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Characterization and Antimicrobial Activities of a Metabolite from a New *Streptomyces* Species from Bangladeshi Soil

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Abstract

A new actinomycete strain was isolated from Western part of Bangladesh and identified as a new *Streptomyces* species on the basis of its morphological, biochemical, cultural characteristics and 16S rRNA data. The present paper describes the isolation and characterization of compound **1** from this new *Streptomyces* species with the help of various chemical and spectroscopic methods. Antimicrobial activity of compound **1** was tested by disc diffusion assay method and compared with that of standard antibiotics (Kanamycin for antibacterial activity and Nystatin for antifungal activity). The compound has been found to exhibit moderate to strong antimicrobial activity against the test organisms. Cytotoxicity of the compound **1** and the pet. ether extract of Czapek Dox (alkaline) broth of *Streptomyces* species was evaluated in brine shrimp bioassay with LC_{50} values of 23.85 µg/ml (ppm) and 19.95 µg/ml (ppm), respectively.

Keywords: *Streptomyces*; Antimicrobial activity; Cytotoxicity.

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1. Introduction

Antibiotic resistant pathogens have been widely and continuously reported. In consequence, novel antibiotics have been isolated from various microorganisms and search for new antibiotics against pathogenic bacteria and fungi are still continued. Soil bacteria and fungi have played a significant role in antibiotic discovery. *Streptomyces* species have been always a source of thousands of bioactive compounds and screening programs have shown that secondary metabolites can be isolated which bind to active sites of enzyme organelles and receptors [1, 2]. Streptomycetes are rich source of bioactive compounds, notably antibiotics, enzyme inhibitors and pharmacologically active agents [3]. As a result, secondary metabolites produced by *Streptomyces* have been the primary source of antibiotics and more recently, are used as herbicides, anticancer drugs,

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immunoregulators and antiparasitic compounds [4]. Recently, research provides a basis for the development of new approaches to combat human diseases [5]. Infectious diseases are leading health problems with high morbidity and mortality in the developing countries [6, 7]. The first systemic search for antibiotics, made by Gratia and Dath around 1924 [8], resulted in the discovery of actinomycetin from a strain of Actinomycetes. At present, 4,000 antibiotic substances obtained from bacteria and fungi have been applied in medicine, out of which about 75% are produced from Gram-positive actinomycetes bacteria such as *Streptomyces* sp. [9, 10]. Most of the antibiotics are extracellular-secondary metabolites which are normally secreted in culture media and serve as intermediates from primary metabolisms as precursors for their biosynthetic process [10].
As part of our ongoing research of microbial metabolites [11, 12], we isolated a new strain of *Streptomyces* species [13] from a soil sample collected in the region of Kushtia. We, herein, report the isolation of compound **1** from this new species as well as the antimicrobial and cytotoxic activities of this compound and crude pet ether extract of Czapek Dox (alkaline) broth of this new species.

2. Materials and methods

2.1. Collection and identification of organism

The organism was isolated from a soil sample collected from Kushtia, Bangladesh at the depth of 0.75m during the month of september 2003, using crowded plate technique [14]. The 16S rDNA sequence generated in this work was compared with the 16S rDNA sequences of other organisms retrieved from the EMBL/GenBank database. The nearest is being *Streptomyces parpurascens*, with 99.3% nucleotide similarity. In addition, the morphological, physiological and biochemical properties of the present isolate was also different from *Streptomyces parpurascens* [13]. On the basis of morphological, physiological, biochemical and sequencing of 16S rDNA studies, the organism was identified as a novel *Streptomyces* species and named as *Streptomyces lalonnensis* [13].

2.2. Fermentation

The strain was grown on modified Czapek Dox (alkaline) agar slant at 37.5^{o}C for 10 days. Spores were collected from a slant culture with 10 ml of the same medium (broth). Cultivation of the strain was made by transferring 1ml (ca. 10^8 cells/ml) of the spore suspension and was incubated at 37.5^{o}C (at 250 rpm) for ten days in 500 ml Erlenmeyer flasks each containing 100 ml of antibiotic medium consisted of 2 % sucrose, 0.25 % yeast extract, 0.1 % K_2HPO_4, 0.05 % $MgSO_4$ .7H_2O, 0.001 % $FeSO_4 7H_2O$ and 0.002% NaCl.

