SCLEROTHRICIN, A NEW BASIC ANTIBIOTIC

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Sclerothricin is a new strongly basic antibiotic obtained from the culture filtrate of *Streptomyces sclerogranulatus* SHIMAZU *et* YONEHARA nov. sp. The antibiotic is active against gram-positive and gram-negative bacteria and some fungi. Sclerothricin ($C_{16}H_{30}O_8N_6$) seems to belong to the streptothricin group. It has two N-methyl, a free carboxyl and two N-terminal groups. No carbamoyl group is present.

During the course of a screening program for antimicrobial antibiotics, a potent broth was obtained from a culture of a *Streptomyces*, No. 7672-MC_4, isolated from a soil sample collected in Ehime, Japan. The active component, produced together with an antibiotic LL-AB 664-like substance, was isolated as an amorphous hydrochloride by the usual isolation method for basic water-soluble antibiotics. Sclerothricin belongs to the streptothricin group of antibiotics, but differs from any previously reported. The antibiotic was named sclerothricin because the producing strain (No. 7672-MC_4) developed sclerotia. The taxonomical features of the strain, named *Streptomyces sclerogranulatus* SHIMAZU *et* YONEHARA nov. sp., are described in another paper[1]. The isolation, characterization and biological properties of sclerothricin are described in the present communication.

Production, Isolation and Purification of Sclerothricin

The production of sclerothricin in jar fermentors was carried out as follows: Fifteen liters of a medium consisting of 1.5 % soybean meal, 0.2 % $(NH_4)_2SO_4$, 0.2 % dry yeast, 2.5 % starch, 0.5 % NaCl and 0.4 % $CaCO_3$ (pH 6.2) were sterilized at 120°C for 20 minutes in a stainless steel fermentor of 30-liter volume. Each jar was inoculated with 600 ml of a seed broth, which was prepared in shake flasks from a culture of *Streptomyces* No. 7672-MC_4 in the same medium for 48 hours at 27°C. Air was supplied at a rate of approximately 1 volume per volume of broth per minute with agitation at 400 rpm at 27~28°C. The time of growth was 68 hours. The final pH of the cultured broth was 6.6.

The broth (60 liters) was adjusted to pH 2.0 with dil. HCl, filtered to remove the mycelium, and the filtrate decolorized with 0.5 % of active carbon. After the carbon

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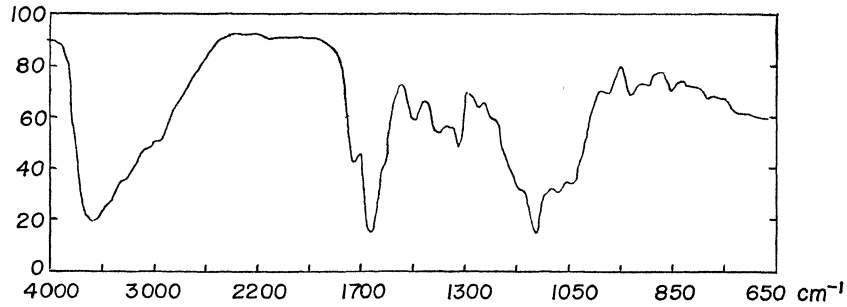
cake was filtered off, the filtrate was adjusted to pH 7.5 with dil. NaOH. The active substances (assayed by the cup plate method using *B. subtilis* PCI 219 as the test microorganism) in the filtrate were adsorbed on active carbon (2 % W/V). The carbon cake obtained by filtration was washed with water (10 liters) and eluted batchwise 3 times with 10 liters of 60 % acidic aqueous acetone (pH 3 with HCl). The eluates were combined, concentrated *in vacuo* to 700 ml and 7 liters of acetone were added. The precipitate which formed from an aqueous acetone solution contained two different active components which were then adsorbed by a column (8 cm × 90 cm) of Amberlite IRC-50 ion-exchange resin (NH_4^+ form). One of the two active fractions was identical with LL-AB 664[7], as judged by comparisons using silica gel or alumina thin-layer chromatograpy. After the column was washed with water (10 liters), sclerothricin was eluted with 7 liters of 0.1 N NH_4OH (LL-AB 664-like substance was not eluted under this condition). The active eluate, which did not contain any of the LL-AB 664 component, was concentrated *in vacuo* to a small volume. The concentrated solution was adjusted to pH 2 with 2 N H_2SO_4 and the active fraction precipitated by the addition of ten parts of acetone. The resultant crude powder (4.5 g) was applied as solution in a mixed solvent, and developed with the same solvent mixture, *n*-propanol - pyridine - acetic acid - water (15 : 10 : 3 : 10 V/V) to a column of crystalline cellulose (Avicel 800 g, 6 cm × 80 cm). The collected active fractions were concentrated *in vacuo*, adjusted to pH 2 with 2 N H_2SO_4 and treated with ten parts of acetone. The resultant precipitate was chromatographed again on the same column as described. An aqueous ethanol (60 %) solution of the active principle was introduced onto a column (4 cm × 40 cm) of acidic alumina, previously treated with aqueous sulfuric acid, and then developed with 60 % aqueous ethanol. The concentrated active principle was converted to the hydrochloride salt by the addition of 3 N HCl and then precipitated with ten parts of acetone. The procedure was repeated, and the resultant hydrochloride salt was then purified on a column of Sephadex LH-20, using a solvent system of water - methanol - acetic acid (89.5 : 10 : 0.5, V/V). The active eluate was concentrated *in vacuo*, adjusted to pH 2 with HCl and treated with ten parts of acetone. The precipitate formed was dissolved in a small volume of methanol and reprecipitated by adding ten parts of ethanol. Three hundred milligrams of sclerothricin hydrochloride were thus obtained as a white powder. The purified sclerothricin was detected as a clear single spot with ninhydrin following several thin-layer chromatographic analyses. These employed Avicel with *n*-propanol - pyridine - acetic acid - water (15 : 10 : 3 : 10), silica gel with chloroform - methanol - 14 % aqueous ammonia (2 : 1 : 1, upper layer) and alumina with 60 % ethanol.

