PURPUROMYCIN, A NEW ANTIBIOTIC ISOLATED FROM *ACTINOPLANES IANTHINOGENES* N. SP.

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Purpuromycin ($C_{26}H_{18}O_{13}$) is a new antibiotic isolated from the culture broth of *Actinoplanes ianthinogenes* proposed as a new species on the basis of its morphological, cultural and physiological characteristics. The antibiotic, obtained as purple crystals, belongs to the naphthoquinone group of antibiotics and resulted to be related to rubromycins. The isolation and purification procedures and the chemico-physical properties of the product are reported. Purpuromycin is active *in vitro* against Gram-positive bacteria at 0.005~0.02 μg/ml, at 1~20 μg/ml on Gram-negative bacteria and at 0.2~0.5 μg/ml on fungi.

A new *Actinoplanes* species has been isolated from a soil sample collected in Blumenau (Brasil) in the course of a screening for antibiotics originated from strains of the family *Actinoplanaceae*. In the present paper we wish to report on the taxonomy of the new strain, number A/1668 in our culture collection, and on the properties of purpuromycin, the active metabolite produced.

Taxonomy of *Actinoplanes ianthinogenes* n. sp.

Macroscopic Examination

Colonies grown for a few weeks on oatmeal agar are 0.5~0.8 cm in diameter; have irregular contorns and the surface is irregularly ridged, with slightly elevated, rough center. Cultural characteristics were determined after 6~14 days of incubation at 30°C and at pH 7.0. The strain was cultivated on various standard media suggested by SHIRLING and GOTTLIEB[1] with

Fig. 1. Sporangia imbedded in the vegetative mycelium (1,200×)

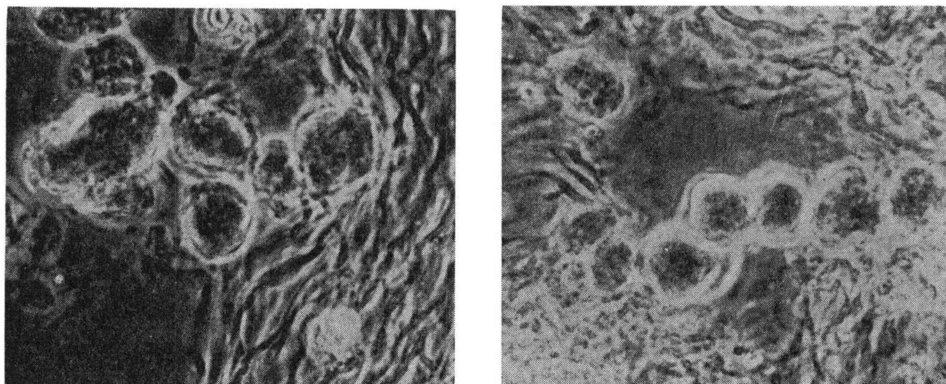
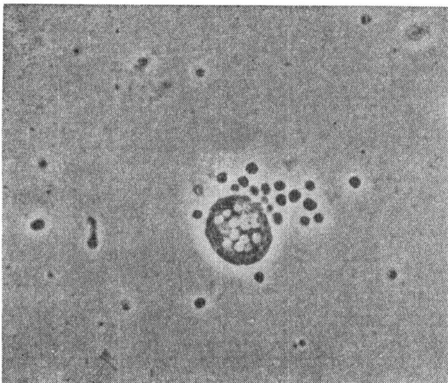


Table 1. Cultural characteristics of strain A/1668.
The number of some of the culture media refer to those given by SHIRLING and GOTTLIEB[1].

