

Chapter-3

Studies on Activity & Mode of Action of the Polyketide

INTRODUCTION

Breakthrough discovery of penicillin had led to revolution in the fight against bacterial pathogens. Several new molecules and their derivatives have been worked out since then and have been introduced in clinical practice. Of them, *Streptomyces spp.* has emerged as single largest source of such antimicrobial molecules.

S. flaviscleroticus is one species, with no reports on its potential antimicrobial activity. During earlier work in the lab, it was found to be source of one or more antimicrobial compounds. Presence of type II polyketide was determined by southern hybridization experiments, using conserved act I probe. Later construction of PKS⁻ deletion mutant had confirmed the results.

This chapter deals with the studies conducted with the polyketide product obtained from *S. flaviscleroticus*. The work encompasses the spectrum over which the molecule spans its activity, determination of minimal inhibitory concentrations, cidal/static nature, post-antibiotic effect and the effectiveness of the antibiotic against pathogenic strains resistant to commercial antibiotics. Interestingly, the mode of action of the polyketide, by which it stops the bacterial activity, has also been deciphered.

When this work was initiated in 2003, chemical characterization of the polyketide was underway. Owing to the bulky nature of molecule, miniscule quantity of production and lack of expertise available in natural product chemistry, deciphering of chemical structure of the molecule took too long. Later, after conducting all the studies including decoding mode of action of the polyketide, chemical characterization studies revealed the polyketide to be Chromomycin A3. It is a well known molecule obtained from *S. griseus* sub *griseus* and has been worked out by Sato and coworkers way back in 1960. Since the identity of molecule was not known when this study was done, chromomycin has been mentioned as 'the polyketide' through out this chapter.

RESULTS & DISCUSSION

3.1 Spectrum of Activity:

Crude ethyl acetate extract from *S. flaviscleroticus* was effective over a broad range of microbes (gram-positive and gram-negative bacteria and fungi). Pure polyketide molecule isolated from the crude extract using column chromatography and subsequent 2-D TLC as per the method standardized in our laboratory (Namita kumari, 2007) was tested for antimicrobial activity, by agar-well assay on solid media as well as in broth. Test results for experiments conducted for a series of concentrations using standardized protocols (Andrews J, 2001) following NCCLS (National Committee for Clinical Laboratory Standards) guidelines, were highly effective against gram positive bacteria but could not inhibit growth of gram-negative bacteria. Also, it did not exhibit any antifungal activity, which was present in crude, when tested against yeast and *Aspergillus* spp. (Table: 3.1).

Typically, antimicrobial agents are classified as being either narrow-spectrum or broad-spectrum depending upon the extent of its application on a range of microbes. Owing to the specificity of the range of microbes that the molecule can affect, it can be classified as narrow spectrum antibiotic. The narrow spectrum of the polyketide should not be interpreted as a limitation, since it has advantages over broad spectrum antibiotics. The microflora of human intestine is a consortium comprising of a diversity of bacterial species (Jernberg et al, 2005). Use of broad-spectrum antibiotics washes off these beneficial bacteria whereas use of host specific narrow-spectrum antibiotics provide an advantage in preserving the microflora that additionally provides reduced risk of superinfection (Sullivan et al, 2001). Secondly, these drugs do not target multiple resistance determinants and thus may be less likely to select for resistance (Nord et al, 2006). Antibiotics such as glycopeptides, macrolides, nitrofurantoin, metronidazole, aztreonam, nalidixic acid, and phosphomycin are typical examples of narrow spectrum antibiotics in commercial use for various treatments.

The minimal inhibitory concentrations on test strains for most antibiotics normally fall in microgram amounts (Andrews J, 2001), but for the polyketide under study the concentrations required to bring about inhibition were in range of nanogram concentrations. The concentrations are significantly low when compared to those of drugs in medical / research use.

Table 3.1: Effect of the polyketide on different groups of micro-organisms.

Microbe	subsp./Strain	MIC (ng/ml)
Gram Positive Bacteria		
<i>Micrococcus luteus</i>	MTCC106	80
<i>Micrococcus roseus</i>	MTCC678	100
<i>Bacillus subtilis</i>	MTCC121	15
<i>Bacillus megaterium</i>	ATCC14945	100
<i>Staphylococcus aureus</i>	aureus	200
	BB255	100
	BB270	250
Gram Negative Bacteria		
<i>E. coli</i>	DH5 α	No inhibition
	S17	No inhibition
<i>Salmonella typhimerium</i>	-	No inhibition
Fungi		
<i>Saccharomyces cerevisiae</i>	uvarum	No inhibition
<i>Aspergillus niger</i>	lab isolate	No inhibition
<i>Aspergillus flavus</i>	lab isolate	No inhibition