Physical and Chemical Properties of Sclerothricin

Sclerothricin hydrochloride is a white amorphous powder and it melts at 214°C with decomposition. It is quite stable in acidic solution but unstable in basic solution. Sclerothricin hydrochloride is readily soluble in water and methanol, slightly soluble in ethanol and insoluble in most of the common organic solvents. Ultraviolet absorption spectroscopy shows no characteristic bands. The infrared absorption spectrum of

Fig. 1. IR Spectrum of sclerothricin HCl (KBr).



sclerothricin measured in a KBr disc is shown as Fig. 1. The optical rotation of the antibiotic is $[\alpha]_D^{23}$ −74° (*c* 1.1, water). The titration equivalent of sclerothricin hydrochloride in water is 273.

Elemental anaylsis of the antibiotic hydrochloride was as follows:

Calcd. for $C_{16}H_{30}O_8N_6 \cdot 2HCl$: C 37.87, H 6.31, O 25.25, N 16.57, Cl 14.00
Found: C 37.88, H 6.38, O 25.40, N 16.60, Cl 13.63

Sclerothricin shows a positive color development in the ninhydrin, sodium ferricyanide, LEMIEUX, BENNEDICT, ELSON-MORGAN (pink) and PAULY (weak) tests, but is negative in the SCHIFF, maltol, MOLISCH, FEHLING, anthrone, TOLLENS, ferric chloride, SAKAGUCHI (slightly colored) and biuret reactions.

The NMR spectrum of sclerothricin hydrochloride in trifluoroacetic acid shows two singlets at δ 3.05 (3H) and δ 3.25 (3H) due to N-methyl groups. Chemical analyses for the N-methyl groups in sclerochricin gave a value of 1.7 mole.

Degradations of Sclerothricin

Acid hydrolysis of sclerothricin was carried out in 6 N HCl at 120°C for 18 hours in sealed tube. On paper chromatographic examination of the hydrolysate, 3 or 4 spots were observed by ninhydrin spraying (Fig. 2). One mole of glycine was detected in the hydrolysate by the amino acid autoanalyser. Separation of the several components in the hydrolyzate of sclerothricin was carried out by the following procedure: The hydrolyzate was introduced onto a cellulose column with a solvent system of *n*-propanol - pyridine - acetic acid - water (15 : 10 : 3 : 10, V/V). Two different fractions appeared in the eluates. One of these was thought to be an amino sugar moiety, because of its positive triphenyltetrazolium chloride reaction. The other was a complex of two different components. The latter fraction was concentrated to dryness *in vacuo*, dissolved in water and then introduced onto an Amberlite CG-50 (Na^+) ion-exchange column. The column was washed with water and a ninhydrin-positive effluent was collected and concentrated to dryness *in vacuo*. The substance not adsorbed on the ion-exchange column was identified as glycine by thin-layer chromatographic and infrared absorption characterization. The substance (I) adsorbed on the ion-exchange column was eluted with 0.5 N HCl and the ninhydrin-positive eluates were collected and concentrated to dryness *in vacuo*. The eluted substance (I) was dissolved in water and an aqueous solution of picric acid was added. The insoluble picrate

