ANTIBIOTIC PRODUCTION BY NEW FORM-GENERA OF THE ACTINOMYCETALES. I

SPORANGIOMYCIN, AN ANTIBACTERIAL AGENT ISOLATED FROM *PLANOMONOSPORA PARONTOSPORA* VAR. *ANTIBIOTICA* VAR. NOV.

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A new antibiotic producing actinomyces belonging to the genus *Planomonospora* is described. Detailed description of its morphological and some of its biochemical characters are given. The antibiotic for which the name sporangiomycin is proposed can be produced successfully under submerged conditions. Sporangiomycin was found to belong to the depsipeptide or peptolide group of antibiotics, and is related to thiostrepton. The antibiotic is highly active *in vitro* against gram-positive bacteria, and protects mice from streptococcal and diplococcal infections.

With minor exceptions, the antibiotics isolated from actinomycetes reported in the literature are almost exclusively produced by representatives of the genus *Streptomyces* and considerably less frequently by the genus *Nocardia*, *Micromonospora* and *Thermoactinomyces*. Only recently did there appear reports on antibiotics formed by some of the other lesser known genera of the Actinomycetales. GERBER and LECHEVALIER[1,2] reported for the first time the production of a phenazine antibiotic (iodinin) from *Microbispora aerata* and OKUDA *et al.*[3,4] described sporaviridin as the first chemically characterized antibiotic produced by the genus *Streptosporangium*. Additional literature references do exist on the *in vitro* antibiotic activity of members of the genera *Streptosporangium*[5,6], *Actinoplanes*[7], *Microellobosporia*[8], and *Microechinospora*[9], however, so far no antibiotics were isolated and described from these genera.

During the last years a screening program directed towards the isolation and study of new form-genera of the Actinomycetales was started in these laboratories. The scope of this work was to conduct a systematic search among these largely unknown genera in the hope of finding among them new antibiotic substances. In the present paper a new antibiotic-producing microorganism is described. The new isolate, strain B987, was found to be akin to *Planomonospora parontospora*[10], from which it can, however, be distinguished by its physiological and biochemical properties and was named *Planomonospora parontospora* var. *antibiotica*. The antibiotic produced by this new isolate was named sporangiomycin.

The Sporangiomycin-producing Organism

Strain B987 was isolated from a soil sample collected in the Province of Buenos

Aires, Argentina, and was shown to be highly active against gram-positive bacteria. Another organism, strain B988, undistinguishable from strain B987 and producing the same antibiotic was isolated from a soil sample received from Venezuela. Most of the procedures used in the taxonomic study of strain B987 were those recommended by SHIRLING and GOTTLIEB[11]. Additional media recommended by WAKSMAN[12] were also used. Soil agar, a medium specially suited for sporangia formation was prepared as already described[13]. The various media were inoculated with 0.05 ml of a twice washed mycelium suspension grown for 48 hours in medium V6 (MARGALITH and PAGANI)[14].

General Characteristics

The microorganism has a mycelium which differentiates into: (a) substrate or vegetative mycelium growing into the agar medium and forming on its surface a compact leathery growth which is usually of a light rose color and (b) aerial or secondary mycelium arising from the substrate mycelium and growing into the air away from the agar surface. The aerial mycelium is white and poorly developed. The sporangia are formed only on the aerial mycelium, each one containing a single sporangiospore which becomes motile only some time after it has been expelled from the sporangium.

Vegetative Mycelium

The vegetative mycelium is approximately 1 μ in diameter, irregularly branched, twisted, occasionally presenting swellings, and usually of a light rose color. Hyphae do not fragment.

Aerial Mycelium

The aerial mycelium is about 1 μ in diameter, hyaline to white, poorly branched and not well developed. The outstanding characteristic of the culture is the formation on the aerial mycelium only, of sporangia containing a single sporangiospore measuring $1.0\sim1.5\times3.5\sim4.5$ μ. They are formed in a double parallel row, and are attached to the sporogenous hyphae not by means of a sporangiophore but directly and by the entire surface of the base of the sporangium, forcing the sporogenous hyphae to become typically bent as can be seen in Plate 1. Sporangiospores in various stages of expulsion from the sporangia can be seen in Plates 2 and 3. The optimum temperature for development of isolate B-987 was found to be at 28~37°C; no growth took place at 45°C.

Plate 1. Aerial mycelium with sporangia. The bending of the sporogenous hyphae is clearly seen.

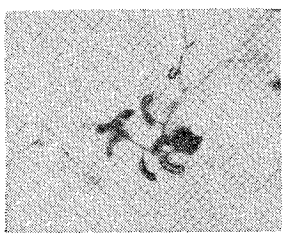


Plate 2. Beginning of the sporangiospore eclosure.

