Precursor-directed combinatorial biosynthesis of cephalosporin analogue by endolithic actinobacterium *Streptomyces* sp. AL51 by utilizing thiophene derivative

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Abstract

Natural products or their derivatives provide a reliable resource for new drugs. The multi-step chemical reaction to produce new drug is not only expensive but also release pollutants. The precursor-based combinatorial biosynthesis (PCB) is, however, a better option to produce novel natural products with potential pharmaceutical applications. The present work is an attempt to synthesize an antibacterial compound by transforming thiophene precursor using endolithic *Streptomyces* sp. AL51. The *Streptomyces* sp. AL51 was isolated from a granite rock sample collected from Myllem, Meghalaya, India. The isolate was identified as *Streptomyces* sp. based on its cultural, morphological, biochemical and molecular characteristics. The bioactive compound CAX1 was extracted from the fermentation broth. The compound was characterized by bioactivity-guided fractionation and identified by infrared, UV–visible, nuclear magnetic resonance and mass spectrometry data and identified as 7-[1-(thiophene-5-yl)-1-formamido]-3-propylenyl-3-cephem-4-carboxylic acid with molecular formula $C_{15}H_{14}N_2O_4S_2$. The purified compound showed considerable in vitro antibacterial activity against both Gram-positive and Gram-negative bacteria showing its broad spectrum property. The obtained results provide promising baseline information for the potential use of endolithic actinobacterium for semisynthetic drug discovery. This is the first report on PCB of broad range antibacterial compound by endolithic *Streptomyces* strain.

Keywords Endolithic · *Streptomyces* · Cephalosporin analogue · Precursor based combinatorial biosynthesis · Antibacterial

Introduction

The increasing frequency of bacterial pathogens with multiple resistance to typically utilized antibiotics are posing challenges to healthcare companies as remedy possibilities are restricted and available solutions in many cases are costly (Walsh 2003). In spite of the presence of various antimicrobial agents, the search of new antibiotics

is essential with increase in drug-resistant pathogens (Huttner et al. 2013). Natural products or their derivatives provide a reliable resource to structural and mechanistic new drug (Berdy 2005). Among those natural sources, bacteria occupy a significant position (Raghava Rao et al. 2017). However, for the discovery of a new drug, only a small percentage of bacteria have been explored till now (Cragg and Newman 2013) and in many cases due to the structural complexity of natural products, it becomes difficult to produce molecules having better medicinal activities. In comparison to multi-step chemical reaction to produce a new drug which is expensive and lead to generation of pollutants, precursor-based combinatorial biosynthesis (PCB) provides an option for the production of novel natural products with potential pharmaceutical significance which is effective and ecologically friendly (Gao and Demain 2002; Kennedy 2007; Sun et al. 2015). In this process, a precursor analogue has to be fed to the producing microorganism to produce a new product (Kennedy 2007). Previously, propargyl-premonensin was produced

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with the addition of propargyl-malonyl-N-acetyl-cysteamine to fermentation broth of *Streptomyces cinnamomensis* A495 (Blicke 1952). There are some known biologically active and medicinally useful heterocyclic molecules which act as precursors in PCB for the synthesis of new drugs (Kennedy 2007; Tran et al. 2012). Thiophene is one of those heterocyclic compounds having five-membered ring. These thiophene derivatives are well known for therapeutic application (Mabkhot et al. 2016). It has been found that some thiophene derivatives can also act as precursors in the biosynthesis of antibiotics when they are supplemented to the fermentation medium (Blicke 1952).

Streptomyces are generally known for their importance in production of biological molecules like antibiotics, enzymes and other bioactive compounds (González et al. 2016). There are approximately 3000 identified antibiotics and more than half of the medically important antimicrobial and antitumor antibiotics are created by *Streptomyces* (Lu et al. 2015). They are well known for their possession of gene clusters which orchestrate antibiotic biosynthesis (Alexander and Jensen 1998). Aminoglycosides, anthracyclines, glycopeptides, β -lactams, macrolides, nucleosides, peptides, polyenes, polyethers and tetracyclines are major types of antibiotics produced by *Streptomyces* (Miyadoh 1993). Cephalosporins are one of the β -lactam antibiotics which are found to be active against vast number of pathogenic bacteria. *Streptomyces* including *S. clavuligerus* and *S. lipmanii* are one of the producers of cephalosporin and its derivatives (Corcoran 1981; Jensen and Vining 1985; Jermini and Demain 1989; Nigam et al. 2007; Antonio et al. 2012; Pana 2012). The occurrence of multi-drug resistant bacterial strains to already existing antibiotics has led to the search of new semisynthetic cephalosporin derivatives with better action (Pana 2012).

