STAPHCOCCOMYCIN, A NEW BASIC MACROLIDE ANTIBIOTIC

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Staphcoccomycin (SCM) is a new member of the basic macrolide family of antibiotics which was isolated from the fermentation broth of *Streptomyces* sp. AS-NG-16. The production, purification and determination of physical and chemical properties of this novel metabolite have been completed. Comparison of the mass fragmentation patterns of SCM and its peracetate with those of angolamycin peracetate suggested a des-mycarosyl derivative of angolamycin. Moreover, the molecular ion peak (*m/e* 771) corresponded to $C_{39}H_{65}NO_{14}$ and the ^{1}H-NMR of SCM was also consistent with the proposed structure.

Streptomyces sp. AS-NG-16 was isolated from a soil sample obtained from Luxor in upper Egypt.

This paper describes the producing organism in brief while the procedure of isolating the active principle and determination of its chemical, physical and biological properties are described in greater detail.

Materials and Methods

SCM producing organism

Streptomyces sp. AS-NG-16 was allowed to grow on starch-nitrate agar medium at 28°C. The morphological, physiological and biochemical characteristics of the organism were investigated according to the system described by WAKSMAN[1].

Production and isolation of SCM

Streptomyces sp. AS-NG-16 was grown in 14-liter fermentors (New Brunswick) aerated with 1 vol. air/1 vol. medium/1 min. The temperature was kept at 28°C with a stirring shaft rate of 600 r.p.m. The production medium has the following composition (g/100 ml): Soluble starch 2.0; soybean flower 1.0; $NaNO_3$ 0.2; K_2HPO_4 0.1; $MgSO_4 \cdot 7H_2O$ 0.05; KCl 0.05 and $FeSO_4 \cdot 5H_2O$ 0.00075; initial pH 7.0. The antibiotic production reached its maximum level (8 mg/liter) after 72 hours of fermentation after which the fermentation broth was filtered and extracted with 30% v/v of ethyl acetate - chloroform mixture (1: 1, v/v) at pH 7.0. The extract was evaporated under vacuum to dryness and the residue was redissolved in a minimum amount of chloroform. Petroleum ether (b.p. 40~60°C) was then added until turbidity started and then left in refrigerator overnight. The precipitate was harvested by centrifugation as a yellow powder.

Purification of SCM

The crude preparation was chromatographed on preparative T.L.C. plates (silica gel GF_{254}) using the lower phase of the following developing mixture: $CHCl_3$ - MeOH - 7% NH_4OH (4: 1: 2, v/v/v). Two biologically active components were separated in the ratio of 9: 1. The spots were scrapped off the plate, extracted with acetone and then evaporated to dryness. The residue was dissolved in chloroform and precipitated with petroleum ether (40~60°C). The major component (Rf 0.32) was purified further using the same T.L.C. technique.

The UV, IR, ^{1}H-NMR and MS analysis were carried out and the results are given below.

The *in vivo* evaluation of SCM is given in Tables 2 and 3.

Results and Discussion

Description and Classification of *Streptomyces* sp. AS-NG-16

The SCM producing organism formed pink-lavender aerial mycelium with pale brown pigments and creamy or pallid brown substrate mycelium on potato and glucose yeast extract media. The organism liquefied gelatin, coagulated and peptonized milk, produced H_2S and reduced nitrate. *Streptomyces* sp. AS-NG-16 produced poor growth on cellulose, inulin, D-sorbitol, D-xylose, L-arabinose and D-raffinose. Better growth was observed on starch, fructose and sucrose. The organism gave a positive melanin test.

Spore chains of strain AS-NG-16 are spirals and monopodially branched (Plate 1) and some show bumps and two show spines (Plate 2). From the aforementioned results *Streptomyces* sp. AS-NG-16 could be classified as a member of the series *Lavendulae* as described and classified by WAKSMAN[1].

