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Study on Bioactive Compounds from *Streptomyces* sp. ANU 6277

KOLLA J.P. NARAYANA[1], PEDDIKOTLA PRABHAKAR[2], MUVVA VIJAYALAKSHMI[1*], YENAMANDRA VENKATESWARLU[2] and PALAKODETY S.J. KRISHNA[3]

[1] Department of Microbiology, Acharya Nagarjuna University, Guntur - 522 510, A.P.
[2] Organic Chemistry Division-I, Indian Institute of Chemical Technology
[3] Biotechnology Unit, Institute of Public Enterprise, Hyderabad - 500 007, A.P., India

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Abstract

An attempt was made to study the bioactive compounds from a terrestrial *Streptomyces* sp. ANU 6277 isolated from laterite soil. Four active fractions were recovered from the solvent extracts obtained from the culture broth of five day-old strain. Three bioactive compounds were purified and identified as 3-phenylpropionic acid, anthracene-9,10-quinone and 8-hydroxyquinoline. The components of the partially purified fourth active fraction were analyzed by gas chromatography-mass spectrometry and identified as benzyl alcohol, phenylethyl alcohol and 2H-1, 4-benzoxazin-3 (4H)-one. Four active fractions were screened for antimicrobial activity against Gram-positive and Gram-negative bacteria, and fungi including phytopathogenic, toxigenic and dermatophytic genera. Among these metabolites, 8-hydroxyquinoline exhibited strong antibacterial and antifungal activity as compared to 3-phenylpropionic acid and anthracene-9,10-quinone.

Key words: *Streptomyces* sp. ANU 6277, antimicrobial activity, bioactive compounds

Introduction

Actinomycetes are Gram-positive bacteria that are wide spread in nature and play a pivotal role in the production of bioactive metabolites (Sanglier *et al.*, 1993). Among actinomycetes, the members of the genus *Streptomyces* are considered economically important because they alone constituted 50% of soil actinomycete population and 75% of total bioactive molecules are produced by this genus (Prabavathy *et al.*, 2006). They continue to be prolific sources of secondary metabolites with biological activities that ultimately find application as antimicrobial, anticancer agents or other pharmaceutically useful compounds (Bibb, 2005). Majority of streptomycetes and other actinomycetes members produce a diverse array of antibiotics including aminoglycosides, glycopeptides, β-lactams, macrolides, nucleosides, peptides, polyenes and tetracyclines (Berdy, 2005). As a result of the increasing prevalence of antibiotic-resistant pathogens and the pharmacological limitations of antibiotics, there is an exigency for new antimicrobial substances (Sahin and Unger, 2003). Taxonomy of the strain ANU 6277 and production and biological properties of 3-phenylpropionc acid were reported earlier (Narayana *et al.*, 2007). In the present study, an attempt was made to explore the other bioactive compounds of the strain and their antimicrobial spectrum.

Experimental

Materials and Methods

Cultivation of the strain. A prevalent actinomycete strain, *Streptomyces* sp. ANU 6277 was isolated from laterite soil sample collected at Acharya Nagarjuna University (ANU) campus by dilution plate technique (Narayana *et al.*, 2007). The strain was maintained on yeast extract-malt extract-dextrose (YMD) agar medium (Williams and Cross, 1971). Actively growing pure culture of the strain was used to inoculate 100 ml of YMD broth in 250 Erlenmeyer flasks. After 48 h incubation at 30°C, YMD culture broth (10%) was used as seed culture to inoculate 500 ml fermentation broth (4% dextrose, 0.9% proteose peptone, 0.1% yeast extract, 0.6 % $CaCO_3$, 0.1% K_2HPO_4, 0.1% $MgSO_4 \times 7H_2O$, pH 7.2) in 2-litre Erlenmeyer flasks and incubated at 30°C for 5 days.

