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Full Length Research Paper

Cytotoxic compounds from a marine actinomycete, *Streptomyces albovinaceus* var. *baredar* AUBN$_{10}$/2

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Marine sediment samples from Machilipatnam (Krishna District, A.P) coast off Bay of Bengal, India were investigated as a source of actinomycetes to screen for the production of novel bioactive compounds. More than 10,000 bioactive compounds have been described from marine actinomycetes, with many different properties, ranging from colour pigments to cytotoxic compounds. The search for cytotoxic compounds is continuing due to the demand for new anticancer drugs. In this work, compound I was isolated from the marine derived actinomycete strain AUBN$_{10}$/2, obtained from marine sediment samples of Bay of Bengal, India. This was obtained by solvent extraction followed by chromatographic purification. The pure compound I was identified from spectroscopic data which was related to the actinomycin D, it showed a potent cytotoxic activity against cell lines HMO2 (gastric adenocarcinoma) and HePG2 (hepatic carcinoma) *in vitro*. It also exhibited antimicrobial activities against gram positive and negative bacteria.

Key words: Marine actinomycetes, phenoxazinone chromophore, actinomycin D, cytotoxic compounds, antibacterial activity.

INTRODUCTION

Actinomycetes are prolific producers of antibiotics and majority of the antibiotics in clinical use today are produced by them. Among the various microorganisms, the actinomycetes have yielded over two-third of the naturally occurring antibiotics and continued to be a major source of novel and useful antibiotics. Apart from antibiotics, actinomycetes also produce other bioactive secondary metabolites (Bérdy, 2005), anticancer (mitomycin and daunomycin) (Galm et al., 2005) and immunosuppressive agents (rapamycin and FK506) (Simmons et al., 2005). Although these organisms are continually studied extensively, it is clear that the rate of discovery of novel metabolites from terrestrial actinomycetes is decreasing and that new source of bioactive substances must be explored (Fenical and Jensen, 2006). One of such resource can be found in marine microorganisms, which are now being explored for their potential as producers of biomedically important metabolites (Krasil'nikov, 1962; Okami et al., 1976 Pisano et al., 1986; Weyland and Helmke, 1988; Williams et al., 1999). In this study, we reported the extraction, purification and structure elucidation of cytotoxic compound I, which can be considered as a new source of cytotoxic antibiotic from the marine-derived actinomycete strain AUBN$_{10}$/2, isolated from the marine sediment samples of Bay of Bengal, India.

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Abbreviations: GI$_{50}$, Drug concentration causing 50% growth inhibition; **LC$_{50}$,** minimum concentration which reduces the initial cell number to half; **TGI,** drug concentration causing 100% growth inhibition; **MIC,** minimum inhibitory concentrations; **I,** purified antibiotic.

MATERIALS AND METHODS

Isolation and maintenance

Strain AUB N$_{10}$/2 was isolated from a marine sediment sample colleted at a depth of 30 m at a distance of 8 km off Machilipatnam

coast of Bay of Bengal. For the isolation, starch casein agar with the addition of rifampicin (Pisano et al., 1989) (2.5 µg/ml) and flucanozole (75 µg/ml) was used to minimize bacterial and fungal contaminations, respectively. The strain was sub-cultured onto starch casein agar slant (medium with 50% sea water) (Weyland, 1981), incubated at 28°C for 5 - 7 days to achieve good sporulation and was preserved in 20% glycerol at -80°C.

Taxonomy

Taxonomic characteristics of the isolate were determined by cultivation on various media as described by Shirling and Gottlieb (1966), and Waksman (1961) and Arai (1975). Morphological characteristics were observed after incubation of the culture at 28°C for 14 days on Oatmeal agar (ISP med 3). Cultural characteristics were determined after growing the culture at 28°C for 14 days. The carbon source utilization pattern was determined as per the ISP protocol (Shirling and Gottlieb, 1966). Cell wall composition was analysed by the method of Lechevalier and Lechevalier (1975), using thin layer chromatography plates as described by Staneck and Roberts (1974).

