Characterization of antibacterial compounds produced by the actinomycetes using NMR spectral analysis

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Abstract

A total of 3 strains were found with potential antibacterial activity. The MMKK5 strain showed moderate, good and excellent growth in GAA broth during 7, 14 and 21 day of incubation respectively while in starch-casein broth weak growth observed in 7 days incubation but finally at twenty one days showed excellent growth. MMKK2 showed moderate to good growth up to 21 days of incubation. Whereas, MMKK2 and BPA showed good growth in both the media in 21 days of incubation. MMKK5 strain found to be highly active against test microorganisms like *Bacillus subtilis, Bacillus megaterium* and *Bacillus stearothermophilus* and was modertately active against *Bacillus cereus* and *Staphylococcus aureus*. MMKK2 showed its high inhibitory activity to *B. cereus* and *B. megaterium* while showed moderate inhibition with *B. subtilis*, *S. aureus* and *B. sterothermophilus*. BPA showed moderately active inhibition to only *Bacillus cereus* and *Micro luteus*, while all other bacteria were passive or with slight activity. The NMR spectra of MMKK5 showed purely aliphatic compounds, the signals were normalized to the integral of the spectral region 8 to 9 shows aldehyde group and showed the compound slightly soluble with methanol, ethyl acetate showed carboxylic groups, showing the aliphatic aminoaldehyde (Gummy solid CH_2 group 3.22 at 2 ppm) and two (CH_3 groups 1.88 and 3.02 correlated at 1 ppm). NMR spectra of MMKK2 showed singlet, doublet peak multiplicity signals showed NH, CH_2, CH_3 groups signal 1.67 and 2.63 correlated 2.44 at 1 ppm of CH_3 group. Singlet signals 2.52 and 3.00 at 2 ppm of CH_2 group. NMR spectra of BPA in DMSO-d_6 showed signal regions were normalized to the integral of the spectral region 8 to 9 ppm showed 1.32 and 2.33 singlet signal at 5 to 6 ppm and 1.97 at 7.5 ppm.

Keywords: Actinomycetes, MMKK-5, MMKK-2, BPA, antibacterial activity, NMR spectra

Introduction

Actinomycetes have been looked upon as a potential source of antibiotics and the past findings proves that actinomycetes are the richest source of secondary metabolites. Actinomycetes hold a prominent position as targets in screening programme due to their diversity and their proven ability to produce novel antibiotic and other non-antibiotic lead molecules of pharmaceutical interest. Since the discovery of actinomycin (Waksmann and Lechevalier, 1962), the first antibiotic from actinomycetes, many commercially important bioactive compounds and anti-tumor agents in addition to enzymes of industrial interest have been produced using actinomycetes (Tanaka and Omura, 1990). It has been estimated that approximately 2/3 of the naturally occurring antibiotics have been isolated from these organisms (Takizawa *et al*., 1993). So far, the terrestrial soil was the most predominant and widely exploited source. The marine sediments as a source of bioactive actinomycetes was less exploited one of the most successful approaches to obtain new types of useful microbial metabolites is to investigate slowly growing (or) until now simply neglected microorganisms from marine sediments. Number of reports is available on the characteristics of actinomycetes and their physiology and biochemistry (Vikineswary *et al*., 1997; Ellaiah *et al*., 1996, 2002).

Actinomycetes have been recognized primarily on their morphological criteria. They are usually considered to be actinomycetes with ability to form branching hyphae at some stage of their development. They are ubiquitous in the world that surrounds us, but they seem to prefer the solid constituents of the earth: litter, humus, dung, soils and even rock surfaces. Actinomycetes are commonly believed to have a role in man's life, in particular for his own purpose where they can be used for the production of antibiotic, nutrient cycle, production of certain enzymes have a role in digestion of some compounds such as protein in a farm of keratin (or) for the production of vitamins (Lechevalier, 1981).

Actinomycetes are saprophytic bacteria that secrete important hydrolytic enzymes, antibiotics and medicinally important secondary metabolites. Between 1988 and 1992 more than hundred different new molecules from actinomycetes were discovered. Approximately 75% of these originated from *Streptomyces* genus (Sanglier *et al*., 1993; Sacromento *et al*., 2004). Therefore, and because of their ability to secrete valuable proteins, *Streptomyces* have been considered as an alternative host organism for producing recombinant proteins (Cruz *et al*., 1992). Induction of mutation and selection to improve the producitivity of cultures has been

strongly established for over fifty years and is still recognized as a valuable tool for many antibiotics (Venkateshwarlu *et al*., 2000). The mutant strains can be achieved by inducing genetic variation in the natural strain with increasing productivity. An actinomycetes strain (*Streptomyces* sp.) isolated from Kuwait tropical desert (Hashem and Diab, 1973), was chosen due its marked stability and tested for its double capacity to produce antimicrobial agents active against some pathogenic with special focus on the cytotoxic activity against brain human tumor cell line. The possibility of producing mutants from the parent strain has been explored and direct cytotoxic activity against brain cancer cell lines was also investigated. The molecular weight and the structure of the isolated proteo-polysaccharides were also investigated of these mutant forms.