* Corresponding author: M. Vijayalakshmi, Department of Microbiology, Acharya Nagarjuna University, Guntur-522 510, A.P., India; **e-mail: profmvl@gmail.com**

Extraction, purification and identification of bioactive compounds from the strain. After 5 days incubation of the culture, the fermentation was stopped and pH of the medium was adjusted to 3.5 with 1N HCl. Cells were removed from fermentation broth by filtration and the culture filtrate was extracted with ethyl acetate. The crude solvent extract was subjected to Silica gel chromatography (22×5 cm, Silica gel 60, Merck) and eluted with gradient solvent system consisting of ethylacetate + hexane. Elutions collected during column chromatography were concentrated and tested for their antimicrobial activity against a Gram-positive (*Bacillus subtilis* MTCC 441) and Gram-negative bacteria (*Pseudomonas aeruginosa* MTCC 424), and yeast (*Candida albicans* MTCC 183) to screen bioactive fractions. Further purification of bioactive fractions was carried out in HPLC preparative column (10 mm* 250 mm, 5 µ using hexane:2-propanol, 8:2). Structural elucidation of pure bioactive compounds from the strain was carried out by EI-MS, ^{1}H NMR and ^{13}C NMR spectral studies.

The partially purified fourth active fraction was analysed by gas chromatography-mass spectrometry (GC-MS). An Agilent GC-MS system equipped with a fused silica capillary column (CW-amine 60 m×0.25 mm I.D., Film thickness 0.5 µm) was used to analyze the compounds in this active fraction. The data was processed with GC/MSD ChemStation (Agilent Technologies 6890-N series GC with 5990 series II MSD). Column condition was programmed as column oven temperature 150°C (4 min) – 4°C/min, temperature of inject port 250°C and detector port 280°C (Roy *et al.*, 2006). The peaks of components in gas chromatography were subjected to mass-spectral analysis. The spectra were analyzed from the available library data, NIST MS Search (version 2.0) (included with NIST '02 mass spectral library, Agilent p/n G1033A).

Biological activity. Minimum inhibitory concentrations (MIC) of bioactive compounds obtained from the strain against different microorganisms including bacteria and fungi were determined by conventional agar dilution method (Cappuccino and Sherman, 1999) using nutrient agar for bacteria and Sabouroud's agar medium for fungi and yeast. Different concentrations of compounds (0 to 1000 µg/ml) were prepared in Dimethyl sulphoxide (DMSO) and assayed against test organisms. The organisms used in this assay are *Bacillus cereus* MTCC 430, *B. subtilis* MTCC 441, *Escherichia coli* MTCC 40, *Klebsiella pneumoniae* MTCC 109, *Proteus vulgaris* MTCC 742, *Pseudomonas aeruginosa* MTCC 424, *P. fluorescens* MTCC 103, *Staphylococcus aureus* MTCC 96, *Aspergillus flavus*, *A. niger*, *Candida albicans* MTCC 183, *Fusarium oxysporum*, *F. udum* MTCC 2204, *Epidermophyton floccosum* MTCC 613, *Microsporum canis* MTCC 2820 and *Penicillium citrinum*.

The antimicrobial activity was observed after 24–48 h incubation at 30°C for bacteria and 48–72 h incubation at 28°C for fungi and yeast. The experiment was performed in triplicates and negative controls were maintained as DMSO without compound. Positive controls were tested with tetracycline for antibacterial activity, carbendazim for phytopathogenic and toxigenic fungi and griseofulvin for dermatophytes. Lowest concentration of compounds that showed antimicrobial activity in terms of growth inhibition zone against test organisms was recorded as MIC value (Hwang *et al.*, 2001).

Results and Discussion

The scheme for the extraction of bioactive compounds from the strain is presented in Figure 1. An amount of 2.54 g crude solvent extract was obtained from 40 liter of fermentation broth after defatted with cyclohexane. Four active fractions were screened, concentrated and designated as AF1, AF2, AF3 and AF4. TLC analysis of the compounds revealed that AF2 and AF3 exhibited pure single distinct band. AF1 with little impurity and AF4 had mixture of three compounds. Purity of AF1 was obtained after preparative HPLC step and determined as 3-phenylpropionic acid by ^{1}H NMR, ^{13}C NMR and EI-MS spectral studies (Narayana *et al.*, 2007).

