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Mutactimycin PR, a New Anthracycline Antibiotic from *Saccharothrix* sp. SA 103

I. Taxonomy, Fermentation, Isolation and Biological Activities

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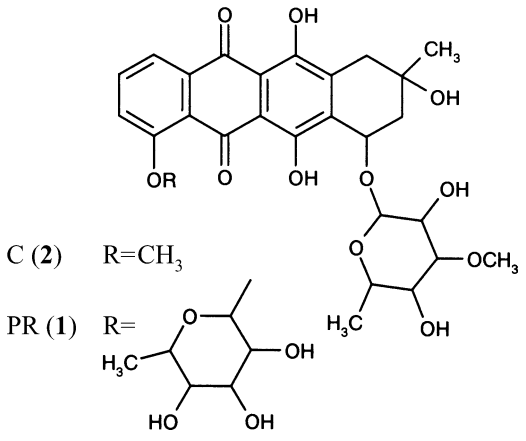
In the course of screening for new antibacterial agents, a new isolate collected from a soil sample of an arid area in south Algeria, produced a red pigment which was shown an antagonistic action against a Gram-positive bacterium *Bacillus subtilis*. The isolate was identified as *Saccharothrix* sp. and named SA 103. The red pigment, eluted by HPLC on reverse phase C_{18} column, contained two compounds of an anthracycline antibiotics group. The structure of the major product (**2**) was characterized as mutactimycin C, and PR (**1**) was a new member of this group, designated as mutactimycin PR. These compounds showed an antibiotic activity against certain Gram-positive bacteria *in vitro*. This is the first report of mutactimycins production by the genus *Saccharothrix*.

Antimicrobial agents are widely used in several fields: human therapy, veterinary, phytopathology, food industries and treatment of leather and wood. Many of these antibiotics are produced by microorganisms, and actinomycetes are likely the most important group of producing organismes. The *Streptomyces* are widely known to obtain antimicrobial agents, but over last several years, important products were obtained from other genera. One of the strategies for the screening of interesting isolates[1~5] and isolation of new antimicrobial agents[6~8] is to explore unusual ecosystems such as deep sea water, hyper saline areas, arid climates, *etc*. Areas of south Algeria represent the widest part of Sahara and can be of interest for these objectives. The few recent studies in these areas indicated an abundance of actinomycetes in soils, and several rare genera were detected.[1] In this context, and during our last study on these soils, we obtained several isolates of the genus *Saccharothrix*. One of these isolates, named *Saccharothrix* sp. SA 103, was investigated for its elaboration of a novel anthracycline antibiotic, designated as mutactimycin PR (**1**), and the known mutactimycin C (**2**) (Fig. 1). In this paper, the taxonomy of the producing strain, fermentation, isolation and biological activities of mutactimycin PR are described. The physicochemical

Fig. 1. Structures of mutactimycins PR (**1**) and C (**2**).



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properties and structure elucidation of mutactimycin PR are described in the following paper.[9]

Materials and Methods

Micro-organism

The producing micro-organism, strain SA 103, was obtained during a taxonomic study of actinomycetes of soils from arid regions of Algeria. It was isolated from a Saharan soil sample of Hoggar (region of Tamanrasset in the south Algeria) by suspending the soil in sterile distilled water and plating on Humic-vitamins B-agar[10] containing 50 μg/ml of actidione. A pure culture of strain SA 103 was preserved by lyophilisation. It was also maintained at 6°C for laboratory use as a slant on ISP medium No 2 (SHIRLING and GOTTLIEB).[11]

Taxonomy

Taxonomic characteristics of strain SA 103 were determined by cultivation on various media described by SHIRLING and GOTTLIEB[11] and WAKSMAN.[12] Morphological characteristics were determined after growth at 30°C for 14 days. The color names and hue numbers were assigned using the ISCC-NBS centroid color chart (U. S. National Bureau of Standard, 1976). Detailed observation of mycelial and spore morphologies was performed with the use of scanning electron microscopy (Hitachi, model S-450). Physiological properties were examined by the methods of GOODFELLOW[13] and WAKSMAN.[12] The type of diaminopimelic acid isomers in the cell wall, and the whole-cell sugars composition were determined by the methods of BECKER *et al.*[14], and LECHEVALIER and LECHEVALIER[15]. Phospholipids and mycolic acids were analyzed by the procedure of MINNIKIN *et al.*[16,17]