Two important factors must be recognized in the attempt to classify the actinomycetes, especially for the purpose of establishing specific differences. These are, first, the ability of the organisms to produce vegetative and aerial mycelium; and second, the great variability of the cultures. To facilitate recognition of these organisms and to establish differences for classification purposes, well-defined media and standard conditions of cultivation must be used.

The filamentous growth and the true branching of the actinomycetes differentiate these organisms from the true bacteria. It is often difficult if not impossible to distinguish between the profuse branching of certain fact that the *Nocardia* produce mycelium consistently in the early stages of their development. There is thus a gradual transition between the mycobacteria and the nocardias. There is also a definite transition between the nocardia and the *Streptomycetes*, due to the constant and marked nature of the serial mycelium of the latter and the transitory and undifferentiated aerial mycelium of the former. In the present investigation, an attempt was made to isolate the actinomycetes from the coal belt regions of Adilabad district, Andhra Pradesh, India and identification of the strains isolated was done using standard procedures mentioned by earlier researchers and the strains have been tested for the antibacterial activity with the gram positive and gram negative bacteria and the antibacterial principle was detected with NMR spectroscopy.

Material and methods

Isolation of Soil Samples

Soil is the habitat for a variety of organisms, including bacteria, viruses, fungi, actinomycetes, protozoa, insects, nematodes, worms and many other animals. The microbial population in soils can be very high. In surface soil the bacterial population can approach 10^8 to 10^9 cells per gram of dry weight. The gram-positive bacteria, which show varied degree of branching and mycelial development, are an important and less studied part of the microbial community. They include the coryneforms, the nocordioforms, and the true filamentous bacteria or actinomycetes. Further, the examination of soil of extreme environments for isolation of actinomycetes are very less. Hence, in the present study an attempt was made to isolate the actinomycetes in coal field soils.

Soil sample were collected from five important coal field regions of Singareni Colleries Company Limited (SCCL), Adilabad, Andhra Pradesh, i.e., Mandamarri Kalyani Khani (MMKK) and Bellampally (BPA) (Plate-I).

Plate -I

SCCL Mandamarri Kalyanakhani 5

Sample collection site of MMKK 5

SCCL Mandamarri Kalyanakhani 2

Sample collection site of MMKK 2

SCCL Bellampally Area

Sample collection site of underground mine area in Bellampally

In the process of sample collection one inch of superficial layer was dug and collected soil was kept in clean polythene bags and carried to the laboratory for isolation of actinomycetes. The standard soil dilution technique procedure was adopted for the separation of actinomycete colonies from the soil. Generally, actinomycetes from the soil were isolated by pour-plate technique on Glycerol-aspargine agar, Starch-casein agar and Inorganic salt-starch agar media after serial dilutions in the distilled water. Dry colonies of actinomycetes were selected and isolated. Thus, isolated colonies were preserved in Glycerol based agar media and stored at 20^{o}C. The actinomycetes of which antimicrobial activity should be determined were revived by streaking on starch-casein agar and incubated at 28^{o}C for 7 days.

Serial dilution technique

Microorganisms were isolated from the soil samples collected from coal mines and made serial dilutions as 10^{-1} (1/10), 10^{-2}(1/100), 10^{-3} (1/1000), 10^{-4} (1/10,000), 10^{-5}(1/1,00,000). One ml portion of the dilutions were inoculated into Glycerol-asparagine, Starch-casein and inorganic salt starch agar media by pour-plate method and usually 10^{-4} and 10^{-5} were used for enumeration and separation of actinomycete colonies.

Selective media

A selective media is the one that contains one or more agents that inhibit the growth of a certain microorganisms and there by encourage or select the microorganism of interest and allow it to grow. Selective

media are very important in primary isolation of a specific type of microorganism from soil samples containing different types of microorganisms of interest and allow it to grow. They enhance isolation by suppressing the unwanted background organisms and favoring growth of the desired ones.

