A NEW ANTIBIOTIC X-5108 OF *STREPTOMYCES* ORIGIN
I. PRODUCTION, ISOLATION AND PROPERTIES

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A new antibiotic, designated X-5108, produced by a new species of micro-organism named *Streptomyces goldiniensis* var. *goldiniensis*, has been isolated in essentially pure but amorphous form. Assay methods, and cultivation and isolation procedures for the antibiotic are described. The antibiotic is active *in vitro* primarily against a number of gram–positive bacteria, whereas *in vivo* it is especially active against *Streptococcus pyogenes* infections of mice and as a poultry growth promotant. It shows a very low toxicity in mice with an LD_{50} of >1 g/kg sbc, and >4 g/kg p.o. Its empirical formula is $C_{44}H_{62}N_2O_{12}$. Structural studies reveal one methoxyl, one N–methyl and six C–methyl groups. UV, IR and NMR spectra and optical rotation of the pure antibiotic are given. The antibiotic appears to belong to a new chemical class.

∙In the course of our search for new antibiotics, a hitherto undescribed actino-mycete, designated X-5108 was isolated from a soil sample obtained in Bermuda. Shake flask cultures possessed substantial antibacterial activity against gram–positive bacteria *in vitro* after 4～6 days. Concentrates of partially purified solvent extracts made from broth filtrates exhibited oral antistreptococcal activity in infected mice. An isolation program was therefore undertaken to obtain pure antibiotic for further evaluations.

Results and Discussion

Culture and Assay Methods

The streptomycete was cultivated by inoculation of spore suspensions or cells in vegetative growth state into 100-ml portions of medium in 500-ml Erlenmeyer flasks, which were then incubated at 28°C on rotary shakers for 4～6 days. Media were adjusted to pH 6.5～7.5 prior to sterilization. On a larger scale, 100～5,000 gallon (400～20,000 liters) fermentors were employed.

Antibiotic activity was generally and most reliably followed by a microbiological agar diffusion cup-plate assay with *Bacillus* E or *B. megaterium* ATCC 8011 as test bacteria. The concentration of antibiotic required for either organism to produce an inhibition zone of about 20 mm in diameter was considered to be 1 unit per ml. Initially, a partially purified sample of the antibiotic complex served as a standard, with potencies of various fractions being computed from a dose-response curve. Eventually, the pure antibiotic was used as standard, and antibiotic yields are expressed in terms of mg of standard. The original unit corresponds to about 1.6 mcg of highly

purified antibiotic. Broths containing the cells were diluted for assay in M/15 potassium phosphate buffer, pH 6.0~6.5. A turbidimetric assay employing *Streptococcus faecalis* has also been developed. A UV chemical assay has been conveniently used for rapid estimation of antibiotic potencies in tank fermentors. Acidic butanol extracts of broth filtrates are suitably diluted, neutralized to pH 7.0 and read in a spectrophotometer at 325 nm, where the pure sodium salt of the antibiotic has an $E_{1cm}^{1\%}$ 435. Bioassay and UV values run fairly parallel for the first 4~5 days of fermentation, but then diverge.

The culture produces at least two other biologically active components in addition to the main antibiotic, with most of the antibacterial activity being found in the broth filtrate.

Thin–layer chromatographic methods have been found useful to qualitatively monitor the effects of changes in media and strains on the distribution of antibiotics produced and to analyze the final product. Due to the UV absorbing chromophores of the antibiotic complex, visualization of the spots could be achieved by the fluorescent indicator method. In these cases, chromatography was performed on silica gel F_{254} glass plates (E. Merck, Darmstadt), 0.25 mm thick, activated by overnight storage at 50°C. Plates are developed with a solvent containing chloroform, methanol and conc. ammonium hydroxide (80 : 20 : 1, v/v). Zones are then visualized under UV light and photographed. For bioautographic detection, silica gel F 254–kieselguhr foils (Brinkman) were used; their coating permitted contact with bioassay agar plates seeded with *Bacillus megaterium* without peeling. For bioautographic detection 5 mcg were usually spotted; to detect trace constituents, up to 50 mcg were often required. Rf values fluctuate considerably from day to day on both chromatography supports. The main antibiotic component on silica gel plates generally has an Rf=0.19, whereas on silica gel–kieselguhr the Rf is 0.29.