Fermentation

A loopful of strain SA 103 from a mature slant culture was inoculated into a 250-ml Erlenmeyer flask containing 50 ml of sterile seed medium consisting of glucose 0.4%, malt extract 1% and yeast extract 0.4% (adjusted to pH 7.2 before sterilization) and cultured on a rotary shaker (250 rpm) at 30°C for 2 days. For the production of antibiotics, 3 ml of the seed culture were transferred into 500-ml Erlenmeyer flasks, each containing 100 ml of the above medium, and cultivated for 10 days using the same conditions. The assay of whole antibacterial activity was carried out on nutrient agar medium, by an agar diffusion assay against *Bacillus subtilis* ATCC 6633. Growth inhibition was examined after 24-hour incubation at 30°C. The antimicrobial activity was estimated by measuring the diameter of inhibitory zone. Mycelium dry weight was determined in Eppendorf cups filled with 1 ml homogenized culture broth and dried at 105°C for 24 hour (PFEFFERLE *et al.*).[18]

Purification of Antibiotics

The culture broth (8 liters) was centrifuged at 12000 rpm for 15 minutes, and the supernatant was extracted twice with an equal volume of *n*-butanol. The organic phase was dehydrated by Na_2SO_4 and dried *in vacuo* using a rotary evaporator. The red crude extract obtained was suspended in a minimum volume of MeOH, filtered through a 45 μm pore size filter and then eluted on HPLC under the following conditions to obtain the pure products: Uptisphere UP5ODB C_{18} column (250×7.8 mm i.d., Interchrom); mobile phase, isocratic at 63% MeOH in H_2O; flow rate, 1.5 ml/minute; UV detection, 220 nm. The active peaks were detected by agar diffusion method against *Bacillus subtilis*.

Biological Assay

The minimum inhibitory concentrations (MIC) of the antibiotics were determined by a conventional agar dilution method using nutrient agar medium. The antimicrobial activity was observed after 24~48 hour incubation at 30°C.

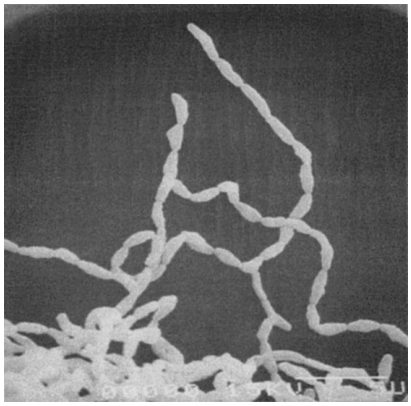
Results and Discussion

Taxonomic Characterization of the Producing Strain

Strain Morphology

The strain SA 103 formed a well-developed pink aerial

Photo 1. Scanning electron micrography of the aerial mycelium of the strain *Saccharothrix* sp. SA 103 grown on ISP No 2 for 10 days at 30°C.



mycelium which fragmented into long straight or flexuous spore chains. The spores were rod-shaped and 1.9~2.9×0.6~0.7 μm in size with a smooth surface (Photo 1). Endospore, sclerotic granules, synnemata and flagelled spores were not observed. The substrate mycelium was brownish red to deep red and showed no or moderate fragments. The strain produced a characteristic abundant dark red pigment that was revealed as antibacterial antibiotics.

Cultural Characteristics

Table 1 shows the cultural characteristics of the strain SA 103 on different media plates. The growth of the strain was abundant on yeast extract - malt extract agar, Bennett and nutrient agar but was moderate on oatmeal agar and inorganic salts - starch agar. The color of mycelia ranged between yellowish pink to light reddish brown for aerial hyphae and brownish orange to very deep red for the substrate mycelium. The strain produced a dark red or brownish orange soluble pigment on all media used, but no melanoid pigments were observed.

Chemotaxonomy

The chemotaxonomic study showed the presence of *meso*-diaminopimelic acid and the absence of glycine (cell-wall type III). The whole-cell sugar pattern consisted in rhamnose and galactose (cell sugar type E)[19] and the characteristic phospholipid was phosphatidyl-ethanolamine (phospholipids type PII). No mycolic acids were detected.

Strain Physiology

The results of physiological tests are reported in Table 2. The strain utilized many organic compounds and was resistant to crystal violet (0.001%), lysozyme (0.005%), phenol (0.05%), potassium tellurite (0.01%), sodium azide (0.001%) and to few antibiotics. Permissive temperature for growth ranged from 15 to 48°C, with the optimal temperature at 30°C.

