TETRENOLIN AND SS/1018 A, ANTIBACTERIAL AGENTS ISOLATED FROM A STRAIN OF ACTINOMYCETALES

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Two metabolites, active against gram-positive bacteria, have been isolated from a strain of Actinomycetales. The chemical structure of one of the two, named tetrenolin, has been previously described[1]. The present paper deals with the description of the producing organism, the isolation and properties of the two metabolites.

Two metabolites, active *in vitro* against Gram-positive bacteria, have been isolated from the culture broth of a strain of Actinomycetales (our code number SS/1018).

The name tetrenolin was given to one of the two metabolites whose structure has been described in a preceeding paper[1] and the name SS/1018 A to the second metabolite.

The characteristic of the producing organism, the isolation and the biological properties of the two metabolites are described here.

Description of the Producing Strain

For the study of the growth characteristics, strain SS/1018, isolated from a soil sample collected in Anzoategui (Venezuela), was grown on a variety of standard media according to GOTTLIEB and SHIRLING[2] and on additional media recommended by WAKSMAN[3].

The cultural characteristics observed are shown in Table 1.

Substrate mycelium is about 1 μ in diameter, branching, twisted and usually of brown color.

Aerial mycelium, abundant on oatmeal agar, is 1 μ about in diameter, white with long branching hyphae. On the top of the hyphal stalks are globose clusters of spore (sporangia-like bodies produced by incurving hyphae) (Figs. 1, 2).

Spores were produced very abundantly on the aerial mycelium under favourable conditions. The spores are spherical to oblong and have diameters of about $1\sim1.3\times1.3\sim1.8\,\mu$. The surface of the spores observed under the electron microscope appears smooth.

Cell-wall composition: For the analysis of the cell-wall hydrolysates the method described by BECKER *et al.*[5] was followed. The results of the chromatographic analysis are given in Table 2.

The optimum temperature range for development of strain SS/1018 is from 28° to

Table 1. Cultural characteristics of strain SS/1018.
The number of some of the culture media refer to those given by SHIRLING and GOTTLIEB[1].

Culture medium	Vegetative mycelium	Aerial mycelium	Soluble pigment
Medium 2	Very good growth, smooth surface, brown	Whitish in traces	Absent
Medium 3	Poor growth, smooth, flat hyaline	Abundant, white with numerous spores	Absent
Medium 4	Very poor growth, thin hyaline	Whitish in traces, with spores	Absent
Medium 5	Moderate growth, wrinkled surface, cream-orange coloured	Absent	Absent
Medium 6	Moderate growth, wrinkled brown	Absent	Absent
Medium 7	Poor growth, thin yellowish	Absent	Absent
Oat-meal agar	Moderate growth, smooth surface, reddish-brown	Abundant, white with numerous spores	Absent
HICKEY TRESNER's agar	Good growth, smooth surface, reddish-brown	Whitish in traces	Absent
BENNETT's agar	Very good growth, wrinkled surface, amber-brown	Moderate, white, with spores	Absent
CZAPEK-glucose agar	Very poor growth, thin hyaline	Absent	Absent
Glucose asparagine agar	Good growth, wrinkled surface, yellowish	Whitish int races, with spores	Absent
Nutrient agar	Good growth, wrinkled surface, light reddish-brown	Absent	Absent
Potato agar	Moderate growth, wrinkled surface, amber	Absent	Absent
Calcium malate agar	Very poor growth, thin brown	Absent	Absent
Skim milk agar	Good growth, smooth surface, light dark-brown	Absent	Violet-brown rather diffusible

Fig. 1. Scanning reflection electron micrograph of strain SS/1018.
Spore cluster on branched stalks produced by incurving aerial hyphae×2,100 (1/1.5).

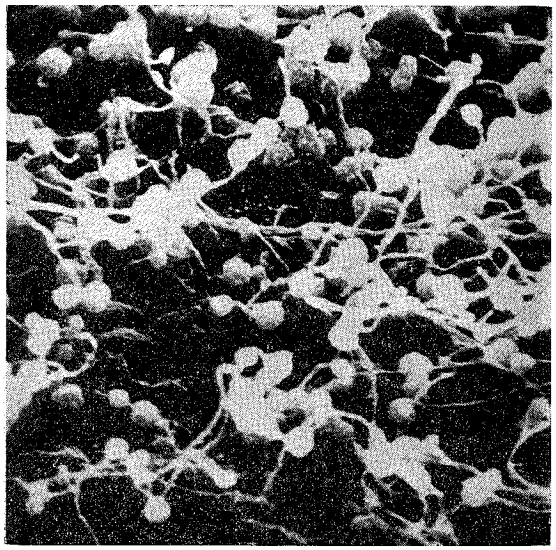

Fig. 2. Scanning reflection electron micrograph of strain SS/1018.
Detail of a cluster with twisted spore chain ×9,000 (1/1.5).

