

# An antibacterial compound Pyrimidomycin produced by *Streptomyces* sp. PSAA01 isolated from soil of Eastern Himalayan foot-hill

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## Article

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# Abstract

Selective isolation of soil Actinobacteria was undertaken to isolate a new class of antibiotics and bioactive molecules. A *Streptomyces* sp. PSAA01 (MTCC 13157), isolated from soil of Eastern Himalaya foot-hill was cultivated on a large scale for the production of the antimicrobials SM02. It has been found that the maximum amount of SM02 produced while PSAA01 was grown in ISP-2 medium (pH 7.0) for 7 days at 30°C. A significant zone of inhibition against *Staphylococcus aureus* has been found with the crude cell-free culture media (50 µL) of 7 days grown PSAA01. After the purification and chemical structural characterization, we found that SM02 is a new class of antibiotic having 747 dalton molecular weight. The compound SM02 contains pyrimidine moiety in it and is produced by a species of *Streptomyces* and thus we have named this antibiotic pyrimidomycin. The antimicrobial spectrum of SM02 has been found to be restricted in Gram-positive organisms with a MIC of 12 µg/mL. SM02 was found active against *Mycobacterium* sp. and also multi-drug resistant Gram-positive bacteria with similar potency and found to disrupt the bacterial cell wall. SM02 also showed significant impairment in the biofilm formation by *S. aureus*. Furthermore, SM02 showed synergy with the most used antibiotic like ampicillin, vancomycin and chloramphenicol. SM02 did not have cytotoxicity towards human cell lines indicating its activity is limited within bacteria.

## Introduction

Actinomycetes are known to be the most common source of antibiotics. *Streptomyces* are a very common genus for antibiotic production [1]. A diverse group of antibiotics derived from actinomycetes is used broadly in medicinal purposes, for example, β-lactams, tetracyclines, macrolides, aminoglycosides, or glycopeptides [2]. Soil has been regarded as a great reservoir of actinobacterial strains [3, 4]. It has been estimated that around 45% of the bioactive compounds and 80% of antibiotics obtained from actinomycetes are derived from the two genera, *Streptomyces* and *Micromonospora* [5, 6]. Actinobacteria generally produce antibiotics when they are exposed to diverse stress conditions. Stress conditions might often appear through nutrient deprivation which induces actinobacteria, especially *Streptomyces* to transform from vegetative mycelial structure to erected sporogenic aerial mycelia [7]. In addition to the nutrient deprivation, the presence of different neighbouring genera is also a limiting factor for the actinobacterial growth and it also induces antibiotic production to make its growth smooth.

*Streptomyces* are in general filamentous, Gram-positive bacteria present in all types of environments with a high G + C content in their genome. It has been estimated that soil itself contains about 90% of total actinobacteria. Due to the presence of diverse *Streptomyces* species on a large scale, the isolation of novel antibiotic-producing species should be very specific [8].

Recently, the treatment of most infectious diseases and control of different pathogenic organisms have become one of the key challenges as the uses of the antibiotics are compromising which makes the pathogens turn resistant against most of the antibiotics and making them multi drug-resistant pathogens (MDR pathogens) [9, 10]. There are several strategies such as efflux followed by most of the MDR

pathogens against the antimicrobial agents [10, 11]. The most common strategy of the MDR pathogens to be resistant against the antimicrobial agent is biofilm formation [12]. The problem of the exponential rise of antimicrobial resistance can be mitigated with antimicrobial agents produced by other microbes as the majority of clinically relevant antibacterial agents (75%) are natural products of the microbial origin or their analogs [10, 13, 14].

There are many types of antibiotics which are having different targets for the inhibitory effects on the microorganisms. Formation of DNA, cell wall, proteins are the major targets for most of the antibiotics to inhibit the organisms. Carbenicillin, penicillin G, cefuroxime, aztreonam, cefoperazone, vancomycin, oxacillin, ampicillin are known as beta-lactam drugs that target the cell wall synthesis of the organisms and inhibit them [15, 16]. On the other hand, oleandomycin, clindamycin, lincomycin, tobramycin, chloramphenicol, gentamycin, streptomycin, etc. block cellular protein synthesis to inhibit the microorganisms [17–20]. Besides these, another category of antibiotics which includes levofloxacin, novobiocin, rifampicin, ofloxacin, nitrofurantoin, etc. target nucleic acid synthesis [21]. However, some antibiotics may affect even the eukaryotic systems by interfering with several cellular functions [22] and thus it is very crucial to check the toxic effect on human cells.

In this study, an antimicrobial producing *Streptomyces* sp. PSAA01 has been isolated along with 25 other actinobacteria from the soil sample collected from Manas National Park, Assam, India. The antimicrobial production has been optimized taking various media, culture time and media pH. The chemical structural elucidation of the compound has also been revealed using CHNS/O analysis, mass spectrometry, FTIR,  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR, UV-Visible and fluorescence spectroscopy. The antimicrobial and antibiofilm properties of the compound have been investigated using multiple test pathogens. The cytotoxicity of the compound has further been checked against the human cell line and probable mode of action has been deciphered.

## Methods

### Selective isolation of actinomycetes.

The soil samples were collected from Manas National Park, Chirang and Baksa (26°65'N-91E; altitude: 200 ft; annual rainfall: 333 cm and a temperature range from 15 to 37° C). In order to enrich and selectively isolate the actinobacteria, the soil samples were subjected to pre-treatment with  $\text{CaCO}_3$  and incubated for 7 days at room temperature followed by incubation for 2 hr at 65°C in a hot air oven [23,24]. 1 g of the pre-treated soil was dissolved in 1 mL of 0.9% NaCl and diluted sequentially up to  $10^{-6}$ . Each 0.1 mL of the diluted sample was spread on a starch casein medium. The medium was supplemented with cycloheximide (50  $\mu\text{g}/\text{mL}$ ) and nystatin (50  $\mu\text{g}/\text{mL}$ ) to inhibit undesired fungal growth. After incubation for 3–4 days at 30°C, the inoculated plates showed different actinobacterial colonies (25). Each of the colonies having different and unique features has been selected and streaked on fresh ISP-2 (International Streptomyces Project) plate [23,26].

# Identification of the PSAA01

An isolate, PSAA01 with differential pigmentation (aerial and substrate mycelium and diffusible pigments) was incubated for 14 days on ISP-2 medium [26]. Various colony morphology like colony size, shape, texture, margin, optical properties was observed on 14 days of incubation on ISP-2 medium. The cellular morphology has been observed under Scanning Electron Microscope following the standard protocol [27]. The 16S rDNA gene was amplified with the help of 8F and 1492R universal primers followed by sequencing of the amplicon and further sequence analysis using BLASTn program (<https://blast.ncbi.nlm.nih.gov>) and EZbiocloud server [28]. 16S rDNA gene sequences of the closest type strains were obtained from the EZbiocloud server [28] and the phylogenetic analyses were performed using Neighbour Joining (NJ) [29] and Maximum Likelihood (ML) [30] algorithms. To reveal the evolutionary status of PSAA01, the distance-based and character-based phylogenetic trees were constructed. The 16S rDNA gene sequence of the strain was deposited in NCBI and the accession number is MT829328.

