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STUDIES ON THE STREPTOMYCES SC_4 :
Chemical Formulation of Antibiotic SC_4-X

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Abstract

A new species of *streptomyces*, namely *streptomyces* SC_4, was isolated from a soil sample collected in Taiwan. An antibiotic, SC_4-X, was isolated and purified as its amorphous sulfate salt from *streptomyces* SC_4. It is active against Gram-positive and Gram-negative bacteria and fungi. This antibiotic is tentatively considered to be a new member of the streptothricin type antibiotics, which contain three molecules of β-lysine, one molecule of aminosugar, and one molecule of streptolidine. The difference in structures between SC_4-X and those other antibiotics is the extents of aminosugar modifications. The purification and elucidation of chemical structure of this basic and water soluble antibiotic is reported.

Introduction

Streptothricins, first isolated by Waksman and Woodruff in 1942, are basic and water soluble antibiotics produced by *Streptomyces lavendulae*. Since then a number of related antibiotics have been identified, including yazumycin by Akasak *et al.* in 1968 and Taniyama *et al.* in 1971, streptothricin S15-1 by Brown *et al.* in 1977 and Kawamura *et al.* in 1976, streptolidine by Carter *et al.* in 1961, roseonine by Goto *et al.* in 1974, sclerothricin by Kono *et al.* in 1969, streptolin by Larson *et al.* in 1953 and Van Tamelen *et al.* in 1952, roseothricin by Nakanishi *et al.* in 1954, streptidine by Peck *et al.* in 1946, streptothricin R4H and racemomycins by Sawada *et al.* in 1974, glycinothricin by Sawada *et al.* in 1977, sclerothricin by Shimazu *et al.* in 1969, citromycin by Taniyama *et al.* in 1972, SF-701 by Tsuruoka *et al.* in 1968, pleocidin, mycothricin, grasseriomycin, grisin, phytobacteriomycin and polymycin by Khokhlov in 1961.

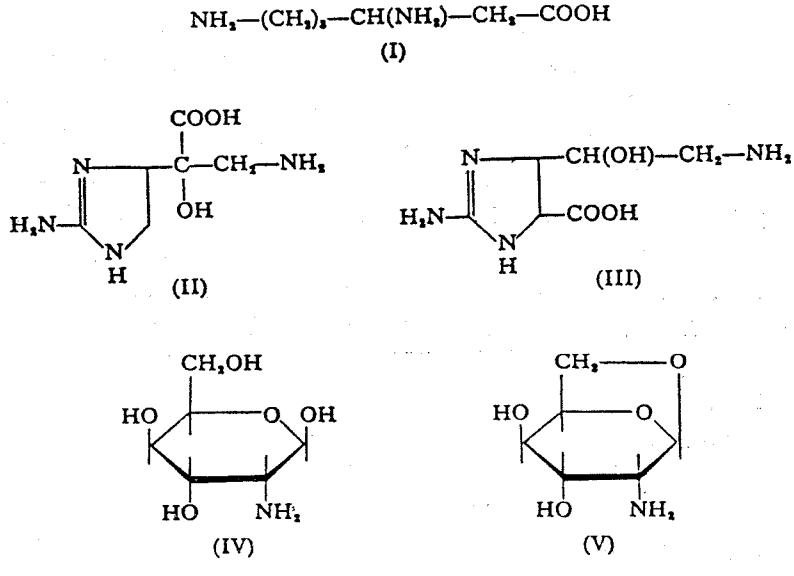
All streptothricins are active against a wide spectrum of Gram positive and Gram negative bacteria and fungi, but are highly nephrotoxic. Most streptothricins were isolated by absorption and solvent extraction. Although it has always been difficult to obtained crystalline compound in free form,

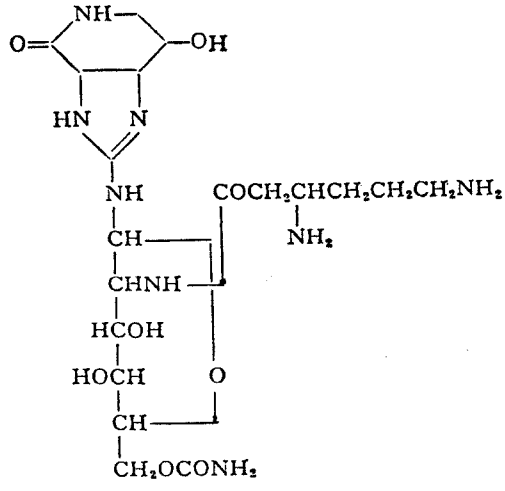
crystalline derivatives have been reported as the reineckate by Fried *et al.* in 1945 and helianthate derivatives by kuehl *et al.* in 1945. Streptothricin picrate (Peck *et al.* in 1946) and sulphate (Carter *et al.* in 1954) have been also used in some isolation procedures.