The structure of AF2 and AF3 was elucidated by ^{1}H NMR, ^{13}C NMR and EI-MS studies. ^{1}H NMR (300 MH_Z, $CdCl_3$) of AF2 showed protons at 7.8 (4H, dd, aromatic-H), 8.3 (4H, dd, aromatic-H) and ^{13}C NMR (300 MH_Z, $CdCl_3$) exhibited peaks at 126 (4-aromatic carbons), 135 (4-aromatic carbons) and 185 (keto carbon). ^{1}H NMR (300 MH_Z, $CdCl_3$) of AF3 depicted protons at 7.1 (1H, d, aromatic-H), 7.3 (1H, d, aromatic-H), 7.5 (2H, d, aromatic-H), 8.2 (1H, d, aromatic-H), 8.3 (1H, S, O-H) and 8.8 (1H, d, aromatic-H). ^{13}C NMR (300 MH_Z, $CdCl_3$) of AF3 exhibited peaks at 110, 117, 121, 127, 128, 135, 138, 147 and 152 (9-aromatic carbons). Molecular weights of AF2 and AF3 were determined by EI-MS analysis. AF2 gave molecular ions in positive mode at *m*/*z* are 50, 63, 76, 126, 152, 180, 208 suggested a molecular weight of 208 g/mol. AF3 gave molecular ions in positive mode at *m*/*z* are 129, 93, 77, 145 indicated a molecular weight of 145 g/mol. Based on the above data, AF2 and AF3 were characterized as anthracene-9,10-quinone and 8-hydroxyquinoline respectively.

The compounds in fourth active fraction (AF4) were analysed by GC-MS (Fig. 2). Three peaks were observed with retention time of 7.74 min (C1), 8.70 min (C2) and 8.85 min (C3) respectively. MS analysis of these peaks suggested M^+ at *m*/*z* 108, 122 and 149 g/mol of molecular weights respectively. By