Identification of Actinomycetes

The isolated actinomycetes were identified using different staining techniques, various biochemical methods and molecular approaches.

Staining of Actinomycetes

Actinomycetes are gram-positive prokaryotes characterized by formation of branched filamentous body and are referred to as mold-like bacteria. During the growth of these isolates sterile cellophane was placed on the surface of solidified nutrient agar medium and incubated the isolates at 30^{o}C till it sporulates. After incubation removed the cellophane bearing growth from the agar surface and stained for 2 minutes in a mixture of stains with 2 parts Bismark brown (0.1% w/v), 2 parts of Toluidine blue (0.1% w/v) and 1 part of saturated ammonium sulphate solution then rinsed in distilled water and allowed it to air dry and mount in canada balsam. The vegetative mycelium stained light yellow and the spores blue with red granules in the aerial hyphae.

Slide culture technique

A thin agar block cut from poured plate is placed on a sterile microscope slide and inoculated and a sterile coverslip is applied. After incubation, in a moist chamber, viewed the slide culture directly on the microscope stage. The aerial mycelium and the substrate mycelium within the agar block was observed and identified.

Biochemical tests

The strains isolated from the soil samples of coal mines, were identified by conventional biochemical tests in accordance with Bergey's Manual of Systematic Bacteriology 2nd edition (Taiwo and Oso, 2004).

Isolation of antibacterial compounds

Antibacterial compound was recovered from the filtrate grown with different actinomycetes strains by solvent extraction method following the process described by Westley *et al*. (1979), Parthasarathi *et al*. (2012). Ethyl acetate was added to the filtrate in the ratio of 1:1 (v/v) and shaken vigorously for 1 hour for complete extraction. The ethyl acetate phase that contains antibiotic was separated from the aqueous phase. It was evaporated to dryness in water bath at 80^{o}-90^{o}C and the residue obtained was weighed. Thus obtained compound was used to determine antimicrobial activity, minimum inhibitory concentration and to perform bio-autography.

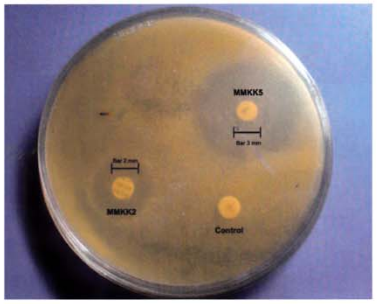
NMR spectra analysis

For the analysis of NMR spectra, a loopful of culture of the strains were inoculated into basal medium g/L with the following composition : yeast extract, 10.0, K_2HPO_4, 1.0, $MgSO_4.7H_2O$, 0.5, $CaCl_2.2H_2O$, 0.04, $FeSO_4.7H_2O$, 0.005, $ZnSO_4.7H_2O$, 0.0005, distilled water 1000 ml and incubated in Erlenmeyer conical flask at 28-30^{o}C for 7, 14 and 21 days. After incubation 100 ml extract was taken and added 100 ml methanol, 100 ml ethyl acetate and shake well by the separating funnel for one hour. The content was kept for the settlement for two layers and upper layer was taken and again added 100 ml ethyl acetate and kept for 30 minutes for separation of two layers. The upper most layer of 100 ml was taken and evaporated in water bath at 100^{o}C. After evaporation, completely dried the extraction and with the sterilized glass rod wrapped the extraction and taken in a polyethane cover and analysed each strain by the di-methyl sulfoxide (DMSO) and did the experiment by the Varian 200 MHz Gemini 2000 for the NMR spectra's analysis of eight strains and recorded the data.

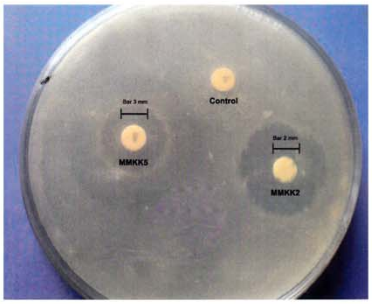
Results and Discussion

Based on the criteria mentioned by different scientists the antibiotic production of actinomycetes isolated from different ecological conditions were surveyed and presented in plates (II) and tables (1-3). From the plates and tables it was evident that the antimicrobial activity of the eight strains of actinomycetes grown on two different media glycerol-broth and starch-casein broth for 7, 14 and 21 days. The MMKK5 strain showed moderate, good and excellent growth in Glycerol broth during 7, 14 and 21 day of incubation while in starch-casein broth weak growth observed in 7 days incubation but finally at twenty one days showed excellent growth. MMKK2 showed moderate to good growth up to 21 days of incubation. Whereas, MMKK2 and BPAit showed good growth in both the media in 21 days of incubation (Table-1).