Fig. 2. Paper chromatography of hydrolysate of sclerothricin.

solvent system: *n*-PrOH - Pyridine - AcOH - H_2O (15 : 10 : 3 : 10)
H Hydrolysate of sclerothricin (6 N HCl 120°C, 18 hours)
S Streptolidine HCl
L β-Lysine HCl

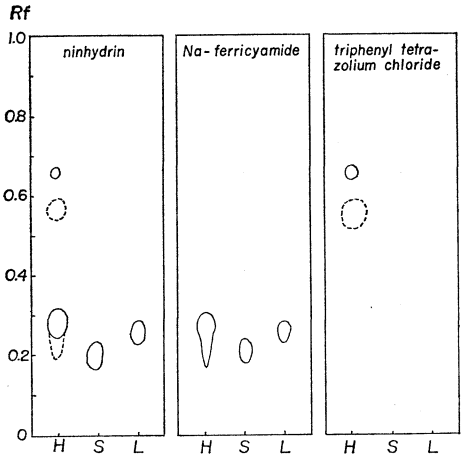
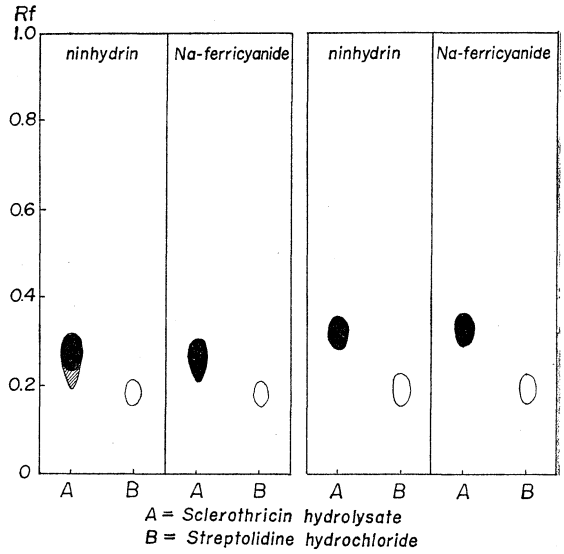


Fig. 3. TLC (avicel) of strong basic moiety of sclerothricin hydrolysate.

n-PrOH - Pyprdine - AcOH - H_2O (15 : 10 : 3 : 10)

BuOH - MeOH - NH_4OH - H_2O (10 : 4 : 3 : 3)



which formed was converted to the hydrochloride by solution in acetone followed by the addition of concentrated hydrochloric acid. The hydrochloride obtained gave a positive sodium ferricyanide and ninhydrin reaction, and was apparently different from streptolidine hydrochloride on the basis of thin-layer chromatographic comparison with an authentic specimen (Fig. 3).

Failure to Detect a Carbamoyl Group in the Unknown Substance (I)

The rapid evolution of carbon dioxide from the carbamoyl group on acid treatment has been found typical of the usual streptothricin group of antibiotics[2a,2b]. Sclerothricin hydrochloride (140 mg) was dissolved in 2 N sulfuric acid and refluxed. Any carbon dioxide evolved was detected as barium carbonate, but after 2 hours the barium carbonate obtained was negligible. A different method of detection of the carbamoyl group in streptothricin[2b] was then applied to sclerothricin. Sclerothricin (48.7 mg) was dissolved in water (9 ml), a saturated aqueous solution of barium hydroxide (6 ml) was added and the mixture kept at room temperature for two days. Again the detectable barium carbonate was insignificant. These results suggested that there is no carbamoyl group in sclerothricin contrariwise in the streptothricins.