Streptothricin type antibiotics are basic substances, soluble in water and dilute solutions of acid and alcohol, and decompose in concentrated acid and alkali. They are insoluble in ether, petroleum ether and chloroform. In the crude preparation, they are sensitive to heat, but withstand heating at 100°C for 15 min after purification. Treatment with proteolytic enzymes does not affect their biological activities.

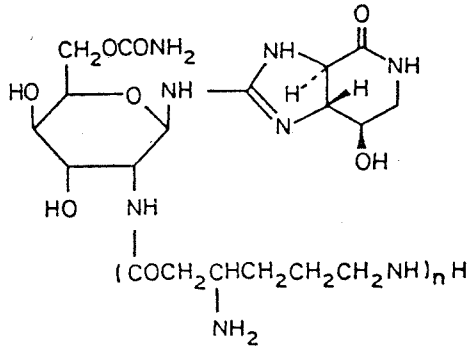
Acid hydrolysis of streptothricin produced three compounds, which upon chromatography on a cellulose column with the solvent system *t*-butanol-acetic acid-water (2:1:1, v/v) were separated and identified as L-β-lysine (I) (Carter, Clark *et al.* in 1961), a cyclic guanidine derivative with the structure 2-amino-imidazoline (II), corrected by Carter and McNary as (III), and amino sugar with the structure 2-amino-2-deoxy-α-D-gulose (D-2-gulosamine) (IV) (Van Tameleon *et al.*) and its 1,6-anhydro-derivative (V) (Johnson and Westley in 1692). Complete structure of streptothricin (VI) was established by Van Tamelen *et al.* in 1961.

More affirmative evidence for the proposed formulae of streptothricins E-A was given by Khokhlov and Shutova. They isolated and tried to identify the products after mild hydrolysis of each streptothricin (E-A). All amide bonds in these compounds were shown to link through ε-amino group of β-lysine residues, whereas their β-amino groups remain free. Uncertainty



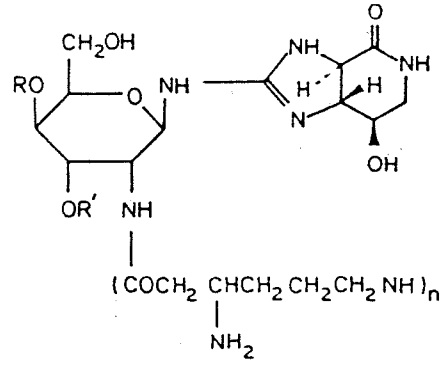


(VI)



E : n = 2
D : n = 3
C : n = 4
B : n = 5
A : n = 6

(VII)



R, R′ = H, $CONH_2$
n = 2,3,4,5,6

(VIIa)

remains that formulae (VII) represent the structures of all streptothricins, there is still no conclusive evidence to confirm that the location of carbamoyl group is at the C-6 carbon of the gulosamine moiety in streptothricin F (F:n=1). It is equally possible that in streptothricin E-A the carbamoyl group is located at C-3 or C-4 of gulosamine. The structures of all streptothricin E-A may therefore be designated by the general formulae (VIIa).

Streptomyces-SC_4 has been isolated from a soil sample collected in Kaohsiung, Taiwan. This organism is capable of producing a series of basic and water soluble antibiotics which are strongly active against the growth of Gram-positive, Gram-negative bacteria and true fungi. A new antibiotic, designated as SC_4-X, has been obtained from the fermentation broth of *streptomyces*-SC_4. This work has been undertaken to compare the antibiotic properties and purification procedures of the aminosugar type antibiotic SC_4-X with the known streptothricin type antibiotics. The physico-chemical characteristics and chemical formulation of SC_4-X are also discussed.

Materials and Methods

Strain

The strain *Streptomyces* SC_4 was isolated from a soil sample collected at Kaohsiung, Taiwan. According to Bergey's key, it was classified into the genus of *Streptomyces*. The organism was kept in the lyphilized form. Slants of stock cultures were maintained on trypton yeast extract glucose (TYG) agar. These slants as well as subsequent cultures used in this study were routinely incubated at 28°C for 7 days before harvest.

Determination of the antimicrobial spectrum

Test bacteria were cultured on the Difco antibiotics medium 1. Human pathogenic fungi were cultured on Difco Sabouraud agar. The Czapek's-Dox medium was used for plants pathogenic fungi. Antibacterial and antifungal activities of the antibiotics were measured by the paper disc method.