Plate 1. Sporophores of *Streptomyces* AS-NG-16. (×400)

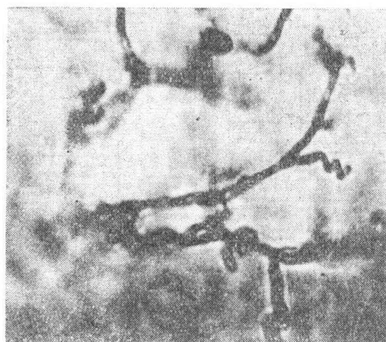
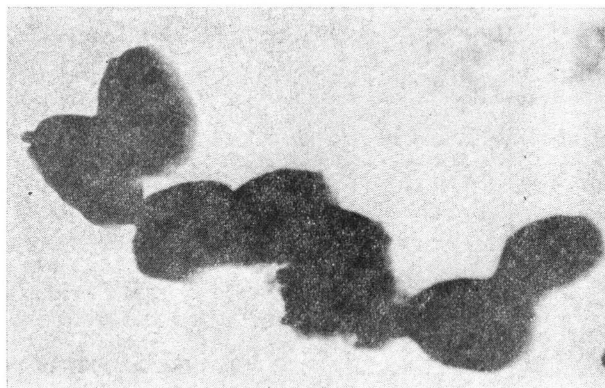


Plate 2. Electronmicrograph of the spores of *Streptomyces* AS-NG-16. (×24,000)

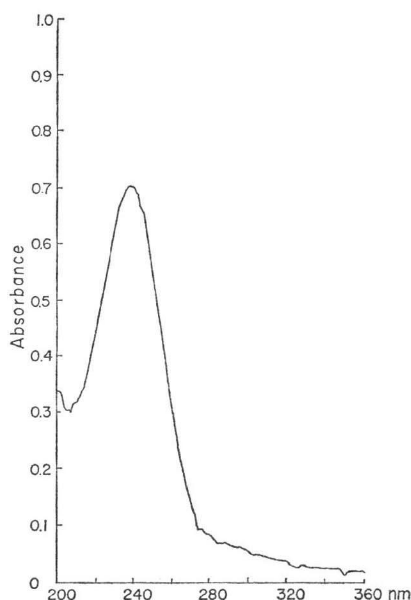


Physicochemical Properties of SCM

SCM was obtained as a pale yellow powder that melts at 117~119°C and is soluble in most organic solvents, slightly soluble in water but almost insoluble in petroleum ether. The elemental analysis gave the following data: C 57.28, H 7.82, N 1.87, and O 33.03% (by difference). The molecular weight was 771 as determined by MS and the empirical formula was suggested to be $C_{39}H_{65}NO_{14}$.

The pure antibiotic had a UV absorption maximum at 240 nm (Fig. 1) ($E^{1\%}_{1cm}$ 165 in ethanol) whereas the IR spectrum in nujol (Fig. 2) showed specific absorption bands at wave numbers cm^{-1} 1060, 1620, 1690 and 1720 which indicate the presence of C–O–C, C–O, –C=C– (conjugated), C=O (conjugated) and C=O of the lactone ring.

Fig. 1. Ultraviolet absorption spectrum of SCM.



The Rf values of SCM on silica gel GF_{254} using different developing solvents were: 0.1 for benzene - acetone (1: 1); 0.32 for lower phase of $CHCl_3$ - MeOH - 7% NH_4OH (4: 1: 2); 0.82 for acetone; 0.58 for BuOH - AcOH - H_2O (3: 1: 1); 0.88 for $CHCl_3$ - MeOH (4: 1) and 0.57 for MeOH - benzene (45: 55).

Assignments of the mass fragmentation patterns of SCM (Fig. 3a) and its peracetyl derivative (Fig. 3b) are given in Charts 1a and 1b respectively, while those of the ^{1}H-NMR signals (Fig. 4) are listed in Chart 2.

Fig. 2. Infrared spectrum of SCM in nujol.