Culture medium	Cultural characteristics
Medium n. 2 (Yeast extract malt agar)	Abundant growth, wrinkled, light amber with violet zones
Medium n. 3 (Oatmeal agar)	Moderate growth, crusty, light violet
Medium n. 4 (Inorganic salts-starch agar)	Abundant growth, smooth surface, deep orange with violet zones
Medium n. 5 (Glycerol-asparagine agar)	Abundant growth, rough surface, amber with violet zones
Medium n. 6 (Peptone-yeast extract iron agar)	Scant growth, smooth surface, opaque, deep brown
Medium n. 7 (Tyrosine agar)	Moderate growth, rough surface, opaque, amber to light brown
Oatmeal agar	Abundant growth, wrinkled, orange with violet zones, weak violet pigment
HICKEY and TRESNER's agar	Moderate growth, crusty, light brown
CZAPEK glucose agar	Scant growth, thin, opaque, hyaline
Glucose asparagine agar	Abundant growth, crusty, light orange with violet zones
Nutrient agar	Scant growth, thin, orange to brown
Potato agar	Moderate growth, crusty, violet
BENNETT's agar	Abundant growth, wrinkled, light brown with surface violet
Calcium malate agar	Very scant growth, thin, light orange
Skimmed milk agar	Abundant growth, wrinkled, violet with orange edges
CZAPEK agar	Moderate growth, crusty, opaque, hyaline
Egg agar	Moderate growth, crusty, opaque, hyaline
Peptone glucose agar	Moderate growth, deep orange
Agar	Very scant growth, thin, hyaline
LOEFFLER-serum	Moderate growth, rough surface, orange with violet zones
Potato	Moderate growth, wrinked, deep orange
Gelatine	Scant growth, crusty, light violet
Cellulose agar	Very scant growth, thin, hyaline

Fig. 2. Spore liberation by means of the unoriented rupture of the sporangia (1,200×).



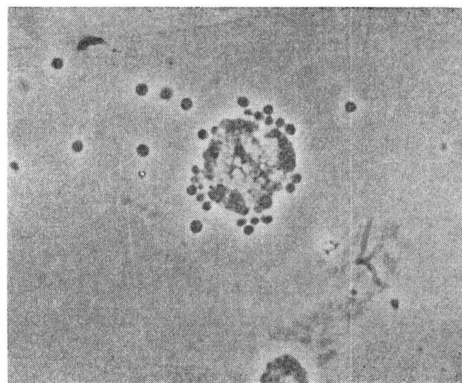


Table 2. Utilization of carbon compounds by strain A/1668.

Carbon source	Utilization*
Inositol	−
Fructose	+
Rhamnose	+
Mannitol	+
Xylose	+
Raffinose	−
Arabinose	+
Cellulose	−
Sucrose	+
Glucose	+
Mannose	+
Lactose	−
Salicin	+

* + = Positive utilization. − = no growth.

Table 3. Physiological characteristics of strain A/1668.

Test	Results
Hydrolysis of starch	positive
H_2S formation	weakly positive
Tyrosinase reaction	negative
Casein hydrolysis	positive
Solubilization of calcium malate	weakly positive
Nitrate reduction	positive
Liquefaction of gelatine	positive
Litmus milk coagulation	negative
Litmus milk peptonization	negative
Cellulose decomposition	negative
Chromogenic action	positive

Table 4. Cell-wall composition of strain A/1668.

Isomers of diaminopimelic acid		Amino acids			Sugars	
LL-DAP	Meso-DD-DAP	Aspartic acid	Glycine	Lysine	Arabinose	Xylose
−	++	−	++	trace	+	+

For the identification of amino acids the disrupted cells have been hydrolyzed in 6 N HCl at 100°C for 18 hours; for the identification of sugars the cells have been hydrolyzed in 2 N H_2SO_4 at 100°C for 2 hours.

The hydrolyzates have been examined by descending chromatography using paper Whatman No. 1.

addition of some media recommended by WAKSMAN[2] and the cultural characteristics are listed in Table 1. The strain grows well on different media with a violet color of the substrate mycelium; on oatmel agar produces a slightly diffusible violet pigment.