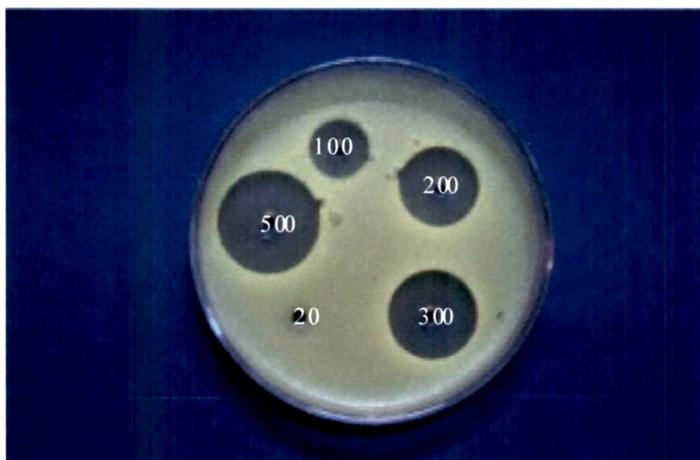
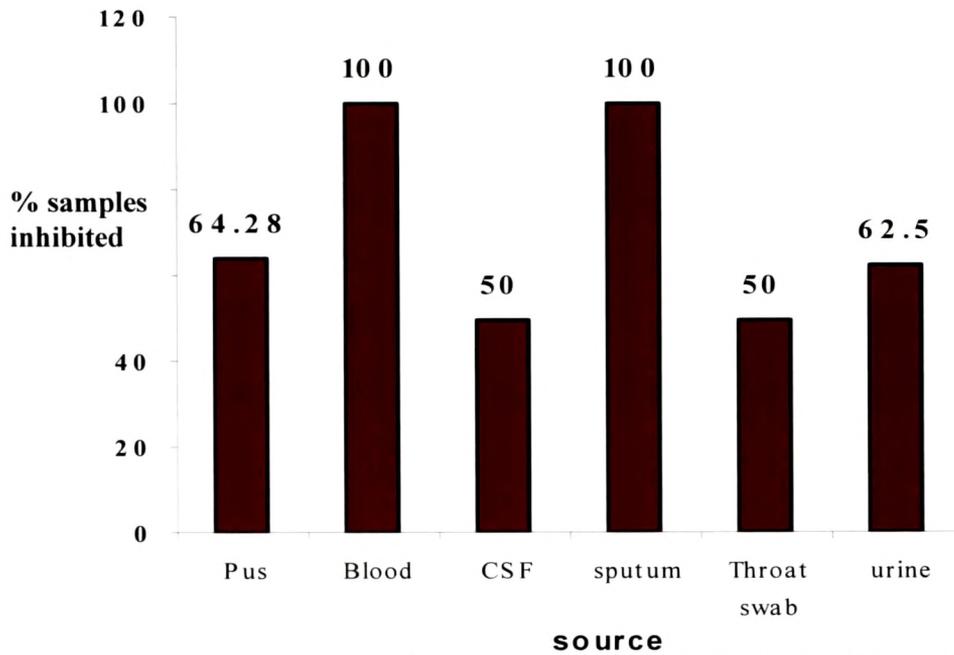


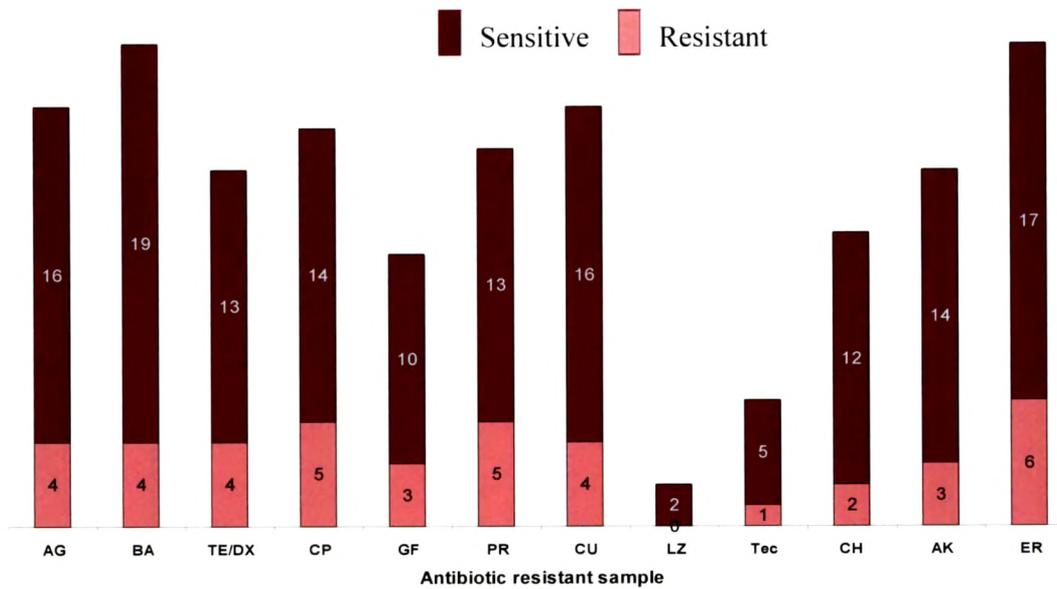
Fig 3.1: Zones of inhibition of *M. luteus* in agar well assay, at increasing (20ng, 100ng, 200ng, 300ng, 500ng) concentrations of the polyketide.