Antimicrobial assay

Bacillus subtilis PCI 219 was used as the indicator bacteria. A series of two-fold diluted antibiotic samples were prepared and each aliquot of 0.005 ml of the test solutions was applied on paper discs (0.6 cm diameter) which were then placed on agar plates seeded with appropriate organisms.

Fermentative production

The composition of the sporulative media included 15 ml glycerol, 10 g asparagine, 5 g tyrosine, 0.5 g K_2HPO_4, 0.5 g $MgSO_4$, 0.01 g $FeSO_4$, 0.5 g NaCl and 15 g agar in 1,000 ml of deionized water. The pH was adjusted to 7.2

with 1 N NaOH before autoclaving. The composition of the seed media contained 5 g tryptone, 3 g yeast extract, 10 g glucose, 1 g K_2HPO_4, 1 g KH_2PO_4 and 15 g agar in 1,000 ml of deionized water. The fermentation media contained 15 ml glycerol, 1 g asparagine, 0.5 g tyrosine, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 5H_2O$ and 0.5 g NaCl in 1,000 ml of deionized water. Each spore suspension slant was inoculated into five 250 ml Erlenmeyer flasks each containing 30 ml of the seed media. After 3 days incubation in a rotary shaker at 28°C, the inoculum (10% by volume) was transferred to 2,000 ml Erlenmeyer flasks each containing 1,000 ml seed medium. After 3 days incubation at 28°C, the inoculum (also 10% by volume) was transferred to a 15-liter jar fermentor containing 12 liters of medium. Fermentation was carried out at 28°C for 7 days under aeration of 20 liters/min and agitation at 200 rpm. During the fermentation period, 50 ml of the cultured fluid was drawn daily for the assay of antibiotic activities. The rate of growth was determined by measuring the volume of mycelia in the broth after centrifugation at 3,000 rpm for 10 minutes. The antibiotic activity of the beer supernatant was measured with *Bacillus subtilis* PCI 219 as the test organism.

Isolation and purification of SC_4-antibiotic

Fermentation broth (24 liters), harvested from two 15-liters fermentors, was adjusted to pH 2.0 with 6 N hydrochloric acid, stirred for 30 minutes and again adjusted to pH 6.8 with 1 N sodium hydroxide before filtration through Whatman No 1 filter paper. The filtrate was passed through Amberlite IRC 50 (H-form) column. After washing with sufficient amount of deionized water, the contents was subsequently eluted with 6 liters of 0.3 N hydrochloric acid or 10% (w/v) NaCl solutions.

The eluate was adjusted to pH 2.0 and decolorized by passing through a column containing active carbon and eluted with 70% methanol at pH 2.0. Fractions containing SC_4-X were pooled and concentrated in vacuo. The concentrated solution was added with five volumes of methanol and the biologically inactive precipitates were removed through filtration. The filtrate was concentrated in vacuo and added into it twenty volumes of ehtanol-acetone mixture (1:5, v/v), yielding brown powdery precipitates containing SC_4-X antibiotic. The crude antibiotic was further purified through a cellulose column (Whatman CF II) by eluting with a solvent system of *n*-propanol-pyridine-acetic acid-water (15:10:3:12, v/v). Active fractions were combined and the solvent evaporated in vacuo to dryness. Then the crude preparation was dissolved in water, passed through a column of Dowex-1 (OH-form), then adjusted the pH value of the solution to 3.0 with 2N

sulfuric acid, and finally added with twenty volumes of ethanol to precipitate SC_4-X. After filtration and drying in a desiccator, 500 mg of partially purified SC_4-Xwas obtained.

Purification

One gram of the partially purified SC_4-antibiotics were applied on a column of Sephadex G-25 (2.5×90 cm) and eluted with deionized water. The active eluate was separated into two major fractions, designated as SC_4-I and SC_4-II. Pike SC_4-I and SC_4-II were concentrated individually in vacuo, and treated with 20 volumes of ethanol at pH 3.0 with 2N H_2SO_4. The later fraction, SC_4-II, was chromatographed again on a carboxymethyl cellulose Whatman CM 32 column (2.5×90 cm) and eluted with a 0 to 0.4 M linear gradiant of sodium chloride. The antibiotic SC_4-II was further separated into two active fractions at this step. The first fraction was designated as antibiotic SC_4-X, and the second fraction was designated as antibiotic SC_4-Y. Each active fraction was concentrated in vacuo and treated with charcoal for desalting. The antibiotics SC_4-X and SC_4-Y were eluted with 70% methanol from charcoal at pH 2.0. The purified SC_4-X and SC_4-Y antibiotics were confirmed as a clear single spot with ninhydrin in several silica gel TLC systems which included *n*-propanol-pyridine-acetic acid-water (15:10:3:12, v/v), wet-BuOH with 2% of *p*-toluene sulfonic acid, *n*-BuOH-acetic acid-water (2:1:1, v/v) and *n*-BuOH-pyridine-acetic acid-water-*t*-BuOH (15:10:3:15:4, v/v).