Fermentation

A full grown slant culture of the strain AUBN$_{10}$/2 on starch casein agar (composition: 1.0% soluble starch, 0.03% Casein, 0.2% KNO_3, 0.2% NaCl, 0.005% K_2HPO_4, 0.002% $CaCO_3$, 0.001% $FeSO_4.7H_2O$ and 2.0% agar) was transferred into Erlenmeyer flasks (2 x 250 ml) containing 50 ml of seed medium (composition: 1.0% Soyabean meal, 1.0% corn steep solids, 0.5% glucose and 0.5% $CaCO_3$ with pH 7.0) and incubated for 3 days at 28°C on a rotary shaker (220 rpm). A 10% level of this inoculum was transferred into 200 ml of production medium in 1 L EM flasks (30 No's). The medium composition is: 1.0% soyabean meal, 0.5% corn steep solids, 1.0% soluble starch, 0.5% glucose and 0.7% calcium carbonate with pH 7.0. The inoculated production flasks were incubated for 72 h at 28°C on a rotary shaker (220 rpm).

The culture broth (6.5 L) was centrifuged at 4000 rpm for 10 min, at 10°C and clear culture supernatant was separated. It was extracted twice with ethyl acetate (2 x 1.2 L) and washed with 500 ml water at pH 7.0. The ethyl acetate layer was concentrated in vacuum at 35°C to give 5.0 g of the crude ethyl acetate extract. A polymer was obtained from the crude ethyl acetate extract, which did not possess any antimicrobial activity. The mycelium was extracted with acetone (1.0 L) and then centrifuged (4000 rpm, 10 min and 10°C). The acetone extract was concentrated in vacuum and lyophilized to obtain 4.5 g of mycelium extract.

The mycelium extract (5.0 g) was chromatographed on a silica gel column (22 X 5 cm) and eluted with dichloromethane/methanol (95:5, 1 L) to give ten fractions (Figure 1). The dried residues of all 10 fractions were dissolved, each in a specified volume of dichloromethane to give 1 mg/ml concentration and tested for their antimicrobial activities using *Bacillus subtilis* as test organism by disc-plate method. Among 10 fractions, fraction VI (41.8 mg) exhibited good activity and was purified by chromatography on Sephadex LH-20 (methanol) to obtain four fractions. The third fraction VIc was found to possess good antimicrobial activity while others have no or negligible activities. The active fraction VIc was further purified by chromatography on Sephadex LH-20 (acetone) resulting in two distinct fractions VIc.I (6.8 mg) and VIc.II (5.5 mg). Fraction VIc.I (1) had good antimicrobial activity (100 µg/ml) but negligible activity was observed with VIc.II. Compound 1 (Figure 2) was found to be pure and was pale yellow solid, which was identified as actinomycin D (Inbar and Lapidot, 1988).

The structure of compound I was assigned by spectroscopy interpretation of ^{1}H NMR, ^{13}C NMR, H,H-COSY, HMQC, HMBC, IR and ESI mass spectra.

Instrumental analysis

Melting points were determined on a Fisher-Johns apparatus. NMR experiments were performed on Bruker DRX-500 (500 MH_Z) and Bruker AMX 300 (300 MH_Z). The chemical shifts were expressed in δ (ppm) using $CDCl_3$ as solvent and TMS as internal reference. The MS spectra were measured on Varian MAT 731 and Finnigan MAT 311 A. UV spectra were recorded on a Kontron Uvikon 860. IR spectrum was measured on a Perkin Elmer Model 298 (KBr discs). TLC was carried out on silica gel 60 F_{254} plates (Merck, 0.25 mm) and column chromatography on silica gel (Macherey and Nagel, < 0.08 mm) or Sephadex LH-20 (Pharmacia). R_f values were determined on 20 x 20 cm plates, the evaluation length was 10 cm. Compounds were viewed under UV lamp at 254 nm and sprayed with anisaldehyde-H_2SO_4 followed by heating.