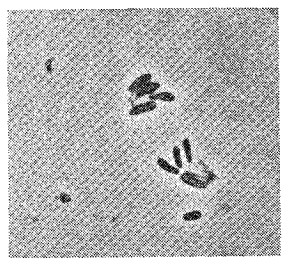
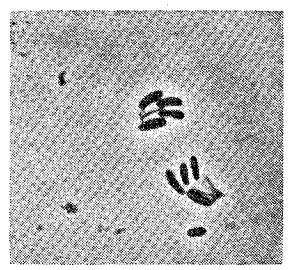


Plate 3. Same as Plate 2. Sporangiospore eclosure practically completed (20 min.).



Appearance on Various Media

The inoculated media were incubated at 28°C and examined after 7, 14, and 21 days. Colors were determined, when necessary, according to MAERZ and PAUL[15]. The number of some of the culture media refer to those given by SHIRLING and GOTTLIEB[11].

Medium n. 2: No growth.

Medium n. 3: Good growth, smooth surface; vegetative mycelium light rose (Pl 2; D9); traces of whitish aerial mycelium. Sporangia present.

Medium n. 4: Very good growth, smooth surface; vegetative mycelium light rose color (Pl 2; D9). No aerial mycelium.

Medium n. 5: Poor growth, colony flat, smooth surface; vegetative mycelium hyaline; traces of white aerial mycelium with sporangia.

Medium n. 6: No growth.

Medium n. 7: Moderate growth, smooth surface; vegetative mycelium rose (Pl 2; G10). No aerial mycelium. Light brown diffusible pigment.

HICKEY and TRESNER's agar: Poor growth, colony flat, smooth surface; vegetative mycelium light rose (Pl 2; D9). No aerial mycelium.

BENNET agar: Good growth, elevated smooth surface; vegetative yellow with rose tinge (Pl 10; B7). Traces of whitish aerial mycelium with sporangia.

CZAPEK agar: Moderate growth, slightly crusty; vegetative light rose (Pl 1; B10). Traces of whitish aerial mycelium with sporangia.

Skim milk agar: Moderate growth, smooth surface; vegetative rose colored (Pl 2; A10). No aerial mycelium.

Glucose asparagine agar: Moderate growth, smooth, flat; vegetative hyaline with a rose tinge; traces of whitish aerial mycelium.

Nutrient agar: Good growth, surface slightly crusty; vegetative light orange (Pl 10; L10). No aerial mycelium.

Potato agar: Moderate growth, smooth surface; vegetative cream colored with traces of orange. Traces of whitish aerial mycelium.

Calcium malate agar: Very poor growth; vegetative hyaline. Traces of whitish aerial mycelium with sporangia.

Carbon utilization: The test was performed using the basal medium according to SHIRLING and GOTTLIEB[11]. The results are summarized in Table 1; the physiological properties of strain B987 are given in Table 2.

Table 1. Utilization of carbon sources

Carbon source	Response
Inositol	−
Fructose	++
Rhamnose	++
Mannitol	++
Xylose	++
Raffinose	−
Arabinose	++
Cellulose	±
Sucrose	+
Glucose (positive control)	++
No carbon (negative control)	−

++: Strongly positive utilization. Growth is similar to or greater than growth on positive control.

+: Positive utilization: growth is significantly greater than "no carbon" although somewhat less than on glucose.

−: Utilization negative: growth is similar to "no carbon" and much less than on positive control.

Table 2. Physiological properties of *Planomonospora parontospora* var. *antibiotica*

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

The microscopic and cultural studies performed on strain B-987 clearly indicate that this isolate belongs to the recently described genus *Planomonospora*[10]. Although the cultural characteristics of strain B-987 are similar to those of *P. parontospora*[10], some properties, such as the formation of melanoid pigments, hydrolysis of tyrosine, production of H_2S as well as the formation of the antibiotic, sporangiomycin, render it different from the former species. Strain B-987 was considered to be variant of *Pl. parontospora* and was therefore named *Planomonospora parontospora* var. *antibiotica* var. nov.

Antibiotic Production

Fermentation conditions suitable for the production of sporangiomycin were studied, and the following medium was found to be useful in shake flask culture and in 4 liter fermentors: meat extract, 4.0 g; peptone, 4.0; NaCl 2.5; yeast extract, 1.0; soybean meal, 10.0; glucose, 50.0; $CaCO_3$ 5.0; tap water 1 liter. Maximum antibiotic activity was obtained after 47~72 hours of fermentation. A paper disk-agar plate assay using *Staphylococcus aureus* and *Bacillus subtilis* as the test organisms was employed for determination of the antibiotic levels in the fermentation broth and the extracts. Expressed in dilution units, antibiotic titers of 1/640 against S. *aureus* and 1/1,280 against *B. subtilis* were obtained.