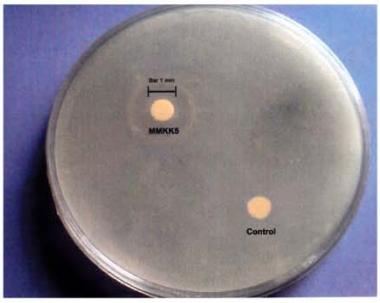
PLATE - II



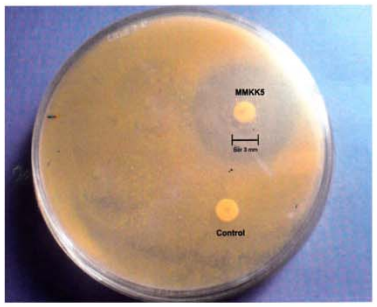
Antimicrobial activity of two strains of actinomycetes against *Bacillus megaterium* ATCC 9885



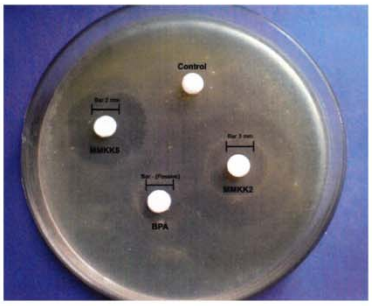
Antimicrobial activity of two strains of actinomycetes against *Bacillus stearothermophilus* ATCC 2328



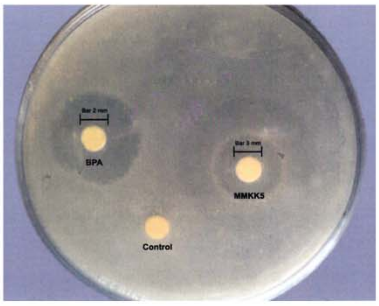
Antimicrobial activity of MMKK5 strain against *Escherichia coli* ATCC 2343



Antimicrobial activity of MMKK5 against *Bacillus subtilis* ATCC 11778



Antimicrobial activity of three strains of actinomycetes against *Bacillus cereus* ATCC 11778



Antimicrobial activity of two strains of actinomycetes against *Micrococcus luteus* ATCC 2170

Table–1 Growthof three strains of actinomycetes in glycerol broth (A) andStarch-casein broth (B)

Strains	A – Days of incubation			B – Days of incubation		
	7	14	21	7	14	21
MMKK5	++	+++	++++	+	++	++++
BPA	+	++	++	+	++	++
MM-KK2	++	+++	+++	++	+++	+++

+ = Weak growth ; ++ = Moderate growth ; +++ = Good growth ; ++++ = Excellent growth

Antibiotic resistance was approximately recorded in 50% of the strains with broad or narrow range (Table-2).

Table–2 Antimicrobial activity of MMKK5, BPA and MMKK2 against Gram positive and Gram-negative bacteria

Test Bacteria	Diameter of the inhibition zone (in mm)		
	MMKK5	BPA	MMKK2
Gram-positive bacteria			
Bacillus subtilis	3	2	2
Bacillus cereus	2	-	3
Bacillus megaterium	3	1	2
Staphylococcus aureus	2	1	2
Micrococcus luteus	3	2	1
Bacillus stearothermophilus	3	1	2
Gram-negative bacteria			
Enterobacter aerogenes	-	1	1
Proteus vulgaris	--	--	--
Klebsiella pneumoniae	--	--	--

The inhibitory effect of the strains was divided into five groups according to their size of the group -- = No zone of inhibition ; - = Passive $\leq$ 10 mm; Group 1 = 11-20, slightly active; Group 2 = 21=33 mm, moderately active; Group 3 = > 34 mm Highly active.

The strains exhibited sensitivity to a number of antibiotics like penicillin, tetracycline, ampicillin, streptomycin, streptothricin, actinomycin A, D, ciprofloxin. MMKK5 showed resistance to HIV drug, Lanostad-N-30, while, Actinomycin C does not showed any action against the strain MMKK5. MMKK2, MMKK5 and BPA showed high range of antibiotic resistance.

The antibacterial activity of three strains of actinomycetes against six gram-positive and four gram-negative bacteria was studied and reported (Table-3).

