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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 5 (2): 201-203, 2002

Isolation of a Yellowish Antibiotic Pigment 4-hydroxy Nitrobenzene from a Strain of *Streptomyces*

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Abstract: Chloroform extract of the culture filtrate of *Streptomyces* sp. and chromatographic analysis has lead to the isolation of an antimicrobial compound. The structure of the compound was identified as 4-hydroxy nitrobenzene from its IR, UV, ^{1}H-NMR, ^{13}C-NMR, EIMS, HMBC, HMQC data. This is the first report of isolation of 4-hydroxy nitrobenzene from natural sources.

Key words: *Streptomyces* and 4-Hydroxy nitrobenzene

Introduction

The pathogenic microbes are gaining resistance by curious mechanisms due to indiscriminate use of antibiotics. Antimicrobial drug resistance is one of the most serious problems for human being and disease like cancer, AIDS etc. are now all around the world. So medical scientists and researchers are searching for newer, safer and more potent and improved antibiotics to combat against drug resistance, mutants as well as to combat the newer diseases. Hence, the search for the isolation of new, more potent and safer antimicrobial compounds are going on and at the same time many of the marketed antibiotics are being withdrawn due to the serious side effects (Roche, 1950). The present work is a part of such an effort. The genus *Streptomyces* is already reported for the production of various antibiotics (Waksman and Woodruff, 1940; Dienstag and Nue, 1972; Dugger, 1948). As a part of our continuing search, an antagonistic *Streptomyces* species was isolated from soil samples, containing *Streptomyces* species. This paper describes the isolation and characterization of an antimicrobial compound, 4-hydroxy nitrobenzene from the culture filtrate of *Streptomyces* species.

Materials and Methods

Instruments: UV spectra were taken in methanol solution using a Perkin-Elmer Lambda 9 UV / Vis/ NIR spectrometer. IR spectra were recorded by preparing KBr disc using Philips 9800 FTIR spectrometer. The ^{1}H-NMR and ^{13}C-NMR spectra were measured on a JEOL JNM-alpha spectrometer (500 MHz), in $CDCl_3$ with an internal reference, TMS and chemical shift are given in δ values (ppm). Assignments of the proton and carbon signals were made by pulse field gradient (PFG) hetero nuclear multiple quantum coherence (HMQC) and PFG heteronuclear multiple bond connectivity (HMBC) experiments. Mass spectra were recorded on a JEOL DX 300 spectrometer and major peaks are indicated, as m/z. Melting points were determined on a Koflet- hot-stage apparatus.

Collection of the organism: An antagonistic organism was isolated from soil sample, collected from Meherchandi (near Fine and Arts Department of the Rajshahi University), Bangladesh, at the depth of 0.75m, using "crowded plate technique" (Hammond and Lambert, 1978). The organism was identified as *Streptomyces* species (John *et al.*, 1994) by morphological and biochemical study (Sathi, 2000).

Preparation of inoculum and isolation of antibiotic: The organism was allowed to grow in yeast extract-glucose medium until sporulation occurred. Matured spores were aseptically transferred to 25ml of sterilized yeast extract-glucose broth medium to make spores suspension, that was again aseptically transferred to 250ml-culture flask containing 100ml medium. This flask was incubated at 37.5°C for 15 days. The maximum antibiotic production was achieved when the colour of the broth medium became dark brown. This culture filtrate was then separated by filtration from its mycelium mat and preserved for the extraction of the antimicrobial compounds. The effect of incubation period, temperature and various physical parameters on the production of antimicrobial compounds were studied against *Shigella shiga* and *Bacillus subtilis* by disc diffusion method (Bauer *et al.*, 1966).

Extraction of antibiotics from culture filtrate: Hundred ml of culture filtrate were taken in 250ml separating funnel and was shaken with $CHCl_3$ (3 × 30ml) for complete extraction. In this way the total filtrate was extracted. The extract thus obtained was evaporated under reduced pressure in a rotavapour at 45°C to get a yellowish mass.

Isolation and purification of active metabolites: The yellowish $CHCl_3$ extract was analysed by thin layer chromatographic technique (Beckett and Stenlake, 1986) on activated silica gel GF_{254} using different solvent systems. The best resolution was obtained by $CHCl_3$: MeOH (10:1) solvent system. Although a number of metabolites have been resolved from $CHCl_3$ fraction but when these fractions were tested against *Shigella shiga* and *Bacillus subtilis* by disc diffusion method (Bauer *et al.*, 1966) only three compounds namely ZS-1, ZS-2 and ZS-3 were found to be active (Sathi, 2000). So the crude extract was found to contain three antimicrobial compounds but only ZS-3 was isolated and the isolation of other bioactive compounds is in progress. The compound ZS-3 was separated from the extract by preparative TLC technique using solvent system $CHCl_3$: MeOH (10:1).
The purity of ZS-3 was checked by TLC using different solvent systems and observing the developed plate under UV light, iodine vapour and also sprayed with 1% vanillin sulphuric acid reagent.

Characterization of the compound ZS-3: The compound ZS-3 was obtained as yellowish needle shaped crystals, melting point 112-113°C. The spot of ZS-3 appeared as bluish under UV lamp at 254nm and brick red when sprayed with vanillin-

Table 1: Production monitoring of fermentation broth from *Streptomyces* species in different days

Incubation period (days)	Diameter of the zone of inhibition (mm)		
	Flask No. 1	Flask No. 2	Average
1	0	0	0
2	0	0	0
3	5	7	6
4	8	8	8
5	12	12	12
6	16	16	16
7	18	20	19
8	20	20	20
9	22	22	22
10	23	24	23.5
11	24	24	24
12	24	24	24
13	25	25	25
14	25	25	25
15	26	26	26
16	24	24	24
17	20	20	20
18	18	19	18.5
19	15	14	14.5
20	12	12	12

Test organism: *Shigella shiga*

Table 2: Effect of carbon sources on metabolite production from *Streptomyces* species.