Cytotoxic activity

The cytotoxic activity of the pure compound was performed on the growth of tumor cells *in vitro* for their cytotoxic or cytostatic activities, according to the NCI guide lines (Grever et al., 1992). The cell lines used were HMO2 (Gastric adenocarcinoma) and HePG2 (Hepatic carcinoma). Cells were grown in 96-well microtitre plates of RPMI 1640 tissue culture medium supplemented with 10% fetal calf serum at 37°C in a humified atmosphere of 50% CO_2 in air. After 24 h of incubation, the pure compounds (0.1 ~ 10.0 µg/ml) were added to the cells. After 48 h incubation, the cells were fixed by addition of trichloracetic acid and cell protein was assayed with sulforhodamine B (Skehan et al., 1990). For compound I tested, the GI_{50} (drug concentration causing 50% growth inhibition), TGI values (drug concentration causing 100% growth inhibition) and LC_{50} (minimum concentration which reduces the initial cell number to half) were determined (Table 2).

Antimicrobial activity

The minimum inhibitory concentrations (MIC) of the purified antibiotic (I) against different test organisms were determined by the broth dilution method (Table 3).

RESULTS AND DISCUSSION

Taxonomy of strain production

The sporophores occurred as short closed spirals with 2 to 4 turns, arranged in groups and formed spiral spore chains which belong to section Spira (S). The cultural characteristics of the strain are summarized in Table 1. The colour of aerial mycelium was medium gray and colour of reverse side was brown. Diffusible pigments were not formed. It utilized glucose, L-arabinose, sucrose, xylose, inositol, mannitol and fructose. The analysis of cell wall hydrolysates showed the presence of LL-diaminopimelic acid and glycine without any characteristic sugar pattern. The taxonomic studies indicated that it belongs to the genus *Streptomyces.* A detailed survey of the literature indicated that it is closely related to *Streptomyces albovinaceus.* The comparison of our isolate with the reference culture established that it is a