Table-3 : Antibiotic resistance of MMKK5 , BPA and MMK2 strains of actinomycetes

Antibiotics	MMKK5	BPA	MMK2
Penicillin	+	+	+
Tetracycline	+	+	+
Ampicillin	+	+	+
Streptomycin	+	+	+
Streptothricin	+	+	+
Actinomycin A	+	+	+
Actinomycin D	+	+	+
Ciprofloxin	+	-	+
Norfloxin	-	-	+
Actinomycin C	-	+	+
Lamostad-N-30	+	-	-
Zidovudine	-	-	-

+ = Positive; - = Negative

The diameter of the bacterial clear zone was measured and the minimum inhibition concentration of the actinomycetes extracts was calculated. The inhibitory effect was divided into three groups, i.e., negative, group one, two and three. The negative inhibition shows passive nature with ≤ 10 mm, group shows one 11-20 mm with slightly active, group two shows 21-33 mm with moderately active while group three shows ≥ 33 mm with highly active inhibition.

All the strains under study (Table-3) showed negative activity to two gram negative bacteria strains, *Enterobacter aerogenes* and *Klebsiella pneumoniae*. MMKK5 strain found to be highly active against test microorganisms like *Bacillus subtilis*, *Bacillus megaterium* and *Bacillus* and *Bacillus stearothermophilvs* and was modertately active against *Bacillus cereus* and *Staphylococcus aureus* (Plate -II). *Micrococcus luteus* and *E. coli* showed slightly active inhibition while *Enterbacter aerogenes*, *Proteus vulgaris* and *Klebsiella pneumonia* showed passive activity. MMKK2 showed its high inhibitory activity to *B. cereus* and *B. megaterium* while showed moderate inhibition with *B. subtilis*, *S. aureus* and *B. sterothermophilus*(Plate-II). BPA showed moderately active inhibition to only *Bacillus cereus* and *Micro luteus*, while all other bacteria were passive or with slight activity (Plate-II). Up onobservation of the immense inhibitory action of the strains confirmed that they are responsible for the production of antibacterial compounds.

The ^{1}H NMR spectra were recorded with a Varian 200 MHz Gemino 2000 FT-NMR spectrometer in DMSO-d_6. The NMR spectra of MMKK5 (Fig.1) showed purely aliphatic compounds, the signals were normalized to the integral of the spectral region 8 to 9 shows aldehyde group and showed the compound slightly soluble with methanol, ethyl acetate showed carboxylic groups, showing the aliphatic aminoaldehyde that (Gummy solid CH_2 group 3.22 at 2 ppm) and two (CH_3 groups 1.88 and 3.02 correlated at 1 ppm). The integral region showed that the concentration of (glutamate signal 2.04 at 5-6 ppm) control spectra were acquired to ensure that the changes seen in the spectra of 5-aminolevulinic acid (ALA) incubated when exposed to light, truly originated from the photosensitization effects.N^{1}-[(methylphenyl)sulfonyl]-2-(1-oxo-2H-phthalazin-2-yl)aceto-hydraziderecrystallized from methanol and ethyl acetate to yield 87%. ^{1}H NMR (DMSO-d_6) showed 2H, N-H, 1H, 3H, CH_3 formed broad singlets, doubled doublet peak multiplicity. Proton and carbon chemical shifts on 1.84, 3.00, 2.04, 1.22 at 0 to 10 ppm. It should be noted that as the total spectrum signal decreased with increasing light exposure.