Extraction and Purification

The antibiotic can be obtained by extracting with ethyl acetate the mycelial cake being previously washed with water and methanol-water mixtures; the extraction is repeated, and the collected solvents are concentrated to a small volume. An amorphous yellowish product is obtained by keeping the concentrated solution at 0°C for a few hours; additional product can be recovered by precipitating the residual solution with petroleum ether.

Sporangiomycin is almost insoluble in methyl alcohol, and a first purification is obtained by treatment of the crude products with an excess of methanol; the product being insoluble in methanol is dissolved in anhydrous chloroform, the solution is decolorized with charcoal, and the antibiotic is precipitated by the addition of hexane. The product is then crystallized twice from chloroform.

Chemical and Chemico-physical Properties

The crystalline product occurs as a whitish powder m.p. 261~262°C and optical rotation $-100°$ ($[\alpha]_D^{25}$ in 0.5% chloroform solution); is insoluble in water, acetone, dichloromethane, methanol and higher alcohols; is slightly soluble in chloroform and soluble in dimethylformamide, diethylenedioxide, dimethylsulphoxide and glacial acetic acid.

The antibiotic contains nitrogen and sulphur and the elemental analysis gives the following values (average of three determinations): C 50.4, H 5.6, N 15.5, O 18.0, S 10.5. No methoxy group is present.

The molecular weight, determined with the dew-point method[16] on a 6% sporangiomycin solution in dioxane is 1,800 (±100). Analytical data and molecular

weight determination indicate a molecular formula $C_{77\sim80}H_{101\sim105}N_{20\sim21}O_{21}S_6$ although slightly different formulas are not excluded.

The paper chromatographic behaviour of sporangiomycin with different solvent systems is shown in Table 3.

The antibiotic gives positive TOLLENS', FEHLING's and biuret reactions; MOLISCH's, anthrone and ferric chloride reactions are negative while ninhydrin reaction is positive only after acid hydrolysis.

Hydrolysis of the antibiotic with 6 N HCl at 120°C for 8 hours in a sealed tube liberated five ninhydrin-positive compounds and one hydroxyacid.

Table 3. Paper chromatographic behaviour of sporangiomycin with different solvent systems.

Solvent system	Rf*
Water saturated butanol	0.88
Water saturated butanol containing 2 % *p*-toluenesulfonic acid	0.83
Water saturated butanol containing 2 % conc. ammonia	0.82
Butanol saturated water	0.1÷0.5
20 % Ammonium chloride	0.0
Phenol - water (75 : 25)	0.98
n-Butanol - methanol - water (40 : 10 : 20) containing 0.75 g methylorange	0.94
n-Butanol - methanol - water (40 : 10 : 30)	0.91
Water - acetone (1 : 1)	0.10
Water saturated ethyl acetate	0.88

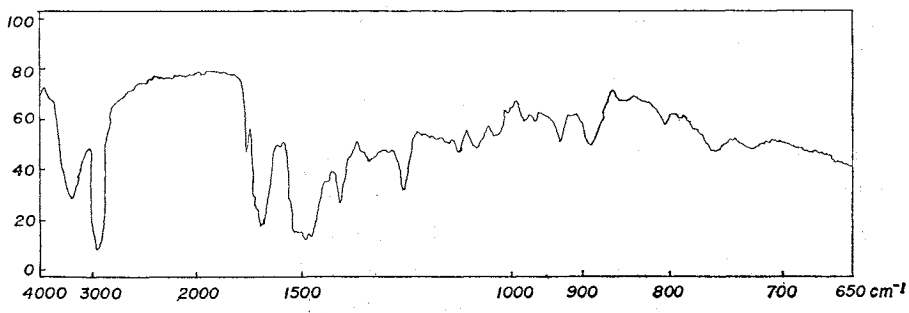
* Antibiotic visualized on agar plates seeded with a suspension of *B. subtilis*.

Sporangiomycin does not show any characteristic ultraviolet absorption spectrum in methanol or aqueous solutions; in 6 N H_2SO_4 it shows a peak at 318 mμ; the infrared spectrum (Fig. 1) shows a weak absorption of an ester band (1,720 cm^{-1}) and strong absorptions of secondary amide bonds (1,660 cm^{-1} and 1,520 cm^{-1}).

From the above reported data a peptidic nature for sporangiomycin is evident, moreover the presence of an ester bond in the infrared spectrum and of an hydroxyacid in the hydrolysate allow the classification of sporangiomycin among the depsipeptide or peptolide class of compounds.

Sporangiomycin is the first antibiotic produced by the recently described genus *Planomonospora*[10]; a comparison with other polypeptide antibiotics produced by actinomycetes revealed a similarity of sporangiomycin with the peptidic antibiotics thiostrepton[17], (thiactin or bryamycin[18]) and with siomycin[19]. A chromatographic analysis of the antibiotic thiostrepton and of its acid hydrolysate showed some differences with sporangiomycin while a comparison with siomycin was not possible for lack of a reference sample. Analysis of acid hydrolysates of sporangiomycin is in progress in order to identify the products of hydrolysis.