Microscopic Examination

Aerial mycelium is absent. The vegetative mycelium reveals branched hyphae with a diameter of ~1 μ. The sporangia abundantly formed on oatmeal agar and CZAPEK glucose agar are globose with irregular surface and diameter ranging from 4.0 to 10.0 μ (Fig. 1). They are supported by a short sporangiophore arising from the vegetative mycelium. Sporangial release is observed after rupture of the wall of sporangium (Fig. 2). The sub-spherical spore having a diameter of 1.4~1.8 μ are motile. On the basis of these characteristics the strain can be assigned to the genus *Actinoplanes* according to J. N. COUCH[3]. In Table 4 the analysis of cell-wall composition according to the method of BECKER *et al.*[4] is reported. The results are in agreement with the proposed assignment being the cell wall of type II and sugar pattern D according to M. P. LECHEVALIER and H. LECHEVALIER[5].

Physiological and Nutritional Characteristics

Utilization of carbon sources was examined according to the method of PRIDHAM and GOTTLIEB[6] and is shown in Table 2. Physiological characteristics are described in Table 3. The strain grows well from 28° to 37°C in media of pH ranging from 6.0 to 9.0 and no growth was observed at 50°C. The mycelium color is not affected by pH variation. Continuous illumination[7] during the growth phase influences slightly the color of the substrate mycelium that changes from violet to brownish-violet. When grown in submerged culture in media containing glucose the strain produces an abundant violet pigment.

Table 5. Chromatographic behavior of purpuromycin.

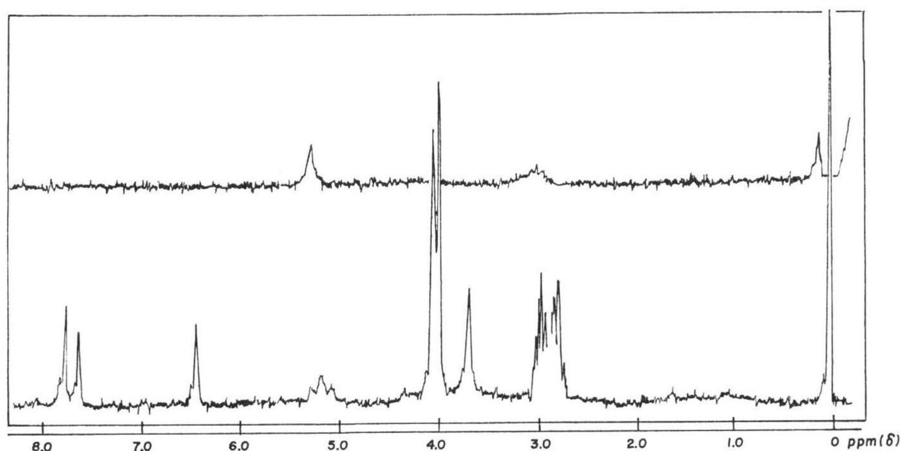
Solvent System	Rf
Water-saturated *n*-butanol	0.15
Water-saturated *n*-butanol+2% *p*-Toluensulphonic acid	0.68
Water-saturated *n*-butanol+2% concentrated ammonia	0.0
Ammonium chloride (20% solution in water)	0.0
n-Butanol - methanol - water (40 : 10 : 20) containing 0.75 g methyl orange	0.75
n-Butanol - methanol - water (40 : 10 : 30)	0.70
Water acetone (1 : 1)	0.48
Water-saturated ethyl acetate	0.98
Chloroform - methanol (95 : 5) (TLC)	0.55

Paper chromatography performed with descending technique on Whatman No. 1, antibiotic visualised on agar plates seeded with *S. aureus*, TLC chromatography performed on silica-gel plates to a distance of 10 cm.