2, 4-Dinitrophenyl Derivative of Sclerothricin

The 2, 4-dinitrophenyl derivative of sclerothricin was prepared with 2, 4-dinitrofluorobenzene as customary for peptides. It was dissolved in 5.7 N hydrochloric acid and hydrolyzed at 105°C for 5 hours in a sealed tube. The hydrolyzate was concentrated to dryness *in vacuo*. It was examined by paper chromatography using a solvent system of butanol - methanol - ammonia (28 %) - water (10 : 4 : 3 : 3 V/V), and by thin

layer chromatography on silica gel using a solvent system of chloroform - *t*-amyl alcohol - acetic acid (70 : 30 : 3 V/V). Three orange spots were observed on the chromatograms; the fastest moving spot was identical with 2, 4-dinitrophenol itself, the middle spot migrated with DNP-glycine and the slowest unknown spot gave a positive ferricyanide reaction. On the chromatograms no positive spot appeared on treatment with the ninhydrin reagent. From previously reported work[5], the hydrolyzates of the DNP derivative of the streptothricin antibiotics gave bis-DNP-β-lysine, free streptolidine and a free hexosamine, indicating that β-lysine was in an N-terminal position in streptothricin. In contrast, the hydrolyzate of DNP-sclerothricin contained two DNP-fractions suggesting the presence of two nitrogen terminal positions in sclerothricin. In the NMR spectrum of sclerothricin in perdeutero dimethyl sulfoxide, a signal was observed at δ 9.92 due to the carboxyl group. Accordingly, the streptolidine moiety in sclerothricin may not have a lactam ring between the carboxyl and amino group, possibly because the latter may be N-methylated.

Discussion

Sclerothricin was compared with the known basic water-soluble antibiotics[2~11], using paper chromatography and thin-layer chromatography as shown in Figs. 4, 5. No antibiotics compared were identical with sclerothricin.

From its properties, sclerothricin shows more resemblance to antibiotics BD-12[11], BY-81[11], LL-AC 541[6] and LL-AB 664[7] than to other antibiotics of the streptothricin group. Sclerothricin, LL-AC 541 and LL-AB 664 have glycine in their structure instead of L-β-lysine. The NMR spectrum of LL-AC 541 shows a signal at δ 3.3 due to the N-methyl group of N-methyl-α-D-gulosamine. Sclerothricin has two N-methyl groups, one of them has a signal at δ 3.25 in its NMR spectrum probably corresponding to the N-methyl group on gulosamine in LL-AC 451. However, none of these four antibiotics are

Fig. 4. Comprative papergrams of sclerothricin with known antibiotics.

solvent system : BuOH - MeOH - NH_4OH - H_2O (10 : 4 : 3 : 3)
test organism : *B. subtilis* PCI-219

SM	Streptomycin sulfate	GR	Grasseriomycin sulfate
ZA	Zygomycin A sulfate	RA	Racemomycin A sulfate
PA	Paromomycin sulfate	Rm	Racemomycin mix sulfate
FR	Fradiomycin sulfate	RC	Racemomycin C sulfate
KM	Kanamycin sulfate	YZ	Yazumycin sulfate
VM	Viomycin sulfate	SF	SF-701 sulfate
		SC	Sclerothricin sulfate

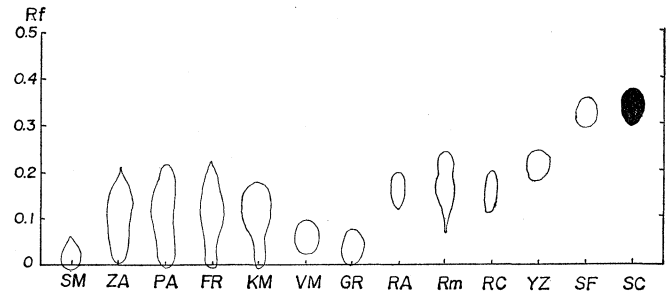


Fig. 5. Comparative thin-layer chromatograms of sclerothricin with known antibiotics (Silicagel G)

solvent system : $CHCl_3$ - MeOH - 14% NH_4OH (2 : 1 : 1 upper layer)
test organism : *B. subtilis* PCI-219