## Preliminary screening for antimicrobial property.

Antimicrobial activity of the strain PSAA01 was examined in Mueller-Hinton (MH) agar media following the standard agar-diffusion method [31]. In this method, 5–7 days old colony of PSAA01 (10 mm in diameter) was picked and inoculated on the plate which is just spread with exponentially grown test organisms such as *Staphylococcus aureus* MTCC 96 and *Bacillus cereus* MTCC 1272 (Gram-positive) and *Escherichia coli* MTCC 1687 and *Pseudomonas aeruginosa* (Gram-negative). For a homogenous distribution of antimicrobial compound, the plates were kept at 4°C for 2 hrs followed by the incubation at 37°C for further 24 hrs [27].

## Production optimization.

The optimal production of the antimicrobial compound produced by PSAA01 has been explored with various media conditions, incubation time and the media pH. Five media AIA [26], ISP-2 [32], ISP-3 [32], tryptic soya broth (TSB) [33], and starch casein [32] were selected by virtue of the differences in their composition [25] while growth parameters like temperature (28°C), pH (8), shaking speed (180 rpm), time (7 days), and inoculum (1%) were kept constant. The medium which showed the maximum antibiotic production was selected for the all-downstream experiments. The timepoint and pH for the maximum antibiotic production in the selected medium were also investigated. The medium which allows the maximum production of antibiotics was checked by measuring the wet weight of the cell mass of the strain PSAA01 inoculated in five different media.

# Determination of antimicrobial potency of SM02 compound.

The efficacy of the antimicrobial activity of the SM02 produced by the isolate PSAA01 was determined against 6 different Gram-positive test organisms (*S. aureus* MTCC 96, *S. pyogenes* MTCC 1928, *B. cereus* MTCC 1272, *B. subtilis* MTCC 441, *S. aureus* MRSA, *Mycobacterium smegmatis* mc<sup>2</sup> 155) and 4 different Gram-negative test organisms (*E. coli* MTCC 1687, *P. aeruginosa*, *Klebsiella pneumoniae*, *Salmonella typhi*) and compared with the standard antibiotics like ampicillin (10 µg) and chloramphenicol (4 µg). To determine the antimicrobial efficacy of the compound, 0.1 mL culture of 0.1 OD (A<sub>600</sub> nm) cell of test organisms were seeded on MH agar medium and the 50 µL of the cell-free extract was applied in the well made in each plate along with the standard antibiotic in independent well [27]. The plates were incubated for 24 hr for all the test organisms and 48 hr for *Mycobacterium smegmatis* mc<sup>2</sup> 155. After the incubation the zone of inhibition was observed.

## Antibiotic sensitivity of PSAA01.

The PSAA01 has been assessed in order to know the probable antimicrobial categories of the compound produced by it, efficacy of thirty standard antibiotics were tested keeping in mind that the producer strain supposed to be resistant towards the compound it produces. 0.1 mL of the freshly grown culture of the strain PSAA01 was seeded on MH agar plate and disc diffusion protocol was followed by using different types of antimicrobial compounds such as (i) cell wall inhibitors: penicillin G (10 U) (P), carbenicillin (10 µg) (CB), cefoperazone (75 µg) (CPZ), cefuroxime (30 µg) (CXM), aztreonam (30 µg) (AT), vancomycin (30 µg) (VA), oxacillin (25) (Oxa), ampicillin (10) (Amp); (ii) nucleic acid inhibitors: levofloxacin (5 µg) (LE), novobiocine (30 µg) (NV), rifampicin (30 µg) (R), ofloxacin (5 µg) (OF) and nitrofurantoin (30 µg) (NIT); (iii) protein synthesis inhibitors: clindamycin (2 µg) (CD), lincomycin (15 µg) (L), tobramycin (10 µg) (TOB), oleandomycin (15 µg) (OL), streptomycin (25 µg) (S), chloramphenicol (30 µg) (C), gentamycin (10 µg) (GEN). After incubation, the antibiotic sensitivity was noted by measuring the diameter of the zone of inhibition. The strain PSAA01 was marked as sensitive or resistant based on the growth pattern on the medium. The appearance of zone around the disc indicated the sensitivity whereas the absence of any zone marked the strain as resistant.

## Production, extraction, and purification.

The strain PSAA01 was inoculated in a freshly prepared sterile ISP-2 medium (volume: 3 L) and incubated at 30°C for 7 days. After the incubation, the medium was centrifuged for 15 min at 13000 rpm to collect the supernatant. An equal volume of ethyl acetate with the supernatant was added to extract the active compounds. After extraction, the active organic phase was collected and dried by a rotary evaporator. The crude sample SM02 was subjected to TLC (R<sub>f</sub>: 0.3) with chloroform and petroleum ether (1:1, v/v) as mobile phase solvent. The concentrated extract was collected from preparative TLC and

further purified through column chromatography using Silica gel 60 (F<sub>254</sub>) and chloroform/petroleum ether (1:1, v/v) as solvent system. The pure product was obtained as light brown solid after the evaporation of the solvent from the eluted fractions through the rotary evaporator. The purified product was then used further for its chemical and functional characterization.

## Estimation of MIC and MBC.

To determine the minimum inhibitory concentration (MIC), 190  $\mu\text{L}$  MH medium and 10  $\mu\text{L}$  stock solutions (7.5, 15, 30, 60, 120, 240, 480,  $\mu\text{g}/\text{mL}$ ) of SM02 compound derived from PSAA01 was mixed into the well of 96-well plate in order to get a final concentration of SM02 at 0.375, 0.75, 1.5, 3, 6, 12, and 24  $\mu\text{g}/\text{mL}$ .  $5 \times 10^5$  cells were used for the inoculation into each experimental set of both MIC and minimum bactericidal concentrations (MBC) assay. After adding SM02, the 96-well plate was incubated at 37°C for 24 hrs at shaking conditions (180 rpm). MIC was recorded as the lowest SM02 concentration where visible growths of the test organism were absent. Minimum bactericidal concentrations (MBC) were analyzed in microplates through the broth dilution method [34]. 45  $\mu\text{L}$  sterile MH broth along with 5  $\mu\text{L}$  (240, 480, 960 and 1920  $\mu\text{g}/\text{mL}$ ) SM02 compound and 50  $\mu\text{L}$  test inoculum was mixed and volume adjusted to 100  $\mu\text{L}$  to get a final concentration of SM02 at 12, 24, 48 and 96  $\mu\text{g}/\text{mL}$ , followed by the incubation at 37°C for 24 hrs shaking condition (180 rpm). After incubation, 10  $\mu\text{L}$  cultures from each well were taken for colony counts and were spread on LA plates and incubated at 37°C for 24 hrs. MBC was noted as a culture with a minimum concentration of the compound SM02 in which no colony was found on the agar plate.