Classification

Based on the morphological and the chemical properties described above, strain SA 103 was considered to belong to the genus *Saccharothrix*.[20] Compared to the nearest species *Saccharothrix syringae* NRRL B-16 468^T, the strain SA 103 differed by the ability to degrade lactose but not hypoxanthine and sodium butyrate, by susceptibility to erythromycin, gentamicin, oxytetracycline and vancomycin, by resistance to penicillin, rifampicin, crystal violet and sodium azide, and by the growth at pH 5.0. Therefore the strain was designated as *Saccharothrix* sp. SA 103.

Fermentation

The time course of the antibacterial activity production

Table 1. Cultural characteristics of *Saccharothrix* sp. SA 103.

Medium	Growth	Aerial hyphae	Substrate mycelium	Diffusible pigment
Yeast extract-malt extract agar (ISP No 2)	Good	Abundant Moderate yellowish pink (29)	Very deep red (14)	Dark red (16)
Oatmeal agar (ISP No 3)	Moderate	Moderate Pale yellowish pink (31)	Brownish orange (54)	Brownish orange (54)
Inorganic salts-starch agar (ISP No 4)	Moderate	Moderate Brownish pink (33)	Brownish orange (54)	Brownish orange (54)
Bennett agar	Good	Abundant Light reddish brown (42)	Dark reddish brown (44)	Dark reddish brown (44)
Nutrient agar	Good	Moderate to abundant Yellowish pink (29)	Deep red (13)	Deep red (13)

Table 2. Physiological characteristics of strain *Saccharothrix* sp. SA 103.

Degradation of		Nitrate reduction		+
Adenine	-	Production of melanoid		
Arbutin	+	pigments		-
Casein	+	Decarboxylation of sodium		
Gelatin	+	Acetate		+
Esculin	+	Benzoate		-
Guanine	-	Butyrate		-
Hypoxanthine	-	Citrate		+
Starch	+	Oxalate		-
Testosterone	+	Propionate		+
Tween 80	+	Pyruvate		+
Tyrosine	+	Succinate		+
Xanthine	-	Tartrate		-
Adonitol	-	Growth at		
L-Arabinose	+	48°C		+
Cellobiose	+	pH 5.0		+
Dextrin	+	pH 9		+
Dulcitol	-	Tolerance to		
Erythritol	-	Crystal violet	(0.001%)	+
D-Fructose	+	Lysozyme	(0.005%)	+
Galactose	+	phenol	(0.05%)	+
D-Glucose	+	Phenol	(0.1%)	-
Glycerol	+	Potassium tellurite	(0.01%)	+
Inositol	-	Sodium azide	(0.001%)	+
Lactose	-	Sodium azide	(0.01%)	-
Maltose	+	Sodium chloride	(5%)	-
D-Mannitol	+	Resistance to		
D-Mannose	+	Chloramphenicol	(25 μg/ml)	-
Melezitose	-	Cycloserine	(10 μg/ml)	-
Melibiose	-	Erythromycin	(10 μg/ml)	-
α-Methyl-D-glucoside	-	Gentamicin	(10 μg/ml)	-
D-Raffinose	-	Kanamycin	(25 μg/ml)	-
L-Rhamnose	+	Novobiocin	(10 μg/ml)	-
Ribose	+	Oxytetracycline	(25 μg/ml)	+
Sorbitol	-	Penicillin	(25 μg/ml)	+
Sucrose	+	Rifampicin	(5 μg/ml)	-
Trehalose	+	Streptomycin	(10 μg/ml)	-
D-Xylose	+	Vancomycin	(5 μg/ml)	-

Fig. 2. Fermentation profile of *Saccharothrix* sp. SA 103.