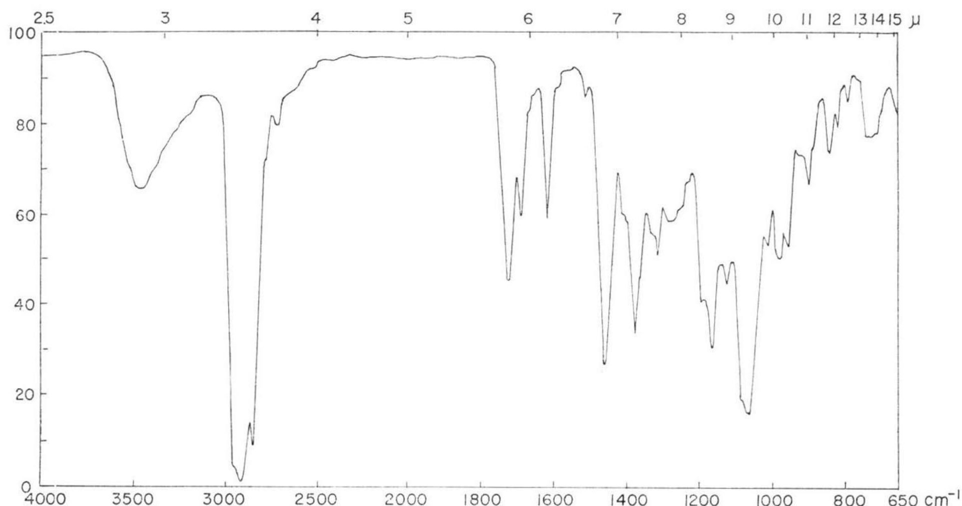


Fig. 3a. MS of SCM.
Instrument: Hitachi RMU-6M Mass spectrometer, Ionizing energy: 50 eV,
Accel. volt.: 1.1 KV, Sample temp.: 190°C.

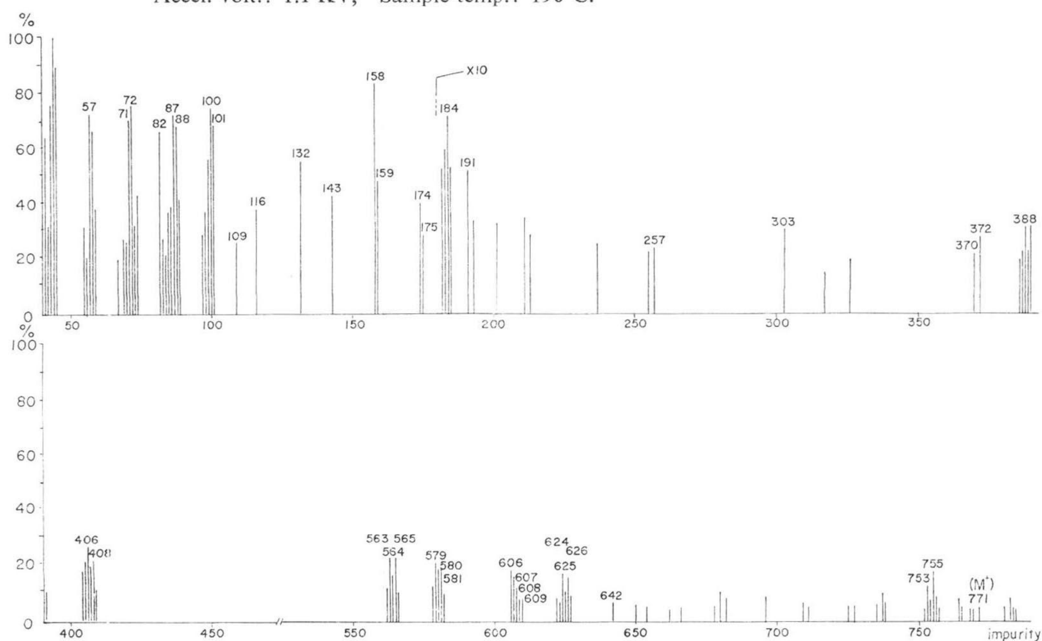
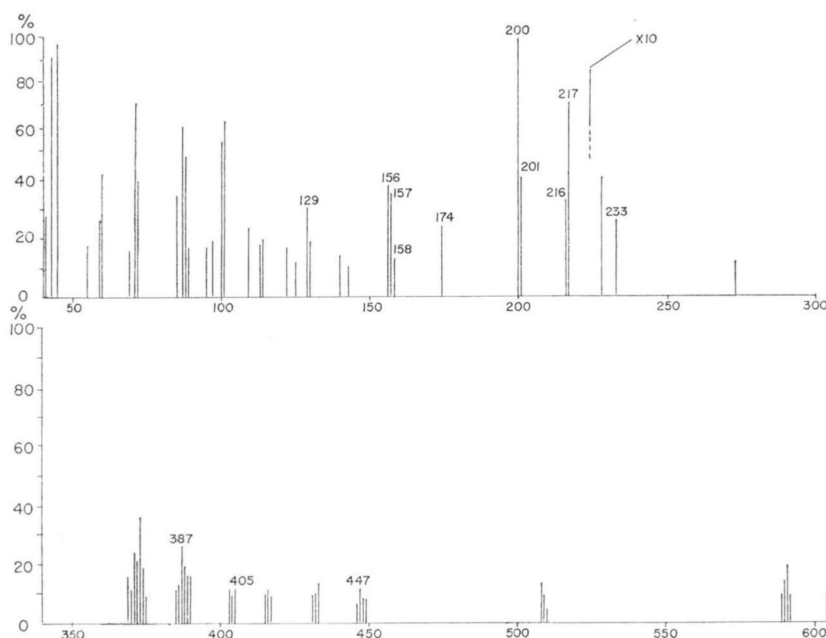