The Culture

Figs. 1 and 2 show photomicrographs of sporophores of the culture taken at

Fig. 1. Light microscope photograph of *Streptomyces goldiniensis* var. *goldiniensis*. Magnification : 1,000 × (1/1.5).
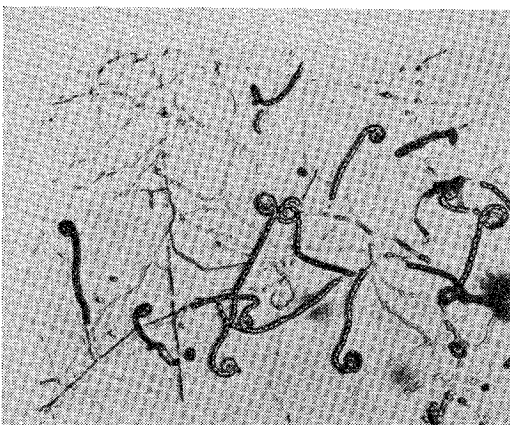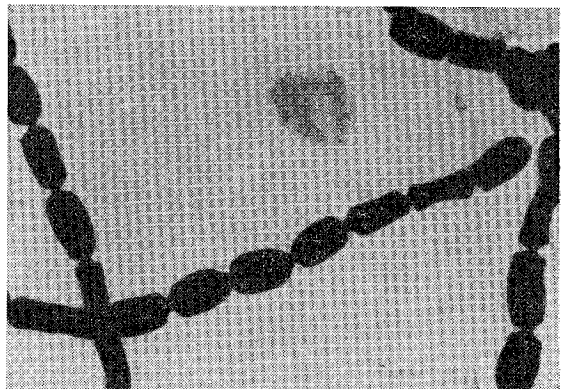

Fig. 2. Electron microscope photograph of spore chain of *Streptomyces goldiniensis* var. *goldiniensis*. Magnification 4,000 × (1/1.5). The culture was grown on tomato paste–peptone agar for 10 days at 28°C.

magnifications of 1,000 and 4,000. The culture produces a well-developed and branched substrate mycelium and characteristic aerial mycelium on many solid media. Its color characteristics place it in the Gray series of PRIDHAM. A detailed morphological and physiological study of the organism showed that it differs sufficiently from members of the same series to be considered a new species. It has been named *Streptomyces goldiniensis* var. *goldiniensis*, and deposited with the American Type Culture Collection as ATCC 21386.

The Fermentation

The organism grows well in shake flasks on a large variety of nutrient media, on many of which it produces the antibiotic in variable amounts. This is illustrated in the data of Table 1. It may be seen that a dozen complex nitrogen sources will support antibiotic potencies of 40~120 mg/liter. The effect on antibiotic yield of variations in concentrations of protein and carbohydrate sources is shown in Table 2. It may be seen that either tomato pomace solids or

Table 1. Effect of nutrient variation on antibiotic yield in shake flasks

All media contained 1 % dextrin, 0.1 % K_2HPO_4 and 0.1 % $CaCO_3$. One ml spore suspension strain 3191-13 used per 100 ml medium in 500-ml ERLENMEYER flasks: rotary New Brunswick shaker: Temp. 28°C ; 5 days incubation.

