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STUDIES ON THE *STREPTOMYCES* SC_4 III. Biological Properties of Antibiotic SC_4-X[1,2]

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Abstract

SC_4-X, a novel antiliotic isolated from ***Streptomyces*** SC_4, inhibits the growth of a variety of Gram-positive, Gram-negative bacteria and plant pathogenic fungi. The antimicrobial activity of SC_4-X was influenced by pH of the test medium. SC_4-X is more stable in neutral and acidic than in alkaline conditions, but it is more active in alkaline than in acidic solutions. SC_4-X survived upon heating at 60°C for 10 hours and under UV irradiation for 24 hours. No degradation of SC_4-X occurred by incubating with homogenates of rat heart, lung and kidney at 37°C for 120 minutes. No cross resistance between SC_4-X, penicillin G and streptomycin was observed. The stepwise type of resistance development was similar to that of penicillin G. SC_4-X was therapeutically effective against experimental ***Staphylococcus aureus*** avian infection in chickens.

Key words: *Streptomyces*; new streptothricin antibiotic; antibiotic activity; biological property.

Introduction

The SC_4-X is a new member of streptothricin antibiotics produced by a novel strain of *Streptomyces*, designated as SC_4. Biological characters of this organism were reported by Wu (1984). Purification and chemical formulation of the SC_4-X was also described (Wu *et al.*, 1983). In this paper, the biological activities and properties of SC_4-X are reported.

Materials and Methods

Antibiotics

Antibiotic SC_4-X was prepared as described previously by Wu *et al.* (1983). Antibiotic SC_4-X was dissolved in phosphate buffer saline at pH 7 for *in vitro* and *in vivo* studies.

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The crystalline dihydrostreptomycin sulfate was purchased from Meiji Seika Kaisha, Japan. Penicillin G was purchased from Banyu Company, Japan.

Determination of Minimum Inhibitory Concentration

Bacteria were cultivated on Difco nutrient agar, trypticase soy agar, antibiotic medium 1 or nutrient broth. The human pathogenic fungi were cultivated on Difco sabouraud medium. The Czapek-Dox medium was used for culturing plant pathogenic fungi and true fungi. Antibiotic SC_4-X and other test agents were dissolved in sterile water for serial two fold dilution with initial concentration at 1,000 mcg/ml. Antibacterial and antifungal activities of the test antibiotics were assayed by the paper disc method. Aliquots of 5 μl solutions were applied on the paper discs, 6 mm in diameter, which were then placed on soft agar seeded with appropriate test organisms. Non-pathogenic bacteria were incubated at 30°C and human pathogenic bacteria were incubated at 37°C for 18–24 h. True fungi were incubated at 28°C for 4 to 5 days. The minimum inhibitory concentration (MIC) was determined as the lowest concentration at which the visible growth of the test organism was completely inhibited. The diameter of each inhibition zone was measured by a caliper.

Influence of Various Conditions on Antimicrobial Activities of SC_4-X

Effect of pH on activity: The MIC of SC_4-X against bacteria at different pH was determined by the paper disc method. The pH of hard agar medium and soft agar medium was adjusted to pH 6, 7, and 8 with HCl or NaOH before sterilization. Six different microorganisms, *Escherichia coli* NIHJ, *Bacillus subtilis* ATCC 6633, *Sarcina lutea* ATCC 9341, *Shigella flexneri* 3B, *Mycobacterium pseudotuberculosis* 607, and *Penicillium digitatum* were used as indicator organisms.

Effect of inoculum size: The MIC of SC_4-X against bacteria at different inoculum size was determined by the paper disc method on nutrient agar with inoculum sizes of 10^2, 10^4, 10^6, and 10^7 viable cells per ml.

Effect of serum: The effect of animal serum on the MIC of SC_4-X against bacteria was determined by paper disc method on trypticase soy agar plates containing 0%, 10%, 20% and 50% by volume of pig serum seeded with 10^6 viable cells per ml of *Staphylococcus aureus* avian or *Escherichia coli* NIHJ.

Stabilitly of SC_4-X in Various Solution at 37°C

SC_4-X was dissolved in phosphate buffer saline at pH 7, trypticase soy broth, human blood or human urine to give concentration of SC_4-X at 100 mcg/ml. Solutions were kept at 37°C for 5 days. The residual activity of SC_4-X in the solution was assayed daily by paper disc method.

