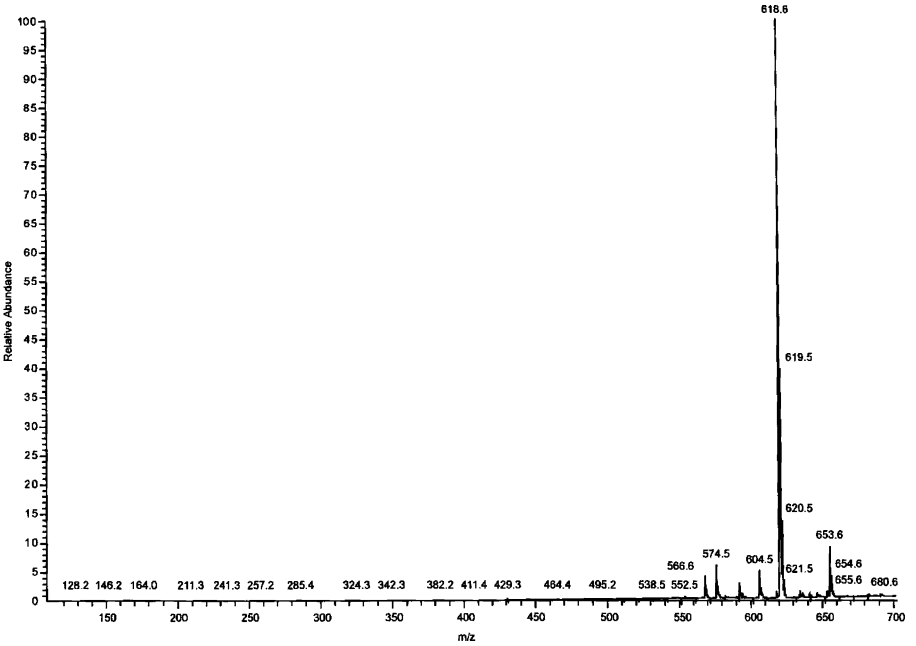

Fig. 6 Mass spectrum of sulfobacin A from *C. gleum* LMG P-22264

which is important in platelet adhesion to wound sites. The structures can be related to the sulfonolipids which is a major components of the cell envelop of gliding bacteria of the genus *Cytophaga* [23]. Takikawa et al. [24] have also reported the synthesis of sphingosine relatives: sulfobacin A, sulfobacin B, and flavocristamide A.

SL Synthesis Inhibition by L-Cycloserine

Studies on use of a known sulfonolipid inhibitor L-cycloserine showed its action on SPT which is the most important enzyme of sulfonolipid biosynthetic pathway. In these studies, it was observed that increase in L-cycloserine concentration from 0 to 60 nM decreased sulfobacin A (A_{452}) synthesis (Fig. 8) and ceased totally at 100 nM. This gave an evidence of presence and involvement of SPT in sulfonolipid biosynthesis of *C. gleum*. Earlier, sphingolipids of fungi *Aspergillus fumigatus* [25] and *Paecilimyces variotii* [26] were observed to inhibit SPT competitively with respect to serine.

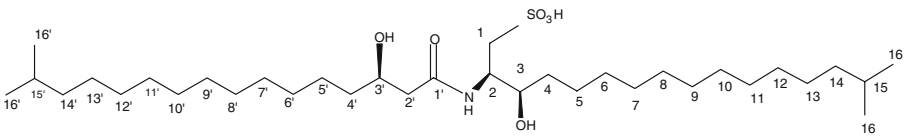

Fig. 7 Proposed structure of sulfobacin A from *C. gleum* LMG P-22264

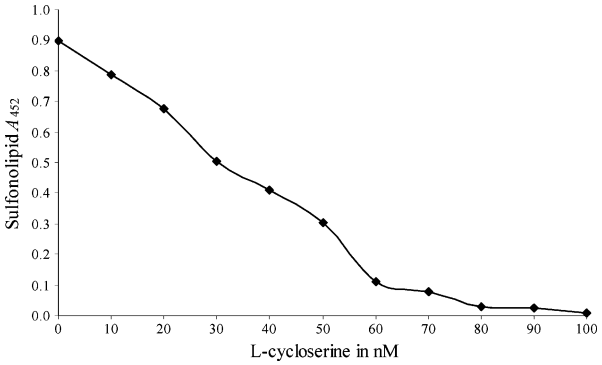

Fig. 8 Inhibition of sulfobacin A from *C. gleum* LMG P-22264 biosynthesis by L-cycloserine