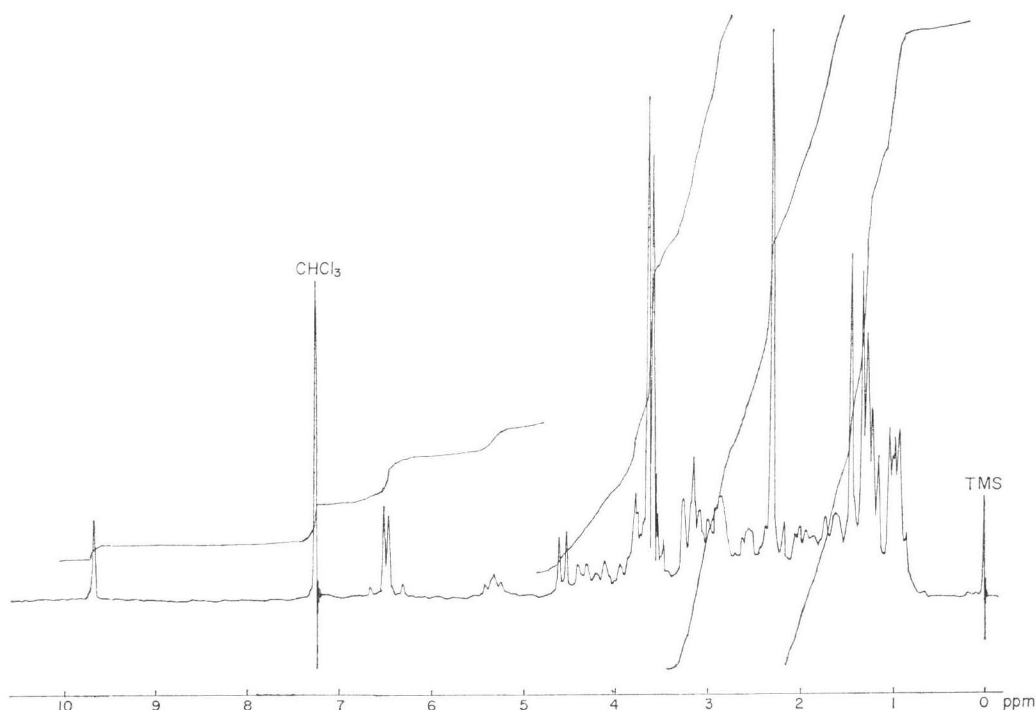
Fig. 3b. MS of SCM peracetate.
Instrument: Hitachi RMU-6M mass spectrometer, Ionizing energy: 50 eV,
Accel. volt.: 1.1 KV, Sample temp.: 170°C.