Table 2. Cell-wall composition of strain SS/1018

Isomers of diaminopimelic acid		Amino acids					Sugars			
LL-DAP	MESO-DD-DAP	Aspartic acid	Glutamic acid	Glycine	Alanine	Lysine	Glucose	Galactose	Mannose	Arabinose
TR	++	+	++	+	++	+	+	+	++	

TR=Trace amount

For the identification of amino acids the disrupted cells were hydrolysed in 6 N HCl at 100℃ for 18 hours; for the identification of sugars the cells were hydrolysed in 2 N H_2SO_4 at 100℃ for 2 hours. The hydrolysates were examined by descending chromatography using Whatman No. 1 paper.

Table 3. Physiological properties of strain SS/1018

Tests	Results
Solubilization of calcium malate	negative
Nitrate reduction	positive
Tyrosinase reaction	negative
Hydrolysis of starch	negative
H_2S formation	weakly positive
Liquefaction of gelatin	negative
Litmus milk	no peptonization no coagulation
Casein hydrolysis	positive

Table 4. Utilization of carbon sources

Carbon source	Utilization
Inositol	+
Fructose	++
Rhamnose	++
Mannitol	++
Xylose	++
Raffinose	−
Arabinose	−
Cellulose	−
Sucrose	+
Glucose	++

++ Abundant growth, + Growth, − No growth.

37°C and no growth takes place at 50°C.

The physiological characteristics are shown in Table 3.

The tests for utilization of carbon sources performed according to PRIDHAM and GOTTLIEB[4] are shown in Table 4.

The genera of the microorganisms belonging to the order of *Actinomycetales* are generally characterized by the arrangements of the spores on the aerial mycelium. On this basis, strain SS/1018 should be assigned to the genus *Actinosporangium*. However the cell wall composition differs from that typical of this genus. The taxonomic position of strain SS/1018 thus remains uncertain.

Antibiotic Production

The fermentation of SS/1018 strain is carried out at 28°C in aerated jars with stirring in a liquid medium containing yeast extract 1.0 g, soybean meal 10.0 g, fish solubles 10.0 g, NaCl 2.5 g, glucose 50.0 g and $CaCO_3$ 5.0 g, 1 liter tap water.

Maximum antibiotic activity is obtained after 144~168 hours of fermentation. The microbiological assay is performed by the agar diffusion method using *Staphylococcus aureus* and *Bacillus subtilis* as the test organisms.

Isolation and Separation of Tetrenolin and SS/1018 A

The two metabolites are selectively extracted from the culture filtrate with butanol, operating at different pH values as tetrenolin is a neutral substance and SS/1018 A an acidic one.

The culture broth of strain SS/1018 is filtered and extracted twice with 50 % butanol at neutral pH, the combined extracts are washed with 10 % the volume of water and concentrated *in vacuo*.

After removal of 90 % of the solvent, the resulting suspension is filtered from an amorphous solid that is microbiologically inactive and the filtrate is poured into a large volume of light petroleum. Crude tetrenolin is thus obtained. The crude powder is suspended into hot chloroform under vigorous stirring, insoluble materials are filtered off and the solution is concentrated *in vacuo* until crystallization of tetrenolin occurs.

After cooling at 4°C for a few hours the crystals are filtered and washed with a small quantity of cold chloroform.

The filtered broth, after the extraction of tetrenolin is brought to acidic pH and extracted twice with butanol. The activity is reextracted into water phase by treating the solvent twice with 20 % the volume of a buffer solution pH 8.5. The water layer, after acidification, is extracted again twice with butanol, the butanol is washed with a small quantity of water at pH 6.0 and concentrated *in vacuo* to a small volume. Amorphous SS/1018 A is obtained by adding a large volume of light petroleum.

Chemico-Physical and Biological Properties of Tetrenolin

The following properties have been determined on a product crystallized twice from chloroform.

Tetrenolin appears as a light yellow substance with m.p. 126~128°C. The elemental analysis gives the following values: C 62.87, H 5.89, O 31.34. The molecular formula $C_{11}H_{12}O_4$ with M.W. 208.2 (theoretical values: C 63.4, H 5.78, O 30.73) has been confirmed by the M^+ peak in the mass spectrum.

Structure I has been established for tetrenolin[1].

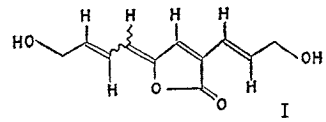

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Fig. 3. Infrared spectrum of tetrenolin
Registered in nujol mull on a Perkin-Elmer mod. 125.