Antiproliferative Activity of the Sulfonolipid

Antiproliferative activity of the sulfonolipid was determined by carrying out the MTT cell proliferation assay for the cell lines Hep-G2, Hep3B, MCF-7, and SiHA. The results are graphically represented in Fig. 9. Sulfobacin A from *C. gleum* LMG P-22264 exhibited antiproliferative activity at 1.0 mg/ml concentration against all cell lines of MCF, SiHA, HepG2, and Hep3B indicating its potential as an anticancer agent. Maximum antiproliferative activity was found against MCF cell line.

Many cytotoxic compounds of therapeutic interest have been isolated from various organisms like marine invertebrates, and some of them have been reported to be of microbial origin. Ascidian *Cystodytes dellechiajei* extracts showed remarkably high antiproliferative activity (IC50 ≤5 μg/ml) in human lung carcinoma A-549, colon

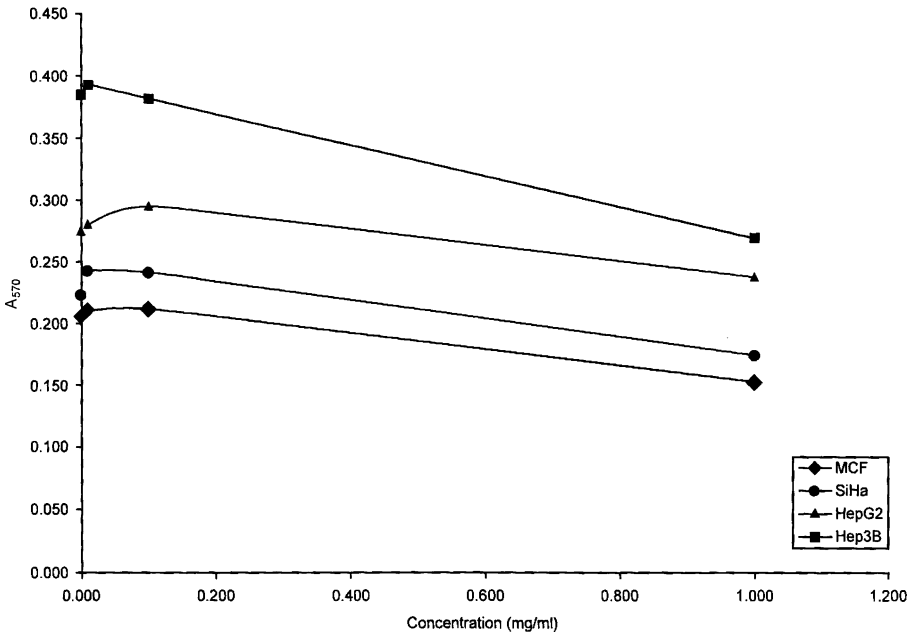

Fig. 9 Antiproliferative activity of the sulfobacin A from *C. gleum* LMG P-22264

adenocarcinoma H-116, pancreatic adenocarcinoma PSN-1, and breast carcinoma SKBR3 cell lines [27]. Cytoplasm fraction of lactic acid bacteria exhibited marked direct antiproliferative activities against colon and gastric cancer cell lines, whereas peptidoglycan was effective for the retardation of colon and bladder cancer cell lines [28]. The authentic components, having antiproliferative activity in cytoplasm fraction, and their action mechanism are unknown at present [29]. The mechanism of action of sulfobacin A from *C. gleum* LMG P-22264 also needs to be studied.

Conclusion

The investigations revealed that an alkaliphilic soil isolate was *C. gleum* and observed to produce a sphingolipid type of compound called sulfobacin A. Inhibition by L-cycloserine confirmed the synthesis of sulfobacin A from *C. gleum* LMG P-22264. Sulfobacin A from *C. gleum* LMG P-22264 showed antiproliferative activity against various cell lines and this potential could be exploited.

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