The polyketide when tested on *Staphylococcus aureus*, as well as its notorious mutant MRSA (Methicillin Resistant *Staphylococcus aureus*) could be easily killed at concentrations below 300ng/ml. It was also effective on other clinical isolates, which included CoPS (coagulase positive), CoNS (coagulase negative) and B-haemolytic *Streptococci*, cultured from various biological sources of human patients.

Due to stringent conditions and practice needs to be followed while handling pathogenic strains, the study was performed in collaboration with Dr. Toprani's Pathological Laboratory, Baroda. The discs impregnated with 1 μ g amount of antibiotic showed inhibition for all the 80 samples tested. So, the polyketide loaded discs were tested with only those samples that were already resistant to more than one drug. Out of total 40 samples tested, sourced from blood, sputum, pus, throat swab, urine and even cerebrospinal fluid the polyketide could effectively work against most of them.



Graph 3.1 : Activity of polyketide on pathogenic strains from various human samples.



Graph 3.2 : Effect of the polyketide on pathogenic samples resistant to other antibiotics

AG= amoxicillin+
clavulanic acid
BA= co-trimoxazole
TE= tetracycline

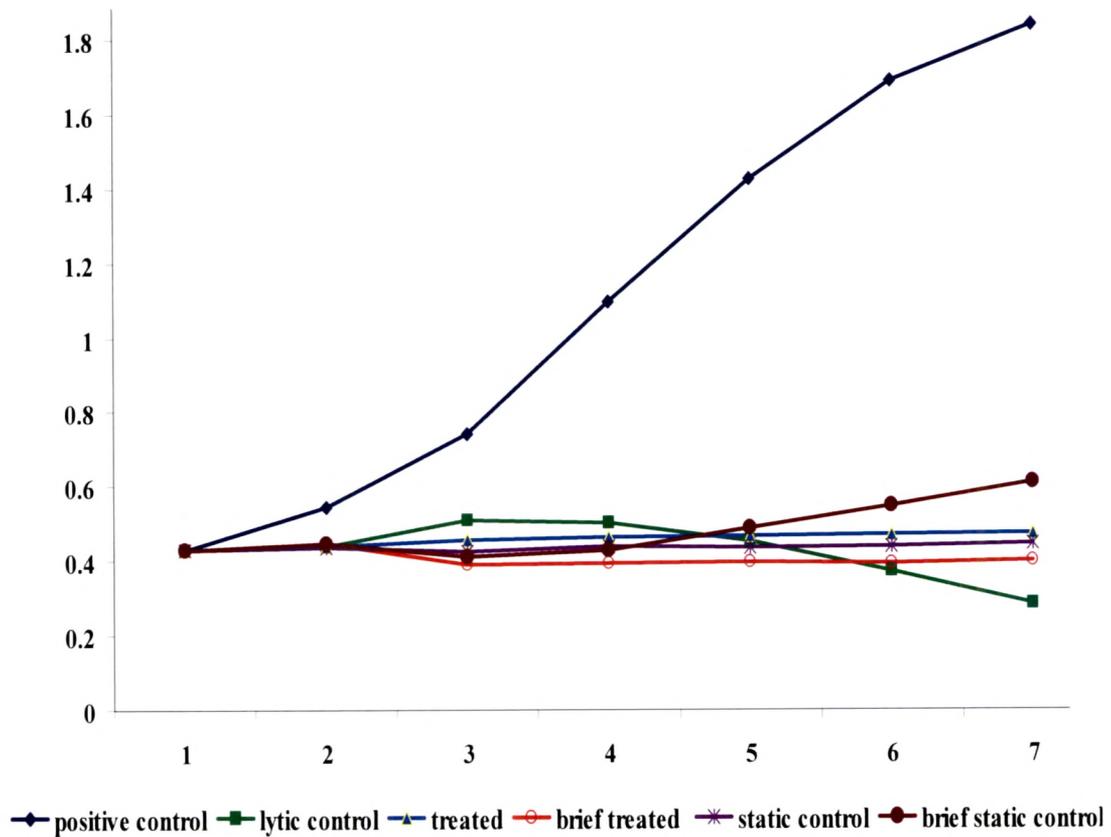
DX= doxycyclin
CP= ciprofloxacin
GF= gatifloxacin
PR= cephalixin

CU= cefuroxime
LZ= linezolid
Tec= teicoplanin
CH = Chloramphenicol

AK= Amikacin
ER= erythromycin

3.2 Growth Curve:

Results of growth curve studies proved instrumental in determining the nature of the antibiotic. The positive control, cultured without any antibiotic pressure, showed an expected exponential growth whereas the sets treated with antibiotics did not show a significant change in O.D. Owing to the lytic nature of ampicillin, at every time interval its culture showed marked drop in cell viability accompanied with loss of cell integrity represented by a decline in the O.D. This was not seen in cultures with tetracycline. The bacteriostatic antibiotic (Chopra and Roberts, 2001) ceased the growth of actively dividing bacteria as efficiently as the lytic antibiotic without damaging the cell structure, which is represented by a horizontal line almost parallel to X-axis in the graph-3.3.