Conclusions

This strain differs clearly from the other *Actinoplanes* strains described in the literature by its characteristics pigmentation. In fact *A. philippinensis*[8], *A. missouriensis*, *A. uthaensis*[3], *A. armeniacus*[9] and *A. brasiliensis*[10] have a yellow or orange vegetative mycelium and *A. italicus*[11] a cherryred vegetative mycelium. *A. taitomyceticus*[12] produces a violet pigment only on some media and differs from A/1668 in some of its morphological and physiological characteristics, as for instance the size of the sporangia. In view of the above-described characteristics and of its ability of producing purpuromycin, the strain A/1668 can be considered a new species of the genus *Actinoplanes* for which the name *Actinoplanes ianthinogenes** is proposed. The type strain A1668 has been

Ffg. 3. PMR spectrum of purpuromycin in DMF at 60 MHz.



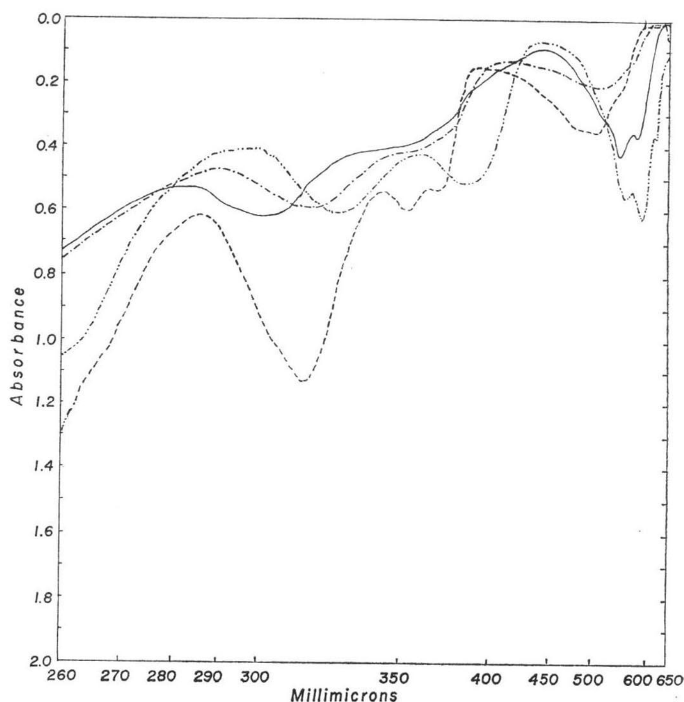
* From greek: *ianthinus*=violet, *ginnao*=to produce; *ianthinogenes*=producing violet.

deposited in the American Type Culture Collection (ATCC) under the number 21884.

Production of the Antibiotic

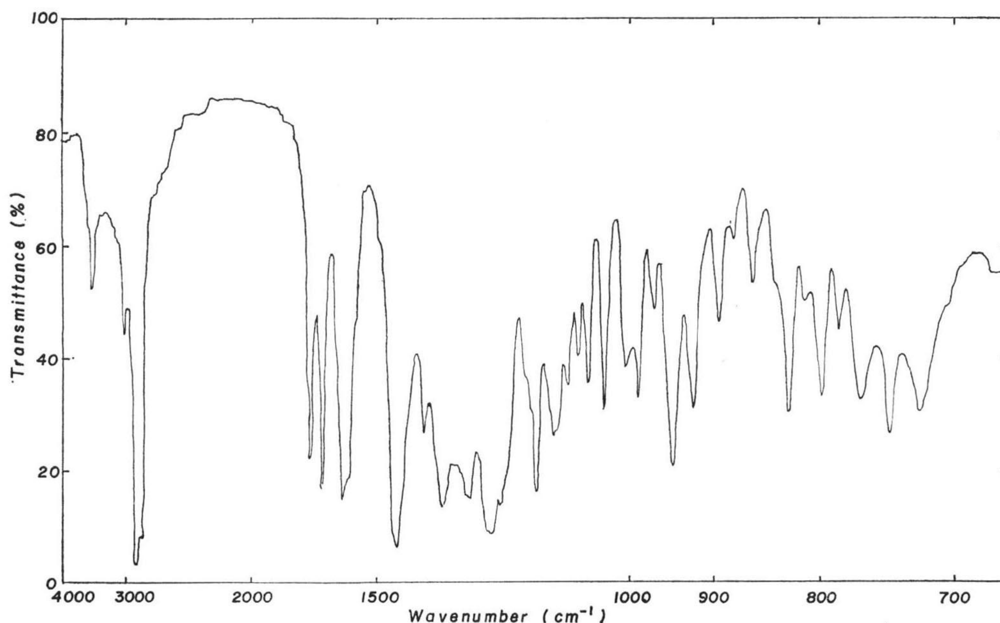
Fermentation conditions suitable for the production of the antibiotic were studied and the following media were found to be useful. Culture medium (g/liter): meat extract 3.0; yeast extract 10.0; starch 25.0; $CaCO_3$ 4.0. Fermentation medium (g/liter): meat extract 4.0; peptone 4.0; yeast extract 1.0; NaCl 2.5; soybean meal 10.0; glucose 50.0; $CaCO_3$ 5.0. For the production of the antibiotic jar fermentors containing 10 liters of fermentative medium were inoculated with one liter of a culture grown for 24 hours and incubated aerobically under