The endolithic bacterial community remains as an unexplored source of novel bioactive compounds. It is expected that the bacteria isolated from such habitats serve as rich source of antibacterial compounds due to their unique potentialities to survive in such extraordinary conditions (Wilson and Brimble 2009). Despite their wide importance, comparatively few studies have reported the microbial composition of endolithic communities (Li et al. 2013; Cao et al. 2016) and no studies have highlighted the exploration of bioactive compounds from such niche. Meghalaya known as the “Meghalaya Plateau” is occupied by varied rocks (Hazra et al. 2010). The rocks of Meghalaya are not fully explored from microbiological point of view. The present study was aimed to isolate bacteria from endolithic niche of Meghalaya and use them for biosynthesis of antibacterial compound with the addition of thiophene precursor to the fermentation media. To our knowledge, this is the first report of biosynthesis of cephalosporin derivatives by bacterial isolate from lithobiontic niches of Meghalaya.

Materials and methods

Sampling and isolation of lithic bacteria

A granite rock sample was collected from Myllem, Meghalaya (25°28.713'N, 91°49.216'E and 1776.46 m msl) using a rock hammer–chisel blade and maintained in sterile plastic bag (HiMedia, India) which was brought to the laboratory in icebox and stored at -20°C until use. For isolation of endolithic bacteria, the collected rock sample was surface-sterilized with 70% alcohol for 30 s followed by washing with sterile water for several times. The surface-sterilized rock sample was then aseptically crushed and grinded by wrapping in triple layer of pre-sterilized aluminium foil and suspended in Ringer's solution supplemented with 0.001% Tween 80 (ratio 1:10 w/v) and shaken vigorously for 1 h (Abdulla 2009). This suspension was then serially diluted and plated on optimized LM10 medium (Bhattacharjee and Joshi 2016) followed by incubation at 30°C for several days till visible growth observed on plates. The isolated colonies which survived heat shock at 55°C (Karuppiiah et al. 2015) were picked, purified by streaking at least three times on modified humic acid-vitamin agar (Hong et al. 2009) plates supplemented with cycloheximide (20 mg l^{-1}) and nystatin (30 mg l^{-1}) and Gram stained. Colonies with confluent growth were purified on ISP-2 medium (HiMedia, India). The spore suspension of those purified colonies were prepared and stored as glycerol stocks at -80°C for long-term preservation.

Precursor based combinatorial biosynthesis (PCB) and microbiological assay

The isolates were subjected to small-scale PCB study with thiophene derivatives as precursor for the selection of appropriate precursor for any worthy bioconversion. The isolates were cultured in medium (per litre): 10 g glycerol, 2 g asparagine, 1 g NH_4Cl , 3.5 g K_2HPO_4 , 0.7 g KH_2PO_4 , 0.6 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$, 20.9 g 3-(N-morpholino) propane sulphonic acid, sodium salt (MOPS) buffer and 1 ml trace salts solution. Trace salts solution contained per litre: 1 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 1 g $\text{ZnSO}_4 \cdot 4\text{H}_2\text{O}$ and 1 g $\text{CaCl}_2 \cdot \text{H}_2\text{O}$. The pH was adjusted to 6.8 prior to sterilization with KOH or HCl as required. The thiophene derivatives were added to fermentation broth after 3 days of growth and sampling was carried out after 10 days of growth. The thiophene derivatives included in this study were 2-Acetyl thiophene, 2-Benzoyl thiophene, 2-(Trifluoroacetyl) thiophene, 3-Methyl-2-thiophene carboxaldehyde and

