LIPIARMYCIN, A NEW ANTIBIOTIC FROM *ACTINOPLANES*

II. ISOLATION, CHEMICAL, BIOLOGICAL AND BIOCHEMICAL CHARACTERIZATION

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Lipiarmycin, a metabolite of *Actinoplanes deccanensis* nov. sp. (PARENTI *et al.*), has been isolated in pure form. It has a molecular formula $C_{52\sim54}H_{74\sim76}Cl_2O_{19}$, (M.W.= 1,073~1,099). From its chemical and physico-chemical characteristics, lipiarmycin can be considered a new antibiotic. Lipiarmycin is highly active against Gram-positive bacteria, including strains resistant to the medically important antibiotics and protects mice experimentally infected with *Streptococcus haemolyticus*. Lipiarmycin inhibits growth of susceptible bacteria by interfering with RNA synthesis.

Lipiarmycin is a metabolite produced by *Actinoplanes deccanensis* nov. sp. active *in vitro* against Gram-positive bacteria. The characteristics of the producing organism and the fermentation studies are reported in a companion paper.[1] In this paper the isolation and purification of the antibiotic together with its physico-chemical, biological and biochemical properties are described.

All the data hereafter reported indicate that lipiarmycin is a new antibiotic substance.

Isolation and Purification

Lipiarmycin is a weakly acidic substance that can be extracted with butanol from the whole culture broth at neutral pH. The solvent is washed with butanol-saturated water, concentrated to a small volume and poured in a large amount of light petroleum. The crude antibiotic obtained is 60 % pure as determined with a spectrophotometric assay and is purified by chromatography on a silica gel column eluted with a chloroform-methanol mixture (9:1). The fractions containing the antibiotic concentrated to a small volume give a white crystalline product with 90 % titer. Pure lipiarmycin is obtained by dissolving the purified product in the minimum amount of methanol, adding ethyl ether until a slight opacity is observed, warming the solution to 40°C and adding light petroleum. After 24 hours at 4°C the antibiotic crystallized out as white plates.

Physico-chemical Properties

Lipiarmycin obtained as described has melting point 173~175°C and specific rotation $[\alpha]_D^{25}$ — 5.5° (*c* 1, 98 % methanol). It is very soluble in methanol, ethanol, pyridine and aqueous sodium carbonate, fairly soluble in higher alcohols, chlorinated hydrocarbons, benzene and acetone, and insoluble in water, sodium bicarbonate and hexane.

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Table 1. Chromatographic behaviour of lipiarmycin.

Solvent system	Rf*
Water saturated *n*-butanol	0.95
Water saturated *n*-butanol+2% *p*-toluensulfonic acid	0.95
Water saturated *n*-butanol+2% concentrated ammonia	0.95
n-Butanol-saturated water	0.00
Ammonium chloride (20% in water)	0.00
n-Butanol - methanol - water (40:10:20) containing 0.75g methyl orange	0.95
n-Butanol - methanol - water (40:10:30)	0.95
Water - acetone (1:1)	0.80
Water saturated ethyl acetate	0.65
Chloroform - methanol (9:1) (TLC)**	0.35

* Paper chromatography on Whatman No. 1, antibiotic visualized on agar plates seaded with *S. aureus*.

** TLC performed on Silica gel HF/UV_{254} plates, spot detected under U.V. light and by spraying with conc. H_2SO_4 containing vanillin at 100°C.

The behaviour on paper and thin-layer chromatography of the pure compound is reported in Table 1. It gives positive TOLLENS, $FeCl_3$ and FOLIN-CIOCALTEU reactions and negative SCHIFF, MILLON and maltol reactions; a blue color is developed by addition of concentrated H_2SO_4.

Fig. 1. Ultraviolet absorption spectrum of lipiarmycin.