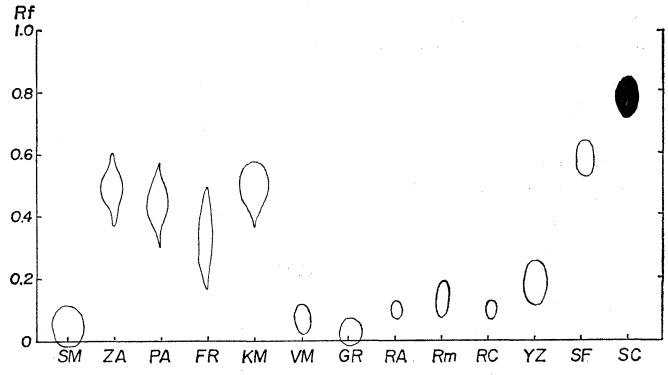
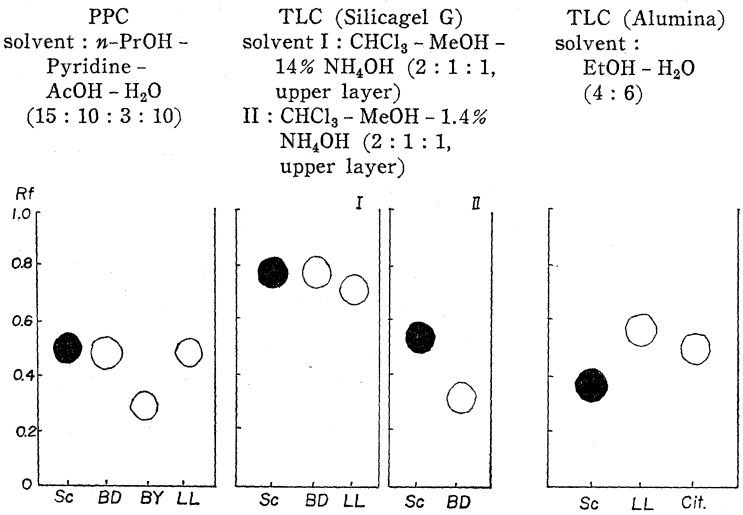


Fig. 6. Comparative PPC, TLC chromatograms of sclerothricin with BD-12, BY-81, LL-AB 664 and citromycin.

test organism : *B. subtilis* PCI-219



identical with sclerothricin, as judged by thin-layer chromatography (Fig. 6). The molecular formula ($C_{16}H_{30}O_8N_6$) of sclerothricin supports the presumption that it may consist of glycine ($C_2H_5O_2N$), an N-methyl-hexosamine ($C_7H_{15}O_5N$) with no carbamoyl group and N-methyl-streptolidine ($C_7H_{14}O_3N_4$).

At this stage, we cannot predict if sclerothricin is a precursor or a biological degradation product of antibiotic LL-AB 664.

Table 1. Antibiotic spectrum of sclerothricin hydrochloride

Test organisms	M. I. C. mcg/ml	Test organisms	M. I. C. mcg/ml
Aerobacter aerogenes IAM-1063	100	*Botrytis fabae* IAM-5125	>100
Agrobacterium tumefaciens B6	>100	*Botrytis cinerea* IAM-5127	12.5
Bacillus subtilis PCI-219	100	*Corticium sasakii* NIAS	>100
Bacillus cereus	50	*Cryptococcus neoformans* IAM-4514	>100
Bacillus circulans	12.5	*Candida utilis* IAM-4215	>100
Corynebacterium xerosis IID	>100	*Cladosporium fulvum* NIAS	100
Escherichia coli	100	*Fusarium lini* NIAS	100
Klebsiella pneumoniae	100	*Gloeosporium kaki* NIAS	50
Mycobacterium phlei IID Timothee	25	*Gibberella fujikuroi* NIAS	12.5
Mycobacterium smegmatis ATCC-607	50	*Gibberella saubinetii* NIAS	50
Pseudomonas fluorescens IAM-1201	>100	*Glomerella cingulata* IAM-8050	25
Pseudomonas tabaci NIAS	50	*Glomerella lagenarium* NIAS	100
Pseudomonas solanacearum NIAS	100	*Helminthosporium sigmoideum*	100
Shigella sonnei IID	100	*Helminthosporium sesamum* NIAS	50
Sarcina lutea NIHJ	25	*Mucor ramannianus* IAM-6128	>100
Staphylococcus aureus FDA-209P	100	*Macrosporium bataticora* IAM-5014	25
Serratia marcescens IAM-1021	>100	*Ophiobolus miyabeanus* NIAS	100
Serratia marcescens var. kilensis ATCC-9986	>100	*Piricularia oryzae* NIAS	100
		Penicillium chrysogenum Q 176	50
Xanthomonas oryzae NIAS	25	*Rhodotorula glutinis* IAM-4757	100
Aspergillus oryzae	>100	*Saccharomyces cerevisiae* NIHJ F-130	100
Alternaria kikuchiana IAM-5005	50	*Trichophyton mentagrophytes* NIHJ 640	50

Biological Properties of Sclerothricin

The biological activities of sclerothricin hydrochloride were examined using the agar streak dilution method. The results are shown in Table 1. Sclerothricin shows inhibitory activity against gram-positive bacteria, gram-negative bacteria and some fungi. Sclerothricin hydrochloride was injected into mice intravenously to determine its toxicity. The LD_{50} was 24 mg/kg, measured after two weeks.

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