4-Methyl-2-thiophene carbaldehyde. Cell-free extract was prepared by following the method as described by Jensen et al. (1982) with minor modifications and were checked for any antibacterial activity against different bacterial representatives including Gram-positive bacteria (*Staphylococcus aureus* MTCC 740, *Staphylococcus epidermidis* MTCC 3615 and *Bacillus subtilis* MTCC 736) and Gram-negative bacteria (*Escherichia coli* MTCC 739, *Pseudomonas aeruginosa* MTCC 1688 and *Klebsiella pneumoniae* MTCC 661) by agar well-diffusion method (Palepu et al. 2015). The isolates were also subjected to small-scale fermentation study without any addition of thiophene derivatives which was used as control. The antimicrobial activity was expressed based on zone of inhibition (ZI). The strain which displayed potent bioactivity on combination with precursor was selected for mass production to produce enough metabolites for purification and characterization of the bioactive compound.

Characterization of isolate

The selected isolate AL51 was subjected to morphological, biochemical, and physiological characterization according to recommendations of the International *Streptomyces* Project (Shirling and Gottlieb 1966), Bergey's Manual of Determinative Bacteriology (Holt 1994), and following the standard protocols as described by Bhattacharjee et al. (2012). For molecular characterization, the selected isolate was cultured on ISP-2 liquid medium at 28 °C for 3–5 days in an orbital shaking incubator (New Brunswick Scientific, USA). Genomic DNA was extracted according to a method described by Selvin et al. (2009) and 16S rDNA gene was amplified by PCR using bacterial primers 27F: 5'-AGA GTT TGA TCC TGG CTC AG-3', and 1492R: 5'-TAC GGY TAC CTT GTT ACG ACT T-3' (Bhattacharjee et al. 2014). The amplified product was sequenced by Big Dye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, USA) and the phylogenetic analysis was performed as per the previous report (Bhattacharjee et al. 2012). The 16S rRNA gene sequence of the isolate and their closely related species were retrieved from NCBI server and aligned using the ClustalW2 program. A distance matrix was generated using Kimura's two-parameter model (Kimura 1980). The tree topologies were evaluated by bootstrap analysis (Felsenstein 1985) based on 1000 replications with MEGA v6.0 program and phylogenetic trees were inferred using the neighbor-joining method (Saitou and Nei 1987). Gaps in alignment were considered as missing data for phylogenetic reconstructions.

Fermentation and extraction of antibacterial product

The selected isolate AL51 was grown on ISP-2 agar and incubated at 28 °C for 7–8 days to prepare a spore suspension (Kieser et al. 2000). The resultant spore suspension (2 ml) was transferred to 25 ml of a sterile seed medium and was incubated on a rotary shaker at 220 rpm, 28 °C for 3 days. The composition of the seed medium was as same as fermentation medium except the addition of 1 g/l yeast extract. The pH was adjusted to 6.8 prior to sterilization with KOH or HCl as required. The seed culture of 2 ml was transferred to Erlenmeyer flasks (total 100) containing 100 ml of the fermentation medium and fermentation was carried out on a rotary shaker (220 rpm) at 28 °C and 2-Acetylthiophene (0.5 g/l) was added after 3 days of growth and sampling was carried out after 10 days of growth. After 10 days of incubation, cell-free extract was prepared by sonication followed by harvesting (centrifugation at 8000×g for 20 min at 4 °C). The pH of cell suspension was adjusted to 2.8 with 50% w/v H₂SO₄. It was thoroughly stirred for 40 min and left to stand at 4 °C for 12 h. This acidified cell-free extract was filtered through filter paper (Whatman No. 1). The filtrate was extracted with an equal volume of methanol and shaken for 2 h in a rotary shaker (New Brunswick Scientific, USA). The solvent was allowed to evaporate in a rotary evaporator (Buchi, Switzerland) at 40 °C (50 rpm) under vacuum to yield a dark brown gummy substance.