Fig. 4. Ultraviolet and visible absorption spectra of purpuromycin in chloroform and water* at different pH.



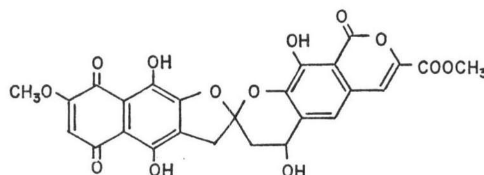
* The solutions in water have been obtained by dilution of an initial solution in DMF.

Fig. 5. Infrared spectrum of purpuromycin in nujol mull.



stirring at 28°C. The antibiotic production was followed with a microbiological assay perfomed by the agar diffusion method using *Staphylococcus aureus* as the test organism; maximum antibiotic activity was obtained after 96~120 hours of fermentation.

Fig. 6. Structure of purpuromycin.



Isolation and Purification of Purpuromycin

The culture broth (60 liters) of *A. ianthinogenes* was adjusted at pH 3.5 with 2% aqueous HCl and extracted with ethyl acetate (30 liters). By concentration of the organic extract at 45°C *in vacuo* a precipitate was obtained and collected by filtration (6 g); by addition of light petroleum to the filtrate, a further amount of crude compound was obtained (4 g). The two combined precipitates were treated with methanol (2 liters) and stirred for about 1 hour at room temperature. The fraction insoluble in methanol (5 g) was collected and dissolved in chloroform-methanol mixture (95 : 5); a small amount of silicagel was added to the solution, the solvent was evaporated from the suspension and the mixture added to the top of a silicagel column buffered with KH_2PO_4 0.5 M; chloroform and chloroform-methanol mixtures were used as eluents. The fraction corresponding to purpuromycin on the basis of color and tlc (see Table 5) was eluted with the mixture chloroform-methanol 98 : 2 and the product obtained was crystallized from the same solvent system (2 g).

Table 6. Antimicrobial activity of purpuromycin.

Microorganisms	Minimal inhibitory concentration μg/ml
Staphylococcus aureus ATCC 6538	0.005
Staphylococcus aureus Tour	0.01
Streptococcus hemolyticus C203	0.02
Diplococcus pneumoniae UC 41	0.02
Staphylococcus aureus Tour with 10% bovine serum	2.0
Clostridium perfringens ISS 30543	0.05
Shigella sonnei ATCC 9290	20.0
Proteus vulgaris X19 H ATCC 881	1.0
Klebsiella pneumoniae ISM	10.0
Escherichia coli SKF 12140	0.5
Escherichia coli MacLeod ATCC 19536	0.5
Salmonella typhimurium Kh.	10.0
Pseudomonas aeruginosa ATCC 10145	>100
Candida albicans SKF 2270	0.5
Trichophyton mentagrophytes SKF 17410	0.2
Mycobacterium tuberculosis H37Rv ATCC 9360	50
Mycoplasma gallisepticum H21 C. Z. B.	5.0
Staphylococcus aureus ATCC 6538 (PC-R)	0.005
〃 〃 〃 〃 (SM-R)	0.005
〃 〃 〃 〃 (TC-R)	0.005
〃 〃 〃 〃 (NB-R)	0.001
〃 〃 〃 〃 (ER-R)	0.002
〃 〃 〃 〃 (CAF-R)	0.001
〃 〃 〃 〃 (RF-R)	0.005