Stability of SC_4-X in Phosphate Buffer at Various pH

Antibiotic SC_4-X was dissolved in phosphate buffer saline at pH 5, 7 and 9 to give concentration of 100 mcg/ml. Solutions were kept at 60°C for 52 h. SC_4-X activity was assayed by paper disc method every 4 h.

Thermostability Test

Antibiotic SC_4-X was dissolved in phosphate buffere saline at pH 7 to 100 mcg/ml and maintained at 60°C, 37°C and 10°C for 48 h in a water bath. At various time intervals till 48 h, 0.1 ml of each sample was withdrawn and assayed for antimicrobial activity by the paper disc method.

UV Stability Test

Antibiotic SC_4-X was dissolved in phosphate buffered saline at pH 7 to a final concentration of 100 mcg/ml and the solution was irradiated by a UV lamp at a distance of 10 cm with constant stirring. SC_4-X residual activity was measured every 60 minutes by paper disc method. Ultraviolet light was emitted from a mercury vapor quartz tube that centered at 2,537 Å with intensity of about 155 μ watts/cm^2 at 44.5 cm distance.

Stability of SC_4-X in Tissue Homogenates

To estimate the stability of SC_4-X in tissue homogenates, male Wister rat about 200 g body weight were used. Twenty percent tissue homogenates each of liver, kidney, lung, heart and small intestine were prepared in 67 mM phosphate buffered saline at pH 7. One ml of 1,000 mcg/ml of SC_4-X antibiotic solution was mixed with 9 ml of the above tissue homogenates, respectively. The mixture was incubated at 37°C for 30, 60, 90, or 120 minutes. Incubation was stopped by adding 2 ml of 99% ethanol to 1 ml of above reaction mixture. The residual activity of SC_4-X in the mixture was determined by the paper disc method.

Development of Resistance

Patterns of development of resistance in *Staphylococcus aureus* 209 p against SC_4-X, streptomycin and penicillin G was studied using Difco trypticase soy broth and trypticase soy agar plates. *Staphylococcus aureus* 209p was incubated in trypticase soy broth overnight and 0.1 ml of culture suspension at 10^7 vc/ml was plated on trypticase soy agar plates containing 1, 2, 4, ... 1,000 mcg per ml of antibiotics. After 48 h of incubation at 37°C, ten colonies on plates with the highest concentration of antibiotic were isolated and inoculated in trypticase soy broth at 37°C for 24 h. The suspensions were reinoculated on the next series of plates. Transfers were made every 48 h from the tube containing the highest concentration of

the antibiotic permitting growth into the next series of plates containing the same and several higher concentrations of antibiotics.

Bactericidal Activity of SC_4-X

The viability of *Staphylococcus aureus* 209p in the presence of SC_4-X was determined by the plate count technique. A 24 h broth culture of *Staphylococcus aureus* 209p was diluted 1,000 times in trypticase soy broth and the antibiotic was added to give concentrations of 1,000, 100, 10, 1.0, 0.1, and 0.01 mcg per ml. One ml of culture broth was withdrawn at intervals of 2, 4, 6 and 8 h from each tube prior to incubation at 37°C. Cells were washed with 10 ml of sterile normal saline three times to remove residual antibiotic from cells. Cells prepared as above were inoculated on trypticase soy agar plates. Colony counts were made after 48 h of incubation.

Cross Resistance

Cross resistance between SC_4-X and other antibiotics was studied with *Staphylococcus aureus* 209p. The bacteria were made resistant to SC_4-X, streptomycin and penicillin G respectively by spontaneous mutation and selection method. These mutants were cultivated on trypticase soy agar plates respectively. The MIC of SC_4-X, penicillin and streptomycin on the cell were then determined by paper disc methods.

Acute Toxicity in Mice

White male ICR mice, 20 grams weight, were used. Animals were divided into 10 groups, each with 10 mice, and injected subcutaneously with various doses of SC_4-X antibiotic. The 50 percent lethal dose (LD_{50}) was calculated from the survival rate of the mice within 7 days of injection.