Graph 3.3: Growth curve of *M. luteus* in absence or presence of different antibiotics

The polyketide supplemented culture also mimicked the pattern exhibited by latter, thereby ruling out the lytic nature of the polyketide.

Set of tetracycline treated cultures when transferred to antibiotic free medium after appropriate washes, showed a gradual resumption of growth after initial lag. This was due to the reversible binding of tetracycline with ribosomes (Chopra et al, 1992), which when removed, allows resumption of cell growth. A post-treatment, small decline in O.D. observed initially, was attributed to the cell loss during phosphate buffer washes.

Growth resumption effect, noted in tetracycline was not observed with the polyketide treated cultures. This was based on an unchanged O.D., even after transferring cells to the antibiotic free medium. It clearly shows that the polyketide molecule binds to its target irreversibly or that the critical time interval before which the effect can be reversed is too small. Data certainly indicates that mode of action of polyketide involves binding to the target in an irreversible manner, thereby bringing about cell death in a non-lytic manner.

3.3 Reasons for limiting nature of Antibiotic:

Many antibiotics which are highly toxic for gram-positive bacteria are lethal to gram-negative bacteria only at high concentration (Nikaido, 1989). In this case, the polyketide could not cause any inhibition even at 2.0 ug/ml concentration, which is 10 times higher than the concentration used for gram positive bacterium. This could be due to the architecture of the cell membranes of gram-negative bacteria that can resist penetration of certain antibiotics. The extra lipopolysaccharide layer that also imparts rigidity to gram negative bacteria forms a physical barrier in the process. (Vaara, 1992)

Outer membrane disorganization and permeabilizing action of EDTA by chelation of Mg^{+2} and Ca^{+2} is well established (Hancock, 1984; Nikaido and Vaara. 1985; Lugtenberg and Alphen. 1983). A final concentration of 5mM EDTA with 100mM Tris was standardized for destabilization of LPS in *E. coli* DH5 α strain as revealed by a significant increase in inhibition zone of control antibiotic (ampicillin). No zone of inhibition could be observed for the polyketide even with the compromised cell wall. (Table-3.2)

Table 3.2: Effect of Tris-EDTA-mediated permeabilization of cell envelope of *E. coli* on sensitivity to polyketide compound.

Variables	Without Treatment	5mM EDTA Treatment	
		+ 50mM Tris	+ 100mM Tris
Control (methanol)	No inhibition	No inhibition	No inhibition
Test (Polyketide)	No inhibition	No inhibition	No inhibition
Ampicillin	8mm	13mm	14mm

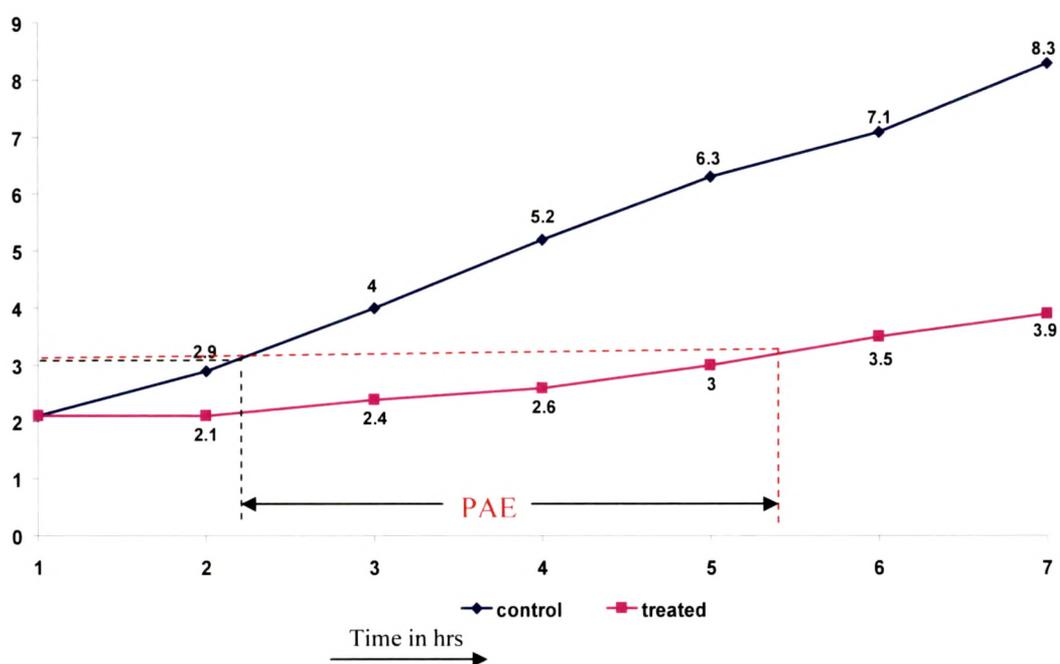
The result suggests that the membrane damage might not be sufficient for the bulky polyketide molecule to cross the barrier. Besides this chemical treatment, an LPS deep rough mutant strain F545 of *E. coli*, procured from Prof. Brandenburg's lab, also showed same results.