Abbreviations: R: resistant against 100 μg of the following antibiotics: PC: penicillin G; SM: streptomycin; TC: tetracycline; NB: novobiocin; ER: erythromycin; CAF: chloramphenicol; RF: rifamycin.

Physical and Chemical Properties

Purpuromycin obtained as described above is a red crystalline substance that decomposes at 212°C and does not melt up to 320°C. The elemental analysis gave C 57.90, H 3.38,

O 38.72 (by difference). The molecular formula is $C_{26}H_{18}O_{13}$ determined on the basis of microanalysis and hydrogen number in the PMR spectrum (Fig. 3). Functional group analysis showed the presence of two methoxyl and four hydroxyl groups. The product appeared to be unitary in paper and thin-layer chromatographic analysis and the Rf values obtained with different solvent systems are reported in Table 5. Purpuromycin is very soluble in aqueous sodium hydroxyde; fairly soluble in aqueous sodium carbonate, trifluoroacetic acid, dimethylformamide; sparingly soluble in aqueous sodium bicarbonate, acetic acid, ethyl acetate, dioxane, chloroform, acetone and insoluble in water and alcohols. The ultraviolet and visible absorption spectrum at different pH is reported in Fig. 4; an acidic function is present in the molecule with a pKa 6.8 spectrophotometrically determined. The IR spectrum is reported in Fig. 5. The product gives a complex with boroacetic acid[13] resulting in a bathocromic shift of the visible maxima and is easily reduced with Zn in dilute HCl and with $Na_2S_2O_4$, the $E_{1/2}$ value is −0.645 V determined in DMF with 50 % buffered solution (Br and Rb) at pH 9.8[14], both these characteristics are indicative of an hydroxyquinone moiety.

The absorption pattern in the visible and ultraviolet region indicates a close similarity of purpuromycin with the metabolites fusarubin and anhydrojavanicin[15] produced by fungi and with the antibiotics actinorhodin[16], griseorhodin[17] and rubromycin[18] produced by *Streptomyces*. The complete structure of the antibiotic has been determined[19] and is reported in Fig. 6.

Biological Properties

Purpuromycin is very active against Gram-positive bacteria and presents a good activity against Gram-negative bacteria and fungi. The minimum inhibitory concentrations against a variety of microorganism are given in Table 6. The product is active also against strains which are resistant to the other antibiotics widely used in the actual chemotherapeutical practice. Purpuromycin injected intraperitoneally into mice showed no toxicity up to 500 mg/kg, however no protection was observed when it was administered orally and subcutaneously at 300 mg/kg dosage in mice infected with *S. aureus*.

No antibiotic has been found in urine and serum, and residual product has been noticed in the site of injection after subcutaneous treatment.

Discussion

Purpuromycin is one of the few antibiotics isolated up to now from *Actinoplanes* and it resulted to be an hydroxy derivative of γ-rubromycin, an antibiotic isolated from *Streptomyces*. Although the two antibiotics are so similar in chemical structure they present a different biological profile. Rubromycins in fact are described[18] with an activity only on Gram-positive bacteria while purpuromycin shows good activity also on Gram-negative bacteria and fungi. The practical interest of the product is anyhow limited by two negative aspects: a tendency of the molecule to bind proteins resulting in a strong inactivation by serum (Table 6) and a very poor solubility that might explain the limited absorption of the product. The preparation of derivatives having a more favorable solubility characteristics and less affected by serum is under way.

Acknowledgements

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