## Observation under Scanning electron microscopy.

To investigate the alteration of the cellular morphology of the test organisms *B. cereus* MTCC 1272 and *S. aureus* MTCC 96 after treatment with the compound SM02. Scanning electron microscopy was performed according to the standard protocol [35]. The test organism was treated with SM02 compound (12  $\mu\text{g}/\text{mL}$ ) in MH medium [31], followed by incubation at 37°C for 24 hr in shaking condition. The treated samples were subjected to centrifugation and fixed by 2.5% glutaraldehyde solution in phosphate buffer for 30 min. The fixed cells were again centrifuged and washed with 0.1 M phosphate buffer (pH 7.2) three times. Then each suspension was serially dehydrated with 25%, 50%, 75%, 90%, and 100% ethanol, respectively. The samples were placed under the microscope and images were captured by ZEISS-EVO-MA-10.

## Biofilm assay.

The antibiofilm property of SM02 was determined using the crystal violet assay. 100  $\mu\text{L}$  of *S. aureus* (O.D was adjusted to 0.5 according to McFarland protocol) was treated with 5  $\mu\text{L}$  SM02 compound of sub-MIC concentrations (1.5  $\mu\text{g}/\text{mL}$ , 3  $\mu\text{g}/\text{mL}$  and 6  $\mu\text{g}/\text{mL}$ ) in MH broth and incubated at 37°C for 24 hr in

shaking condition. After incubation, planktonic cells were discarded and washed with sterile water. Then 100  $\mu\text{L}$  of 0.1% crystal violet solution was added and incubated for 10-15 min at room temperature. The excess crystal violet was washed 3-4 times with sterile water and dried for a few hours. The O.D was documented at 550 nm in a 96-well plate reader after diluting these ten-fold [36].

## Cytotoxicity assay

HuH-7 cell lines (RRID: CVCL\_0336) were prepared from the continuous culture in Dulbecco's Modified Eagle's Medium (DMEM, Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen), penicillin (100  $\mu\text{g}/\text{mL}$ ), and streptomycin (100  $\mu\text{g}/\text{mL}$ ). Cells were initially propagated in a 75  $\text{cm}^2$  polystyrene, filter-capped tissue culture flask in an atmosphere of 5%  $\text{CO}_2$  and 95% air at 37°C in a  $\text{CO}_2$  incubator. When the cells reached the logarithmic phase, the cell density was adjusted to  $1.0 \times 10^5$  per/well in culture media. The cells were then used to inoculate in a glass-bottom dish, with 1.0 mL ( $1.0 \times 10^4$  cells) of cell suspension in each dish. After cell adhesion, the culture medium was removed. The cell layer was rinsed twice with phosphate-buffered saline (PBS) (pH 7.0). Cells were centrifuged at 1200 rpm for 10 min after treatment with trypsin. The supernatant has been discarded and the fresh medium was added to the pellet to adjust  $10^4$  cells in 100  $\mu\text{L}$  medium followed by incubation overnight. All tests have been repeated at least three times. Overnight grown cells were treated with different concentrations of SM02 compound and further incubated overnight. Upon incubation 20  $\mu\text{L}$  MTT reagents (5 mg/mL) were added in each set and incubated at shaking condition for 4 hrs. The supernatants were discarded and 150  $\mu\text{L}$  DMSO was added to dissolve formazan, formed due to the reaction of the MTT dye and NAD(P)H-dependent cellular oxidoreductase present only in viable cells. The absorbances were then measured at  $A_{590}$  nm [37].

## Antimicrobial efficacy of Pyrimidomycin in combination with other antibiotics

To determine the combinatorial effect of pyrimidomycin with the most common antibiotics used in the present treatment regimen, we have used the preliminary checkerboard method according to the standard protocol [38]. The final concentrations of all antibiotics were MIC to 1/4 MIC value in different a 96-well plate. The final concentration of this test was as follows for ampicillin 0.1, 0.2, 0.4  $\mu\text{g}/\text{mL}$ ; vancomycin 0.25, 0.5, 1  $\mu\text{g}/\text{mL}$ ; chloramphenicol 1, 2, 4  $\mu\text{g}/\text{mL}$ ; and pyrimidomycin 3, 6, 12  $\mu\text{g}/\text{mL}$ . This test was performed in triplicate on 96 well plate with MH broth. Total MH broth volume taken was 100  $\mu\text{L}$  in each well. 5  $\mu\text{L}$  test compound (SM02) from different stock solutions, 5  $\mu\text{L}$  test compound (another antibiotic), 50  $\mu\text{L}$  of freshly diluted respective cells and 40  $\mu\text{L}$  of MH broth was added in each well. The final inoculum was  $5 \times 10^5$  CFU/mL. Also, each well contain only the respective individual antibiotics along with inoculum. The cells were incubated in shaking conditions for 16 hrs at 37°C. After that OD was taken at 600 nm. In vitro interaction was measured as the fractional inhibitory concentration index (FICI), the

formula of FICI calculation is (MIC of drug A in combination/MIC of drug A alone) + (MIC of drug B in combination/MIC of drug B alone). FICIs were interpreted as follows: <0.5, synergy; 0.5–0.75, partial synergy; 0.76–1.0, additive effect; 1.0–4.0, indifference; and >4.0, antagonism. The varying levels of synergy between two given agents were determined [38]. All experiments were repeated three times.

## Structural characterization of SM02

The solvents used were distilled and dried according to the standard procedures [39]. The high-resolution mass spectrometry (HRMS) was performed using a micromass Q-TOF Micro™ instrument by using methanol as a solvent. <sup>1</sup>H-, <sup>13</sup>C- NMR spectra were collected at 400 and 100 MHz, respectively, on a Bruker DRX spectrometer. For NMR spectra, CDCl<sub>3</sub> was used as solvent using TMS as an internal standard. Chemical shifts are expressed in δ ppm units and <sup>1</sup>H–<sup>1</sup>H and <sup>1</sup>H–<sup>13</sup>C coupling constants in Hz. The following abbreviations are used to describe spin multiplicities in <sup>1</sup>H NMR spectra: s = singlet; d = doublet; t = triplet; m = multiplet. KBr pellets have been used to record the FTIR spectra using a spectrophotometer. Fluorescence spectra of the compound SM02 were recorded on a Perkin Elmer Model LS 55 spectrophotometer whereas the UV-Vis spectra were recorded on a SHIMADZU UV-3101PC spectrophotometer. Elemental analysis of the SM02 compound was carried out on CHNS/O analyzer.