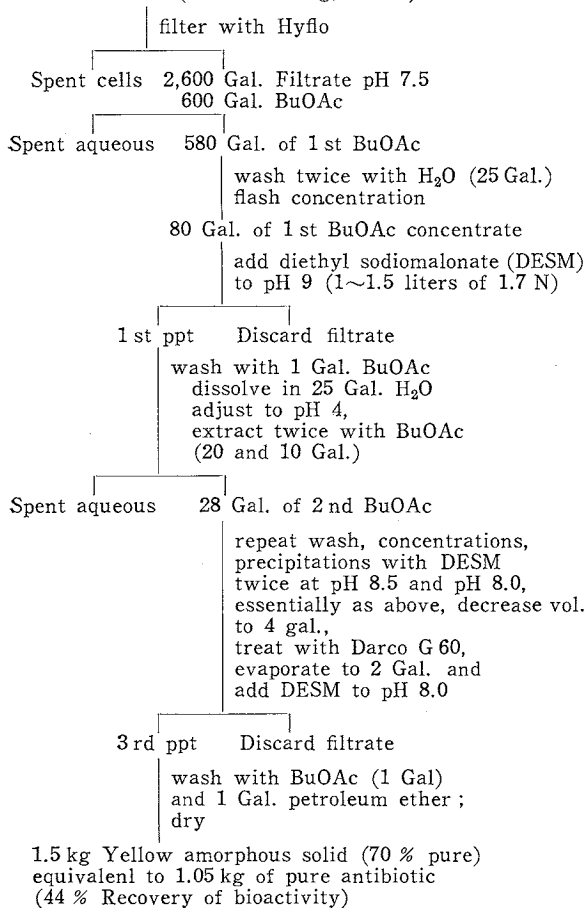| Complex N source added, at 1 % level	Antibiotic yield, in mg/liter
Corn distillers dried grain with solubles	120
Distillers dried solubles (Soludri)	82
Fermentation residues (BY-100)*	72
Tomato pomace solids	91
Protein coconut meal	80
Protein peanut meal	65
Cottonseed flour defatted (Proflo)	37
Soybean flour, defatted	48
Fishmeal	40
Milk sugar-albumin mix. (2 %)	80
Dried torula yeast	61
Cornsteep dried solids	42

* Commercial Solvents Corp.

Table 2. Effect of nutrient variations on antibiotic yield in flasks and tanks

Unless otherwise indicated, each medium contained 0.5 % $CaCO_3$ and 0.1 % of K_2HPO_4 ; Strain 3425-10 was used throughout ; 50~600 gal. tanks.

Item No.	Composition of medium, in %				Antibiotic yield, mg/liter	
	Tomato pomace solids	Distillers dried solubles	Corn-starch	Glucose	Flasks 7 days	Tanks (Duplicate runs) 6 days
1	1	1	1	0.5	240	160, 190
2	2	0	1	0.5	160	110, 74
3	0	2	1	0.5	150	140, 190
4	1	1	1.5	0	220	240, 200
5	1	1	0	1.5	290	200, 200
6	1	1	0	0	87	Not done
7	1 Cottonseed flour, 1 starch, 0.1 CaCO$_3$, 0.1 K$_2$HPO$_4$				150	160 (4 days)

distillers' dried solubles at 2 % level (items 2 and 3) permitted reasonably good yields, both in shake flasks and in tank fermentors, when fortified with corn-starch and glucose. Superior yields, however, were obtained with a mixture of the nitrogen sources plus 1.5 % of carbohydrate, supplied as single or mixed source from starch and glucose.

The antibiotic is also produced on a number of synthetic media but with considerably lower potencies.

Isolation of the Antibiotic

The antibiotic is a weak acid and has not been obtained crystalline. A number of methods for isolating and purifying the antibiotic as its sodium salt are available. In one of them, the antibiotic is isolated from the filtrate of harvested culture broth

Fig. 3. Isolation of antibiotic X-5108
3,000 Gal. Whole broth (6 days), 200 mg/liter
(Total : 2.28 kg, 100 %)

| filter with Hyflo

Spent cells   2,600 Gal. Filtrate pH 7.5
              600 Gal. BuOAc

Spent aqueous   580 Gal. of 1st BuOAc

| wash twice with H₂O (25 Gal.)
| flash concentration

80 Gal. of 1st BuOAc concentrate

| add diethyl sodiomalonate (DESM)
| to pH 9 (1~1.5 liters of 1.7 N)

1st ppt   Discard filtrate

| wash with 1 Gal. BuOAc
| dissolve in 25 Gal. H₂O
| adjust to pH 4,
| extract twice with BuOAc
| (20 and 10 Gal.)

Spent aqueous   28 Gal. of 2nd BuOAc

| repeat wash, concentrations,
| precipitations with DESM
| twice at pH 8.5 and pH 8.0,
| essentially as above, decrease vol.
| to 4 gal.,
| treat with Darco G 60,
| evaporate to 2 Gal. and
| add DESM to pH 8.0

3rd ppt   Discard filtrate

| wash with BuOAc (1 Gal)
| and 1 Gal. petroleum ether ;
| dry

1.5 kg Yellow amorphous solid (70 % pure)
equivalenl to 1.05 kg of pure antibiotic
(44 % Recovery of bioactivity)

Fig. 4. Countercurrent distribution graph
of antibiotic X-5108, Lot 230, mea-
sured by UV absorption at 325 nm.