Proposed Structure of SCM

The above mentioned physicochemical properties of SCM, especially UV maximum at 240 nm, IR absorption at 1720 cm^{-1} $(-\overset{\text{O}}{\overset{\|}{\text{C}}}-\text{O})$ and low nitrogen content (less than 2%) suggest that SCM is a basic macrolide antibiotic possessing the epoxy-enone structure in the aglycone moiety. As described later, the mass fragmentation pattern supported this assumption. The presence of two anomeric protons at δ 4.36 and 4.57 in the ^{1}H–NMR spectrum of SCM (Fig. 4) shows that SCM contains two sugar moieties. Mass spectrometry is often used for the elucidation of the structure of macrolide antibiotics. In the mass spectrum of a macrolide, a pair of fragmentation peaks appearing at regions lower than about *m/e* 200 with 16 mass units difference are diagnostically important. These peaks are ascribed to a sugar molecule and the 16 mass units difference is due to the oxygen atom connecting the glycoside. The two pairs of peaks at *m/e* 175 and 191, and *m/e* 158 and 174 shown in the mass spectrum of SCM (Fig. 3a) are therefore ascribed to the two sugar moieties suggested from the ^{1}H–NMR spectrum. Following peracetylation, these peaks changed to the pairs of peaks at *m/e* 217 and 233, and *m/e* 200 and 216. This change shows that both of the sugar moieties have one hydroxy group capable of being acetylated. Fragmentation peaks at *m/e* 175 and 191 are ascribed to the $C_8H_{16}O_5$ sugar and those at *m/e* 158 and 174 are to the $C_8H_{17}NO_3$ sugar. Among the numerous sugars found in actinomycetes antibiotics ,the $C_8H_{16}O_5$ sugar may be mycinose as in angolamycin[2] and other macrolides, labilose as in labilomycin[3], 2,4-di-O-methyl-α-L-rhamnose as in steffimycin B[4], novobiose as in novobiocin[5] or the sugar as in isoquinocycline[6]. Of these possibilities, the sugars in novobiocin and isoquinocycline were eliminated, because they have a total of three hydroxy groups. Two O-methyl signals in the ^{1}H–NMR spectrum of SCM may reflect the two-O-methyl functions in the remaining sugars. The following

Fig. 4. ^{1}H-NMR signals of SCM.
Instrument: JEOL PS-100, Freq.: 100 MHz, Nucleus: ^{1}H, Solvent: $CDCl_3$, Reference: TMS internal, Conc.: 25 mg/0.4 ml.

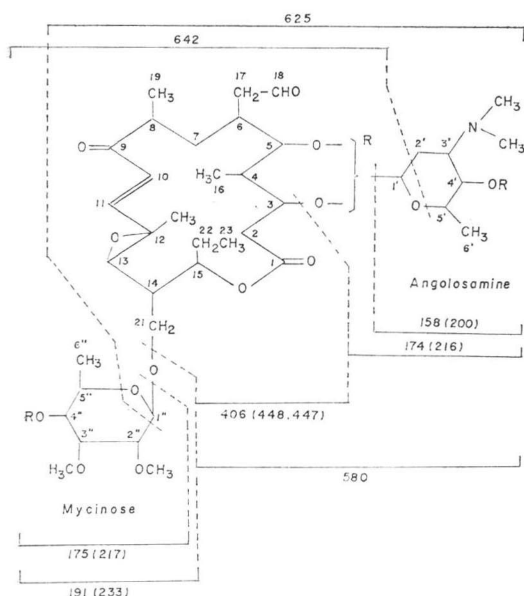


sugars correspond to a formula of $C_8H_{17}NO$: rhodosamine in the anthracyclines, desosamine in many macrolides, angolosamine in angolamycin and megosamine in megalomycin. Among these, desosamine may probably be differentiated from the sugar of SCM, because, in the ^{1}H–NMR spectrum, an anomeric proton due to desosamine would appear at δ 4.2～4.5 as a doublet with $J_{1,2}=7$. Sugars other than those given above could not be distinguished from the sugar in SCM *via* physicochemical means.