## Results

### Isolation and identification of SM02.

A *Streptomyces* sp. PSAA01 (MTCC 13157) was isolated from soil collected from Manas National Park, Assam, India. *Streptomyces* is the most prevalent actinomycetes present in the soil and have a huge contribution towards the development of pharmaceuticals. The strain was grown in the ISP-2 medium. The colony of the isolated strain has been found to be irregular, blackish and rough (Fig. 1a). Scanning electron micrograph of PSAA01 shows that the filamentous mycelium and the spore surfaces are rough (Fig. 1b). The 16S rDNA gene sequence (MT829328) of the strain PSAA01 (MTCC 13157) is having a 99.72% similarity with *Streptomyces melanosporofaciens* DSM 40138, as analysed with EZBioCloud. Phylogenetic analysis with Neighbour Joining (NJ) algorithm shows that the isolate originated from the same ancestor as *Streptomyces melanosporofaciens* DSM 40138, but based on the branch length, it can be assumed that the isolate might be quite different from its closest neighbour, and could be a new species (Fig. S1). It has been found to secrete a bioactive compound SM02, which is having antimicrobial property.

### Production optimization of the compound SM02

ISP-2 medium was found to be the best-tested medium for SM02 production and showed the highest inhibitory effect against *S. aureus*. To estimate the time-point in which the maximum production of the

compound SM02 has occurred was checked by incubation of the strain in ISP-2 medium up to 15 days. The production was found to be maximum after 7 days of incubation (Fig. S2a). Similarly, it has been found that the optimum pH was 7.0 for SM02 production (Table S1; Fig. S2b).

**Antimicrobial activity of SM02:** The zone of inhibition of different test organisms was observed after performing the disc diffusion experiment with the SM02 compound. Among the tested organisms, 7 Gram-positive test organisms (*S. aureus* MTCC 96, *S. pyogenes* MTCC 1928, *B. cereus* MTCC 1272, *B. subtilis* MTCC 441, *S. aureus* MRSA, *M. smegmatis* mc<sup>2</sup> 155) has been found to be sensitive against SM02 compound, whereas the no zone of inhibition was observed against any of the 4 Gram-negative (*E. coli* MTCC 1687, *P. aeruginosa*, *K. pneumoniae*, *S. typhi*) organisms which indicates that SM02 is active against Gram-positive bacteria (Fig. 2; Fig. S3).

**Antibiotic sensitivity test by disc diffusion method:** The category of the isolated compound, SM02, can be assumed from the data of disc diffusion assay of different antibiotics. The effect of those used antibiotics was checked on the isolated *Streptomyces* sp. PSAA01. From this experiment, it has been found that no zone of inhibition was formed in the case of penicillin, rifampicin, ampicillin and aztreonam, which hints that SM02 might be related to these classes of antibiotics and thus the producer organism gets resistant, if not acquired by such trait due to horizontal gene transfer or random mutagenesis (Table S2).

## Structural elucidation of SM02 compound.

3 liters of SM02 culture were extracted with ethyl acetate and the ethyl acetate fraction was dried in a rotary evaporator. The crude mixture was subjected to load on a preparative thin layer chromatography (TLC) plate (Silica gel 60 F<sub>254</sub>) using PET:CHCl<sub>3</sub> (1:1,v/v), R<sub>f</sub>= 0.30. The collected TLC-spot with desired activity was then run into flush column chromatography consisting of silica gel bed (60-120 mesh) with the same eluent ratio of the solvents to purify the compound. Structural characterization was performed by CHNO/S analysis, mass spectrometry, FTIR, <sup>1</sup>H NMR, <sup>13</sup>C NMR, UV-Visible and fluorescence spectroscopy. The mass spectrum of SM02 showed a base peak [M+H]<sup>+</sup> at m/z 747.4687 (Fig. S4) (calculated mass: 746.3250) (Table 1). The molecular formula was determined to be C<sub>42</sub>H<sub>46</sub>N<sub>6</sub>O<sub>5</sub>S based on HRMS analysis data by considering the number of protons and carbons from NMR spectrum (Table 2). Sulfur atom is predicted to be present in SM02 as we performed special elements test by lassaigne test. IUPAC name of SM02 is (2E,5E)-6-(2-((Z)-2,3-dimethyl-4-(6-((6-methyl-5-oxo-4,5-dihydropyrazin-2-yl)methyl)-4-((E)-2-(((E)-4-oxobut-2-en-2-yl)sulfinyl)vinyl)pyridin-2-yl)but-2-en-1-yl)-6-((6-methyl-5-oxo-4,5-dihydropyrazin-2-yl)methyl)pyridin-4-yl)-4-ethylhexa-2,5-dienal (Fig. 3).

The compound showed absorption maxima at 280 nm and emission maxima at 400 nm (Fig. S5, S6). Absorption at 3354, 2934, 2832, 1710, 1661, 1456, 1380, 1027, 928, 744 cm<sup>-1</sup> in the FTIR spectrum suggests the presence of N-H stretching (Amide), C-H stretching (Alkane), C-H stretching (Aldehyde), C=O stretching (Aldehyde), C=O stretching (Amide), C-H bending (Alkane, methyl group), C-H bending

(Aldehyde), S=O stretching (Sulfoxide), C=C bending (Alkene, trans disubstituted), C=C bending (Alkene, trisubstituted) (Table S3, Fig. S7). The detailed NMR studies ( $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR, DEPT-135, COSY and HMBC) (Fig. S8-S12) were performed to establish the structure of SM02.  $^{13}\text{C}$  NMR spectrum (Fig. S9) showed 26 signals that were assigned to 6 methyl, 3 methine, 8 methylene and 15 quaternary carbons. DEPT-135 NMR spectrum (Fig. S10) showed 17 signals in which 14 positive signals and 3 negative signals that suggest the presence of 14 types of CH/CH<sub>3</sub> carbons and 3 types of CH<sub>2</sub> carbons in SM02. The total structure of SM02 consists of substructures A and B (Fig. 3).

**Table 1**

Physicochemical properties of SM02

<b>Appearance</b>	<b>Gummy brown solid</b>
Molecular formula	C <sub>42</sub> H <sub>46</sub> N <sub>6</sub> O <sub>5</sub> S
Molecular weight	746.9270
CHN analysis	C:67.53; H:6.23; N: 11.26; O:10.70; S:4.28
HRMS m/z	(M+H) <sup>+</sup>
Calcd	746.3250
Found	747.4687
UV (Methanol)	280 nm
IR (KBr) cm <sup>-1</sup>	3354, 2934, 2832, 1710, 1661, 1456, 1380, 1027, 958, 744

**Substructure A:** Substructure A (Fig. 3) is the one hand of SM02. It contains a pyridine ring and a pyrimidine moiety attached with a methane (CH<sub>2</sub>) group. The ortho-coupled aromatic protons at  $\delta$  7.24 (H10, 13) and  $\delta$  6.14 (H8) were connected by the COSY spectrum (Fig. 4; Fig. S11).