Solvent system : ethyl acetate/2-propanol/
0.1 M aq. dibasic sodium phosphate solution,
12 : 9 : 20, v/v.

at pH 7.5 by solvent extraction, using butyl acetate. As may be seen from Fig. 3, repeating the cycle of extraction of the antibiotic into butyl acetate and anhydrous precipitation of the sodium salt with diethyl sodiomalonate reagent dissolved in butyl a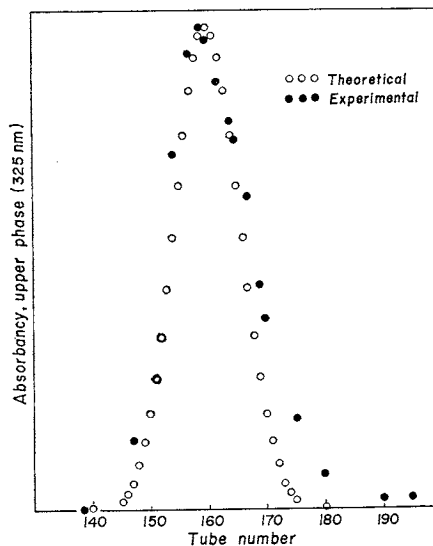cetate yielded a dry yellow amorphous solid, with an estimated 60~70 % purity, and an overall 44 % recovery of bioactivity.

Material of this purity could be further purified by countercurrent distribution, or by gel permeation chromatography in a non-aqueous system. Using a solvent system of water-acetone-methyl ethyl ketone-ligroin (60~90°) in a ratio of 3 : 9 : 2 : 6, in the absence of light and in a nitrogen atmosphere, up to 1,300 transfers were carried out by recycling in a 200-tube CRAIG distribution train. The distribution pattern, determined by UV absorption, showed the separation of a main peak, constituting ~70 % of the original weight, which deviated from the theoretical distribution curve. Evidence was obtained during the extended run that the antibiotic was slowly decomposing. Therefore, a second solvent mixture was used, consisting of ethyl acetate, isopropanol, 0.1 M aqueous disodium phosphate solution, 12 : 9 : 20, v/v. Here, the main component exhibited a distribution ratio of 3.88 (Fig. 4). It was isolated from tubes 150~170 in approximately 90~95 % purity after 200 transfers. Thin-layer chromatographic examination of the product in tubes 150~170 revealed the major component (Rf=0.19) with traces of impurities (Rf=0.23, 0.50, 0.68).

To achieve final purification of the antibiotic, we have used chromatography of the Na salt of the antibiotic on Sephadex LH–20. An ethanolic solution of 300 mg of a sample of the antibiotic, previously purified by counter–current distribution, was adjusted to pH 8~9 with sodium methoxide solution and applied to a Sephadex LH–20 column (290×41 mm), equilibrated with 3 A alcohol (ethanol–methanol, 100 : 5, v/v). The column was developed with 3 A alcohol, the major antibiotic zone emerging at an effluent volume of 950~1,200 ml. Later fractions contained a number of colored zones with negligible biological activity. The active fractions were pooled and evaporated to a small volume to which ether and petroleum ether were added to precipitate the antibiotic. The resulting yellow solids, the sodium salt of antibiotic X–5108, exhibited one major spot upon thin–layer chromatography (tlc), Rf = 0.19 (silica gel F–254, detection by UV light) and Rf = 0.29 (silica gel/kieselguhr, bioautographic detection).

Fig. 5 illustrates tlc and bioautographic analyses of the purest preparation, lot 306, in comparison with earlier cruder preparations. The presence of multiple active spots at various Rf values in crude lots is obvious, whereas fraction 306, estimated to be 90~95 % pure, appears as one–spot material. The free–acid form of the antibiotic was prepared by solvent extraction of an acidified aqueous solution of the sodium salt.