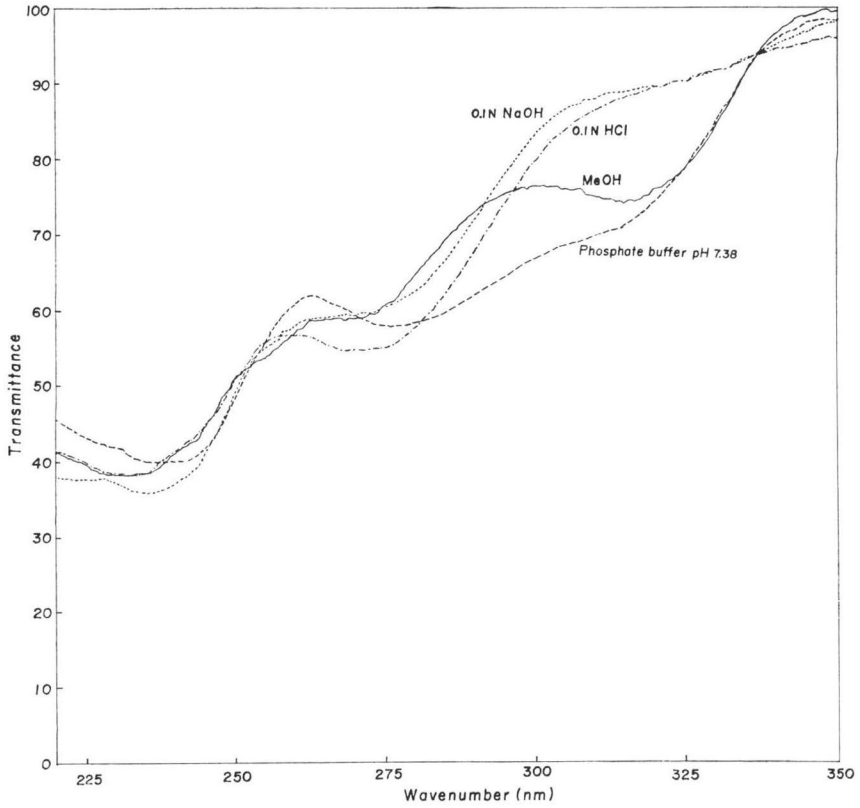


Fig. 2. Infrared absorption spectrum of lipiarmycin (nujol mull).

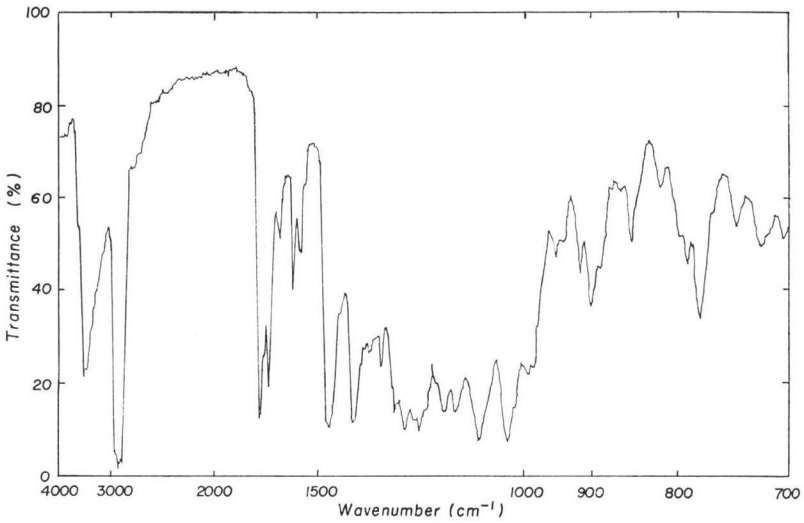
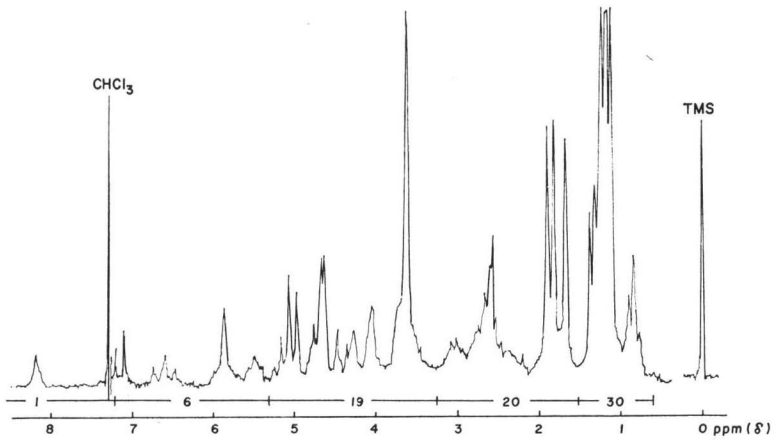