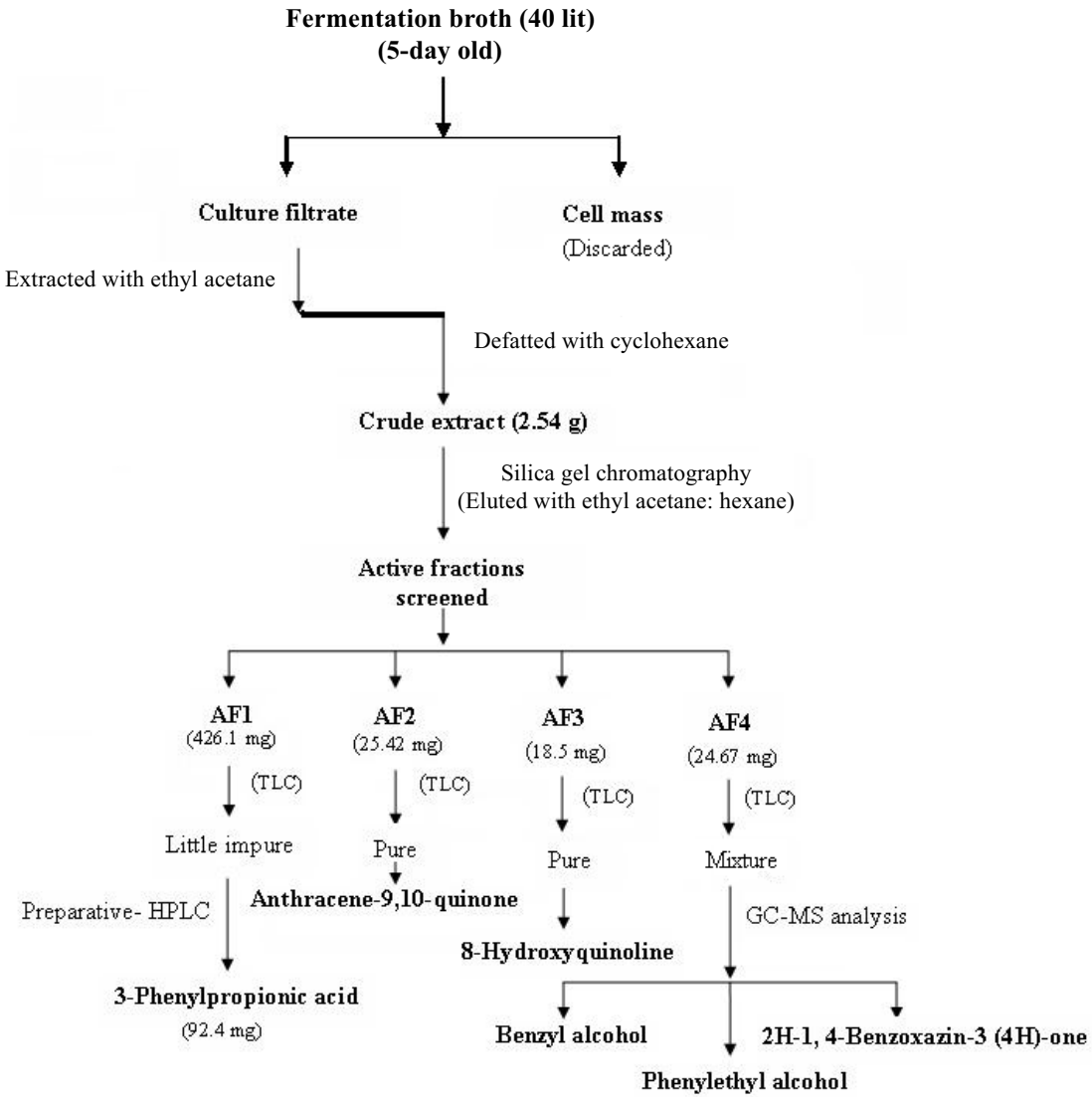

Fig. 1. Extraction of bioactive compounds from the strain ANU 6277.

using available library data, C1, C2 and C3 in AF4 were determined as benzyl alcohol, phenylethyl alcohol and 2H-1, 4-benzoxazin-3 (4H)-one respectively. Claeson and Sunesson (2005) analyzed many volatile compounds including benzyl alcohol and phenylethyl alcohol from *S. albidoflavus* grown on tryptone-glucose medium by GC-MS. A bioactive compound, dibutyl phthalate has been reported in culture broth of *S. albidoflavus* by GC-MS (Roy *et al.*, 2006). No reports were found on 2H-1, 4-benzoxazin-3 (4H)-one from microorganisms especially actinomycetes.

Three bioactive compounds (AF1, AF2 and AF3) and a partially purified active fraction (AF4) of the strain were tested against different microorganisms including bacteria and fungi. Data on their antibacterial activity are presented in Table I. The compound, AF3 (8-hydroxyquinoline) exhibited strong antibacterial activity against all the test bacteria including Gram-positive and Gram-negative ones as compared to AF1, AF2 and AF4. AF3 also exhibited good antimicrobial activity over tetracycline (positive control). Among the bacteria tested, *P. aeruginosa* was highly sensitive to the metabolites of the strain. MIC values of AF1 (10–100 µg/ml), AF2 (75–500 µg/ml), AF3

Table I
MIC (µg/ml) of bioactive compounds (AF1-AF4) from the tested strain against different bacteria

Bacteria	AF1*	AF2	AF3	AF4	Tet**
Bacillus cereus	75	250	25	500	75
B. subtilis	50	100	10	250	50
Escherichia coli	50	100	10	250	25
Klebsiella pneumoniae	100	500	50	500	50
Proteus vulgaris	50	250	25	250	25
Pseudomonas aeruginosa	10	75	25	100	50
P. fluorescens	10	100	50	100	50
Serratia marcescens	25	100	10	250	25
Staphylococcus aureus	100	500	25	500	50

* Narayana *et al.*, 2007; ** Tet-tetracycline (µg/ml)