<div align="center">Characterization of the Purified Antibiotic</div>

The purified antibiotic, which is a yellow amorphous solid, gives the alkali metal

Fig. 5. Thin–layer chromatography of various preparations of antibiotic X–5108.
Layer : Silica-gel/Kieselguhr
System : Chloroform, methanol, conc. ammonium hydroxide, 80 : 20 : 1
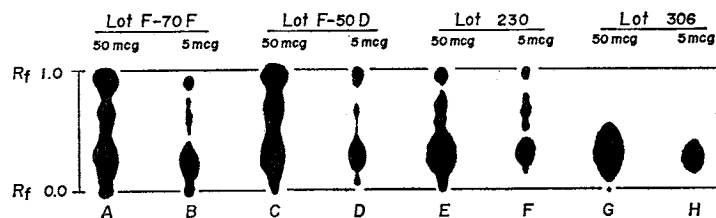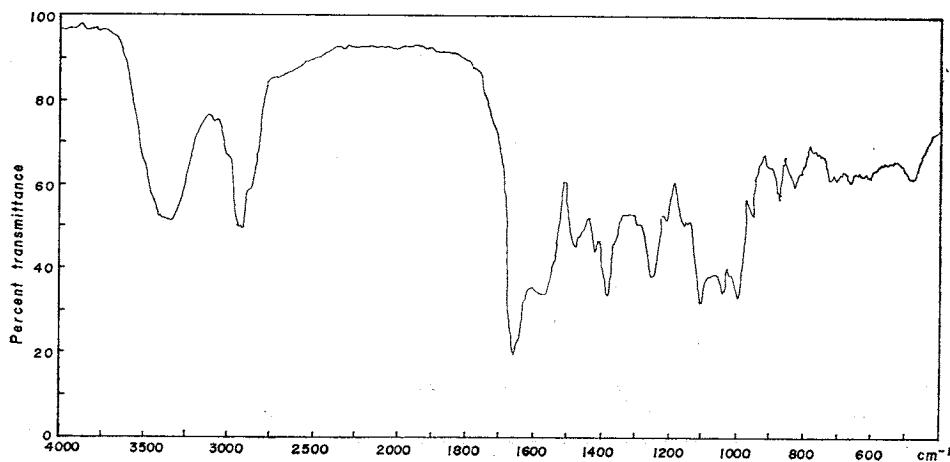Bioautography : *Bacillus megaterium*

Fig. 6. IR spectrum of antibiotic X–5108.

salts when reacted with aqueous sodium hydroxide and aqueous potassium hydroxide. The free-acid form is insoluble in water, but soluble in methanol, ethanol, 1- and 2-propanol, tert-butyl alcohol, ethyl acetate, amyl acetate, butyl acetate, chloroform, acetone and methylene chloride. The sodium salt of the antibiotic is very soluble in water, methanol, ethanol, isopropanol, butanol, N,N-dimethylformamide and dimethyl-sulfoxide; it is slightly soluble in amyl alcohol, tetrahydrofuran and dioxane, and insoluble in benzene, chloroform, ethyl ether and petroleum ether.