2.3. Extraction and isolation

The culture broth (50 × 200 ml) of *S. lalonnensis* was partitioned with pet ether and ethyl acetate (50 × 60 ml) respectively and concentrated to dryness by using a rotary evaporator

under vacuum at 40°C. We could not purify the ethyl acetate extract due to limited facilities and scarcity of time. Preparative TLC of the pet ether soluble part (1.25 gm) over Si gel 60 PF_{254} gel using mobile phase, pet. ether:chloroform (10:1) yielded compound **1** (12 mg purple color powder, mp. 110-120 ^{o}C).

2.4. Antibacterial and antifungal screening

Both antibacterial and antifungal activities of compound **1** and pet ether extract were observed by disc diffusion assay [15, 16]. A total of five Gram positive and nine Gram negative bacteria were used in this antimicrobial screening. Compound **1** and pet. ether extract (100μg/disc and 30μg/disc) were prepared by dissolving with pet. ether. To compare the antibacterial activity, kanamycin (30μg/disc) was used as standard antibiotic. As a negative control, a blank disc impregnated with solvent followed by drying off was used. The antifungal activity of the compound **1** and pet ether were tested against eight pathogenic fungi at a concentration of 200 μg/disc. Potato dextrose agar (PDA) media was used for this purpose. The activity was determined after 72 hours of incubation at room temperature (37 ºC). Nystatin was used as standard at a concentration of 20 μg/disc.

2.5. Minimum inhibitory concentration (MIC) assay

The minimum inhibitory concentrations (MIC) were determined by serial dilution technique [17] in the presence of standard Kanamycin (for bacteria) and Nystatin (for fungi). Bacterial and fungal inocula were prepared at 5×10^6 - 5×10^7 cfu/ml. Final adjustment were made using optical density measurement for bacteria (absorbance 0.05 at a wavelength of 660 nm).

2.6. Cytotoxicity screening

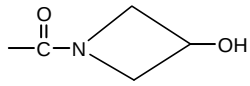
The eggs of the brine shrimp, *Artemia salina*, were collected from an aquarium shop (Dhaka, Bangladesh) and hatched for 48 hr to mature shrimp called nauplii. [18, 19] The test samples (extracts and compound) were prepared by dissolving them in DMSO (not more than 50 ml in 5 ml solution) plus sea water (3.8% NaCl in water) to attain concentrations- 2.5 mg/ml, 5 mg/ml, 10 mg/ml, 20 mg/ml, 40 mg/ml and 80 mg/ml. A vial containing 50 ml DMSO diluted to 5 ml was used as a control. Then 20 matured shrimps were applied to each of all experimental vials and control vial. The number of the nauplii that died after 24 hr was counted. The findings were presented graphically by plotting log of concentration versus percentage of mortality of nauplii from which LC_{50} was determined by extrapolation. The assay was performed in duplicate and the result was calculated as an average of two determinations. The cytotoxicity of the compound **1** was compared with the standard gallic acid and also with the anticancer agent bleomycin.

3. Results and discussion

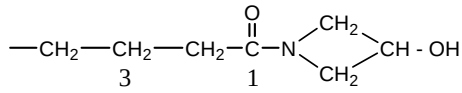
3.1. Characterization of the compound 1

The compound **1** with R_f value 0.92 (Si gel GF_{254}, solvent system, pet. ether: chloroform= 10:1) was isolated from pet. ether extract of the metabolites of a new *Streptomyces* species and was obtained as purple powders with mp. 110-120°C. The compound was found to be odorless and it appeared as a yellowish spot on TLC plate when viewed under UV light (254 nm).