However, among the numerous macrolide antibiotics, angolamycin (II) alone contains $C_8H_{16}O_5$ (mycinose) and $C_8H_{17}NO_3$ (angolosamine) in addition to mycarose ($C_7H_{14}O_4$). Accordingly, the mass fragmentation patterns (Chart 1a) of SCM and its peracetate (Chart 1b) were compared with that of angolamycin peracetate reported in the literature[2]. It was found that, by assuming the structure of SCM (I) as des-mycarosyl derivative of angolamycin, the molecular ion peaks (*m/e* 771) corresponded to $C_{39}H_{65}NO_{14}$, and the mass fragmentation pattern of SCM was consistent as shown in Chart 1. ^{1}H–NMR signals of SCM were also consistent with the proposed structure as shown in Chart 2.

To verify this assumption by chemical means, SCM was then directly compared with the des-mycarosyl angolamycin obtained by treating angolamycin (shincomycin A) with 0.3 N HCl at room temperature for 3 hours. Both compounds showed good agreement in thin-layer chromatography carried out in two solvent systems as shown in Fig. 5. Thus, SCM was identified as des-mycarosyl derivative of angolamycin. Hitherto, the basic macrolide whose structure was shown by structure (I) has not been reported. It may be necessary to refer to shincomycin B which was presumably produced along with shincomycin A (angolamycin). The reported IR spectrum of shincomycin B[7] is very similar to that of SCM except for minor differences. However, it was impossible to compare the identity

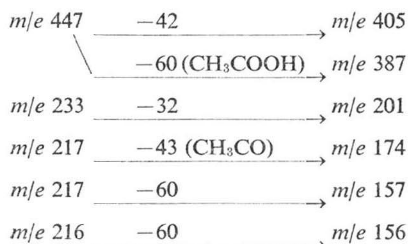
Chart 1.

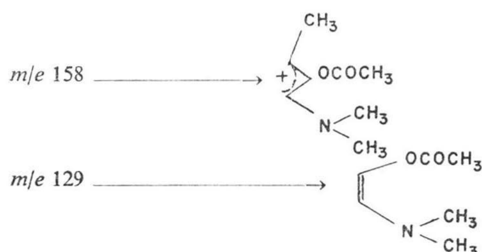


Instrument: Hitachi RMU-6M Mass spectrometer, Ionizing energy: 50 eV. Accel. volt.: 1.1 KV. Sample temp.: 190°C.

Chart 1b. Assignments of the mass fragmentation pattern of peracetyl derivative of SCM.

Peracetyl derivative ($R = -COCH_3$)

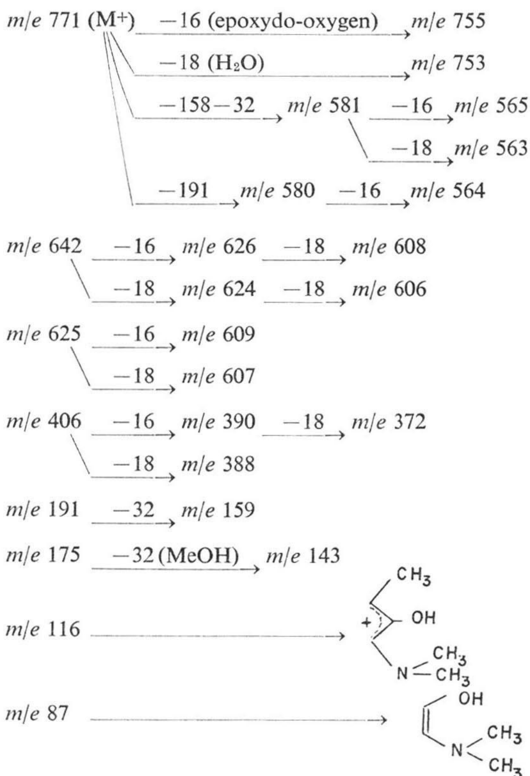




(M+ is not observed).
Sample temp.: 170°C.