Experimental Infection of Chieken

Staphylococcus aureus (avian type) was cultivated on trypticase soy agar slants for 20 h. Cells were then harvested and washed 3 times with saline and finally suspended in normal saline to make cell suspensions of 5×10^7 per ml. Each 0.2 ml cell suspension was injected subcutaneously into chickens. At the same time, each inoculated chicken was administered subcutaneously with various dose of SC_4-X antibiotic. The symptoms were observed by eyes.

Results

Antibacterial Spectrum

The antimicrobial spectrum and minimum inhibitory concentration of SC_4-X against bacteria, animal pathogenic bacteria and fungi are summarized in Table 1.

Table 1. *Minimal inhibitory concentrations of antibiotic SC_4-X*

Test organism	Medium	MIC in mcg/ml	Incubation time
Staphylococcus aureus ATCC 65389	Antibiotic medium 1	15.1	24 h at 37°C
Sarcina lutea ATCC 9341	Antibiotic medium 1	1.4	24 h at 37°C
Bacillus subtilis PCI 219	Antibiotic medium 1	15.6	24 h at 37°C
Bacillus subtilis ATCC 6633	Antibiotic medium 1	3.9	24 h at 37°C
Corynebacterium xeloses	Antibiotic medium 1	3.9	24 h at 37°C
Shigella flexneri 3B	Antibiotic medium 1	3.9	24 h at 37°C
Escherichia coli NIHJ	Antibiotic medium 1	1.95	24 h at 37°C
Proteus vulgaris	Antibiotic medium 1	100	24 h at 37°C
Pseudomonas aeruginosa	Antibiotic medium 1	100	24 h at 37°C
Klebsiella pneumoniae ATCC 10031	Antibiotic medium 1	100	24 h at 37°C
Mycobacterium pseudotuberculosis 607	Antibiotic medium 1 with 5% glycerin	3.9	5 days at 37°C
Escherichia coli swine	Trypticase soy agar +10% pig serum and +10% yeast extract	15.6	24 h at 37°C
Staphylococcus aureus avian	Trypticase soy agar +10% pig serum and +10% yeast extract	7.8	24 h at 37°C
Bordetella bronchiseptica	Trypticase soy agar +10% pig serum and +10% yeast extract	31.2	24 h at 37°C
Hemophilus pleuropneumonia	Trypticase soy agar +10% pig serum and +10% yeast extract	7.8	24 h at 37°C
Pasteurella hemolytica	Trypticase soy agar +10% pig serum and +10% yeast extract	62.5	24 h at 37°C
Aeromonas hydrophila	Trypticase soy agar +10% NaCl	62.5	24 h at 28°C
Vibrio anguillarum	Trypticase soy agar +10% NaCl	100	24 h at 28°C
Saccharomyces cerevisiae	Sabouraud agar	7.8	5 days at 30°C
Cryptococcus neoformans	Sabouraud agar	32	5 days at 37°C
Candida albicans	Sabouraud agar	32	5 days at 37°C
Aspergillus niger	Czapek-Dox agar	100	5 days at 28°C
Colletotricum musae	Czapek-Dox agar	100	5 days at 28°C
Colletotricum lageunarium	Czapek-Dox agar	31.2	5 days at 28°C
Fusarium moniliforus	Crapek-Dox agar	62.5	5 days at 28°C
Gibberella fujikuroi	Czapek-Dox agar	15.6	5 days at 28°C
Penicillium citrinum	Czapek-Dox agar	15.6	5 days at 28°C
Penicillium digitatum	Czapek-Dox agar	15.6	5 days at 28°C
Penicillium italicum wehmer	Czapek-Dox agar	31.2	5 days at 28°C
Gloeosporium papayae	Czapek-Dox agar	31.2	5 days at 28°C

Inoculum size: 10^6 vc/ml.
Method: paper disc method.

Antibiotic SC_4-X showed strong antimicrobial activity against several bacteria, such as *Sarcina lutea, Corynebacterium xelosis, Escherichia coli* NIHJ, *Mycobacterium pseudotuberculosis* 607, *Staphylococcus aureus* avian and some fungi such as *Saccharomyces cerevisiae, Gibberella fujikuroi* and *Penicillium citrinum etc.* On the other hand, SC_4-X showed poor antimicrobial activity against Gram-negative bacilli, such as *Proteus vulgaris, Pseudomonas aeruginosa* and *Klebsiella pneumoniae.*

Influence of Culture Conditions on Antibacterial Activity

The MIC of SC_4-X against various organisms under various conditions were observed.