Purification of antibacterial product and bioautography

The residual dark brown gummy substance was subjected to column chromatography using silica gel (60–120 mesh; SRL, Mumbai) packed using ethyl acetate:water (1:1, v/v) and eluted with a stepwise gradient of ethyl acetate:*n*-hexane (1:1 to 2:1, v/v) to obtain fractions. The fractions so obtained were concentrated to dryness *in vacuo* and were further separated by silica gel thin layer chromatography (TLC) using solvent system *n*-Butanol–pyridine–acetic acid–water (34:24:12:30). The compounds separated were visualized by exposing to iodine vapours and under UV radiation. The antibiotic potentialities of the fractions were detected by bioautography (Zheng et al. 2005) against *E. coli* MTCC 739 and *Bacillus subtilis* MTCC 736. The presence of inhibition, as evidenced by the clear zones around the spots where active components were present, was determined. The retention factor (R_f) of the bioactive spot was measured and recorded. Further, preparative TLC on silica gel G (SRL, Mumbai) was performed for the active spot containing fraction using the same solvent system. The active fraction was physically scraped from the bands, extracting with ethanol, concentrating the extracts and again subjecting to TLC and

second-column chromatography (100–200 mesh; SRL, Mumbai) using the same solvent system, thereby obtaining the fraction in substantially pure form. This active fraction was further subjected to Sephadex LH-20 (Sigma-Aldrich) column chromatography by eluting with water:methanol (1:1, v/v) used with gravity flow. The fractions were concentrated *in vacuo* and lyophilized to get almost pure material. The partially purified active metabolite was further purified using semi-preparative HPLC. The apparatus used was a Waters Breeze 2 HPLC System with a 2998 Photodiode Array (PDA) detector, 1525 Binary HPLC pump and a μ Bondapak C18 column (300 mm $\times$ 3.9 mm, 10 μ m) (Waters Associates, Milford, Mass.). Sample (20 μ l) from the fraction was analyzed at a flow rate of 1.5 ml min⁻¹ at room temperature with detection at 254 nm. The elution was done with a mobile phase of 2/20/78 acetic acid/acetonitrile/water to obtain compound CAX1. The lyophilized compound was dissolved in 10% DMSO and checked for antibacterial activity by agar well-diffusion method.

Characterization of antibacterial compound

Purified active compound CAX1 was subjected to partial characterization using a series of spectroscopic methods such as FT-IR, UV-Visible, NMR and MS. Elemental analysis was performed on a Perkin-Elmer-2400 CHN/S analyzer. For UV-Visible spectrophotometer (CARY-100 BIO UV-visible spectrophotometer; Varian Inc., USA), the obtained fraction was scanned to find out their absorption spectra of UV-visible light at wavelengths ranging from 200 to 800 nm. The obtained spectral data were analyzed with the help of Spekwin32 v.1.71.6.1. A Nicolet 6700 FT-IR spectrometer equipped with OMNIC software v.7.0 (Thermo Electron Scientific Instruments LLC, USA) was used to obtain the infrared spectra of the compound. The spectra was recorded in the 4000–400 cm⁻¹ range and plotted as % transmittance (%T) versus wave number (cm⁻¹). Prior to display, baseline correction and data normalization were performed using Spekwin32 v.1.71.6.1 and interpreted according to infrared frequencies described by Coates (2000). The proton (¹H) and carbon (¹³C) nuclear magnetic resonance (NMR) spectra of the compound were obtained using dimethyl sulfoxide (DMSO-*d*₆) as solvent in a Bruker Advance II 400 MHz NMR spectrometer considering tetramethylsilane (TMS) as an initial standard and chemical shift values were presented in delta parts per million (δ ppm) values. The obtained NMR spectral data were analyzed and structure of the compound was built with ChemBioDraw Ultra 12.0 software (PerkinElmer Inc., Cambridge soft, USA). A high resolution Mass spectrum (HRMS) of the purified active compound CAX1 was recorded on a Q-T of APCI-MS HAB 273 instrument by atmospheric pressure chemical ionization (APCI) method. The samples were introduced

into the ion source by the infusion method at the flow rate 1.0 ml min⁻¹. The capillary voltage of the mass spectrometer was 33 V, with source voltage 4.98 kV for the mass scale (m/z 200–1000).