Fig. 3. Proton magnetic resonance spectrum.
Varian HA-100 (100 MHz) $CDCl_3$ solution



Lipiarmycin contains chlorine and microanalytical data give a formula $C_{52\sim54}H_{74\sim76}Cl_2O_{19}$; the exact molecular weight could not be determined because no molecular ion is visible in the mass spectrum. It is a monobasic acid with pKa 6.1. The equivalent weight 1,076, potentiometrically determined, is in good agreement with the weights calculated from the microanalytical data (M.W. 1,073~1,099).

Functional group analyses show the presence of ten C-methyl groups, two methoxy groups and seven hydroxyl functions. No acetyl groups are present in the molecule. By reaction with diazomethane lipiarmycin gives a dimethyl derivative thus showing that two of the seven hydroxyl functions present in the molecule have an acidic character; the positive $FeCl_3$ and FOLIN reactions indicate that at least one can be a phenolic hydroxyl.

The ultraviolet, infrared and proton resonance spectra of lipiarmycin are reported in Figs. 1, 2 and 3 respectively. The infrared spectrum shows the presence of a saturated carbonyl function (1730 cm^{-1}) and of at least two carbonyl groups conjugated to double bonds (1710,

1690 cm^{-1}). The ultraviolet maxima are in accordance with the presence of carbonyl functions conjugated to double-bond systems. The pmr spectrum, shows that the molecule contain many branched chains both saturated and unsaturated as shown by the signals of methyl groups on saturated (δ 0.7~1.5) and unsaturated (δ 1.8~2.0) carbons. The presence of signals of methyne and methylene groups α to oxygen, the high number of alcoholic hydroxyls and the positive TOLLENS reactions all suggest the presence of a sugar moeity. Aromatic hydrogens are absent; the signals in the region δ 6.6~7.2 have a coupling constant of 16 Hz and so are attributed to hydrogens trans to a conjugated double bond, if an aromatic nucleus is present, as suggested by the high value of double bond equivalents of the molecule, it has to be a fully substituted nucleus.

Homodichloro-orsellinic acid has in fact been isolated from lipiarmycin by hydrolysis. Details on the products obtained by acid and alkaline hydrolysis will be given elsewhere.

Biological Properties

Lipiarmycin is active against Gram-positive bacteria including strains resistant to commercially useful antibiotics. No activity was observed against Gram-negative bacteria or eukariot microorganisms (yeast, fungi, protozoa). The antibiotic is only slightly inhibited by serum. The minimal inhibitory concentrations (MIC) against a variety of microorganisms medically important are shown in Table 2. Lipiarmycin shows no cross-resistence with the following antibiotics: rifampin, penicillin, streptomycin, tetracycline, novobiocin, neomycin, erythromycin, chloramphenicol, cephaloridin, streptothricin, bacitracin and oleandomycin.

Lipiarmycin injected intraperitoneally into mice showed an approximate LD_{50} value of

Table 2. Activity of lipiarmycin in dilution test.

Test organism	Medium*	MIC (mcg/ml)
Staphylococcus aureus ATCC 6538	PS	2
S. aureus Tour	PS	2
S. aureus Tour+10 % bovine serum	PS	5
Streptococcus haemolyticus C203	BH+S	10
Diplococcus pneumoniae UC 41	BH+S	50
Streptococcus mutans ATCC 25175	BH	1.25
Streptococcus mutans ATCC 25175	J	0.75
S. mutans IB-1600	BH	0.65
S. mutans IB-1600	J	0.75
S. mutans 21-Typ.	BH	0.65
S. mutans 21-Typ.	J	1.50
S. mutans ATCC 27607	BH	10
S. mutans ATCC 27607	J	1.25
Mycobacterium tuberculosis $H_{37}R_v$	K	50
Mycoplasma gallisepticum LZB	PPLO	50
Escherichia coli SKF 12140	PS	>100
Candida albicans SKF 2270	PS	>100
Trichomonas vaginalis		>100

* PS=Difco Penassay; BH=Difco Brain-Heart; BH+S=Brain-Heart+Serum; J=JORDAN; K=KIRCHENER broth; PPLO=Difco PPLO.