Stability studies have shown that the sodium salt is quite stable in the solid state, while the free acid is considerably less stable. Antibiotic X-5108 is unstable in both acidic and basic solutions, although it can be kept in aqueous solution at pH 7~9 for 4 hours at 25°C without significant loss of activity. Solutions kept at 25°C or heated to 56°C are two to ten times more stable in methanol than in water or pH 9 borate buffer. Whereas UV absorption at 325 nm was found to be a convenient and fairly reliable method to measure antibiotic production during a fermentation, it was a poor and unreliable criterion for
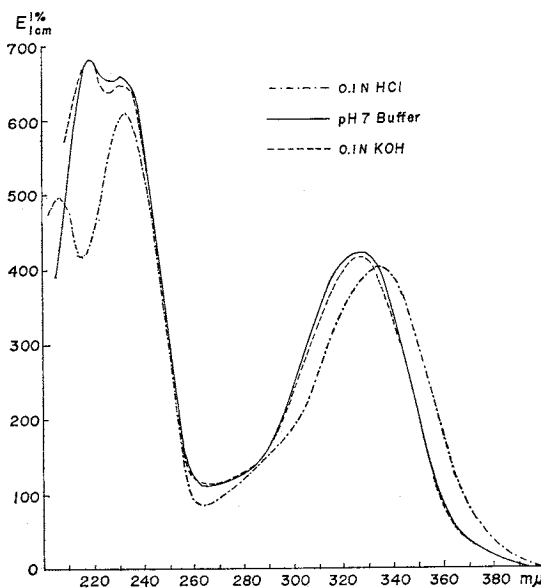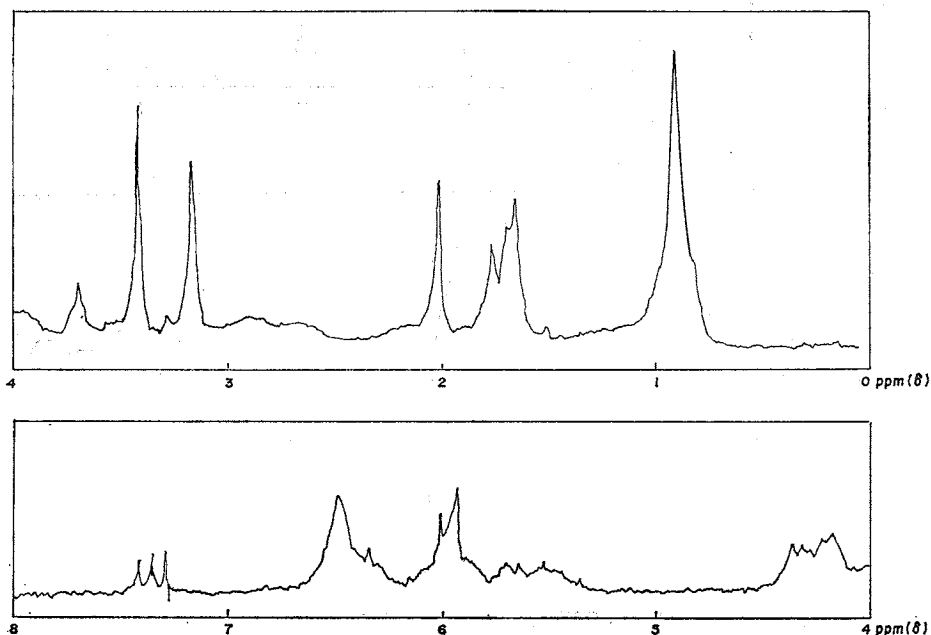
Fig. 7. UV spectrum of antibiotic X-5108.

Fig. 8. NMR spectrum of antibiotic X-5108.

measuring instability of antibiotic solutions, revealing, for example, only 20 % apparent destruction as against 60~95 % losses shown by bioassay.

The elemental analysis of the free antibiotic, C 63.63, H 7.81, N 3.48, O (by difference) 25.08, leads to a calculated empirical formula of $C_{44}H_{62}N_2O_{12} \cdot H_2O$, with a molecular weight of 810. From thermoosmotic measurements, the molecular weight is calculated to be 798 ± 12. A pKa of 6.1 was determined by UV spectra obtained at various pH values. The optical rotation of the sodium salt of the antibiotic is $[\alpha]_D^{25}$ $-82.8°$ (c 0.52, ethanol).

The infrared absorption spectrum of the free acid in KBr is shown in Fig. 6, UV spectra are shown in Fig. 7, and the nuclear magnetic resonance spectrum in Fig. 8. The latter was obtained on a Varian HA–100 spectrometer in $CDCl_3$ with tetramethylsilane as internal reference. The spectrum clearly reveals the complexity of the antibiotic.

In the absence of a suitable crystal for X-ray crystallographic studies, work is being carried out on structure elucidation using classical degradation methods. The antibiotic has been found to have one methoxyl–, one N–methyl– and six C–methyl groups. Catalytic hydrogenation indicated a hydrogen uptake of 6~7 moles with loss of the yellow color. The antibiotic gives a green color with methanolic HCl and a brown–red color with methanolic $FeCl_3$.

Studies in progress indicate that the antibiotic belongs to a new chemical class. More complete data bearing on chemical structure will be presented in a later publication.

Antimicrobial Activity in Vitro

In vitro, antibiotic X–5108 is active primarily against gram–positive bacteria; it is weakly active against some gram–negative bacteria, and inactive against yeasts and fungi. Table 3 presents a comparison with lincomycin, using an agar diffusion cup-plate assay. Both antibiotics are active mostly against gram–positive bacteria, showing the same general order of activity. Against *Bacillus megaterium* and *Streptomyces cellulosae*, antibiotic X–5108 is considerably more active than lincomycin, but the reverse is true against *Bacillus subtilis* and *Staphylococcus aureus*. A major difference between the antibiotics is the small but consistent activity of antibiotic X–5108 against five gram–negative bacteria. In other tests, antibiotic X–5108 was slightly more active than novobiocin or streptolydigin against 3 gram–positive bacteria, but only 1/17~ 1/50 as active against 3 other bacteria.