Determining bioactivity of CAX1

The minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of the active antibacterial compound (CAX1) against both Gram-positive and negative bacterial strains were determined using the 96-well microplate broth-dilution method according to Clinical and Laboratory Standards Institute (CLSI) standards with slight modifications (Wiegand et al. 2008). For determination of minimal bactericidal concentration (MBC), these bacterial samples were transferred from wells that had bacterial concentrations equivalent to and higher than the MIC, with the aid of a sterile swab to a Petri dish containing Mueller Hinton Agar (HiMedia, India) and incubated at 37 °C for 24 h.

Fluorescence microscopic study of drug susceptibility

The antibacterial activity of the purified compound CAX1 (5 μ g/ml) was evaluated by a fluorescence based LIVE/DEAD bacterial viability assay using AO/EB dye system on the growth of *E. coli* (MTCC 739). The bacterial cells were stained with acridine orange (AO) and ethidium bromide (EB) (Sigma, USA) according to the method of Cui et al. (2012) with some minor modifications and observed using an epifluorescence microscope (Leica DM 4000B equipped with Leica DFC310 FX camera; Leica Microsystems, Germany). The untreated bacterial cells were used as a control.

Result

Isolation of lithic bacteria

A total of approximately 2×10^3 total viable bacteria were obtained per gram of granite after 15 days of incubation. A total of 5 isolates survived after heat shock treatment were selected and purified several times on modified humic acid-vitamin agar and were grown overnight on ISP-2 medium. The prepared spore suspensions of the isolates were stored as glycerol stocks at – 80 °C for long-term preservation.

Precursor based combinatorial biosynthesis (PCB) and Microbiological assay

Following screening of 5 thiophene precursors on fermentation in the designated media using 5 endolithic isolates, only

1 isolate (AL51) showed potent antibacterial activity with 2-Acetylthiophene (ACTH) as precursor (Supplementary material, Table S1). In case of control, the isolates were subjected to small-scale fermentation without any addition of thiophene derivatives which did not show any potent antibacterial activity against the tested pathogens.

Characterization of the isolate

The isolate AL51 was characterized by morphological, biochemical, physiological and molecular studies. The isolate was aerobic, Gram-positive with vegetative mycelium (Table S2 Supplementary material). The 16S rRNA gene sequence of the isolate encompassed between two primer-binding sites yielded a band of the expected size of ~ 1500 bp. An almost-complete 16S rDNA sequence containing less than 1% undetermined positions were obtained for the isolate AL51. The sequence was deposited in GenBank database under accession number KU258247. The isolate belonged to the genus *Streptomyces* and was supported by treeing algorithms and showed a high bootstrap value in the neighbor-joining analysis (Fig. 1) with higher degree of taxon separation in Bayesian phylogeny (Supplementary material, Fig. S1).

Fermentation, extraction, purification, and structural elucidation of antibacterial compound

Streptomyces sp. AL51 sporulated optimally on ISP-2 agar plates and grew well in seed and fermentation medium as a liquid culture and yielded a dark brown gummy methanolic extract (29.0 g). The purification of bioactive compound was achieved by combination of column and thin layer

chromatography techniques. The methanolic extract was subjected to purification by silica gel column chromatography and yielded nine fractions (A–I). All the fractions were further separated by silica gel TLC and yielded five fractions. The only one fraction (i.e. C) exhibiting good antibacterial activity against tested bacteria gave three distinct spots on TLC plate with varying R_f value after exposed to iodine vapour or under UV. The R_f value of the fraction which displayed positive bioautography was calculated as 0.79. This fraction was further subjected to preparative silica gel TLC and second-column chromatography to obtain the fraction in substantially pure form. The further purification of this active partially purified sub-fraction was done by chromatography over Sephadex column to give two fractions. Out of these two fractions, only one fraction showed antibacterial activity against tested bacteria (data not shown). This fraction was pooled, concentrated *in vacuo* and lyophilized to obtain 11.1 mg of almost pure compound and further purified by semi-preparative HPLC (4.6 mg, t_R = 14.2 min) with antibacterial activity and was designated as compound Cx1.