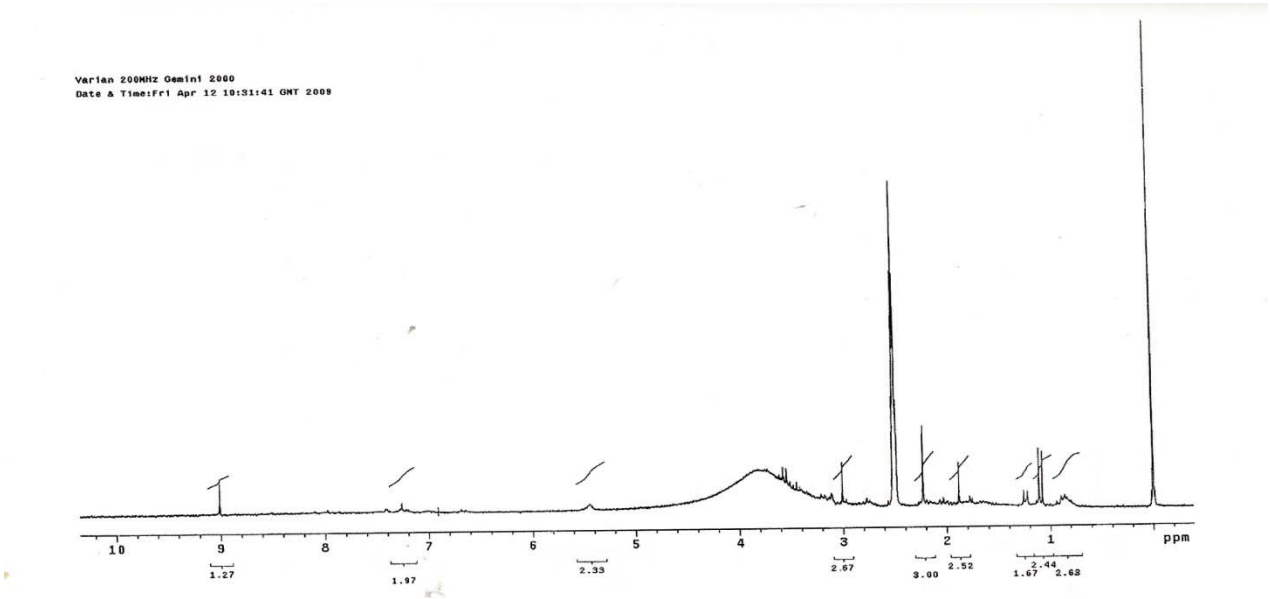


Fig-1. NMR spectra of the antibacterial compounds of the strain MMKK5 in DMSO-d_6

NMR spectra of MMKK2 (Fig.2) showed purely aliphatic. Gummy solid in DMSO-d_6 MMKK2 recrystallized with methanol and ethyl acetate showed singlet, doublet peak multiplicity signals showed NH, CH_2, CH_3 groups signal 1.67 and 2.63 correlated 2.44 at 1 ppm of CH_3 group. Singlet signals 2.52 and 3.00 at 2 ppm of CH_2 group. Slightly NH group with 2.63 at 3 ppm showed the concentration of aspartate and two signal regions 2.33 and 1.97 at 5 to 6 ppm and 7 to 7.5 ppm and singlet peak signal region 1.27 at 9 ppm. NMR spectra of BPA in DMSO-d_6(Fig. 3) showed signal regions were normalized to the integral of the spectral region 8 to 9 ppm showed 1.32 and 2.33 singlet signal at 5 to 6 ppm and 1.97 at 7.5 ppm. Gummy solid CH_2 group showed 2.52, 3.00 at 2 ppm and CH_3 group 1.67 at 1 ppm. Minimum inhibitory concentration (MIC) values of the synthesized compounds were determined by culture broth. The synthesized compounds were dissolved in DMSO/H_2O (50%) at a concentration of 500 μg/ml. MIC values were recorded as the lowest concentrations of the substances that had no visible turbidity. DMSO concentration in the final solutions was not above 12.5% for antibacterial activity.