Inoculum size: The activity of SC_4-X was obviously influenced by the inoculum size. Table 2 indicated clearly that the antibacterial activity increased as the inoculum size decreased.

Table 2. *Effect of inoculum size on antibacterial activity of SC_4-X*

Organism	MIC in mcg/ml			
	Inoculum size vc/ml			
	10^7	10^6	10^4	10^2
Salmonella paratyphi B.	7.75	3.9	1.95	1.95
Sarcina lutea ATCC 9341	31.25	15.5	1.95	1.95
E. coli NIHJ	15	7.75	7.75	1.95
Bacillus subtilis PIC 219	7.75	3.9	0.95	0.95

Medium: trypticase soy agar (Difco) at pH 7.
Method: paper disc method.
Incubation: at 37°C for 18 to 24 h.

pH effect: The results are shown in Table 3. In most of the organisms tested, the MIC of SC_4-X antibiotic at pH 6 were about ten times higer than that at pH 8.

Binding with serum protein: Effect of the addition of serum to the activity of SC_4-X is shown in Table 4. Antimicrobial activity against *Staphylococcus aureus* avian and *Escherichia coli* NIHJ was the same as the medium in the presence of pig serum up to 10%. This shows that the antibiotic SC_4-X does not severely bind with serum proteins.

Stability of SC_4-X Under Various Experimental Conditions

Figure 1a shows the residual activity of SC_4-X in various aqueous conditions at 37°C. SC_4-X was very stable in phosphated buffer at pH 7.0 and 37°C up to 5 days. About 50% SC_4-X activity in trypticase soy broth and 95% in human urine were inactivated after incubating at 37°C for 4 days.

Table 3. *Effect of medium pH on antimicrobial activity of SC_4-X*

Organism	Medium	M.I.C. in mcg/ml		
		pH 6	pH 7	pH 8
Escherichia coli NIHJ	A	31.2	7.8	2.0
Bacillus subtilis ATCC 6633	A	31.2	15.6	3.9
Sarcina lutea ATCC 9341	A	15.6	3.9	2.0
Shigella flexneri 3B	A	31.2	7.8	3.9
Mycobacterium pseudotuberculosis 607	NG	31.2	1	1
Penicillum digitatum	C	125	15.6	15.6

A: Difco antibiotic medium 1.
C: Czapek-Dox agar.
NG: Difco nutrient agar with 5% of glycerol.
Inoculum size: 5×10^6 vc/ml.
Paper disc method: Common bacteria were incubated at 37°C for 24 h. Mycobacterium was incubated at 37°C for 5 days. Penicillum was incubated at 28°C for 5 days.

Table 4. *Effect of pig serum concentration in medium on antibacterial activity of SC_4-X*

Organism	Serum (%)	MIC in mcg/ml
Staphylococcus aureus avian	0	7.75
	1	7.75
	10	7.75
	20	7.75
	50	7.75
Escherichia coli NIHJ	0	7.75
	1	7.75
	10	7.75
	20	7.75
	50	7.75

Inoculum size: 10^6 vc/ml.
Method: paper disc method.
Medium: nutrient agar.
Staphylococcus aureus (avian type) was isolated from poultry farm.

The residual activity of SC_4-X in phosphate buffer of different pH at 60°C was shown in Fig. 1b. Antibiotic SC_4-X was more stable in neutral and acidic than in alkaline conditions. The activity of SC_4-X in solution remained constant at pH 5 and 7 up to 12 h but diminished rapidly at pH 9.

The stability of SC_4-X at different temperatures is shown in Fig. 1c. SC_4-X

maintained high stability at 10°C and only 25% of activity was loss after heating at 60°C for 10 h.

As shown in Fig. 1d, SC_4-X was stable under UV irradiation. It indicates that the SC_4-X is insensitive to UV light.

As shown in Fig. 1e, no degradation of SC_4-X was occurred by incubating it with animal heart, lung and kidney homogenates for 120 minutes, while about 25% of SC_4-X activity was inactivatied in intestinal homogenate.