Proton and C-13 NMR spectra were obtained on a Joel 100 MHz spectometer. All NMR spectra were recorded at ambient temperature in D_2O and are reported as parts per million downfield from Me_4Si ($\delta=0$). Routinely DSS is used as an internal standard. Infrared spectra were taken by using a Perkin-Elmer 283 B spectrophotometer as KBr pellets.

Analytical HPLC was performed by a Waters Model 6,000 A Pump equipped with Model 401 differential refractometer detector. Samples were introduced into a μ-Bondapak-C_{18} Column through a U-6K injector. Conditions for separation of SC_4-X and A249 were specified in the results section.

Amino acid analysis was carried out by Dr. W.C. Chang, Institute of Biological Chemistry, Academia Sinica. Hydrolysate of standard protein was run for comparison.

Results

General properties of SC_4-X

Antibiotic SC_4-X sulfate salt is a white amorphous powder which melts at 180–185°C with decomposition. It is quite stable in acidic solution but unstable in strong alkaline solution. It is hydrophilic, readily soluble in

water, slightly soluble in methanol and ethanol but insoluble in most of the common organic solvents. Ultraviolet absorption spectroscope shows no characteristic band above 220 nm. Its basic nature was indicated by paper electrophoresis. The antibiotic shows positive response to ninhydrin and Molish reaction but is negative to Benedict, Bials, Biuret, and Sakaguchi reactions.

Biological activities of SC_4-X

Table 1. *Antibacterial activity of SC_4-X antibiotic under different pH condition*

Test organisms	MIC (mcg/ml)		
	pH 4.8	pH 7.0	pH 8.2
Escherichia coli ATCC-10536	125	64	32
Bacillus subtilis ATCC-6633	64	32	16
Staphylococcus aureus ATCC-9144	125	4	1
Sarcina lutea ATCC-9341	64	4	1
Micrococcus flavus ATCC-10240	32	16	4
Klebsiella pneumoniae ATCC-10031	500	16	16
Mycobacterium pseudotuberculosis ATCC-607	64	1	1

Paper disc method: 0.005 ml of the test solutions were applied on Whatman No 1 filter paper disc, 0.6 cm diameter.
Common bacteria: Antibiotics medium 1. 37°C for 24 hours.
Mycobacterium: 5% glycerin nutrient agar, 28°C for 7 days.

Table 2. *Antifungal activity of SC_4-X antibiotic*

Test organisms	Medium	MIC (mcg/ml)
		pH 7.0
Penicillium citrinum	C. A.	64
Helminthosporium oryzae	C. A.	4
Colletotrichum lagenarium	C. A.	32
Penicillium italicum W.	C. A.	32
Gibberella fujikuroi	C. A.	16
Candida albicans	S. A.	16
Cryptococcus neoformans	S. A.	32

Paper disc method.
Method: 0.005 ml of the test solutions were applied on Watman No. 1 filter paper disc, 0.6 cm diameter.
Plant pathogenic fungi were incubated at 28°C for 48 hours.
Human pathogenic fungi were incubated at 37°C for 48 hours.
Medium: Sabouraud agar (S. A.), Czapek's-Dox agar (C. A.)

The antimicrobial activities are shown in Table 1. SC_4-X is active against Gram-positive, Gram-negative bacteria and Mycobacteria. The activitity of SC_4-X is higher in alkaline than in acidic conditions. The antifungal activities of SC_4-X are summarized in Table 2. SC_4-X is not only active against the

Table 3. *The Rf values of SC_4-X and streptothricin group antibiotics*

Solvent system	Rf values			
	SC_4-X	S15-1	A 249	SF 701
I. *n*-propanol-pyridine-acetic acid-water (15:10:3:12)	0.19	0.18 0.07	0.16	0.25
II. Chloroform-methanol-28% ammonia (2:1:1)	0.44	0.42 0.36	0.35	0.63
III. *n*-propanol-pyridine-acetic acid-water (1:1:1:1)	0.09	0.44 0.43	0.44 0.32	0.69

Detection: Ninhydrin. Bioautography with *B. subtilis PIC 219*.
TLC-plates silica gel F254 pre coated layer thickness 0.25 mm.

agricultural pathogenic fungi, *Helminthosporium oryzae*, and *Gibberella fujikuroi* but also capable of inhibiting human pathogenic fungi, *Candida albicans* and *Cryptococcus neoformans*.