Table 3. Antimicrobial spectrum of antibiotic X-5108 and lincomycin

Test organism	Diameter of inhibition zone in mm, at 1 mg/ml	
	X-5108, Lot 306	Lincomycin
Bacillus megaterium	40.3	30.3
Streptomyces cellulosae	33.0	23.8
Bacillus subtilis	17.0	22
Staphylococcus aureus	15.8	21.8
Sarcina lutea	29.7	33.0
Bacillus E	37.2	35.3
Mycobacterium phlei	20.2	14.8
Escherichia coli	18.0	trace
BODENHEIMER's *Bacillus* PCI-3	21.7	0
Serratia sp. 101	16.7	10
Pseudomonas aeruginosa	15.0	trace
Aerobacter aerogenes	14.3	trace
Paecilomyces varioti, P. digitatum, S. cerevisiae, C. albicans	0	0

Table 4. Activity of antibiotic X-5108 in dilution tests

Test organism	MIC in mcg/ml
Bacillus megaterium	0.06
Bacillus E	0.5
Bacillus anthracis	0.6
Pneumococcus (2 species)	3~12
Streptococcus (2 species)	3~30
Staphylococcus (2 species)	60~250
Mycoplasma hominis	3~10
Mycoplasma laidlawii	100
Salmonella typhosa	200~500
Escherichia coli	200~500

Table 5. In vivo activities of antibiotic X-5108

Test	mg/kg	
	SC	PO
LD_{50} in mice	>1,000	>4,000
CD_{50}, Streptococcus pyogenes	71	82
Diplococcus pneumoniae	177	382
Klebsiella pneumoniae	395	> 500
Escherichia coli	> 500	> 500
Proteus vulgaris	> 500	> 500
Endamoeba histolytica (rat)	—	424

Promotes poultry growth when incorporated into diet at 50 p.p.m.

In broth dilution tests, illustrated in Table 4, antibiotic X-5108 produces a 50 % inhibition of growth against *Bacillus megaterium* at a 0.06 mcg/ml concentration. Against pathogenic strains of pneumococcus, streptococcus and staphylococcus, minimal active concentrations are 3~60 mcg/ml broth. Two mycoplasma species, *M. hominis* and *M. laidlawii*, are inhibited in broth dilution assays by 10~100 mcg/ml concentrations, respectively. Paper discs impregnated with 1 mcg of antibiotic produce substantial growth inhibition zones on agar plates of *Neisseria meningitidis* and *N. gonorrhoeae*.

For resistance studies, a culture of *Bacillus* E was made resistant in the laboratory to antibiotic X-5108 by repeated subculture in broth containing increasing amounts of the antibiotic. This resistant strain was still highly sensitive in cup–plate assays to novobiocin, streptolydigin, oleandomycin, streptomycin, erythromycin and penicillin G. Antibiotic X-5108 permitted a moderate degree of resistance (64-fold) to develop comparatively slowly in pneumococci and streptococci after 45 serial passages in broth. Using the 64-fold resistant pneumococcus strain, cross-resistance was *not* observed with penicillin G, oleandomycin, carbomycin, erythromycin, novobiocin, chlortetracycline, tetracycline, streptomycin, neomycin and chloramphenicol.

Activity *in Vivo*

When administered by both the oral and subcutaneous routes, antibiotic X-5108 shows good activity against *Streptococcus pyogenes* infections and a lesser effect against *Diplococcus pneumoniae* infections in mice. Against *Klebsiella pneumoniae*, it is slightly active when given subcutaneously but has no effect when given orally. It is otherwise without activity against the bacterial infections listed in Table 5 as well as many others which are not listed.

In protozoal infections, it shows slight activity orally against *Endamoeba histolytica* in rats. When injected subcutaneously, it has good activity against *Trichomonas vaginalis* in mice (89 mcg/ml) but is without effect when administered orally. It has no effect against helmintic, viral and fungal infections.

The sodium salt of antibiotic X-5108 substantially promotes the growth of chickens when incorporated into their diet at a level of 50 ppm. This result compares favorably with the activity of an equal level of procaine penicillin. More complete data on this subject will be presented in a later publication.