Cross Resistance

Cross resistance between SC_4-X, penicillin G and streptomycin was studied on *Staphylococcus aureus* 209p which was made resistant to Sc_4-X, penicillin G or streptomycin respectively by serial subculture in trypticase soy agar plates containing various concentrations of each antibiotic. Table 5 shows that SC_4-X maintoined similar activity against microorganisms, which was made resistant to other antibiotics and SC_4-X resistant organism was still sensitive to other antibiotics. It indicates that no cross resistant occurred among penicillin G, streptomycin and SC_4-X antibiotic.

Table 5. *Cross resistance test among SC_4-X, pencillin G and dihydrostreptomycin*

Organism	MIC		
	SC_4-X	Pe.	Strep.
Sta. aureus 209 P parent type	15.6	0.1	20
R-SC_4-X	30	0.1	20
R-penicillin G	15.6	31	20
R-streotomycin	15.6	0.1	500

Inoculum size: 10^6 vc/ml.
Method: paper disc method.
Medium: trypticase soy agar (Difco).

Development of Resistance

The development of resistance of *Staphylococcus aureus* 209p to SC_4-X, penicillin G and streptomycin was compared. The progression and degree of resistance to 3 kinds of antibiotics are shown in Fig. 2. For streptomycin resistance, the MIC of streptomycin increased from 50 mcg/ml to 500 mcg/ml needs only one transfer and was not affected by further transfer. Patterns of gradual development of resistance against SC_4-X and penicillin G are similar. But resistant against penicillin G was more rapidly developed than those against SC_4-X.