Characterization of SC_4-X by TLC

The Rf values of SC_4-X and other known streptothricin-type antibiotics on silica gel TLC are shown in Table 3. The Rf value of SC_4-X is different from other known streptothricin antibiotics. The Rf value of SC_4-X on silica gel TLC developed with the solvent of *n*-propanol-pyridine-acetic acid-water (1:1:1:1, v/v) is only 0.1 which is much smaller than those of streptothricin antibiotics S15-1, A249 and Sf 701.

Chromatographic characterization of SC_4-X by HPLC

A comparison of purity and identities of SC_4-X with other known streptothricin type antibiotics by HPLC is shown in Fig. 1. A249 and S15-1 each contains at least three peaks. In SC_4-X, the predominant component is peak A which has similar Rf value with a minor component in S15-1. Small amount of highly purified SC_4-X was obtained by repeated injection and collection of the predominant peak in SC_4. The purity of SC_4-X thus collected was shown in Fig. 1, which indicates that the impurity peak is trivial. The negative peak in this chromatogram is due to solvent.

Hydrolysis of SC_4-X and S15-1 by 6 N HCl in sealed bomb tubes at 110°C were carried out at various time intervals. Result from amino acid analysis

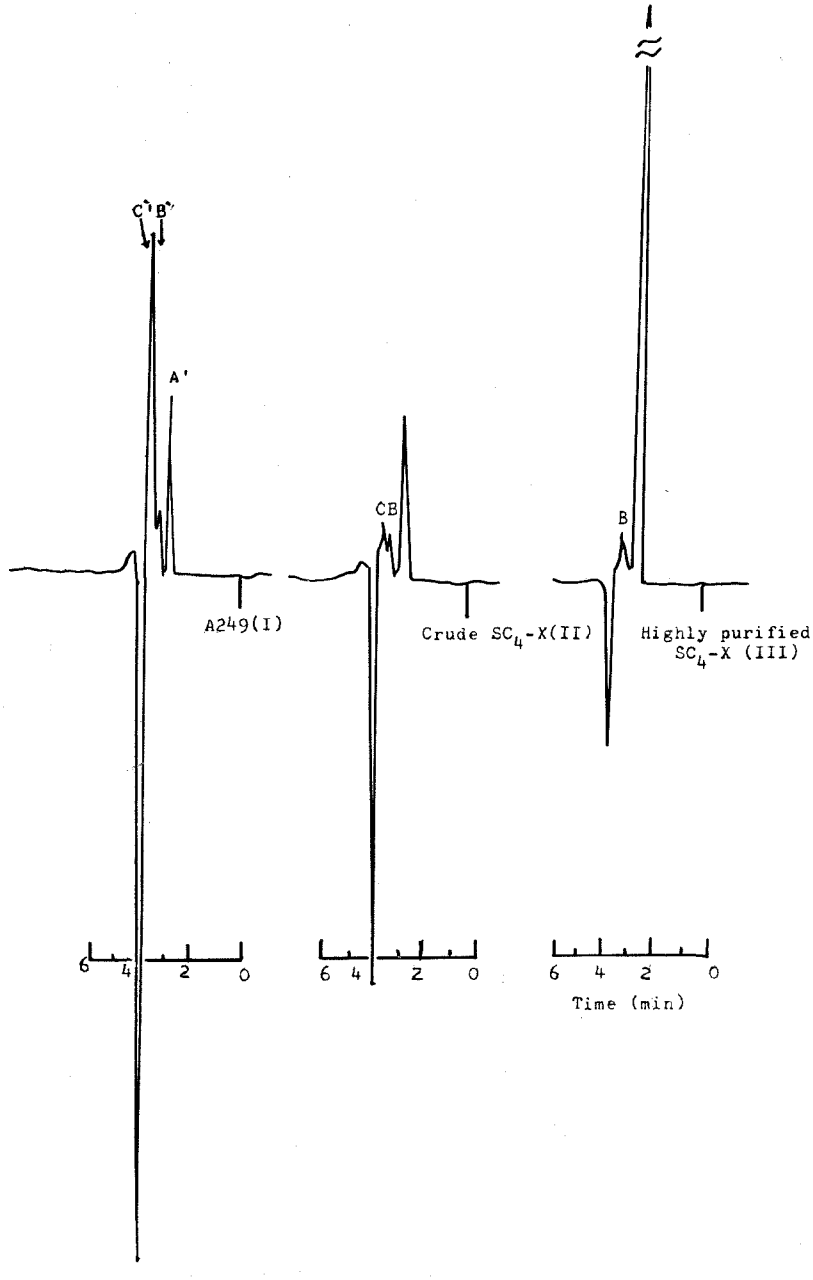