Characterization of antibacterial compound

The purified antibacterial compound Cx1 with the R_f value of 0.79, was isolated as an off white amorphous powder has been identified according to FT-IR, UV-visible, ^1H NMR, ^{13}C NMR spectral analysis and mass spectrometry. The elemental analysis data of the compound Cx1 acknowledged the following calculated values of C = 51.41%, H = 4.02%, N = 7.99%, O = 18.26% and S = 18.30% which appeared close to experimental values C = 51.31%, H = 4.09%, N = 8.10%, O = 18.15% and S = 18.28%. The mass spectrum (HRMS) of the compound Cx1 showed a base peak at m/z 350.1180 representing the molecular formula $\text{C}_{15}\text{H}_{14}\text{N}_2\text{O}_4\text{S}_2$ (Supplementary material, Fig. S2). Consequently, the UV spectrum of the purified antibacterial compound Cx1 from *Streptomyces* sp. AL51 indicated the presence of chromophore group $\text{O}=\text{C}-\text{N}=\text{C}=\text{C}-$ intact β -lactam ring bound to a dihydrothiazine ring with a maximum absorption at λ_{max} of 258 nm (Fig. 2). In addition to this normal absorption maximum in UV range, the compound had two extra absorption maximum at 348 and 369 nm, which were presumptively associated with the substituent at position 3 (Fig. 2). The infrared spectrum of the Cx1 (Fig. 3) showed hydroxyl ($-\text{OH}$) units (bands at 3502 and 3443 cm^{-1}), aromatic ($\text{C}-\text{H}$) group (bands at 3041 cm^{-1}), carbonyl ($\text{C}=\text{O}$) group (bands at 1770 and 1686 cm^{-1}), amine ($-\text{N}-\text{H}$) group (bands at 1605 cm^{-1}), CH_3 , CH_2 and/or CH groups (bands between 3041 and 2969, between 1386 and 1361 and between 989 and 873 cm^{-1}) and aromatic ($\text{C}=\text{C}$) bending (bands between 1573 and 1457 cm^{-1}). The bands between 1334 and 1118 cm^{-1} indicated the presence of ether ($\text{C}-\text{O}$) bonds

Fig. 1 Phylogenetic relationships among the isolates based on 16S rRNA gene sequences of selected strains and their closest phylogenetic neighbours. The tree was constructed using neighbor-joining (NJ) method. *Streptosporangium album* DSM 43023 was included as outgroup. NCBI accession numbers are given in brackets. Bootstrap values, expressed as percentage of 1000 replications and are indicated at the nodes; only values > 50% are given. Scale bar represents nucleotide substitutions per site. Filled circle and filled square represent the studied isolate and outgroup, respectively

Fig. 2 Normalized UV-Vis absorption profile of purified CAX1 produced by endolithic isolate *Streptomyces* sp. AL51 dissolved in methanol. The normalized UV-Vis absorption showed maxima at 258 nm indicated the presence of chromophore group $O=C-N-C=C-$ intact β -lactam ring bound to a dihydrothiazine ring and two extra absorption maximum at 348 nm and 369 nm which were presumptively associated with the substituent at position 3

Fig. 3 Fourier transform-Infrared FT-IR spectrum of the CAX1 produced by endolithic isolate *Streptomyces* sp. AL51. The spectra was recorded in the 4000–400 cm^{-1} range and plotted as % transmittance %T versus wave number cm^{-1}

as well as amine (C–N) groups. The bands at 1078 cm^{-1} indicated the presence of alcohol (C–OH) group. The bands between 847 and 720 cm^{-1} revealed the presence of CH in a benzenic ring and bands between 707 and 696 cm^{-1} revealed the presence of thiol in a cyclic ring. The ^1H NMR chemical shifts (400 MHz, $\text{DMSO}-d_6$, δ , ppm) were: 2.49 (2H, s, $-\text{CH}_2-$), 3.41 (2H, s, $-\text{S}-\text{CH}_2-$), 4.80 (2H, s, $=\text{CH}_2$), 4.89 (1H, s, Ar–H), 5.29 (1H, s, Ar–H), 5.40 (1H, s, $=\text{CH}-$), 7.33 (1H, d, $J = 13.17$ Hz, $=\text{CH}-$), 7.44 (1H, d, $J = 7.01$ Hz, $=\text{CH}-\text{S}-$) and 7.55 (1H, d, $J = 7.08$ Hz, $=\text{CH}-$) (Supplementary material, Fig. S3). The ^{13}C NMR chemical shifts (400 MHz, $\text{DMSO}-d_6$, δ , ppm) were: 136.38