Table 3. Effect of pH of assay medium on antimicrobial activity of lipiarmycin.

Test organism	MIC (mcg/ml)		
	pH 6	pH 7	pH 8.6
Bacillus megatherium	0.75	3.1	12.5
Bacillus subtilis	0.35	6.25	>25
Bacillus cereus	0.35	1.5	6.25
Bacillus sp. Schering/35	1.5	6.25	25
Corynebacterium simplex	0.35	6.25	>25
Corynebacterium equi	0.15	12.5	12.5
Corynebacterium sp. *chromogenes*	0.075	3.1	3.1
Flavobacterium deidrogenans	<0.015	0.35	6.25
Flavobacterium polyglutammicus	>25	>25	>25
Micrococcus glutammicus	<0.015	3.1	3.1
Micrococcus lysodeikticus	N.G.	0.035	1.5
Staphylococcus aureus	1.5	6.25	25
Sarcina lutea	<0.015	<0.015	0.75
Streptococcus faecalis	0.75	12.5	>25
Escherichia coli	>100	>100	>100

NG=no growth
Agar diffusion assay using a multipoint inoculator. Difco Pennassay medium. Growth 28°C overnight.

500 mg/kg. An approximate ED_{50} of 150~200 mg/kg was observed when the compound was administered subcutaneously to mice challenged with *Streptococcus haemolyticus*.

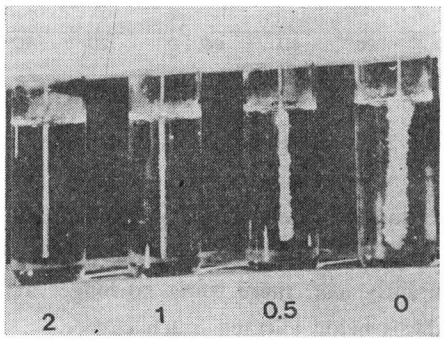
The effect of the pH of the assay medium on the MIC of lipiarmycin against a series of test organisms is shown in Table 3. Clearly at pH 6 it is 2~10 time more active than at pH 7 and 100 times more active than at pH 8.6.

To ascertain whether the pH effect is irreversible or not lipiarmycin (1 mg/ml) was incubated at different pH's for up to 72 hours and, at intervals, the MIC against *S. aureus* was determined at the fixed pH 6.7. No effect of the pH of incubation was observed for up to 48 hours.

The stability of lipiarmycin at different pH's suggests that the effect of the pH of the assay on the activity of lipiarmycin is reversible and so does not involve covalent bond cleavage.

Whether the pH dependency reflects a variation on the permeation of lipiarmycin or on its intrinsic activity is unknown at the moment. Likewise, unknown is the nature of the change in the molecule brought about by the variation in pH.

Plate 1. *Streptococcus mutans* is maintained in thioglicolate broth at 28°C without shaking. The plaque inhibition assay is performed in JORDAN medium supplemented with 5% (w/v) sucrose, sterilized separately. Sterile 20 gauge stainless steel wires incubated in 10 ml medium inoculated with 0.1 ml of an overnight culture in BH+2% glucose. Every day, for 4 days, the wires were transferred to fresh medium which was freshly inoculated.
The number below the test tubes refer to lipiarmycin concentration in mcg/ml.