Fig. 1. Chromatogram of A249 (I), Crude SC_4-X (II), and highly purified SC_4-X (III).
Separation was carried out by a μ-Bondapak C-18 column (30×0.4 cm) solvent: 1% HOAc. Peaks were detected by a refractometer.
flow rate: 1 ml/min

of SC_4-X hydrolysate is shown in Fig. 2. It evidently shows the absence of any other amino acid except two peaks. The first peaks moves with Rf value very similar to histidine and the second one has almost identical Rf value with β-lysine. The peak comes out last from the column is found to be ammonia. The ratio of the peak areas of these three components is approximately 1:3:1 in forty-eight hour hydrolysate. Result of the hydrolysate by HPLC is reported in Fig. 3.

Spectroscopic characterization

Since SC_4-X sulfate is very hydroscopic, infrared spectrum of SC_4-X was taken in KBr pellet immediately after prolonged drying in vacuo (Fig. 4). It shows the presence of –NH, C–OH, and amides which are also present in the IR spectrum of S15–1 (Fig. 5).

Chemical shift and tentative assignment of C-13 NMR spectra of SC_4-X

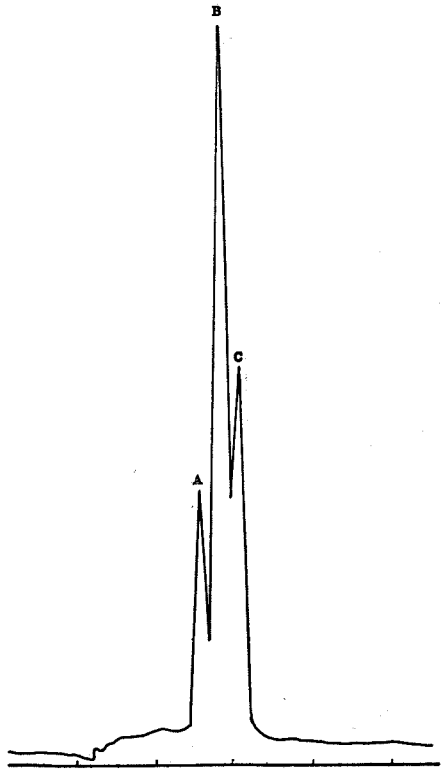


Fig. 2. Chromatogram of the hydrolysate of SC_4-X antibiotic by using an amino acid analyzer.
Peak A: Streptolidine.
Peak B: β-lysine.
Peak C: Ammonia.