Chart 1a. Assignments of the mass fragmentation pattern of SCM.

SCM (R=H)



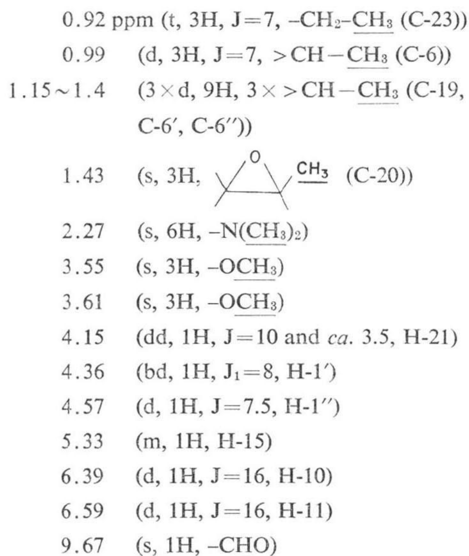
of both antibiotics because of the lack of necessary information about shincomycin B. Moreover, an authentic sample of shincomycin B was not available for direct comparison.

Biological Properties of SCM

The minimal inhibitory concentrations (MICs) of SCM against a variety of bacteria are given in Table 1 which demonstrate that the antibiotic is active mainly against *Staphylococcus aureus* sp. including strains which acquired multi-resistance to other antibiotics. The antibiotic is also active against *Micrococcus luteus*.

Effective curative doses and acute toxicity of SCM are recorded in Tables 2 and 3, respectively.

Chart 2. Assignments of ^{1}H-NMR signals of SCM.



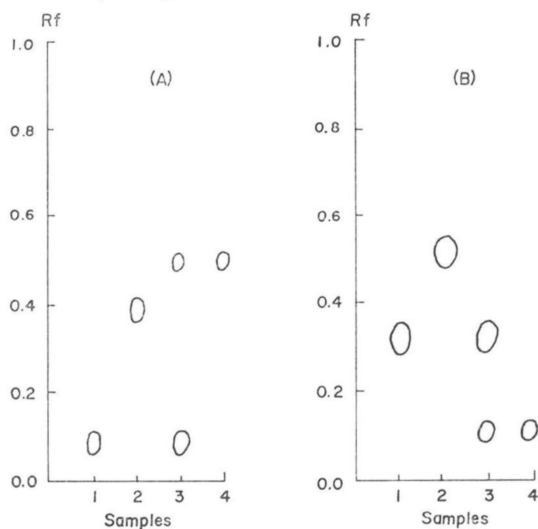
0.92 ppm	(t, 3H, J=7, $-CH_2-\underline{CH_3}$ (C-23))
0.99	(d, 3H, J=7, $>CH-\underline{CH_3}$ (C-6))
1.15~1.4	(3×d, 9H, 3×$>CH-\underline{CH_3}$ (C-19, C-6′, C-6″))
1.43	(s, 3H, epoxide $\underline{CH_3}$ (C-20))
2.27	(s, 6H, $-N(\underline{CH_3})_2$)
3.55	(s, 3H, $-O\underline{CH_3}$)
3.61	(s, 3H, $-O\underline{CH_3}$)
4.15	(dd, 1H, J=10 and *ca*. 3.5, H-21)
4.36	(bd, 1H, J_1=8, H-1′)
4.57	(d, 1H, J=7.5, H-1″)
5.33	(m, 1H, H-15)
6.39	(d, 1H, J=16, H-10)
6.59	(d, 1H, J=16, H-11)
9.67	(s, 1H, $-C\underline{H}O$)

Instrument: JEOL PS-100, Freq.: 100 MHz, Nucleus: ^{1}H, Solvent: $CDCl_3$, Reference: TMS internal, Concentration: 25 mg/0.4 ml.