Fig. 1. Infrared spectrum of sporangiomycin in nujol mull.



Antimicrobial Spectrum

The minimum inhibitory concentration of sporangiomycin against a variety of microorganisms was determined, unless stated otherwise, by the serial agar plate dilution method. To obtain the maximum concentrations of the antibiotic used in the agar plate dilution method (100 μg/ml), a standard solution of sporangiomycin (1,000 μg/ml) solubilized with dimethylformamide was used. In this way solubility of the product was assured at 100 μg/ml. As shown in Table 4, sporangiomycin is highly active only against Gram-positive bacteria, and it presented no cross-resistance with any of the antibiotic resistant staphylococci used. The incorporation of 50 % of bovine serum to the medium had no significant effect on the antibiotic activity.

Table 4. Antimicrobial spectrum of sporangiomycin

	Test organisms	MIC μg/ml
Gram-negative bacteria	*Escherichia coli*	>100
	Pseudomonas aeruginosa	>100
	Salmonella typhimurium	>100
	Proteus morgani ATCC 9237	>100
	Proteus rettgeri ATCC 9918	>100
	Proteus mirabilis ATCC 8259	>100
	Proteus vulgaris ATCC 881	>100
	Klebsiella pneumoniae ATCC 10031	>100
Fungi	*Saccharomyces cerevisiae* ATCC 9763	>100
	Candida albicans ATCC 10231	>100
	Trichophyton mentagrophytes ATCC 8757	>100
	Fusarium oxysporum	>100
	Aspergillus, sp.	>100
	Penicillium chrysogenum ATCC 10002	>100
Gram-positive bacteria	*Streptococcus faecalis* ATCC 7080	0.035
	Streptococcus pyogenes C 203	0.001*
	Sarcina lutea ATCC 9341	0.015
	Bacillus cereus ATCC 9634	0.300
	Bacillus megatheriuum NRRL 10778	0.150
Gram-positive bacteria	*Bacillus subtilis* ATCC 6633	0.035
	Diplococcus pneumoniae UC 41	0.001*
	Mycobacterium phlei ATCC 10142	0.600
	Mycobacterium tuberculosis H 37 Rv ATCC 9360	5.0*
	Staphylococcus aureus 209 P ATCC 6538	0.005*
	Staphylococcus aureus (CS-R)	0.035
	Staphylococcus aureus (GN-R)	0.015
	Staphylococcus aureus (OL-R)	0.007
	Staphylococcus aureus (PL-R)	0.007
	Staphylococcus aureus (K-R)	0.007
	Staphylococcus aureus (SM-R)	0.007
	Staphylococcus aureus (PC-R)	0.075
	Staphylococcus aureus (RF-R)	0.075
	Staphylococcus aureus (BC-R)	0.035
	Staphylococcus aureus (TC-R)	0.015
	Staphylococcus aureus (LC-R)	0.035
	Staphylococcus aureus (ER-R)	0.015
	Staphylococcus aureus (CAF-R)	0.015
	Staphylococcus aureus (STR-R)	0.035
	Staphylococcus aureus (NE-R)	0.015
	Staphylococcus aureus Tour	0.010*
	Staphylococcus aureus Tour+50% serum	0.050*

* MIC determined in liquid medium.

Abbreviations: R: resistant against 500~1,000 μg of the following antibiotics; CS: cycloserine; GN: gentamicin; OL: oleandomycin; PL: polymyxin; K: kanamycine; SM: streptomycin; PC: penicillin; RF: rifamycin; BC: bacitracin; TC: tetracycline; LC: lincomycin; ER: erythromycin; CAF: chloramphenicol; STR: streptothricin; NE: neomycin.

Toxicity and *In vivo* Activity

Due to the low absorption of the product, the acute toxicity of sporangiomycin could not be determined, neither by subcutaneous nor by intraperitoneal routes. At 1,000 mg/kg, unabsorbed material was found to be present at the site of application, however, at this level, no delayed toxicity was observed.

The *in vivo* activivty of sporangiomycin was tested in mice against experimental

infections with *Staphylococcus aureus*, *Streptococcus hemolyticus* and *Diplococcus pneumoniae*. The product was administered subcutaneously for three consecutive days as a suspension in carboxymethyl-cellulose. No protection was obtained against *S. aureus* even at 200 mg/kg whereas against *Streptococcus hemolyticus* and *Diplococcus pneumoniae*, an approximate ED_{50} of respectively 20 mg/kg and 8 mg/kg was obtained. Sporangiomycin gave no protection by oral route.

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