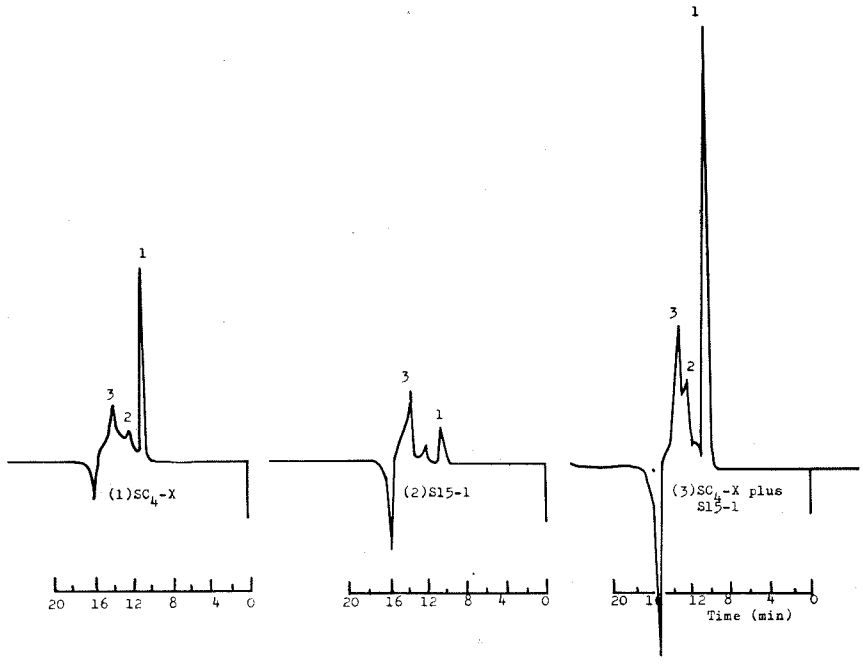


Fig. 3. HPLC analysis of the hydrolysates of SC_4-X, S15-1 and SC_4-X plus S15-1.
Hydrolysates of 0.5 mg antibiotic with 6N HCl at 110°C for 24 hours in a sealed bomb.
Detector: Δ IR×32, Solvent: 1% HOAc/H_2O, Column: μ-Bondapak C_{18},
Flow rate: 1 ml/min, Chart speed: 0.1 in/min.

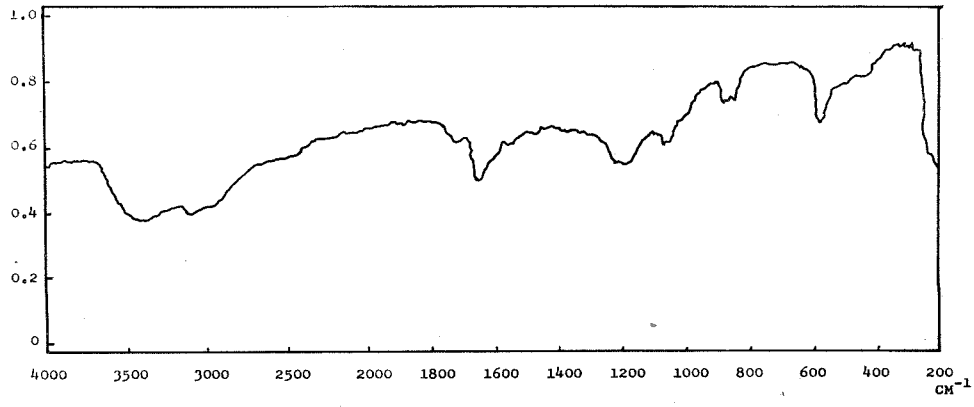


Fig. 4. Infrared spectrum of SC_4-X in KBr pellet.

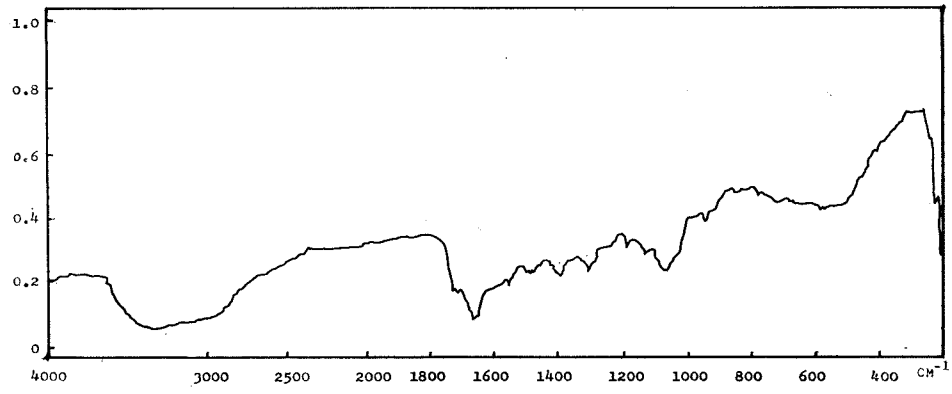


Fig. 5. Infrared spectrum of S15-1 in KBr pellet.

Table 4. *Chemical shifts and tentative assignment of C-13 magnetic resonances of SC_4-X, A249 and S15-1*

	SC_4-X	A249	S15-1
β-lysine	49.4	49.2	49.4
	37.4	37.1	37.4
	29.9	29.9	29.9
	23.8	23.8	23.8
	39.8	39.8	39.9
Sugar	55.2	55.2	55.2
	61.7	61.7	61.7
	68.6	67.6	67.6
	70.8	70.8	70.8
	74.3	74.3	74.3
	79.6	79.5	79.6
Streptolidine	49.8	50.1	49.8
	50.1	50.3	50.1
	61.2	61.1	61.2
	61.5	61.5	61.5
Carbamoyl and Quanidino $\rangle C{=}O$	158.3	158.4	158.8
	—	163.2	163.7
	172.2	172.6	172.8
	172.4		
	172.8		

1) Chemical shifts are reported in ppm, δ (Dioxane=67.4)
2) The resonances of streptolidine D-glucosamine, carbamoyl, guanidino and carbomyls have not yet been satisfactorily assigned.
3) 100 MHz Joel FT-NMR Spectrometer.

and other related streptothricins are shown in Table 4. The presence of peaks corresponding to β-lysine is particularly grouped at the upper portion. Values of the chemical shift of β-lysine and streptolidine portion were found identical with other streptothricin antibiotics such as S15-1 and A249.