The compound **1** was assigned on the basis of a series of 1D and 2D NMR data (Table 1) and mass spectra. The molecular formula of compound **1** was established as $C_{14}H_{23}NO_3$ from a molecular ion peak at 253 in the mass spectra. The ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) showed two doublet of doublet at 4.17 (J = 1.53) and 4.32 (J = 1.35), a multiplet at 5.26. In COSY spectrum, these protons showed interaction among themselves. The ^{13}C-NMR spectrum (125 MHz, $CDCl_3$) showed one methylene (δ_C 62.3 directly connected to δ_H 4.32 and δ_H 4.17 from HSQC) and one methine (δ_C 69.1 directly connected to δ_H 5.26 from HSQC) peaks. In the HMBC experiment, the proton at δ 5.26 showed ^2J correlation to the methylene carbons δ_C 62.3 while protons at 4.17 and 4.32 showed both direct and long range (^3J) correlation to the same carbon (δ_C 62.3). This means that the carbon chemical shift at 62.3 must be accounted from two methylene carbons corresponding to protons at 4.17 and 4.32. The latter protons showed ^2J correlation to methine at δ 69.1 and ^3J correlation to a carbonyl at 173.5. From this finding the partial structure of the compound can be drawn as

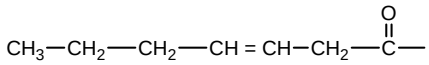


The ^{1}H-NMR spectrum showed a series of methylene peaks. Among these peaks, the multiplet at 1.62 (δ_C 25.1 from HSQC) showed ^2J connectivity to carbons at 34.2 (δ_H 2.32 from HSQC) and 29.3 (δ_H 1.29 from HSQC) and ^3J correlation to carbonyl at 173.5. So this methylene was assigned as H-3 starting from carbonyl at 173.5 (C-1). H-3 showed direct connectivity to H-2 and H-4 in ^{1}H- ^{1}H COSY experiment. Thus the above partial structure can be extended to



The ^{13}C-NMR spectrum showed two olefinic methine carbons at 129.9 and 130.2. Both of these carbons correspond to a multiplet signal at 5.34 in ^{1}H-NMR spectrum which in turn showed connectivity to carbonyl at 173.4 and methylene at 27.4 (δ_H 2.02 from HSQC) and 29.9 (δ_H 1.28 from HSQC). A methyl signal appeared as triplet at 0.89 in ^{1}H-NMR spectrum exhibited HMBC correlations to methylene carbons at 22.9 (δ_H 1.30 from HSQC) and 27.4 (δ_H 2.02 from HSQC). The methylene protons at 2.02 was connected to

olefinic protons (δ_H 5.34) and methylene at 1.30 in COSY experiment. The latter methylene was connected to methyl signal. So the structure of this part of the molecule can be drawn as



So combination of these two partial structures gives the complete structure of **1**. Thus compound **1** is identified as (*E*)-1-(3-hydroxyazetidin-1-yl) undec-7-ene-1,5-dione (Figure 1). To our best knowledge this is a new compound.

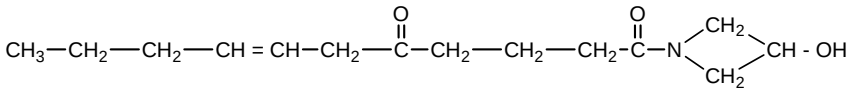


Fig. 1. Structure of compound **1.**

Table 1. ^{1}H (500 MHz), ^{13}C (125 MHz) and ^{1}H- ^{13}C HMBC (500 MHz) data of the compound **1** in $CDCl_3$.

Positions	^{1}H	^{13}C	HMBC Key Correlation	
	δ_H	δ_C	^{2}J	^{3}J
1	-	173.5	-	-
2	2.32, *m*	34.2	173.5 (CO), 25.1	29.3 (CH_2)
3	1.62, *m*	25.1	34.2 (CH_2), 29.3 (CH_2)	173.5 (CO)
4	1.29, *m*	29.3	NS	NS
5	-	173.4	-	-
6	1.28, *m*	29.9	-	-
7, 8	5.34, *m*	129.9, 130.2	27.4 (CH_2), 29.9 (CH_2)	173.4
9	2.02, *m*	27.4	130.2 (CH)	-
10	1.30, *m*	22.9	12.4 (CH_3)	-
11	0.89, *t*, *J* =1.74	12.4	22.9 (CH_2)	27.4 (CH_2)
2′, 4′	4.17, *dd*, *J* =1.53 4.32, *dd*, *J* =1.35	62.3	69.1 (CH)	62.3 (CH_2), 173.5 (CO)
3′	5.26, *m*	69.1	62.3 (CH_2)	

3.2. Antibacterial activity

Both the compound **1** and pet. ether extract showed remarkable biocidal activity (100 µg/disc) against the tested Gram positive and Gram negative bacteria (Table 2). In case of some pathogenic bacteria, pet. ether extract was more active than compound **1** which indicated the presence of more bioactive compounds in the extract. When the concentration was 30 µg/disc, none of the test materials showed a remarkable antibacterial activity against the tested bacteria in comparison with the standard kanamycin. In the

present investigation we found that, both the compound and extract showed comparatively better antibacterial activity against the Gram positive bacteria than the Gram negative bacteria.