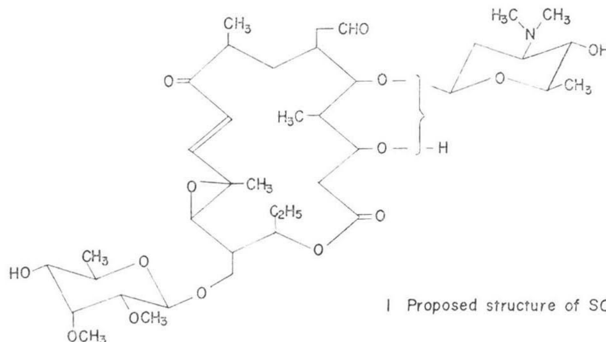
Fig. 5. Comparative TLC of staphcoccomycin with shincomycin A. (Detection by 40% H_2SO_4 spraying followed by heating).

A: Silica gel GF_{254} (benzene - acetone (1:1 v/v))

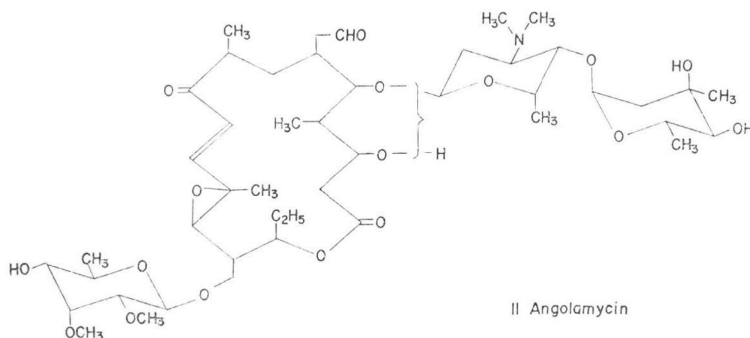
B: Silica gel GF_{254} (a 1:1 mixture of MeOH and lower phase of $CHCl_3$ - MeOH - 7% NH_4OH (4:1:2)).



Samples 1: Staphcoccomycin
2: Shincomycin A
3: Shincomycin A acid hydrolyzate
4: Mycarose



I Proposed structure of SCM



II Angolamycin

Table 1. MIC (mcg/ml) of SCM against Gram-positive bacteria.

Test organism	MIC mcg/ml 48 hrs
1. *Staphylococcus aureus* FDA 209 P	1.56
2. *S. aureus* Terashima	3.12
3. *S. aureus* Smith	3.12
4. *S. aureus* 252 R	3.12
5. *S. aureus* 199 R	25
6. *S. aureus* 664 R	1.56
7. *S. epidermidis* 10131 R	0.78
8. *S. epidermidis* Kawamura	3.12
9. *Streptococcus faecalis*	100
10. *Streptococcus faecalis* Urayama R	100
11. *Bacillus subtilis* PCI 219	12.5
12. *Micrococcus luteus* PCI 1001	0.39

Activity against Gram-negative bacteria was not observed at 100 mcg/ml.

Serial agar dilution method in heart infusion agar was used.

The plates were incubated at 37°C for 48 hours.

S. aureus 252 R: resistant to tetracycline, and kanamycin.

S. aureus 199 R: resistant to tetracycline, streptomycin, kanamycin, neomycin, penicillin, chloramphenicol, erythromycin, and other macrolides.

S. aureus 664 R: resistant to tetracycline.

S. epidermidis 10131 R: resistant to leucomycin.

Table 2. Curative effect (minimum curative dose CD_{100}*) of SCM against *Staphylococcus aureus* Smith.

	MCD* mg/kg
i.m.	6.25
p.o.	12.5

* CD_{100} no animals died at indicated doses.

Observation period: 2 weeks

Mice: strain ddy (male), 4 weeks age, 20 ± 1 g.

Treatment: i.m. and p.o. administration of the test solution prepared by suspending the antibiotic in 0.3% CMC.

Table 3. Acute toxicity of SCM.

Dose (mg/kg)	Mouse survival/mouse treated
50	10/10
100	10/10
200	10/10
300	10/10

Mice: Strain ddy (male), 4 weeks age, 20 ± 1 g.

Treatment: i.v. administration of the test solution prepared by suspending the antibiotic in sterile saline solution.

Observation: 1 week.

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