Discussion

SC_4-X antibiotic is a water-soluble basic antibiotic. As shown in Table 5, it is different from known antibiotics. SC_4-X inhibits not only *Mycobacterium*, Gram-positive and Gram-negative bacteria but also human and plants pathogenic fungi. It is therefore not identical with streptomycin, viomycin, neomycin and kanamycin. The chemical tests, Rf value of TLC or HPLC chromatography, and antimicrobial spectrum of SC_4-X suggested us that it is probably a new member of streptothricin-like antibiotics.

Streptomyces SC_4 produces a series of compounds with broad spectrum of antibiotic activities. We have concentrated in this report on the structural elucidation of SC_4-X.

Result from the amino acid analysis of SC_4-X hydrolysate indicates the presence of compound A, lysine analog (B), and ammonia. The compound (B) is eventually confirmed by NMR to be β-lysine. The compound A, which behaves like histidine in amino acid analysis, is actually streptolidine. In the forty-eight hours hydrolysate of SC_4-X by 6 N HCl. The product ratio of β-lysine: Streptolidine: NH_3 is approximately 3:1:1. In conjunction with other chemical tests, SC_4-X is evidently a streptothricin type antibiotic. Interpretation of proton and C-13 NMR spectra further support that the lysine moieties are β-lysine and an intact streptolidine is present. The C-13

Table 5. *Comparison of SC_4-X with other known antibiotics*

Antibiotics	Color reaction			m.p.	Rf values*
	Maltol	Ninhydrin	Sakaguchi		
SC_4-X	−	+	−	180-185	0.44
Yazumycin	−	+	+	230-236	0.3-0.33
Streptothricin	+	+	−	213-217	0.26
Zygomycin A					0.62
Viomycin					0.11
Paromomycin					0.68
Aminosidin					0.68
Glebomycin		−			

* Rf value by silica gel T.L.C.
Solvent: the upper layer of $CHCl_3$:MeOH:17% NH_4OH (2:1:1, v/v).

NMR assignment of β-lysine and streptolidine is reported.

The presence of a highly modified six-membered ring sugar moiety is also confirmed by NMR and chemical tests. Difference in 50–75 ppm region of C-13 NMR exists particularly between S15–1 and SC_4-X implies that the configuration and/or modification, including linkage of β-lysine and carbamyl moieties, could be different.

Streptothricin type antibiotics are extremely difficult to purify to homogeneity. Traditional separation based on size and charge is hard to distinguish cluster of compounds with structural difference merely on the number of β-lysine molecules and positions of sugar modification. In our comparison study for purity by HPLC of some characterized streptothricin type antibiotics, namely A249 and S15–1, we found that A249 and S15–1 each contains at least three compounds. The HPLC profiles of S15–1 and SC_4-X are almost identical except the peaks corresponding to β-lysine reflect the numbers of β-lysine moieties of individual antibiotics.

In general, we have confirmed that SC_4-X is a streptothricin type antibiotics which is comprised of one modified sugar, one streptolidine and 3 molecules of β-lysine. Our results is not sufficient to clearly assign the stereochemistry and linkage of the subtituents of pyranose moiety. Further improvement of purification techniques for larger scale sample is definitely a obligatory step to secure complete assignment of the fine structure of SC_4-X.

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鏈黴菌 SC_4 之研究

SC_4-X 抗生素之化學組成

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Streptomyces sp.（簡稱爲鏈黴菌 SC_4）爲由臺灣土壤中分離而得之新抗生素產生菌。其所產生的抗生素抗菌譜甚廣，對革蘭氏陽性、陰性菌及動植物病原性黴菌等具有生長抑制之作用。SC_4-X 抗生素可將其培養液，經離子交換及層柱分析法予以純化。經紅外光譜，氫及碳13核磁共振譜，高效液相及薄層層析法及胺基酸分析法之結果得知 SC_4-X 之化學結構與 streptothricin 抗生素之 A249 及 S15-1 類似但不儘相同。SC_4-X 之分子內含有 3 分子之 β-lysine，1 分子之胺醣及 1 分子之 streptolidine 但可能在胺醣化學修飾及立體化學上略有不同。本文即探討 SC_4-X 之生物活性及其化學結構。