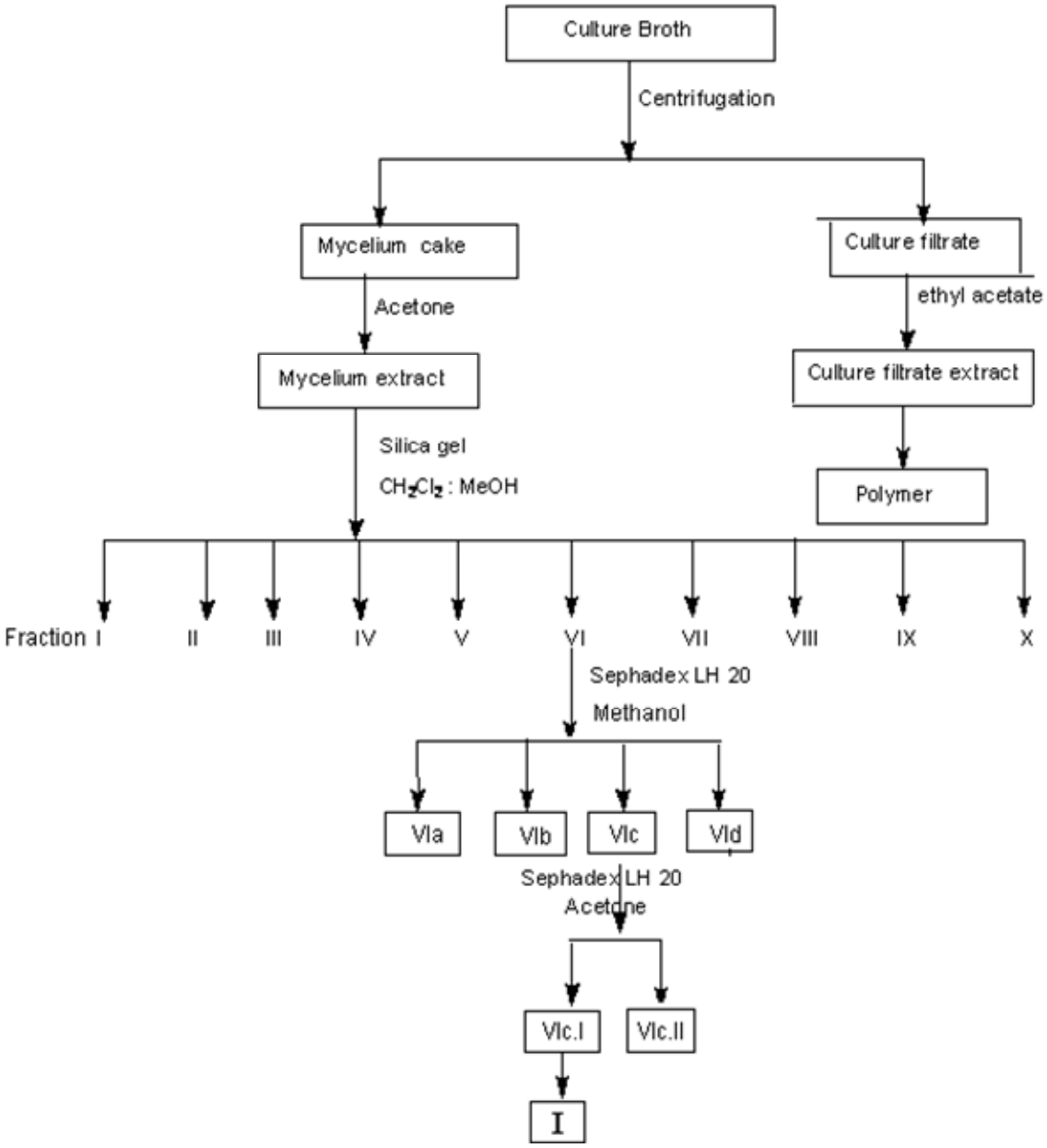

Figure 1. Scheme for the isolation of pure fractions (compound I).

variant of *S. albovinaceus* and was designated as *S. albovinaceus var. baredar.*

Structure elucidation of compound 1

The molecular formula of 1 was determined as $C_{62}H_{86}N_{12}O_{16}$ on the basis of the EI-MS (70eV), which gave a ([M^+], 40) ion at *m/z* 1278.4; IRV_{max} (KBr) cm^{-1}3400, 3250, 2950, 1740, 1650, 1590, 1485 and 1192 cm^{-1}.

The ^1H NMR spectrum of compound I in dimethyl-sulfoxide (DMSO) with 300 MHz showed two *ortho* coupled protons at δ_H 7.66 and 6.73 of a 1,2,3,4-tetrasubstituted aromatic ring, and two 3H singlets at δ_H 2.02 and 1.95 of methyl groups in *peri*-position of an aromatic system. This is characteristic for the phenoxazi-none chromophore (Figure 3) in various actinomycins.

In addition, the spectrum showed overlapping NMR signals between δ_H 9.00-0.46 as it is indicative for two pentepeptide cyclic ring (α and β ring). Four broad doublet of amide proton at δ_H 8.97, 8.70, 8.18 and 7.66, twelve hydrogen signals of α-amino acid protons were observed at δ_H 6.40 (d), 6.27 (d), 5.16 (dd), 5.04 (m), 4.61 (d), 4.41 (d), 4.14 (dd), 4.10 (dd) 2.87 (d) and 2.81 (d), eight hydrogen signals of β-amino acid protons were observed at δ_H 5.81 (dq), 5.76 (dq), 3.40 (dd), 2.95 (m),