Table 2. Antibacterial activity of pet. ether extract, compound **1** and Kanamycin.

Test organisms	Strain No.	Diameter of zone of inhibition (mm)				
		pet. ether extract 30μg/disc	pet. ether extract 100μg/disc	comp.**1** 30μg/disc	comp.**1** 100μg/disc	Kanamycin 30μg/disc
Gram positive						
Sarcina lutea	QL-166	11	20	13	22	20
Bacillus megaterium	QL-38	10	20	11	22	25
Bacillus subtilis	QL-40	10	24	11	23	26
Streptococcus-β-haemolyticus	CRL	12	21	12	21	24
Staphylococcus aureus	ATCC-259233	11	19	13	21	22
Gram negative						
Salmonella typhi	-	13	24	12	19	20
Shigella dysenteriae	AL-35587	15	30	13	20	22
Shigella boydii	AL-17313	14	23	12	20	24
Shigella sonnei	AJ-8992	13	20	13	25	23
Shigella flexneri	AL-30372	10	21	13	27	25
Shigella shiga	ATCC-26107	11	24	14	24	24
Escherichia coli	FPFC-1407	9	16	14	20	25
Pseudomonas aeruginosa	CRL	12	25	11	20	23
Klebsiella species	-	12	23	10	18	24

3.3. Antifungal activity

The antifungal activities of the compound **1** and pet ether extract were determined at the concentration of 200 μg/disc against pathogenic fungi. It was found that the pet ether extract had greater activity than the compound **1** against all of the pathogenic fungi (Table 3) which may be due to the presence of more bioactive compounds in the crude extract.

Table 3. Antifungal activity of pet. ether extract, compound **1** and Nystatin.

Test Organism	Diameter of zone of inhibition (in mm)		
	pet. ether extract 200 μg/disc	comp.**1** 200 μg/disc	Nystatin 20 μg/disc
Candida albicans	20	11	22
Aspergillus fumigatus	17	8	19
Aspergillus flavus	23	14	23
Aspergillus niger	22	13	24
Epidermophyton floccosum	18	9	21
Trichoderma species	22	-	30
Fusarium species	19	8	26
Bipolavis species	20	8	24

3.4. Minimum inhibitory concentration (MIC)

The MIC values of the compound **1** and pet. ether extract against *Bacillus subtilis*, *Staphylococcus aureus*, *Salmonella typhi*, *Shigella dysenteriae*, *Escherichia coli*, *Streptococcus β haemolyticus*, *Candida albicans*, *Aspergillus flavus* and *Aspergillus niger* were shown in Table 4. The MIC values of the compound **1** and pet. ether against the tested microorganisms were maximum which indicated that the compound **1** were less active against the bacteria and the fungus. For compound **1** the MIC values were 64,128, 128, 128, 64, 128, 64, 128 and 64 µg/ml, respectively against the organisms; whereas the pet. ether extract showed MIC values between 32-128 µg/ml which was indicative of their potent antibacterial properties than the compound **1**.

Table 4. Results of MIC values (in µg/ml).

Test organisms	comp. **1**	pet. ether extract	Kanamycin	Nystatin
Bacillus subtilis	64	32	2	-
Staphylococcus aureus	128	64	8	-
Salmonella typhi	128	128	4	-
Shigella dysenteriae	128	64	2	-
Escherchtia. coli	64	32	4	-
Streptococcus β-hemolyticus	128	64	-	8
Candida albicans	64	64	-	4
Aspergillus flavus	128	64	-	8
Aspergillus niger	64	32	-	4

3.5. Cytotoxicity assay

The mortality rate of brine shrimp nauplii was found to increase with increasing the concentration of complexes. Table 5 summarizes the LC_{50} values of the pet ether extract

Table 5. Cytotoxic effect of compound **1**, pet.ether extract and standard bleomycin and gallic acid.