Chemical treatment as well as genetic mutant approach both shows that hydrophobic nature and bulky size of the polyketide is the limiting factor for its entry to the cell even with relatively permeabilized outer membrane. Similar result could have been obtained if an antibiotic manages a slow influx across the damaged outer membrane, which gets counterbalanced by the dilution resulting from the expansion of the growing cell (Nikaido, 1989). But for the polyketide that possibility is ruled out since the entry of small fraction of molecules in the cell would suffice for effective lethal action. The outer membrane does not form a barrier for other small molecules such as β -lactams, since they utilize predominantly the porin pathway. Due to the nonspecific nature of these porin channels, other small, hydrophilic agents like the tetracyclines, aminoglycosides and chloramphenicol could also get entry into the cell (Komatsu et al, 1991). Hydrophobic quinolones first disorganize the bilayer by chelation of divalent cations and then diffuse through it (Chapman et al, 1988).

3.4 Post-antibiotic effect:

Post antibiotic effect (PAE) is the term used to describe the suppression of bacterial growth that persists after a brief exposure of organisms to antimicrobials. Precisely, the period of time after complete removal of an antibiotic, during which there is no growth of the target organism (Zhanel, 1991). The PAE appears to be a feature of most antimicrobial agents and has been documented with a variety of common bacterial pathogens.

There was no change in the O.D. of polyketide treated culture even after a prolonged period of incubation in fresh medium under appropriate conditions. The high bactericidal



Graph 3.4: Growth of *S. aureus* culture post-incubation with the polyketide (treated) or without polyketide (control).

action of the molecule would have killed most of the bacteria as was observed in MIC studies described earlier. Since the increase in small number of left over cells would go unrecorded in the noise of large number of dead cells in the culture, there was no change in the OD over a period of time. To overcome it, same cultures were also studied for increase in cell number over period of time by cell count method using dilution plating technique. This method would eliminate noise of dead cells, and only an increase in CFU number over a period of time would be obtained. In past various methods such as impedance measurement (Baquero et al, 1986; Gould et al, 1989), bioluminescence assay of bacterial ATP (Isaksson et al, 1988; Winstanley and Hastings, 1989), spectrophotometry (Rescott et al, 1988), electronic particle counting (Nadler et al, 1989) and even morphology (Hanberger et al, 1990) have been used to measure post-antibiotic effect. But the method of viable count (Craig and Gudmundsson, 1996) has been most popular (Jacobs et al, 2003) due to its simplicity and accuracy.

$$\begin{aligned} \text{PAE}^* &= \text{T} - \text{C} \\ &= 265 - 70 \\ &= 195 \end{aligned}$$

*refer method 2b.6.5 (pg. 86) for details

A PAE of three hours was calculated from the colony count data (Graph -3.4). The significant result of this *in vitro* experiment suggests that pathogens would require a lot of time to recover from the deleterious effect of the antibiotic. Though there can be several other factors that influence PAE including the type of organism, concentration of antimicrobial, duration of antimicrobial exposure, and antimicrobial combinations (MacKenzie and Gould, 1993).

3.5 Effect of polyketide on stationary phase culture

The effectiveness of an antimicrobial agent is measured by its ability to inhibit and kill bacteria. However, tests of this ability are usually performed on log-phase bacteria or those in a very rapid-growth phase in media supplying all of the necessary nutrients for optimal growth (NCCLS, 1990). Clinically, microorganisms in some infected tissues may be

walled off quickly by host leukocytes, followed by fibrin deposits, and thus the growth of the bacteria may be less optimal and controlled by the limited access to nutrition.

Also, a deliberate slow-growth phenomenon has been reported in pathogenic bacteria like *Staphylococcus aureus* (Chuard et al, 1997) and *Helicobacter pylori* (Millar and Pike, 1992), to reduce sensitivity to antibiotics. Isolates of *S. aureus* from chronic subcutaneous cage infections in rats were shown to be in a state of dormancy (Churd et al, 1991). In these scenarios, the organisms multiply at a less-than-optimal rate and the effectiveness, rather lack of effectiveness of antibiotics, against organisms in such a growth state is just being recognized. It has been observed that most antibiotics are ineffective against this trick of pathogens.