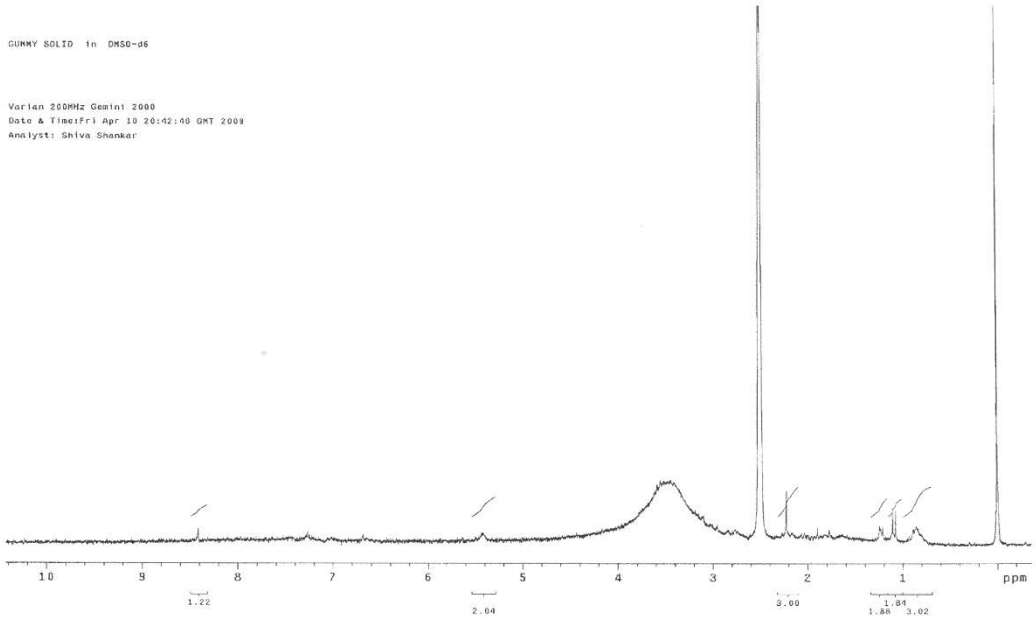


Fig-2. NMR spectra of the antibacterial compounds of the strain MMKK2 in DMSO-d_6

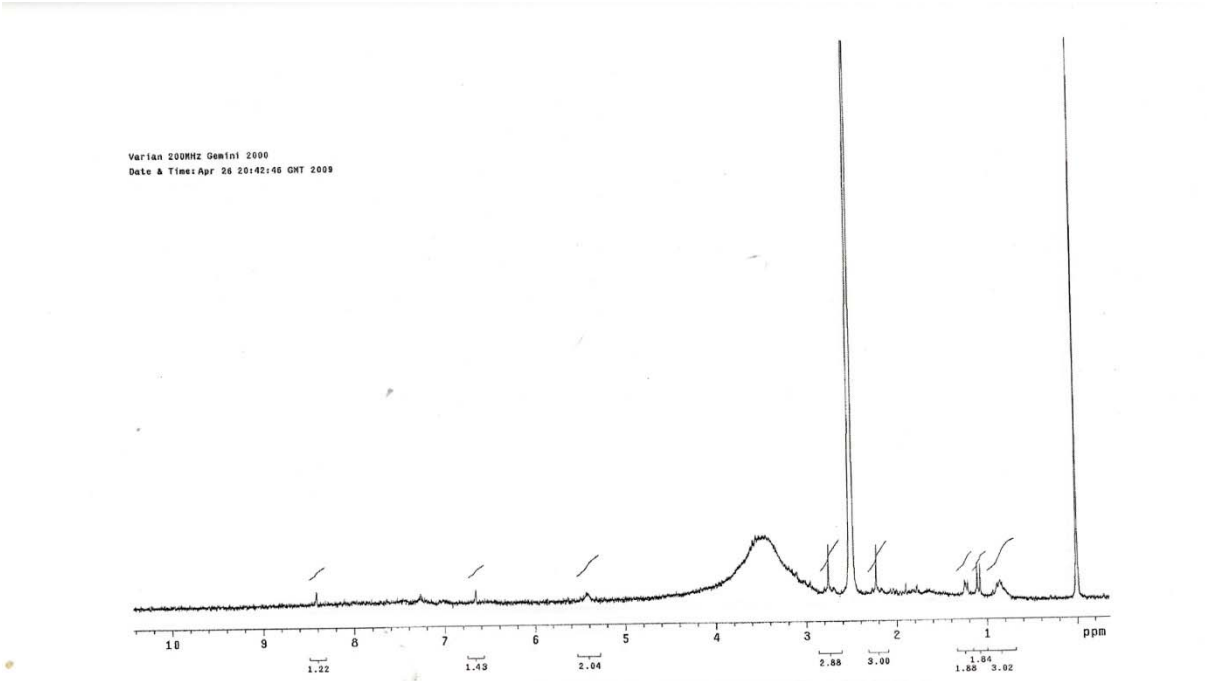


Fig-3. NMR spectra of the antibacterial compounds of the strain BPA in DMSO-d_6

The effectiveness of reference deconvolution in reconstructing a spectrum relies on the selection of an appropriate reference signal. Several factors contribute to the suitability of a signal for use as a reference. The avoid errors resulting from division by very small numbers, the decay envelop for the chosen signal should not drop to zero anywhere in the field that is being processed. The signal should also be well-separated from any other signals to ensure absorption or dispersion components (Morris, 1997). Kjeldstad *et al.* (1984) did not make an effort to find red LED's (Light Emitting Diodes) with an optimum wavelength in the red part of the spectrum demonstrated the NMR effects by choosing a blue light LED that was efficient in producing spectral changes. Glycine betaine and proline are known osmolytes (Rudulier *et al.* 1984; Boyaval *et al.*, 1999) and glycine betaine has been extensively studied in *E. coli* and been proven to have osmoprotective abilities (Rudulier *et al.*, 1984). K^+ efflux in photodynamic therapy (PDT) treated bacteria and erythrocytes during photo-induced porphyrin sensitization has also been reported for many other cell types and photo-sensitizers (Murphy, 1988; Paardekooper *et al.*, 1995). Based on ^{1}H NMR spectra of the target compounds and the literature suggested that the target compounds had rotamers due to hydrogen bonding between N-H next to carboxyl at the side chain and the carbonyl group of pyridazinone rings. That is the target compounds had rotomers one of which was with hydrogen bonding and the other without hydrogen bonding. Magic angle spinning (MAS) NMR spectra is typically carried out by spectral stimulation or comparison with the spectra of model compounds and quadrupole parameters can be derived from the structural parameters by calculation methods (Mains, 2001).