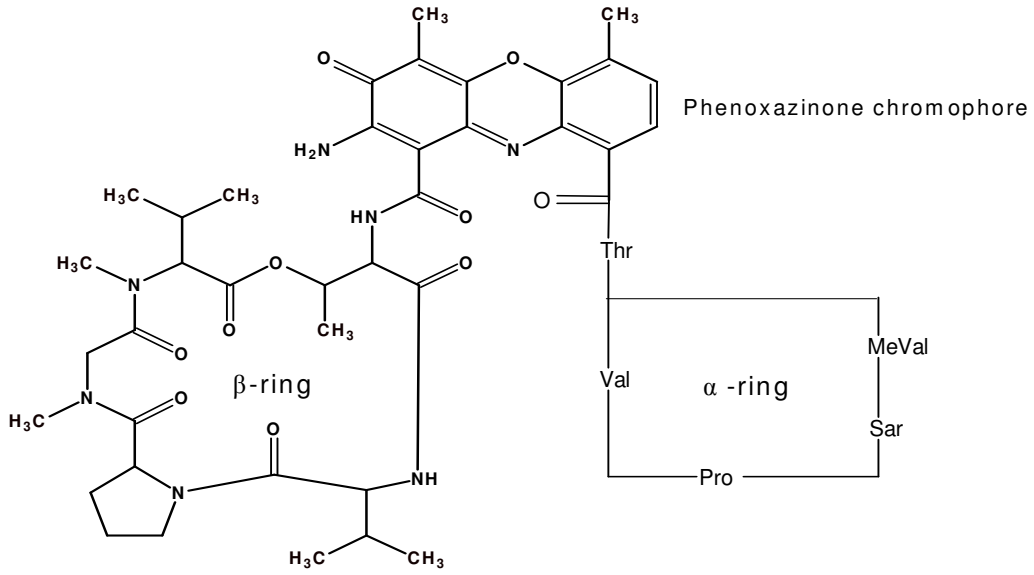

Figure 2. The structures of compound I.

Table 1. Cultural characteristics of strain $AUBN_{10}/2$.

Medium	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigment
Yeast extract- malt extract agar (ISP2)	Good	Gray	Reddish brown	None
Oatmeal agar (ISP3)	Poor	Grayish white	Yellowish brown	None
Inorganic salts- starch agar (ISP 4)	Good	Gray	Brown	None
Glycerol asparagine agar (ISP 5)	Good	Grayish white	Reddish brown	None
Tyrosine agar ((ISP 7)	Poor	Light gray	Brownish gray	None

Table 2. Cytotoxic activities of compound I.

Cell line HMO2			Cell line HePG2		
GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}
0.006	0.009	0.011	0.009	0.011	0.016

All the concentrations are in µg/ml; GI_{50} = concentration which produces half of the maximum cell growth Inhibition; TGI = concentration which produces complete growth inhibition; LC_{50} = minimum concentration which reduces the initial cell number to half (24 h growth).

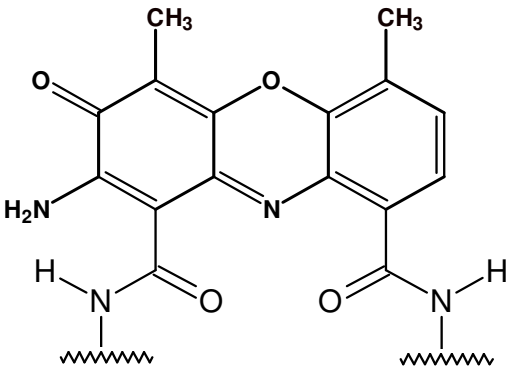

Figure 3. Phenoxazinone chromophore.

2.90 (dd), 2.77 (m), 2.58 (m) and 2.55 (m), twelve hydrogen signals of γ-amino acid protons were observed as ten doublet methyl proton at δ_H 1.66, 1.60, 1.58, 1.52, 1.34, 1.32, 0.96, 0.94, 0.47 and 0.46 and two methylene proton at δ_H 1.92 and two hydrogen signals of δ-amino acid protons were observed as δ_H 4.05 (d) and 3.98 (d).