The results obtained with the cells under natural stationary phase also revealed the same fact. The polyketide that could easily work on gram positive bacterium at nanogram concentrations could not effectively act on the stationary phase cultures even at a very high concentration of 5ug/ml. Alternatively, when log phase cultures (0.6 O.D.) were maintained in nongrowing conditions of phosphate buffer for two hours, and then exposed to the

MM

Table- 3.3: Response of natural and induced stationary phase culture of *M. luteus* to 10X MIC concentration of antibiotics.

Treatment (1hr)	Natural Stationary Phase Culture	Induced Stationary Phase Culture
Control (50ul Methanol)	1.0×10^6	1.0×10^6
Tetracycline (150 ug/ml)	8.0×10^5	8.0×10^5
Test (2.5ug/ml)	9.8×10^5	200
Ampicillin (1mg/ml)	1.0×10^6	9.5×10^5

μ-

polyketide an interesting result was obtained. There was a significant reduction in viable count, though the cells were not dividing (Table- 3.3).

Similar results were obtained on lowering antibiotic concentrations that were comparable to one obtained with actively growing cultures. The results (Table- 3.4) showed that most of the cells were killed in first 20 min. of incubation and the lethality increased over the time. Loss of viability due to sudden starvation in phosphate buffer reported earlier (Greenwood, 1991) was ruled out as there was an insignificant change in cell viability of control cultures spread after three hours of incubation, time equivalent to starvation and treatment period of the treated cultures. At the same time inability of other two antibiotics, namely ampicillin (inhibitor of cell wall synthesis) and tetracycline (inhibitor of protein synthesis), to act even at high concentrations ensured that the cells were perfectly under non-

Table 3.4 : Effect of polyketide at MIC, on nongrowing *M. luteus* over a period of time.

Treatment	Incubation time in Phosphate buffer (in min.)	Duration of Treatment (in min.)	Cell count
Control	0	No treatment	7.82×10^7
	180	No treatment	4.48×10^7
Ampicillin (100ug/ml)	120	20	4.48×10^7
	120	40	3.36×10^7
	120	60	3.10×10^7
Tetracycline (15ug/ml)	120	20	5.10×10^7
	120	40	4.09×10^7
	120	60	4.30×10^7
Test (250ng/ml)	120	20	1.64×10^3
	120	40	9.54×10^2
	120	60	3.74×10^2

growing condition. The residual colonies obtained from the spread plates of polyketide treated cultures, when tested for resistance in presence of the polyketide, were found to be polyketide sensitive. Thus they must have developed from the cells that could have simply escaped interaction with polyketide and that starvation did not lead to development of antibiotic resistance phenotype as obtained in other cases against pathogens like *Mycobacterium* (Betts et al, 2002), *Enterococcus faecalis* (Giard et al, 1996), *Salmonella typhimurium* (McLeod et al, 1996) and *Pneumococci* (Tuomanen and Tomasz, 1990).

The difference in the results of the two non-growing cultures is ascribed to the difference in their treatments. In first case, culture was allowed to gradually enter stationary phase, and it had sufficient time as well as resources to prepare for adverse conditions, by incorporation of necessary changes in cell envelope. It is known that gradual nutrient limitation engenders important physiological changes including decreased drug permeability across the outer or cytoplasmic membranes, and alterations in the structure of peptidoglycan such that it resists degradative enzymes (Lewis, 2002). Whereas in the second case actively growing cells were suddenly exposed to starving conditions which did not provide cell either necessary nutrients or sufficient time to transform itself for the resting phase.

Ability of the polyketide to act on induced stationary phase culture narrows down the search for the target on which the polyketide molecule acts. Especially, under the given conditions, the starving cells cease most of its biosynthetic activities to save limiting energy and allow only vital activities like respiration and minimal transcription of most essential genes. This leaves very few effective targets, which antibiotics can act upon, and cause lethality. The candidate targets that can be hypothesized for the effect shown by polyketide are the processes that are very critical for cell survival even when bacteria are not replicating, such as transcription, allowing drugs like fluoroquinolones (ciprofloxacin and rifampin) to retain some activity (Bahl et al, 1997). Recent studies have shown respiration, essential process for aerobic bacteria, being targeted by phenothiazines (Boshoff et al, 2004; Weinstein et al, 2005.). Some molecules bring about damage or alterations in the existing membranes of the non-growing cells (Xie et al, 2005).