Plaque Inhibition Tests

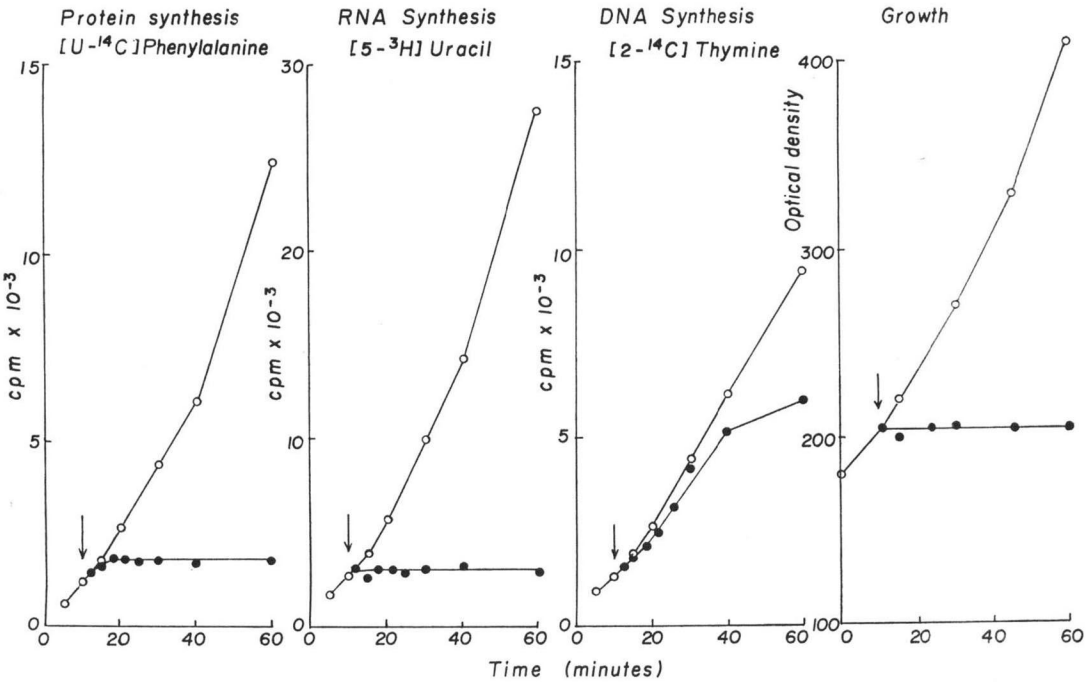
Lipiarmycin is particularly active against

strains of cariogenic *Streptococcus mutans* (Table 2) suggesting that it could be used as an antiplaque agent. Plate 1 shows the effect of lipiarmycin on plaque formation on stainless steel wires.[2] A complete inhibition was found at 1μg/ml equivalent to the MIC, substantial inhibition being observed, however, at half this concentration.

Mechanism of Action

The effect of lipiarmycin on macromolecular synthesis has been tested by adding the antibiotic to exponentially growing cultures of *Bacillus subtilis* containing appropriate radioactive precursors (Fig. 4). At a concentration ten times the MIC lipiarmycin gave a pattern of inhibition very

Fig. 4. Effect of lipiarmycin on macromolecular synthesis in *B. subtilis*.
Radioactively labelled precursors were added at zero time to cultures of *B. subtilis* thy$^-$ growing logarithmically in a defined medium. Antibiotic (1μg/ml) was added 10 minutes later and the incubation continued for a generation time (~48 min.). Incorporation of radioactivity into the cold 5% trichloroacetic acid insoluble fraction of cells was determined at suitable intervals before and after addition of lipiarmycin and also in a control culture containing no antibiotic. Growth was determined by measuring the absorbance at 575 nm in 1 cm cuvettes. Acid-insoluble material was collected on membrane filters and the radioactivity estimated by liquid scintillation counting. The radioactive precursors used were as follows: [U-^{14}C]phenylalanine for protein synthesis (0.66μCi/ml, 5μg/ml); [5-^{3}H] uracil for RNA synthesis (0.1μCi/ml, 5μg/ml); [2-^{14}C] thymidine for DNA synthesis (0.2μCi/ml, 10μg/ml).



similar to that obtained for known inhibitors of DNA dependent RNA polymerase *e.g.* rifampicin[3]. A rapid suppression of RNA synthesis (uracil incorporation) occurs, followed by an inhibition of protein synthesis (phenylalanine incorporation) and, finally, DNA synthesis (thymine incorporation) is effected after about one generation. At higher concentrations the specifity is apparently lost, there being no longer a lag in the inhibition of protein synthesis and DNA synthesis being affected much earlier.

Acknowledgments

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