An analysis of HMBC spectroscopic data provided further structural information on substructure A (Fig. 3, 4). H16 (at  $\delta$  7.75) showed a long-range correlation to  $\delta$  165.53(C 15) and  $\delta$  170.28 (C12). The cross peak from  $\delta$  7.21 (NH a) to  $\delta$  165.53 (C15) and  $\delta$  166.13 (C18), also from H<sub>3</sub>-19 ( $\delta$  2.03) to C18 supported the partial structure of the center ring. A long-range coupling from the proton signal of the pyridine unit at  $\delta$  7.24 (H13,10) to the methylene carbon  $\delta$  110.97 (C7) and  $\delta$  126.38 (C8) was observed. The presence of long-range coupling from the proton signal of olefinic proton  $\delta$  6.14 (H8) to methylene carbon at  $\delta$  111.94 (C3) and proton  $\delta$  5.46 (H7) to methyl carbon at  $\delta$  12.66 (C6) has also been revealed. In HMBC spectra there is three-bond cross coupling between C4 ( $\delta$  51.82)-H<sub>3</sub> 6 ( $\delta$  0.96) and four-bond cross coupling between C4 and aldehydic hydrogen H1 ( $\delta$  9.61), indicating the presence of substructure A.

**Table 2**<sup>1</sup>H and <sup>13</sup>C NMR spectral data of SM02 in CDCl<sub>3</sub>

Position	$\delta_{\text{H}}$ , J(Hz)	$\delta_{\text{C}}$	Position	$\delta_{\text{H}}$ , J(Hz)	$\delta_{\text{C}}$
1	9.61-9.63(d, J=8)	209.05	23	–	133.57
2	5.46-5.47(d, J=4)	156.49	24	1.59 (s)	21.16
3	7.14-7.18 (d, J=16)	153.34	25	3.55(s)	38.57
4	3.11-3.15 (t, J=16)	51.82	26	–	166.13
5	1.38-1.41(d, J=12)	29.81	27	–	166.13
6	0.96-0.98 (t, J=8)	12.66	28	7.24 (s)	110.97
7	7.10-7.12(d, J=8)	126.38	29	–	153.47
8	7.03-7.05(d, J=8)	130.53	30	7.24 (s)	110.97
9	–	153.47	31	7.21-7.22(d, J=4)	146.18
10	7.24 (s)	110.97	32	6.32-6.33(d, J=4)	145.48
11	–	166.13	33	–	170.28
12	–	166.13	34	6.63-6.65(d, J=8)	143.43
13	7.24 (s)	110.97	35	9.61-9.63(d, J=8)	200.34
14	3.55(s)	45.29	36	2.53(s)	14.44
15	–	111.94	37	3.55(s)	45.29
16	8.55(s)	122.03	38	–	111.94
17	–	182.54	39	8.55 (s)	122.03
18	–	165.53	40	–	182.54
19	2.03(s)	23.14	41	–	165.53
20	3.55(s)	38.57	42	2.03 (s)	23.14
21	–	133.57	NH <sup>a</sup>	7.75-7.77(d, J=8)	
22	1.59(s)	21.16	NH <sup>a</sup>	7.75-7.77(d, J=8)	

**Substructure B:** Substructure B (Fig. 3) contained a pyridine ring and a pyrimidine moiety attached with a methane (CH<sub>2</sub>) group. The ortho coupled aromatic protons at  $\delta$  7.24 (H28, 30) and  $\delta$  6.32 (H31) were

connected by the COSY spectrum (Fig. 4; Fig. S11). From the HMBC spectroscopic analysis the presence of pyrimidine moiety (in substructure B) attached with a pyridine via CH<sub>2</sub> group has also been determined (same as substructure A) (Fig. 3, 4). A long-range coupling from the proton signal of the pyridine unit at  $\delta$  7.24 (H28,30) to the methylene carbon  $\delta$  130.53 (C31) and  $\delta$  133.57 (C32) was observed. The presence of long-range coupling from the proton signal of olefinic proton  $\delta$  6.63 (H32) to  $\delta$  143.43 (C33) and methyl carbon at  $\delta$  21.16 (C36) has also been revealed. The cross coupling of methyl group  $\delta$  1.38 (H<sub>3</sub> 36) to C34 ( $\delta$  146.18) along with the aldehydic carbon C35 ( $\delta$  209.05) indicated the presence of substructure B.

In HMBC spectra, it has been observed that there are four-bond cross coupling between H10,30 ( $\delta$  7.24)-C21,23 ( $\delta$  122.03) and C11,26 ( $\delta$  170.28) -H22,24 ( $\delta$  1.59), which evident that the two substructures are connected through C21-C23 carbon as shown in figure 4.

## Pyrimidomycin inhibits growth and biofilm formation of Gram-positive pathogen

The MIC and MBC values of the pyrimidomycin against various sensitive test organisms were determined (Table S4, Fig. S3). The MIC against the Gram-positive organisms like *S. aureus*, *S. epidermidis*, *S. pyogenes*, *B. cereus*, *S. aureus* MRSA, *M. smegmatis* was 12  $\mu\text{g}/\text{mL}$ . However, we found that the MBC values are relatively higher and are more than 50  $\mu\text{g}/\text{mL}$ . The cellular alteration was observed after the SM02 compound treatment to the test organism. The cellular morphology of the untreated *B. cereus* MTCC 1272 and *S. aureus* MTCC 96 was intact (Fig. 5a, c) whereas the treated cells exhibited altered morphology (Fig. 5b, d), particularly the cellular membrane anticipated to be damaged severely. This data help to reveal the mode of action of the isolated compound, SM02. Further, the antibiofilm property of the pyrimidomycin was determined in sub-MIC value (1.56, 3 and 6  $\mu\text{g}/\text{mL}$ ) by crystal violet assay. In the case of positive control (untreated cells) the optical density was shown to be highest. The optical density was found to be decreased with the increasing concentration of pyrimidomycin indicating that the compound inhibits the biofilm formation by the test pathogen and thus could inhibit the colonization of the pathogen if applied (Fig. 6).

The inhibitory effect of SM02 compound with different concentrations (12, 24, 48, 96 and 192  $\mu\text{g}/\text{ml}$ ) was also checked on human cell line (HuH-7) through MTT assay. The compound did not show any significant toxic effect on the cell line and can be predicted as non-toxic to humans (Fig. 7). However, the standard experiments on the mammalian models and the human subjects will confirm on its suitability for future use.

## Fractional inhibitory concentration index of Pyrimidomycin

As we determine the MIC of pyrimidomycin is 12  $\mu\text{g}/\text{mL}$  but when combined with other individual antibiotics, it shows its MIC much lower than its actual individual MIC value. The individual MIC value of ampicillin, chloramphenicol, vancomycin are 0.4  $\mu\text{g}/\text{mL}$ , 4  $\mu\text{g}/\text{mL}$ , 0.5  $\mu\text{g}/\text{mL}$ , respectively. When 1/4 MIC

of pyrimidomycin was combine with 1/4 MIC of ampicillin, chloramphenicol and vancomycin it showed complete growth inhibition of the test organism. FICI value of both shows equal to 0.5 which indicates pyrimidomycin has a synergistic effect (Table. 3).