Test samples	LC_{50} (ppm)	95% confidence limit (ppm)		Regression equation	χ^2 (df)
		lower	upper		
Compound **1**	23.85	2.08	6.02	Y=3.98+1.85X	3.38(2)
Pet. ether extract	19.95	4.15	10.15	Y=3.17+2.27X	0.35(2)
Standard bleomycin	0.41	0.27	0.62	Y=3.16+2.98X	0.62(2)
Gallic acid	4.53	3.33	6.15	Y=3.93+1.62X	1.25(2)

and the compound **1**. The values were found at 19.95 µg/ml (ppm) and 23.85 µg/ml (ppm), respectively. The standard anticancer drug bleomycin and gallic acid gave LC_{50} value at 0.41 µg/ml (ppm) and 4.53 µg/ml (ppm), respectively.

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References

1. D. H. Williams, M. J. Stone, P. R. Hauck and S.K. Rahman, J. Nat. Prod. **52**, 1189 (1989). doi:10.1021/np50066a001
2. D. H. Williams, Stone, M .J. Mortishire, R. J. Smith and P. R. Hauck, J. Biochem. Pharmaco. **40**, 27 (1990). doi:10.1016/0006-2952(90)90174-J
3. J. Berdy, Biotechnologia **7-8**, 13 (1995).
4. C. J. Thompson and S. H. Bialphos, (In: L. C. Vining and C Stuttard, editors) Genetics and Biochemistry of antibiotic production, Butterworth Heinemann Bio/Technology, Oxford, 1995). pp. 197–222.
5. W. R. Jack and A. R. Gennaro, Remington's Pharmaceutical Sciences, 8th Edition (Mack Publishing Company, Easton Pennsylvania, 1990).
6. R. E. Black, K. D. Brown, S. Becker and M. Yunus, Am. J. Epidemiol. **115**, 305 (1982).
7. J. A. Walsh and K. S. Warren, New Engl. J. Med. **301**, 967 (1979).
8. L. J. Hanka and A.Dietz, J. Antimicrob. Agents Chemother. **3**, 425 (1973).
9. J. Dienstag and H. C. Nue, J. Antimicrob. Agents Chemother. **1**, 41 (1972).
10. A. Argoudelis, D. L. Baczyneky, M. T. Duo, A. l. Laborde, O. K. Sebek, S. K. Truesdel and F. B. Shilliday, J. Antibio., **40**, 750 (1987).
11. A. Jabbar, A. P. Shresta, C. M. Hasan, and M. A. Rashid, Nat. Prod. Sci. **5**, 162 (1999).
12. M. S. Hossain, M. A. Hossain, M. M. Rahman, M. A. Mondol, M. S. A. Bhuiyan, A. I. Gray, M. I. Flores, and M. A. Rashid, Phytochem. **65,** 2147 (2004). doi:10.1016/j.phytochem.2004.10.001
13. M. A. A. Akhanda, M.Pharm. Thesis, Rajshahi University, Rajshahi, Bangladesh, 2004.
14. S. M. Hammond and P. A. Lambert, Antimicrobial Action, (Edward Arnold Ltd. London, 1978).
15. A. W. Bauer, W. M. M. Kirby, J. C. Sherris, and M. Turck, Am. J. Clin. Pathol. **44**, 493 (1966).
16. A. L. Barry, Principle and Practice of Microbiology (Lea and Fabager, Philadelphia, 1976).
17. R. Reiner, Antibiotics: An Introduction (Roche Scientific Service, Switzerland, 1982).
18. J. L. Mclaughlin, Bioassays for discovery of Antitumor and Antiviral Agent from Natural Sources Proceedings of NIH workshop, Bethesda, **18,** 1988.
19. B. N. Meyer, N. R. Ferringni, J. E. Puam, L. B. S. Laeob, D. E. Nichois, and J. L. Mclaughlin, Planta Med. **45**, 31 (1982). doi:10.1055/s-2007-971236