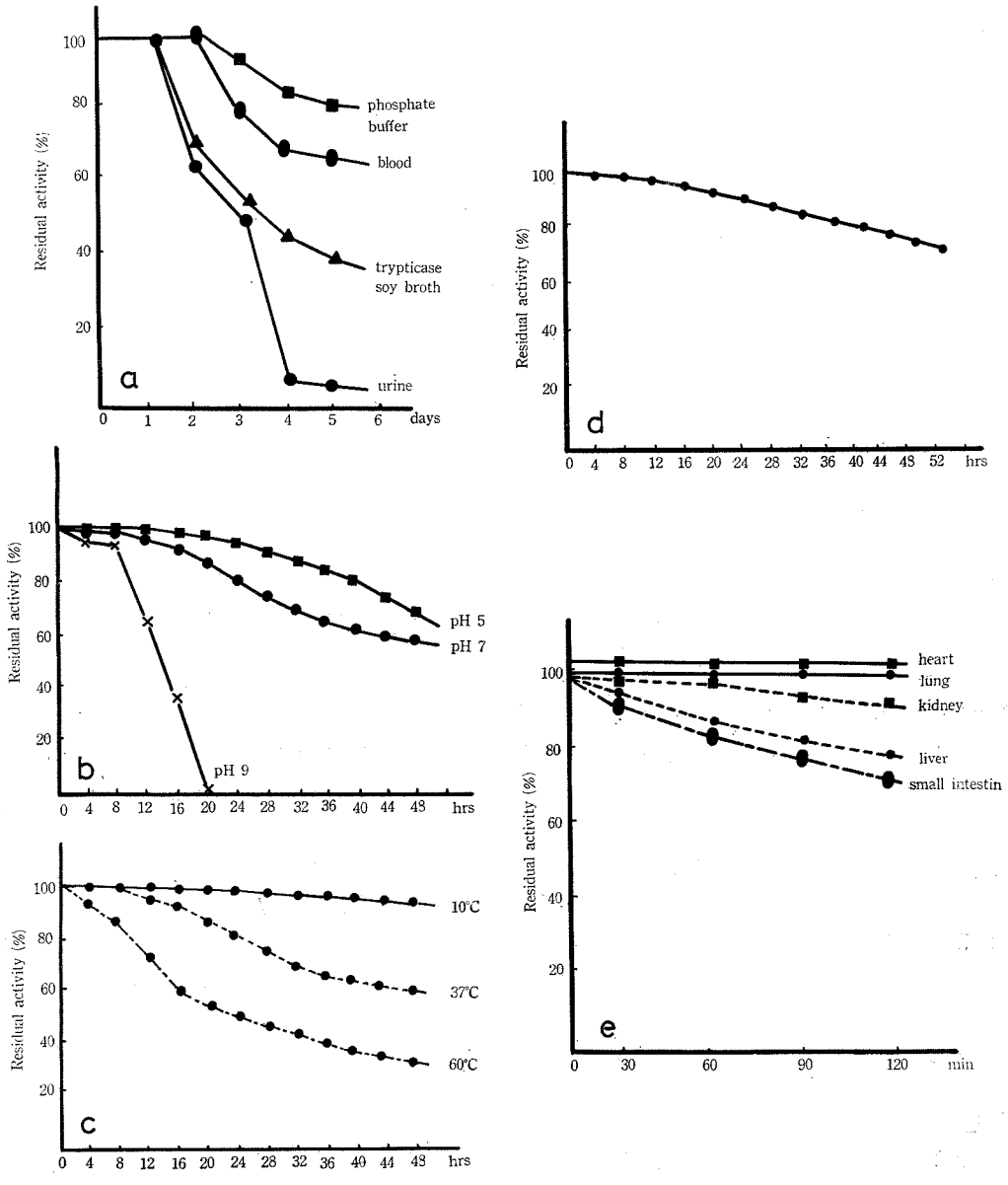

Fig. 1. Stability of SC_4-X under various experimental conditions.
(1a) Residual activity of SC_4-X in various aqueous conditions at 37°C.
(1b) Residual activity of SC_4-X in phosphate buffer of different pH at 60°C.
(1c) Residual activity of SC_4-X at different temperatures. The antibiotic SC_4-X was dissolved in phophate buffer at pH. 7.0.
(1d) Sensitivity of SC_4-X to UV illumination.
(1e) Residual activity of SC_4-X in animal tissue homogenates.

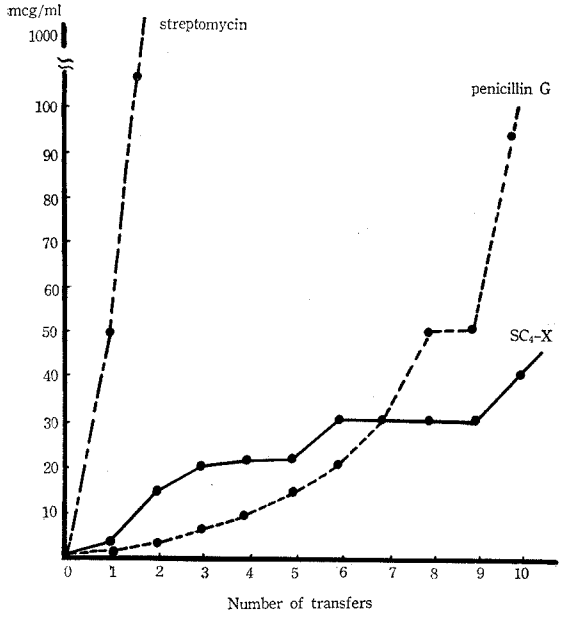

Fig. 2. Patterns of development of resistance of *Staphylococcus aureus* 209 P to SC_4-X, stretomycin and penicillin G.

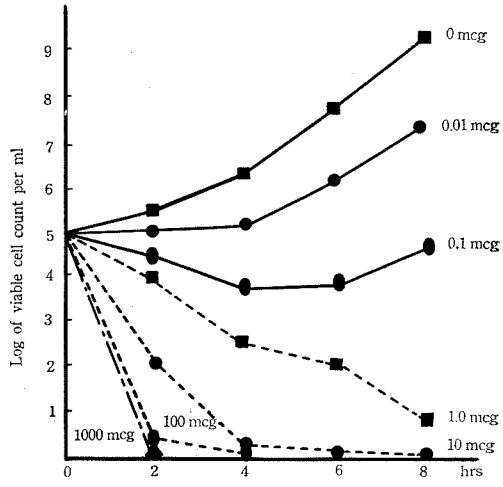

Fig. 3. Bactericidal effects of SC_4-X against *Staphylococcus aureus* 209 P.