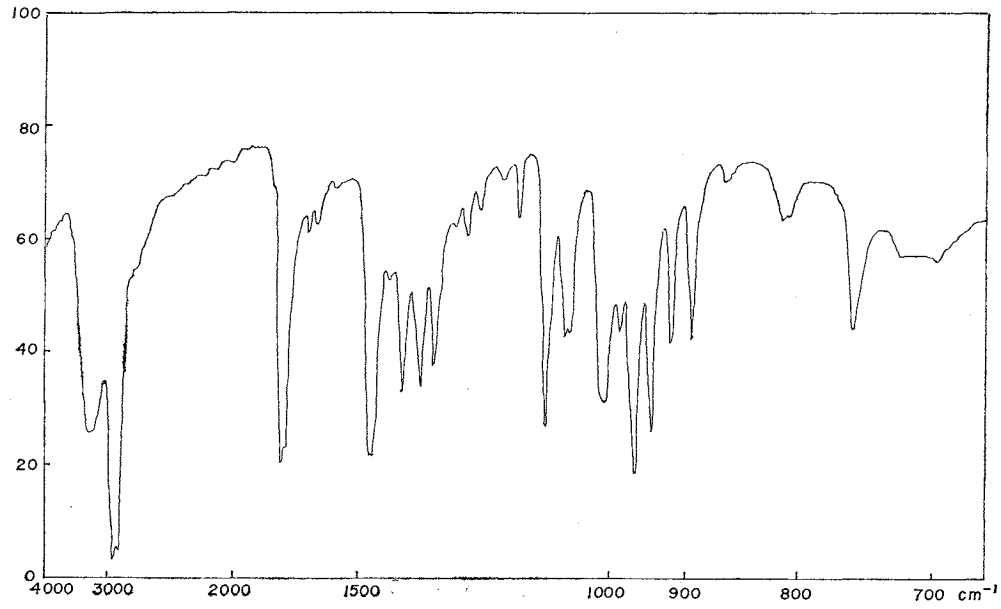

Table 5. Paper chromatographic behaviour of tetrenolin and SS/1018 A with different solvent systems

Solvent system	Tetrenolin Rf*	SS/1018 A Rf*
Water-saturated butanol	0.63	0.0
Water-saturated butanol containing 2 % *p*-toluene sulphonic acid	0.65	0.80(0.60)
Water saturated butanol containing 2 % conc. ammonia	0.0	0.0
Butanol-saturated water	0.0	0.0
20 % Ammonium chloride	0.0	0.0
Phenol - water (75 : 25)	0.95	0.95
n-Butanol - methanol - water (40 : 10 : 20) containing 0.75 g methyl orange	0.80	0.08
n-Butanol - methanol - water (40 : 10 : 30)	0.86	0.13
Water-acetone (1 : 1)	0.0	0.16
Water-saturated ethyl - acetate	0.90	0.0

* Antibiotic visualized on agar plates seeded with a suspension of *B. subtilis*.
Analysis performed by descending paper chromatography on Whatman No. 1 paper

The ultraviolet spectrum shows maxima at 208 mμ (ε 12.300) and at 340 mμ (ε 42.900). The infrared spectrum is reported in Fig. 3. The paper chromatographic behaviour of tetrenolin with different solvent systems is shown in Table 5.

The product is highly unstable at alkaline pH and microbiological inactivation is accompanied by the disappearance of the absorption maximum at 340 mμ.

Table 6. Antibacterial spectrum of tetrenolin and SS/1018 A

Test organism	MIC (mcg/ml)	
	Tetrenolin	SS/1018 A
Staphylococcus aureus 209 P 6538	20	5
S. aureus Tour	50	10
S. aureus Tour+30 % serum	10	10
Streptococcus hemolyticus C 203	5	2
Diplococcus pneumoniae UC 41	5	5
Proteus vulgaris X 19 ATCC 881	>50	>100
Escherichia coli SKF 12140	>50	>100
Pseudomonas aeruginosa ATCC 10145	>50	>100
Candida albicans SKF 2270	>50	>100
Trichophyton mentagrophytes SKF 17410	>50	>100
Mycobacterium tuberculosis H 37 Rv ATCC 9360	50	>100

Minimal inhibitory concentrations have been determined according to the serial dilution method in Difco Pennassay broth.

Tetrenolin is active *in vitro* against gram-positive bacteria and inactive against gram-negative bacteria and fungi; a slight activity is shown on *Mycobacterium tuberculosis*. The antibacterial spectrum is reported in Table 6.

The acute toxicity in mice by the intraperitoneal route is 150 mg/kg.

Chemico-Physical and Biological Properties of SS/1018 A

The product could not be crystallized and was obtained as an amorphous whitish powder. The molecule contains nitrogen and sulphur*, it is soluble in methanol and water, slightly soluble in chloroform and higher alcohols, insoluble in esters, ethers, benzene and acetone. It gives positive reaction to FEHLING and TOLLENS reagents and decolorize $KMnO_4$ in neutral solution.

The ultraviolet spectrum shows maxima at 256 mμ ($E^{1\%}_{1cm}$ 90), in buffer solution at pH 7.3, at 261 mμ ($E^{1\%}_{1cm}$ 95) in 0.1 N NaOH, at 251 mμ ($E^{1\%}_{1cm}$ 105) in 0.1 N HCl and at

* The product was not pure enough for microanalytical determination; sulphur and nitrogen have been determined qualitatively.

262 mμ ($E^{1\%}_{1cm}$ 90) in methanol.

The paper chromatographic behaviour of SS/1018 A in different solvent systems is shown in Table 5.

The product is active *in vitro* against Gram-positive bacteria and inactive on Gram-negative bacteria, mycobacteria and fungi. The antibacterial spectrum is reported in Table 6. No toxicity on mice has been revealed up to 1,000 mg/kg of product injected by the intraperitoneal route.

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