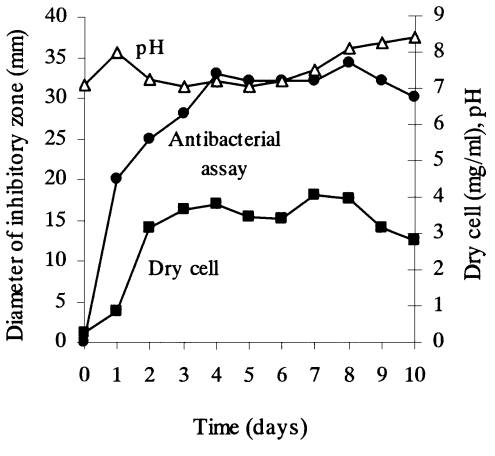
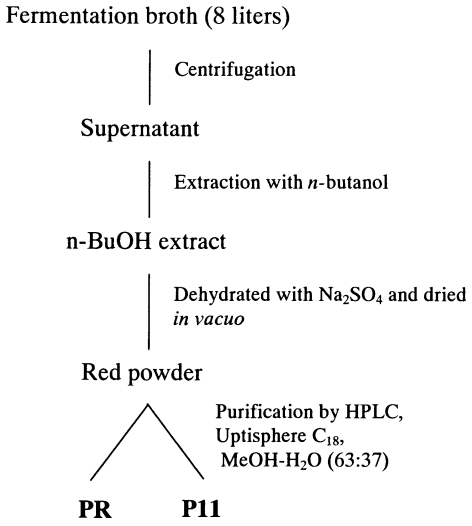


Fig. 3. Bioassay-guided isolation of active products from strain SA 103.



by *Saccharothrix* sp. SA 103 is presented in Fig. 2. The production of biological activity against *B. subtilis* started during the first day and peaked at day four to a stable level. The biomass increased during the first three days, remained stable, then decreased after day eight. The pH kinetic showed a little variation to alkali during the first day, then returned in the neutral, and increased at the end of the fermentation. Generally the production of secondary metabolites by micro-organisms occurs during stationary phase but in our case the production of biological activity was strongly correlated to the growth and was observed all along the culture time course. The same production kinetic has been observed for dithiolopyrrolone antibiotics production by *Saccharothrix* sp. SA 233[6] and clavulanic acid production by *Streptomyces clavuligerus*.[21]

Purification of Antibiotics

The different steps of antibiotics isolation and purification are summarized in Fig. 3. Eight liters of the culture broth obtained were extracted twice with an equal volume of *n*-butanol to extract the entire active products. The elution profile of the red butanolic extract on HPLC revealed two peaks, P11 and PR. The major component P11 was eluted at a retention time of 35.65 minutes and identified as mutactimycin C, an antibiotic produced by the mutant strain 113 of *Streptomyces* sp.[22,23] The component PR was eluted at a retention time of 31.74 minutes, and was

Table 3. Antimicrobial spectrum of mutactimycin PR and C.

Test organisms	MIC (μg/ml)	
	PR (1)	C (2)
Bacillus subtilis ATCC 6633	75	40
Micrococcus luteus ATCC 9314	50	5
Staphylococcus aureus CIP 7625	>100	>100
Staphylococcus aureus CIP 53156	50	10
Listeria monocytogenes CIP 82110	>100	50
Mycobacterium smegmatis ATCC 607	>100	75
Klebsiella pneumoniae CIP 82.91	40	5
Escherichia coli ATCC 10536	>100	>100
Pseudomonas syringae No 1882	>100	>100
Agrobacterium tumefaciens No 2410	>100	>100
Mucor ramannianus NRRL 1829	>100	>100
Saccharomyces cerevisiae ATCC 4226	>100	>100

found to be a new derivative, named mutactimycin PR. The mutactimycins were anthracycline antibiotics group produced by the mutant strain 113 of *Streptomyces* sp.,[22~24] *Nocardia brasiliensis*[25], *Streptomyces* sp. 80-115,[26] *Nocardia transvalensis* and *Streptomyces* sp. GW 60/1571.[27] This is the first report of mutactimycins production in the genus *Saccharothrix*. All described mutactimycins contain one sugar moiety, but as demonstrated in the accompanying publication PR contained two sugars.[9]

Antimicrobial Assay

The antibacterial activity of the new antibiotic PR (**1**) compared with mutactimycin C (**2**) is shown in Table 3. The antibiotics showed the same activity spectrum as some other known mutactimycins.[23] They exhibited moderate activity against Gram-positive bacteria, except *Staphylococcus aureus* CIP 7625 was resistant. No activity was observed against Gram-negative bacteria (except *Klebsiella pneumoniae*), *Saccharomyces cerevisiae* and *Mucor ramannianus*. The compound **1** exhibited lower activity than the compound **2**.

Acknowledgements

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