Bactericidal Activity

The viability of *Staphylococcus aureus* 209p cultivated in trypticase soy broth containing various concentrations of SC_4-X was determined by plate count, as illustrated in Fig. 3. The logarithm of viable cell counts were plotted against exposure time to antibiotic. When the antibiotic was added simultaneously, weak bacteriocidal action was observed at concentration of 10 mcg/ml. At concentration of 1 mcg/ml, the viable cell counts did not change through a period of 8 h. The increase of viable cell count at the concentration of 0.01 mcg/ml of SC_4-X was only slightly less than that in control. These results reveal that SC_4-X is rather bacteriostatic than bacteriocidal.

LD_{50} Value

At a dosage of 250 mcg/g body weight, SC_4-X was not lethal to mice, but animals were severely ill even on the 5th day after injection. All mice received 300 mcg/g body weight died within 5 days. So, the LD_{50} value for SC_4-X to mice ICR was 275 mcg/g body weight.

Experimental Infection of Chicken

Antibiotic SC_4-X was simultaneously administered to chicken and challenged intramuscularly with the strain of *Staphylococcus aureus* avian type. Positive effect

was obtained with a dose of 10 mcg/g body weight of SC_4-X in the chicken, exhibiting significantly preventive effect. SC_4-X was not harmful to chicken at concentration of 50 mcg/g body weight.

Discussion

Since streptothricin F was discovered by Waksman and Woodruff in 1942, investigation of streptothricins have been processed rather slowly. After prolonged investigations by American, British and Japanese scientists, the structures of five first ninhydrin-positive compounds isolated from the total and partial hydralysates of streptothricins were established by Khokhlov *et al.* in 1972. Accordingly, all streptothricins and streptothricin-like antibiotics are active against a wide spectrum of Gram-positive, Gram-negative bacteria and fungi, but are highly nephrotoxic to animals (Khokhlov *et al.*, 1961), which limited their practical use in medicine. Their application in agriculture seems more promising both for suppression of some phytopathogenic microorganisms and as supplement into animal feeds.

In our continuous search for antibiotics of anti-fungi and animal feed additive, SC_4-X was isolated from *Streptomyces* SC_4 as a new member of water-soluble streptothricin-type antibiotics. *In vitro* tests show that SC_4-X is active against common bacteria, phytopathogenic fungi and animal pathogenic bacteria.

Although many antibiotics are available for the treatment of infectious disease in livestock and poultry, prolonged treatment causes resistance. We are particularly aware of infection of *Staphylococcus* in chicken in Taiwan, and its gradual development of resistance that causes penicillin less effective. SC_4-X is effective against *Staphylococcus aureus* in chicken and have the potiential to treat *Staphylococcus aureus* infection in poultry. SC_4-X is reasonably stable at elevated temperature, under UV irradiation and in incubation with animal tissue homogenates. Although SC_4-X is more stable under acidic conditions it is more active as its free base form in alkaline solution. This trend of stability is advantageous for SC_4-X in its practical application. Animals normally can maintain quite well under neutral or slightly alkaline physiological conditions to give SC_4-X long acting period. SC_4-X is less likely to develop resistance than streptomycin from *Staphylococcus aureus* 209p. The stepwise type of resistance development in *Staphylococcus* against SC_4-X is characteristic and no cross resistance between SC_4-X, penicillin G and streptomycin were observed in our studies. From these points of view, SC_4-X has great potential to develop as antibiotic for animal use.

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鏈黴菌 SC_4 之研究

III. SC_4-X 抗生素之生物特性

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鏈黴菌 SC_4 是由臺灣土壤中分離而得之新抗生素產生菌。SC_4-X 抗生素可將 SC_4 菌培養液，經離子交換及層柱分析法予以純化。SC_4-X 抗生素抗菌譜甚廣，對革蘭氏陽性菌，陰性菌及植物病原性眞菌等具有生長抑制的能力。SC_4-X 之活性受培養基之酸鹼度所影響，其活性在鹼性下較酸性中爲强，但安定性則反之，在酸性下之抗生素較在鹼性中爲安定。高溫處理及紫外光之照射均不影響 SC_4-X 抗生素之活性。SC_4-X 不受白老鼠之心臟，肝臟及腎臟磨碎液破壞。SC_4-X與盤尼西林G及鏈黴素之間無交叉抗藥性。葡萄球菌對 SC_4-X 之抗藥性與盤尼西林類似，須經數次之接移才能產生。SC_4-X 抗生素對鷄葡萄球菌的感染預防及治療有良好的藥效。