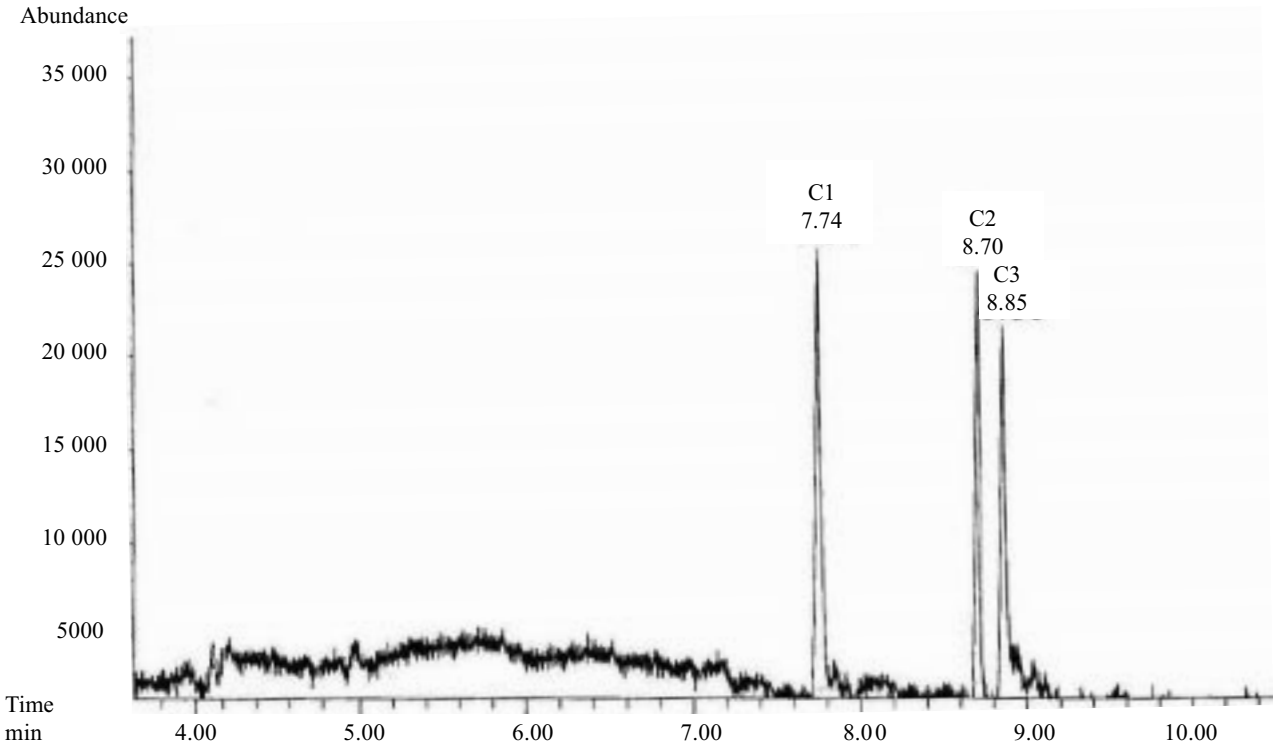

Fig. 2. GC-Spectra of an active fraction, AF4 collected from culture broth of the strain.
Components C1, C2 and C3 with retention time 7.74 min, 8.70 min and 8.85 min are determined as benzyl alcohol, phenylethyl alcohol and 2H-1,4-benzoxazin-3 (4H)-one respectively by mass spectral analysis.

(5–50 µg/ml) and AF4 (100–1000 µg/ml) were observed against the test bacteria.

Among different phytopathogenic and toxigenic fungi, *F. udum* was highly susceptible to the metabolites of strain followed by *A. flavus, P. citrinum* and *A. niger.* But *F. oxysporum* exhibited resistance to AF4 and sensitivity to AF1, AF2 and AF3. Among the four active fractions of the strain, AF3 proved to possess strong antifungal activity against phytopathogenic and toxigenic fungi (Table II). MIC values of these metabolites against the molds ranged from 2–10 µg/ml (AF3), 10–50 µg/ml (AF1), 100–1000 µg/ml (AF2) and 250–1000 µg/ml (AF4), whereas positive control carbendazim showed MIC values ranging from 1–5 µg/ml. Dermatophytes such as *C. albicans*, *E. floccosum* and *M. canis* were found sensitive to all the active fractions of the strain (Table III). AF3 exhibited strong antifungal activity against dermatophytes with MIC values ranged from 2–10 µg/ml as compared to the antifungal antibiotic, griseofulvin with MIC ranging between 10 and 50 µg/ml. MIC values of AF1, AF2 and AF4 ranged from 50–100 µg/ml, 100–250 µg/ml and 250–500 µg/ml respectively.