(C-2), 128.34 (C-3), 30.82 (C-4), 57.57 (C-6), 58.43 (C-7), 169.49 (C-8), 161.87 (C-10), 144.35 (C-11), 128.06 (C-12), 127.33 (C-13), 128.34 (C-14), 169.64 (C-16), 26.87 (C-17), 136.38 (C-18) and 109.65 (C-19) (Supplementary material, Fig. S4). Based on the spectral data, the structure of the compound CAX1 extracted from *Streptomyces* sp. AL51 was identified as 7-[1-(thiophene-5-yl)-1-formamido]-3-propylenyl-3-cephem-4-carboxylic acid and the molecular formula was determined as $\text{C}_{15}\text{H}_{14}\text{N}_2\text{O}_4\text{S}_2$. The structure of the compound is illustrated in Fig. 4.

Bioactivity of CAX1

The MIC and MBC results revealed that the compound CAX1 exhibited broad spectrum antibacterial activity against indicator Gram-positive and negative bacteria. The MIC and MBC of CAX1 ranged from 0.25 to > 64 $\mu\text{g}/\text{ml}$ and 1.00 to > 64 $\mu\text{g}/\text{ml}$ respectively. The compound CAX1 had the lowest MIC (0.25 $\mu\text{g}/\text{ml}$) for *Bacillus subtilis* and the highest MIC (> 64 $\mu\text{g}/\text{ml}$) for *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*. The MIC values for the remaining microorganisms ranged between 0.50 and 4.0 $\mu\text{g}/\text{ml}$ (Table 1).

Fluorescence microscopic study of drug susceptibility

The antibacterial activity of CAX1 was demonstrated by a fluorescence-based LIVE/DEAD bacterial viability assay using AO/EB dye system. Fluorescence micrographs of *E. coli* before and after treatment with CAX1 are shown in Fig. 5. The results suggest that almost all of the bacteria were viable when cultured without the antibacterial compound (control) and number of viable bacterial cells decreased after addition of the compound CAX1.

Fig. 4 Chemical structure of compound CAX1 from *Streptomyces* sp. AL51

Table 1 Antibacterial activities of compound CAX1 against test bacterial strains were determined using the 96-well microplate broth-dilution technique

Test organism	MIC µg/ml		MBC µg/ml	
	Penicillin G	CAX1	Penicillin G	CAX1
Gram-positive				
<i>Staphylococcus aureus</i> MTCC 740	32	0.5	64	2
<i>Staphylococcus epidermidis</i> MTCC 3615	64	> 64	> 64	> 64
<i>Bacillus subtilis</i> MTCC 736	0.5	0.25	4	0.5
Gram-negative				
<i>Pseudomonas aeruginosa</i> MTCC 1688	> 64	> 64	> 64	> 64
<i>E. coli</i> MTCC 739	4	4	16	8
<i>Klebsiella pneumonia</i> MTCC 661	16	4	64	16

MIC minimum inhibitory concentration, MBC minimum bactericidal concentrations

Discussion

Since the Earth was first inhabited by microbes, rock represents the earliest niche for microbes (Gorbushina 2007). To date, the best-studied examples of endolithic communities are those of halite from Atacama Desert (Vitek et al. 2014), Limestone from Maya Archaeological Site (McNamara et al. 2006), Granite (Fajardo-Cavazos and Nicholson 2006; Abdulla 2009), Ca-sulfate crusts in the Atacama Desert (Wierchos et al. 2011), granite rock (Li et al. 2013), seafloor basalts (Cockell et al. 2010), limestone (Cao et al. 2016) and rock from Antarctica (Van Goethem et al. 2016). Meghalaya, located in the Eastern Himalayan region is one of the mega biodiversity hotspots of the world, with diverse rock deposits (Hazra et al. 2010) which has not been explored so far in respect of endolithic microbial community and has pristine natural ecosystems which offer scope for the bioprospection of novel organisms hitherto unknown to scientific world (Bhattacharjee and Joshi 2014). In the present work, effort has been made to isolate endolithic bacteria from granite rock. The heat pretreatment and the serial dilution methods were applied successfully to isolate the actinobacterium from granite rock (Karupiah et al. 2015).