Name of the carbon source	Diameter of the zone of inhibition (mm)		
	Flask No. 1	Flask No. 2	Average
Sucrose	28	26	27
D-Glucose	26	25	25
D-Fructose	30	29	28
Mannitol	33	32	31
D-(+)Galactose	29	29	29
Xylose	27	26	25
Lactose	20	22	21
Rhamnose	22	20	21
D-(+)Mannose	28	26	27
Resorcinol	18	17	16

Test organism: *Shigella shiga*

Table 3: H-NMR and ^{13}C-NMR spectrum (500MHz, $CDCl_3$) of compound ZS-3

Position of C/H	^{1}H-NMR	^{3}C-NMR at δ in ppm of phenol	^{13}C-NMR
1	-	121.2	141.6
2	6.93(1H.d.j =8.7 Hz)	115.8	115.7
3	8.18 (1H.d.j= 8.7 Hz)	127.9	126.3
4	-	155.4	161.4
5	8.18 (1H.d.j= 8.7 Hz)	127.9	126.3
6	6.93 (1H.d.j= 8.7 Hz)	115.8	115.7

Table 4: ^{1}H-^{13}C correlation of ZS-3 using HMBC.

^{1}H –NMR data (chemical shift δ_H)	^{13}C- NMR (chemical shift δ_C)
6.93 (each H, d, J= 8.7Hz, 2H)	141.6
8.18 (each H, d, J= 8.7Hz, 2H)	161.4

Table 5: ^{1}H-NMR and ^{13}C-NMR correlation using HMQC of compound ZS-3

^{1}H –NMR	^{13}C- NMR
6.93(2H.d.j=8.7Hz)	115.7
8.18(2H.d.j=8.7Hz)	126.3

sulfuric acid, followed by heating at 110°C for 5 minutes. ZS-3 is soluble in methanol, ethyl acetate, partially soluble in chloroform, insoluble in petroleum ether, benzene and n-hexane. It showed single spot on TLC using different solvent systems. The elemental composition was C = 51.80, H = 3.62, N = 10.07 and O = 34.50, obtained from the elemental analysis.

UV λ_{max} = 306nm (presence of unsaturation), 227nm (aromatic compound).

IR λ_{max} = 1410cm^{-1} (-C-O), 1500 cm^{-1} (-N=O), 3630cm^{-1} (-O-H) and at 1230cm^{-1} (-C-N).

FAB + MS: Molecular ion peak at m/z = 139.11 and $M^+ + 1$ peak at 140.15, molecular formula $C_6H_5NO_3$.

^{1}H, ^{13}C-NMR, HMBC and HMQC are given in Tables 3, 4 and 5.

4-Hydroxy nitrobenzene

Results and Discussion

The chloroform extract of the culture filtrate of *Streptomyces* species afforded ZS-3. The structure of the isolated metabolite was deduced from its spectral data.

It was found that 15 days of incubation (Table 1), 37.5°C temperature and pH 7 were the most suitable conditions for the higher production of antibiotics. Mannitol was proved to be a better carbon source (Table 2) and 3% NaCl concentration was found to give maximum antibacterial activity.

In ^{1}H-NMR spectrum (Table 3), the compound ZS-3 showed proton peaks at δ-8.18 (each H. d. J = 8.7Hz, 2H), 6.93(each H. d. J = 8.7Hz, 2H), which may be ascribed to aromatic (Ar-H) or olefinic protons, corresponding to carbon peaks at δ-126.3 (two) & 115.7 (two) and 115.7 (two) (^{13}C-^{1}H Cosy, Table 3). Coupling constant of proton remain in the expected range (J = 7-10Hz) of aromatic protons, indicating that these protons are aromatic. From the ^{1}H-^{1}H Cosy analysis aromatic proton peaks at δ-8.18 (each H, d, J = 8.7Hz, 2H) give correlation peak with aromatic proton peaks at δ-6.93 (each H, d, J = 8.7Hz, 2H). On the other hand the presence of no other correlation peaks indicates that these protons are free from proton of adjacent carbon on both sides which indicates that these two sets of aromatic protons are separated by two tertiary aromatic carbons at δ-141.6 & 161.4, (From HMBC, Table-4). This suggests the presence of a disubstituted (at 1, 4 position) of six membered aromatic ring.

The IR spectrum, showed the absorption bands at 1500 and 1230 cm^{-1} which may be due to the presence of O=N and -C=N stretching respectively. In addition, qualitative test gave the positive response for nitro (NO_2) group and carbon peak at 141.6 deshilded by the presence of an electronegative atom. Therefore the compound contain a phenolic group and a nitro group. The proton peak at 6.93 each (H, d, J = 8.7 Hz, 2H) gives long range (HMBC, Table 4) correlation, with carbon peak at 141.6 and the proton peak at δ-8.18 (H, d, J = 8.7 Hz, 2H) gives long range coupling with carbon peak at 161.4. This

indicates that the carbon of 141.6 and 161.4 are ortho-para to each other (Pretsch, 1983). Therefore the structure of the compound ZS-3 is determined as 4 hydroxy nitro benzene. This is the first report of the isolation of 4-Hydroxy nitrobenzene from *Streptomyces* species as well as biological system.

Acknowledgments

The authors wish to thank the institute of Nutrition and Food Science, Dhaka University, Bangladesh and ICDDRB'B, Dhaka Bangladesh for supplying the test organism.

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