**Table 3**

Synergistic relationship of pyrimidomycin with highly used popular antibiotics like ampicillin, chloramphenicol, vancomycin.

Test organism	Antibiotics	MIC of antibiotics (µg/mL)	FICI*	Interpretation
<i>Staphylococcus aureus</i>	SM02	12	0.5	Synergy
	Ampicillin	0.4		
	SM02 + Ampicillin	3		
	Ampicillin + SM02	0.1		
	SM02	12	0.5	Synergy
	Chloramphenicol	4		
	SM02 + Chloramphenicol	3		
	Chloramphenicol + SM02	1		
	SM02	12	0.5	Synergy
	Vancomycin	1		
	SM02 + Vancomycin	3		
	Vancomycin + SM02	0.25		

\*Fractional inhibitory concentration index (FICI) = (MIC of drug A in combination/MIC of drug A alone) + (MIC of drug B in combination/MIC of drug B alone). FICIs were interpreted as follows:  $\leq 0.5$ = synergy; 0.5–0.75= partial synergy; 0.76–1.0= additive effect; 1.0–4.0= indifference; and  $>4.0$ = antagonism.

## Discussions

The studied *Streptomyces* was isolated from the soil sample collected from Manas National Park, Assam, India. The isolated strain PSAA01 has been identified as *Streptomyces* sp. which is capable of producing an antimicrobial compound named pyrimidomycin. The 16S rDNA sequence of the strain PSAA01 has been showing 99.72% similarity with *Streptomyces melanosporofaciens* DSM 40138. Small scale fermentation of the strain has been performed in different media with different temperatures, pH and incubation time to optimize the production of the pyrimidomycin. The used media contains different

major nutritional sources such as carbon, nitrogen. ISP-2 contains yeast extract, malt extract, dextrose; oat meal is present in ISP-3; casein is contained by starch casein medium; TSB contains peptic digest of soyabean meal, dextrose; sodium propionate, sodium caseinate, L-asparagine and glycerol is present in AIA medium. We have found that ISP-2 medium is the optimum medium for the production of the compound by the strain PSAA01 pH 7.0. The structural elucidation has been also done which reveals that the compound contains two substructures, substructure A and B. The molecular weight of this compound is 746.9270. The compound contains pyrimidine moiety and is yielded by *Streptomyces* sp., thus we named it pyrimidomycin.

Nowadays, multidrug resistance (MDR) is a challenging aspect in the clinical area as the control of the resistant strains becomes unsuccessful with most of antibiotics. This is responsible for most of the life-threatening diseases in humans [9, 40]. The isolated compound is specifically effective against Gram-positive organisms as well as exhibits an inhibitory effect on the methicillin resistant *S. aureus* (MRSA), this indicates the inhibitory potency of the newly found antibiotic. MRSA strains show their resistance property against typical beta-lactam drugs which include methicillin, amoxicillin, penicillin, nafcillin, oxacillin and cephalosporin [41]. The minimum inhibitory concentration of the compound is 12 µg/mL against *S. aureus*, *S. pyogenes*, *B. cereus*, *S. aureus* MRSA, *M. smegmatis* and 24 µg/mL for *B. subtilis*. The antibiofilm activity of the compound has been recorded at the sub-MIC values. The SEM assay has been showing the cell wall and cell membrane rupture after the treatment of *B. subtilis* or *S. aureus* with the MIC value (12 µg/mL). The cytotoxic assay has been showing that the pyrimidomycin does not affect tested human HuH-7 cell lines up to the concentration of 48 µg/ml concentration. From all these experiments and their outcomes, it can be demonstrated that the identified strain is a novel antibiotic producer. The SM02 compound has been successfully characterized and the presumable mode of action is also indicated in this study. The inhibitory effect of the compound on drug-resistance strain is a promising outcome. Furthermore, as pyrimidomycin shows a synergistic relationship with very common existing antibiotics like ampicillin, vancomycin and chloramphenicol, it might be a choice of antimicrobials in the future for its combinatorial use.

## Declarations

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### ADDITIONAL INFORMATION

**Author contribution statement:** PD designed and performed all the microbiological experiment, prepared the tables and figures and wrote the initial manuscript; SK designed and executed the chemical analysis of the antibiotic, prepared the tables and figures and wrote the initial manuscript; PKM helped isolation of

the bacterium; PS and SM conceptualized the research, designed the experiments and wrote and edited the main manuscript text.

### Data availability statement

The bacterial culture has been submitted to MTCC, India with the accession number MTCC 13157. The 16S rDNA gene sequence of the strain was deposited in NCBI and the accession number is MT829328. All the data associated with the research are available as supplementary files.

### Competing interests

The authors declare that there are no conflicts of interest

### Ethical approval

The research is not associated with any prior ethical approval.

### Consent of publication

The data used for the manuscript is original and does not require any consent from third party for publication.

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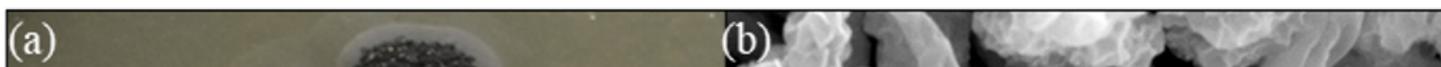
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## Figures