The chances for the polyketide to bring about severe damage to the cell membrane as caused by polymixins (Fotergill and Smith, 1981) was ruled out simply on the basis of growth curve experiment discussed earlier, where the non-lytic bactericidal nature of the drug was confirmed with no time dependent decline in O.D. of the treated culture. The chances of antibiotic altering the permeability of the bacterial membrane, without any conspicuous membrane damage, to cause lethal efflux of potassium ions as shown by enterocin (Herranz et al, 2001) and Daptomycin (Steenbergen et al, 2005) could not be ruled out.

3.6 Changes in membrane fluidity/rigidity:

Diphenyl Hexatriene (DPH) is a fluorescent probe routinely used for measuring mitochondrial membrane fluidity. It is hydrophobic in nature and associates with the lipophilic tails of phospholipids without disturbing the membrane structure. The technique adopted for studying changes in bacterial membrane fluidity was found to be appropriate. Very recently, membrane fluidity changes in *Listeria monocytogens* were reported using same method (Najjar et al, 2007).

A clear difference in the membrane fluidities of the cells in growth phase against that in stationary phase could be observed. But there was no marked change in the membrane

Table 3.6: Difference in membrane rigidity as indicated by marked difference in values of control and test at different growth stages.

Phase		P value
Log	Control	0.225 ± 0.004
	Test	0.230 ± 0.006
Stationary	Control	0.283 ± 0.005
	Test	0.279 ± 0.009

fluidity of growing cells on addition of the polyketide. Absence of change in anisotropy values measured fluorimetrically on spectrofluorimeter (Shimadzu, RTZ750), before and after addition of the polyketide clearly indicated membrane intactness and thus, any action of the polyketide on the membrane to bring about even minor changes to cause leaky membrane could have been detected using this sensitive probe, but the results suggest absence of any such damage.

3.7 DNA binding assay:

The self fluorescing ability of the polyketide molecule was exploited for studying another probable target of the polyketide action, the nucleic acid. This unique feature among *S. flaviscerloticus* metabolites was earlier used as a marker, during isolation of molecule (Namita Kumari, 2008) and studying PKS⁻ mutant (Dave, 2008), in our lab. The molecule could emit fluorescence between 500-650nm with a peak at 540nm, when provided with an excitation wavelength of 470nm. The fluorescence feature has been documented to be exhibited by other naturally occurring DNA binding molecules like chromomycin and mithramycin (Waring, 1981) as well as synthetic molecules like Propidium Iodide (PI) and Ethidium Bromide (EtBr) popularly used as dyes. not quoted!

The prominent fluorescence of polyketide was considerably diminished immediately, on addition of magnesium ions. Probably divalent cation acted as quencher of the fluorophore as reported for mithramycin (Sastry and Patel, 1993). Fluorescence could be restored to a considerable level when DNA was added to the system, providing a direct indication of DNA binding nature of the molecule, thus deciphering the mode by which the polyketide acts on bacterial cells to bring about cell death. The properties of the polyketide exactly mimicked that of chromomycin, produced by *S. griseus* sub. *griseus* used as positive control. The results also satisfy the nanogram concentration requirement of the drug for killing the bacterium.