In this study, an actinobacterial isolate capable for biosynthesis of an antibacterial compound with 2-Acetylthiophene (ACTH) as precursor was identified as *Streptomyces* sp. AL51. The 2-Acetylthiophene (ACTH) is a pharmaceutical intermediate having an acetyl group conjugated with the thiophene ring which is present in various pharmacologically active compounds (Mabkhot et al. 2016). The phylogenetic analysis of the 16S rRNA gene revealed that the isolate shared 99% sequence similarity with the type strain *Streptomyces tyrosinilyticus* (Zhao et al. 2015). However the *Streptomyces* sp. AL51 differed from its closest type strain *S. tyrosinilyticus* (Zhao et al. 2015) in few of its growth characteristics. Previous studies have demonstrated that various *Streptomyces* sp. have been proven to be a good source of antibiotics and other biologically active secondary metabolites. Many of these compounds are being used directly or

Fig. 5 Fluorescence microscopic images of the LIVE/DEAD bacterial viability assay using AO/EB dye system (a) control bacteria cells (no treatment) of test pathogen *E. coli* MTCC 739, (b) bacteria cells of test pathogen *E. coli* MTCC 739 treated with CAX1 (5 µg/ml). The living bacteria with intact cell membranes exhibited green fluorescence and dead bacteria with disrupted cell membranes as red fluorescence. Scale bar represents 10 µm

with minor modifications as antimicrobial drugs (Sletta et al. 2014), which makes perpetual search and isolation for novel species of this genus highly significant.

An important step towards industrialization of an active substance is separation and purification. Here, we successfully purified the methanolic extract of the antibacterial compound CAX1 from the fermentation broth of *Streptomyces* sp. AL51 using silica gel packed column chromatography and TLC (Bajpai and Kang 2011). In both techniques, competition between solute and the mobile phase plays an important role. On testing different solvents, n-Butanol–pyridine–acetic acid–water gave successful results from separation point of view. TLC-bioautography is a significant tool to localize antibacterial activity which was also employed in our research work to locate R_f value of the compound (Choma and Grzelak 2011). The antibiotic was further purified by preparative TLC, Sephadex LH-20 column (Choma and Grzelak 2011; Lee et al. 2003) and semi-preparative HPLC (Vaca et al. 2011).

The structure of the compound was determined by FT-IR, UV–visible, ^1H , ^{13}C NMR spectral analysis and mass spectrometry. The high resolution mass spectrometry (HRMS) analysis of the antibiotic compound showed the peak at m/z 350.1180 implies a cephem compound with molecular formula $\text{C}_{15}\text{H}_{14}\text{N}_2\text{O}_4\text{S}_2$ and was identified as 7-[1-(thiophene-5-yl)-1-formamido]-3-propylenyl-3-cephem-4-carboxylic acid with thiophene and propylenyl substitutions at 7 and 3 positions respectively. The MIC and MBC of the active compound suggesting its efficient broad spectrum antibacterial activity. The fluorescence microscopic study of drug susceptibility further confirmed that the antibacterial compound CAX1 can rapidly and efficiently kill bacteria. Under a fluorescence microscope, living bacteria with intact cell membranes exhibited green fluorescence and dead bacteria with disrupted cell membranes as red fluorescence (Coldham et al. 2010). To the best of knowledge this predicted structure with potential antibacterial activity was not reported earlier as per the DrugBank (Wishart et al. 2006), PubChem (<https://pubchem.ncbi.nlm.nih.gov/>) and other databases, which indicated that it is a novel antibacterial compound.

The present work describes a new and feasible process of utilising rock inhabiting *Streptomyces* sp. for precursor-based combinatorial biosynthesis with 2-acetyl thiophene as precursor leading to a cephalosporin derivative, 7-[1-(thiophene-5-yl)-1-formamido]-3-propylenyl-3-cephem-4-carboxylic acid, which opens scope for furthering research on semisynthetic drugs. It can also be used as a lead compound for designing and development of more efficient and effective compounds similar to well-known cephalosporins.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Research involving human and animal participants This article does not contain any studies with human participants or animals performed by any of the authors.

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