The ^1H NMR spectrum of compound I showed that four singlets methyl that were found at δ_H 2.75, 2.76, 2.06 and 2.24, may be due to N-methyl groups (Table 4). The ^{13}C NMR spectra exhibited 11 signals between δ_C 100 - 150 and one at δ_C 178.6, which are attributed to the twelve ring-carbon atoms of the phenoxazinone system. Twelve carbonyls were observed around δ_C 165 - 175 and are characteristic to those of amide and lactone-carbonyl

Table 4. ^{1}H NMR and ^{13}C NMR data of compound I compared with Actinomycin D (C_6D_6).

Residue	Function group	Chemical shift (ppm)			
		α-ring		β-ring	
		^{1}H	^{13}C	^{1}H	^{13}C
Threonine	C=O	-	169.2	-	167.2
	$C_\alpha H$	5.04	55.9	5.16	55.5
	$C_\beta H$	5.81	75.6	5.76	75.8
	$C_\gamma H_3$	1.66	18.0	1.60	19.4
	NH	7.66	-	8.18	-
Valine	C=O	-	167.8	-	169.7
	$C_\alpha H$	4.10	59.4	4.14	59.2
	$C_\beta H$	2.55	32.6	2.58	32.3
	$C_\gamma H_3$	1.52	21.9	1.32	19.6
	$C_\gamma H_3$	1.58	19.4	1.34	18.9
	NH	8.97	-	8.70	-
Proline	C=O	-	173.3	-	173.8
	$C_\alpha H$	6.27	57.1	6.40	56.9
	$C_\beta H$	3.40	31.5	2.90	31.1
	$C_\gamma H_2$	1.92	23.4	1.92	23.2
	$C_\delta H_2$	2.59	48.1	2.55	47.8
Sarcosine	C=O	-	166.7	-	166.8
	$C_\alpha H_2$	4.41, 2.87	51.1	4.61, 2.81	51.0
	N-CH_3	2.76	34.6	2.75	34.6
MethylValine	C=O	-	166.3	-	167.7
	$C_\alpha H$	2.37	71.1	2.48	71.3
	$C_\beta H$	2.77	27.5	2.95	27.6
	$C_\gamma H_3$	0.91	21.6	0.46	18.4
	$C_\gamma H_3$	0.96	19.7	0.47	19.0
	N-CH_3	2.06	38.8	2.24	38.8
Phenoxazinone chromophore	1	-	101.5	-	-
	2	-	148.1	-	-
	3	-	178.6	-	-
	4	-	112.6	-	-
	4a	-	144.4	-	-
	5a	-	140.3	-	-
	6	-	126.8	-	-
	7	6.75	129.7	-	-
	8	7.65	125.9	-	-
	9	-	129.2	-	-
	9a	-	132.9	-	-
	10a	-	146.3	-	-
	4-CH_3	1.95	7.4	-	-
	6-CH_3	2.02	14.5	-	-
	1-CO	-	173.3	-	-
	9-CO	-	174.3	-	-

groups of a peptide system. The α-carbon atoms of the amino acids are shown in the region (δ_C 50 - 72). In addition, two signals of oxygenated methine carbons are at δ_C 75 - 76.

The spectral information of the compound was searched in different databases like Chapman and Hall (2002), SciFinder, Beilstein and Antibase (Laatsch, 2000) and hints were observed in the above databases. This indicated that compound I is a known compound which was earlier reported from terrestrial actinos (Eckardt et

al., 1970, 1972; Haupt and Eckardt, 1972; Hofle and Wolf, 1983) but not reported so far from marine sources (Figure 2). Our report can be considered as a new source of information of this cytotoxic antibiotic.

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