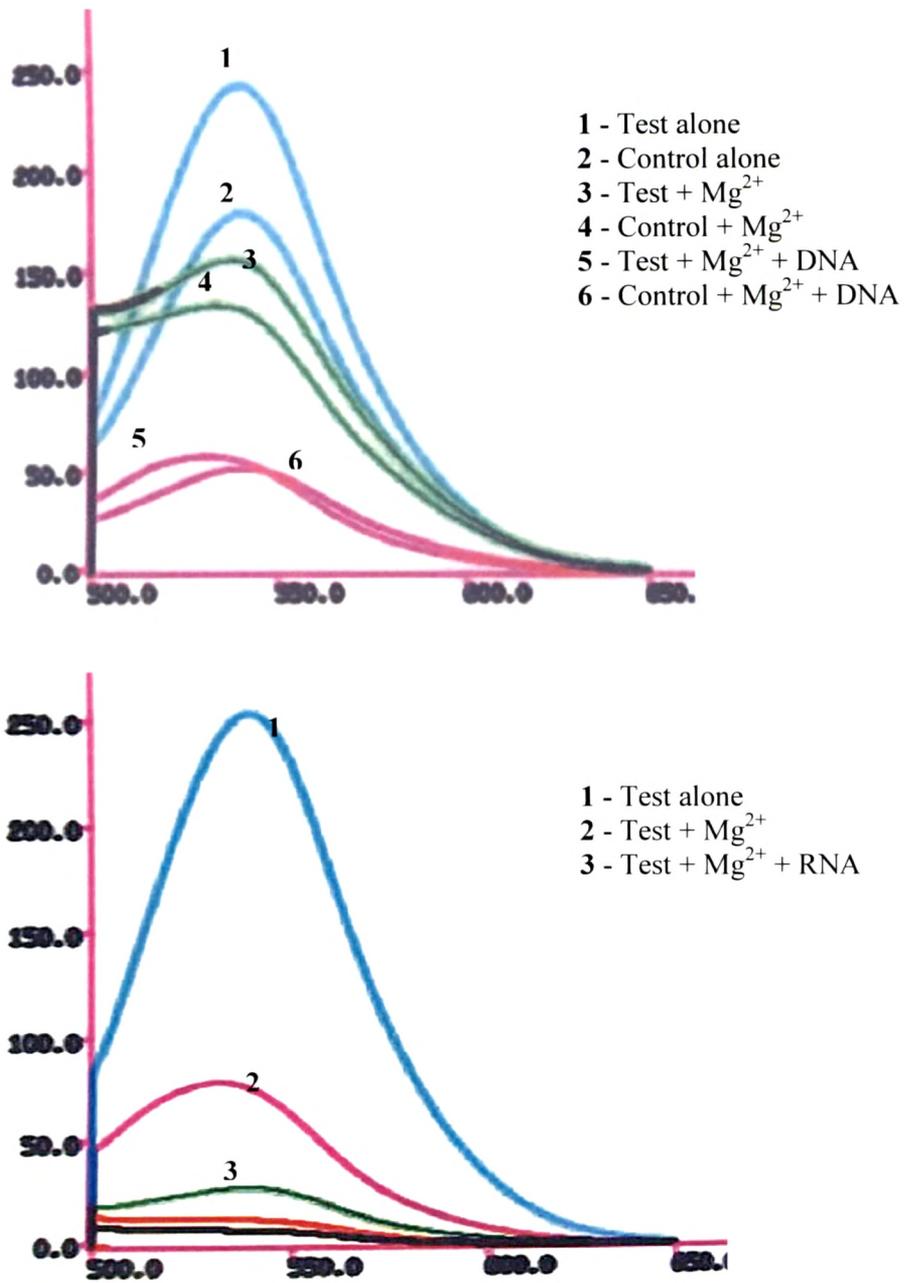


Fig. 3.2: Nucleic Acid binding study of the polyketide (Test) in relation to known DNA binding molecule, Chromomycin (Control)

No x-axis axis!
Is this fluorescence!?

In another experiment, there was no restoration of fluorescence, when RNA was used as source of nucleic acid (instead of calf thymus DNA) against drug - magnesium ion complex. This proves that the drug identifies and binds particularly with DNA, and does not affect RNA. The feature distinguished the polyketide from other DNA binding agents like Ethidium Bromide and Propidium Iodide, which nonspecifically binds to DNA as well as RNA by intercalation. Further, the drug must be exploiting the architecture of DNA for its action.

In vitro DNA cleavage assay result showed total absence of damage to DNA on incubation with the polyketide as evidenced by an intact DNA band, without any smearing pattern, in the agarose gel. Thus the action of the polyketide appears to be limited to binding with DNA and do not cause any breakage by generating reactive oxygen species as in case of amoxicillin (Li et al, 2007) or by directly acting as molecular scissors like calicheamycin (Maraschio et al, 2001). Binding of the polyketide to DNA could curtail two vital processes namely replication and transcription. Replication inhibition would restrain further cell proliferation while transcription inhibition would lead to death of existing cells.

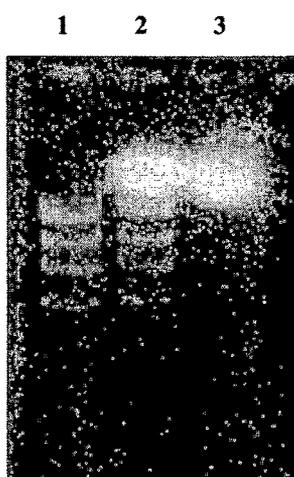


Fig 3.3: Agarose gel electrophoresis of DNA incubated with or without polyketide. Lanes 1) Lamda-H DNA alone; 2) Lamda-H DNA preincubated with the polyketide; 3) fluorescent polyketide alone

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Interestingly, after electrophoresis, when the gel was viewed under uv-light, a prominent fluorescent glow of the polyketide could be obtained in the gel. This shows the overall negative charge of the molecule as it runs towards anode in the electrophoresis unit. This would probably explain the requirement of Mg^{+2} in DNA binding assays. Both, DNA as well as the polyketide being negatively charged would repel from each other and might not be directly able to come in contact unless a positively charged ion mediates to abridge them.

2x

CONCLUSION

The polyketide sourced from *S. flaviscleroticus*, after isolation and purification was found to be highly effective against gram positive bacteria. The bactericidal molecule could prove effective even at nanogram concentrations, over pathogenic isolates resistant to more than one antibiotic. The ability of molecule to act on non-dividing cells could be useful for opportunistic pathogens like *Mycobacterium* that colonizes human respiratory system and escape antibiotic treatment by staying under dormant conditions for long time. The DNA, but not RNA, binding nature of the molecule could make it a potential candidate for studying its role as an anticancer compound.

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