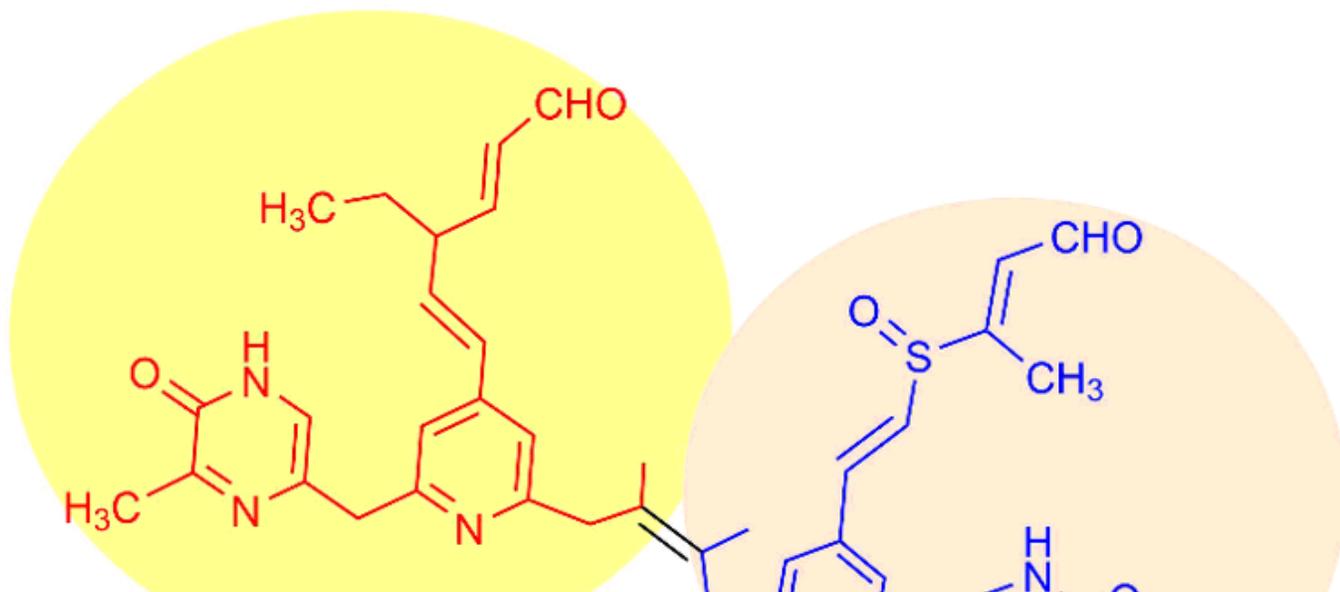


### Figure 1

Morphology of the isolate PSAA01. (a) The colony morphology of the strain PSAA01; (b) the cell morphology of the filamentous actinomycete strain PSAA01 as observed under scanning electron microscopy in 25000X magnification.

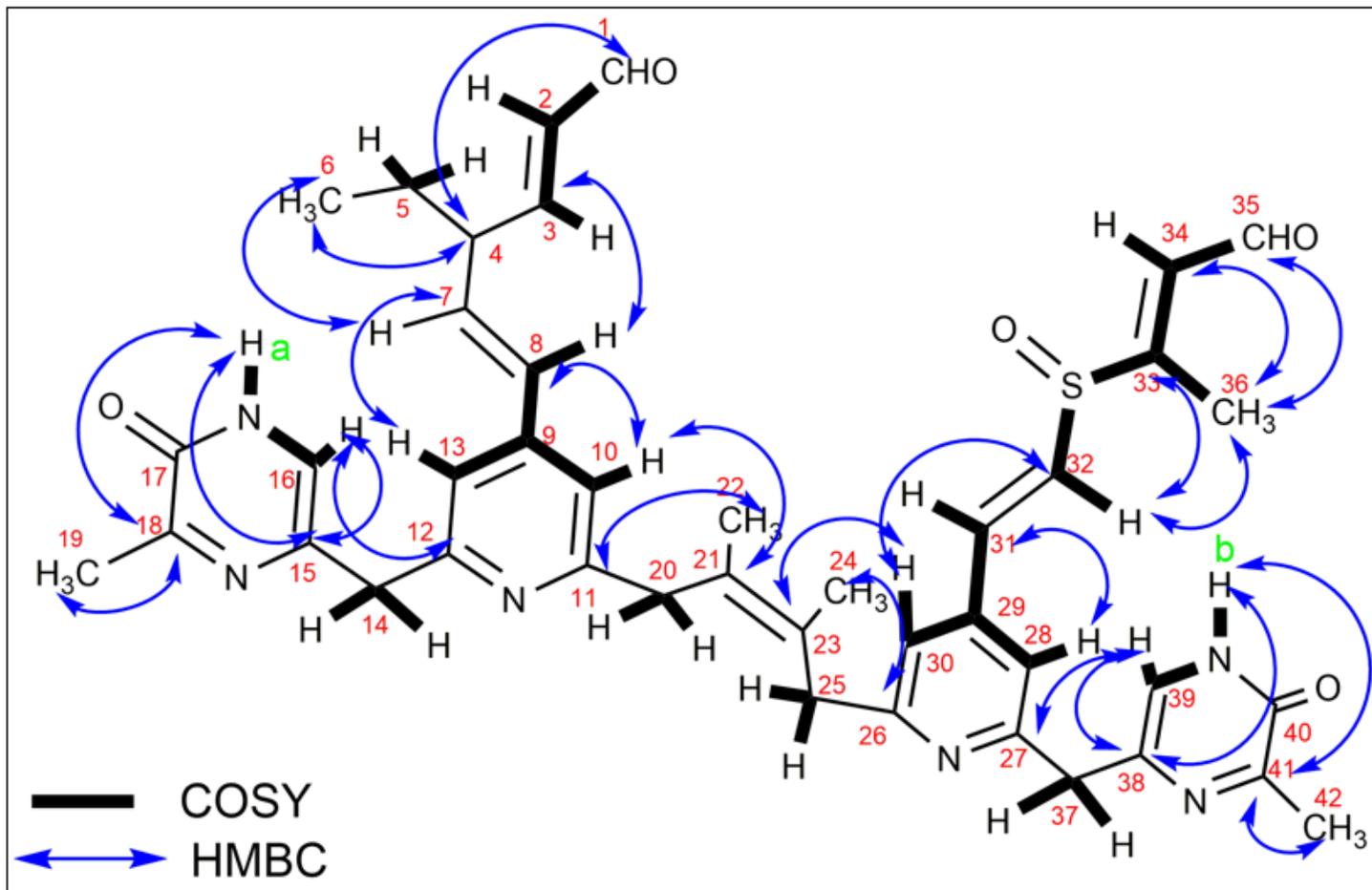
### Figure 2

Antimicrobial efficacy of crude SM02. Efficacy detected by zone of inhibition against different organisms using SM02. (a) = *S. aureus* MTCC 96, (b) = *S. aureus* MRSA, (c) = *B. cereus* MTCC 1272, (d) = *B. subtilis* MTCC 441, (e) = *S. pyogenes* MTCC 1928, (f) = *M. smegmatis* mc<sup>2</sup> 155; and 1 = SM02 crude, 2 = ampicillin (10 µg), and 3 = chloramphenicol (4 µg)



**Figure 3**

The total structure of pyrimidomycin with substructure A (in red) and B (in blue)



**Figure 4**

2D NMR (HMBC) correlation of pyrimidomycin

**Figure 5**

Pyrimidomycin induced membrane disruption of the test organisms *B. cereus* MTCC 1272 (a, b) and *S. aureus* MTCC 96 (c, d). (a and c) SEM images of untreated bacteria with smooth surface; (b and d) images showing cell compartment disruption (arrow-heads) visualized when the cells were treated with pyrimidomycin.