Among the compounds of the strain, only AF3 (8-hydroxyquinoline) was found to be exhibited a better antibacterial as well as antifungal activities over standard antibiotics like tetracycline and griseofulvin respectively. AF3 was proved to be strong bioactive compound of the strain followed by AF1 (3-PPA). You *et al.* (1999) described 8-hydroxyquinoline as a potent lipophilic metal chelator whose 8-hydroxyquinoline copper chelate could be used as a fungicide in agriculture. Antibacterial activity especially antistaphylococcal activities of 8-hydroxyquinoline and its derivatives were reported by Biswas (2003) and Short *et al*. (2006). Hydroxyquinoline and its derivatives

Table II
MIC (µg/ml) of bioactive compounds (AF1-AF4) from the tested strain against phytopathogenic and toxigenic molds

Phytopathogenic and toxigenic molds	AF1*	AF2	AF3	AF4	CZM**
Aspergillus flavus	25	250	5	500	2
A. niger	50	500	5	1000	5
Fusarium oxysporum	50	1000	10	>1000	5
F. udum	10	100	2	250	<1
Penicillium citrinum	25	500	5	500	2

* Narayana *et al.*, 2007; ** CZM – carbendazim (µg/ml)

Table III
MIC (µg/ml) of bioactive compounds (AF1-AF4) from the tested strain against dermatophytes

Dermatophytes	AF1	AF2	AF3	AF4	GF**
Candida albicans	100*	250	10	500	50
Epidermophyton floccosum	50	100	2	250	10
Microsporum canis	75	100	5	500	25

* Narayana *et al.*, 2007; ** GF – griseofulvin (µg/ml)

have been reported from different microorganisms. Mack and Zeeck (1987) detected the calcium salt of 3-hydroxyquinoline-2-carboxylic acid in culture filtrate of *Streptomyces griseoflavus* (Go 3592). A 4-hydroxyquinoline derivative having anti-staphylococcal activity was reported from a soil isolate of *Pseudomonas* sp. KUH-001 (Hwang *et al.*, 1998). Kenawy (2001) found that the polymers containing 8-hydroxyquinoline moiety were inhibitory to *E. coli*, *B. subtilis* and *Trichophyton rubrum.* Sugaya *et al.* (2005) reported a bioactive water soluble substance, 2-hydroxyquinoline from *Comamonas* sp. TKV 3-2-1.

Bioactive metabolites such as AF2 (anthracene-9,10-quinone) and AF4 were found to be showed moderate antimicrobial spectrum against test organisms. Different actinomycete strains have been reported to produce anthracene-9,10-quinone (anthraquinone) compounds. A new anthraquinone derivative has been reported from the culture broth of *Streptomyces griseorubiginosus* that inhibited the binding of activator protein-1 to the recognition sites (Goto *et al.*, 1998). A new anthraquinone, blanchaquinone along with known analogue anthraquinone reported from an Australian *Streptomyces* sp. exhibited cytotoxicity activity but not antibacterial activity (Clark *et al.*, 2004). A new anthraquinone, 1, 8-dihydroxy-2-ethyl-3-methylanthraquinone from a marine *Streptomyces* sp. Fx-58 showed cytotoxicity against HL-60 cells (Huang *et al.*, 2006). The volatile compounds such as benzyl alcohol, phenylethyl alcohol and 2H-1, 4-benzoxazin-3 (4H)-one might be responsible for biological activity of AF4.

In the present study, three bioactive compounds such as 3-phenylpropionc acid, anthracene-9,10-quinone and 8-hydroxyquinoline were purified from the culture broth of the strain and biological activities were studied against a wide range of bacteria and fungi. The bioactive compounds in a partially purified fraction were analyzed by GC-MS. Among the three bioactive compounds identified, 8-hydroxyquinoline showed strong antibacterial and antifungal properties. The compounds 8-hydroxyquinoline and 2H-1, 4-benzoxazin-3 (4H)-one produced by the strain are not reported earlier from actinomycetes.

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