NMR spectroscopy can be used to both identify and quantify chemicals from complex mixtures and highlights some of the strengths, limitations and quantitative metabolonics (David and Wishart, 2008). The recent melamine crisis in China has pointed out as a serious deficiency in current food control systems (Dirk *et al.*, 2009).Interestingly, our chemistry to further elaborate 5 to 1 produced intermediates that show virtual coupling that depends on both the nature and site of aromatic ring substitution. Nitration of 5 produced a 3:2 mixture of isomers 6 and 7, which were difficult to separate (Panigot, 1994). In one instance, small quantities of pure 6 and 7 were obtained by preparative thin layer chromatography (TLC).

Because both the O-nitrophenyl and O-aminophenyl isomers 7 and 9 fail to show the virtual coupling present in 5, 6 and 8 which bear a C_2-symmetric substituent at C-1, it seems plausible that this lack if virtual coupling results from steric interaction of the O-substituent with the axial H-1 or H-2 proton. In support of this concept, none of the ortho nitro or amino C-benzyl analogues 10 or 11 (3) that have an interposed methylene unit show evidence of virtual coupling in the 250 MHz ^{1}H NMR spectra in $CDCl_3$. That other more subtle influence such as electronics may also play a role in suggested by inspection of the spectrum of the O-tolyl analog 12, which were prepared serendipitously during efforts to synthesize 2. In the $CDCl_3$ ^{1}H NMR spectrum of 12, the H-2, H-3, and H-4 resonances overlap extensively, unlike any of the other compounds reported here. However, the H-5 resonance at 4.16 ppm shows some evidence of much less extensive virtual coupling than for 5, implying that the impact of the O-methyl substituent is insufficient to change $\Delta\nu/J_{3,4}$ enough to eliminate virtual coupling under these spectroscopic conditions. Furthermore, it was observed that the 1-β-azido

glucuronide 13, previously prepared demonstrated virtual coupling in the ^1H NMR spectrum in $CDCl_3$, which is nearly identical to that of 5. This coupling is absent at 400 MHz and in the 250 MHz acetone-d_6, benzene-d_6, CD_2Cl_2, CD_3OD, pyridine-d_5 and tetrahydrofuraN-d_9 DMSO-d_6 spectra of 13 and also in the $CDCl_3$ spectrum of the amine prepared by reduction of 13 as well as its acylated derivatives. Once again, linear, symmetrical azide substitution results in virtual coupling while reduction product do not show this property, suggesting, perhaps, that the hybridization of the C-1 attached atom may play a role in causing this phenomenon. The spherically symmetrical, sterically undemanding methyl substituted compound also demonstrates this virtual coupline (Panigot, 1994).

Oleandomycin derivative having a macrolactone of which biosynthesis does not follow the genetic architecture of the oleandomycin. The molecular formula of the compound was suggested as $C_{35}H_{59}O_{11}$ on the basis of the analysis of NMR data (Boem Seok Kim, 2005). A model for how the consensus binding domain peptide from the HMG-I protein binds to the minor groove of AT rich DNA has been proposed on the basis of molecular modeling (Reeves, 1990). These amides might be expected to be less accessible to solvent and potentially may participate in hydrogen-bonds with the carbonyl groups.

The NMR studies on the metabolites extracted from the eight strains of actinomyctes was carried out. NMR spectra of GDK compounds recrystalized from methanol and ethyl acetate to yield 26.2% of spectral region produced slightly. Black solid in DMSO-d_6 showed only NH and slightly CH_3 group 6.00 and 3.13 at 1 ppm and 5-6 ppm. The NMR spectra of SPL-OC and GDK-OC in DMSO-d_6 showed completely absence of the peak signals from 1 to 10 ppm. The minimum inhibitory concentration values of synthesized compounds were determined and based on the efficiency of the strains determined.

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