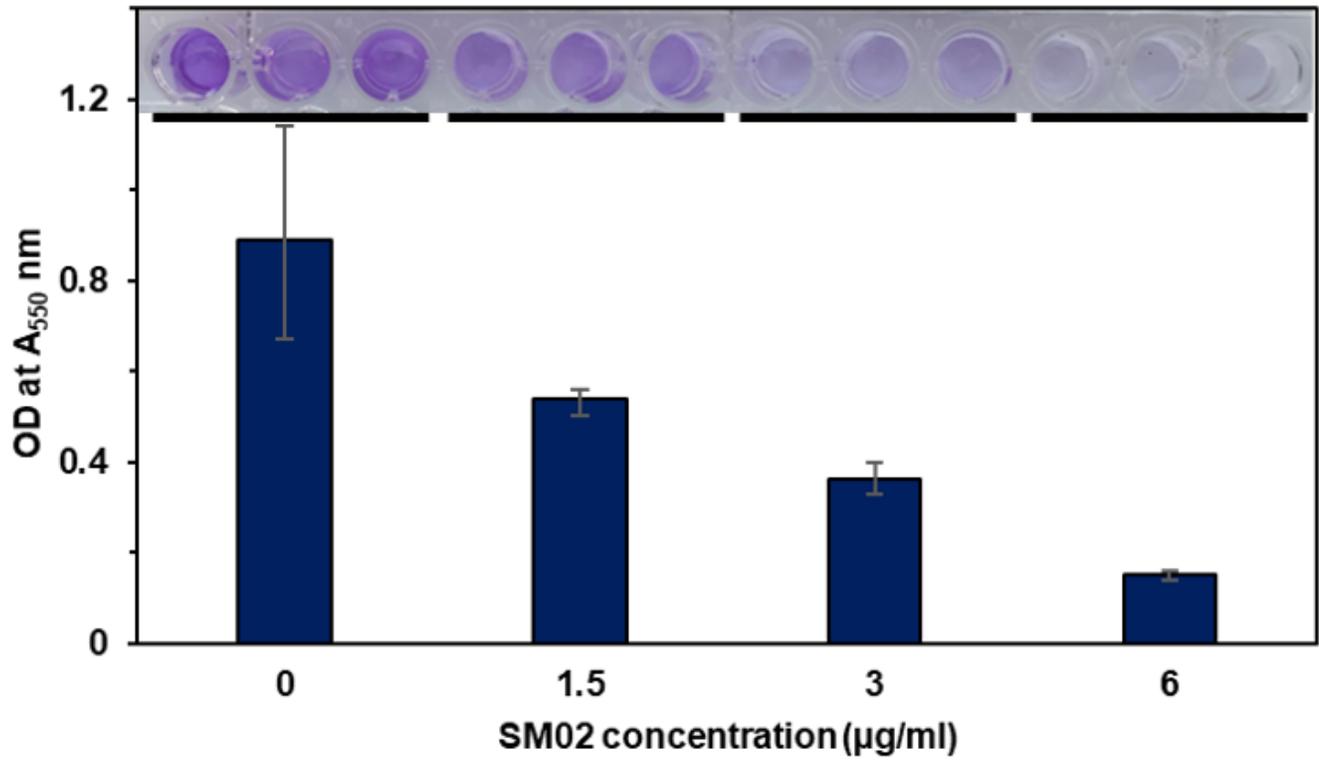


Figure 6

Antibiofilm properties of the compound pyrimidomycin. Increasing concentration leads to reduction of the biofilm formation.

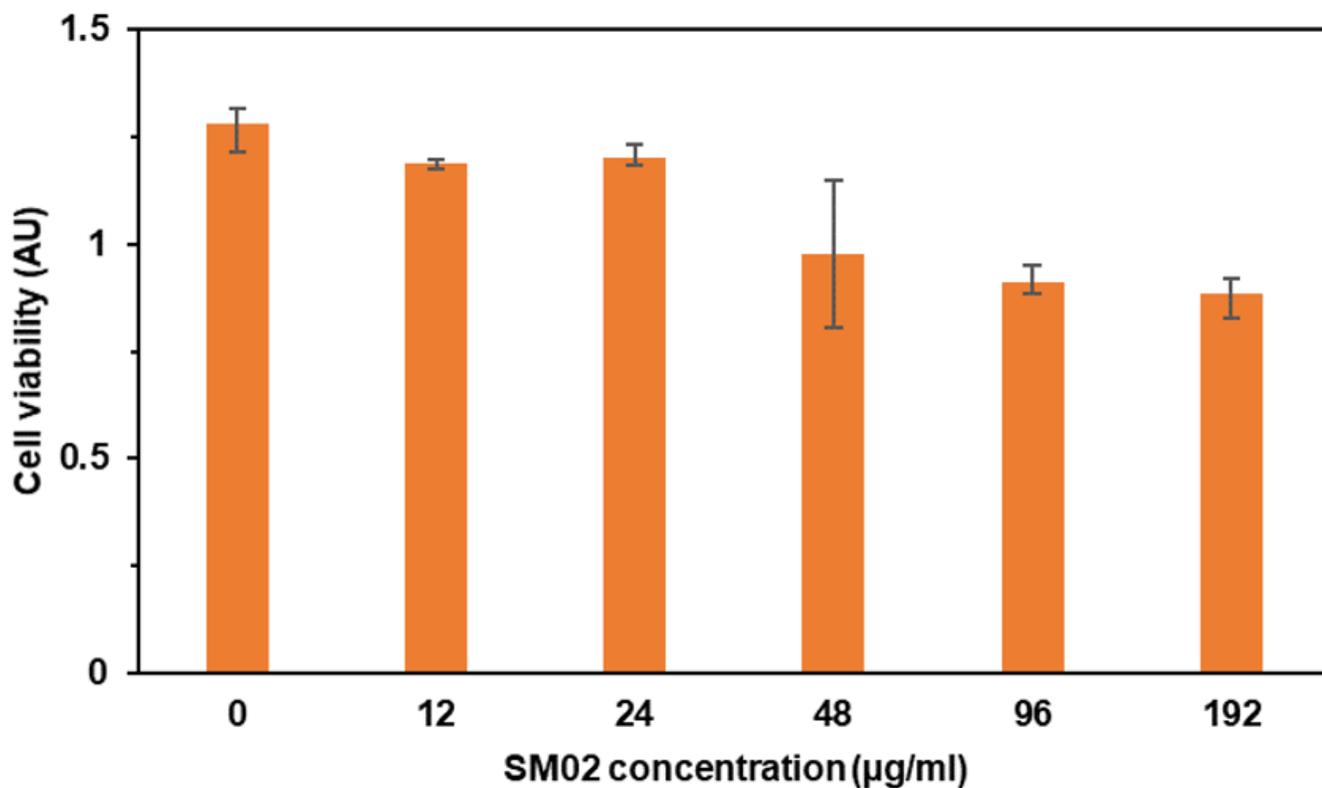


Figure 7

**Cytotoxicity assay of pyrimidomycin against HuH-7 human cell line.** Various concentration of pyrimidomycin was added in respective well having cells. Each concentration point was taken in triplicate.

## Supplementary Files

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