VOL. 55 NO. 8, AUG. 2002 THE JOURNAL OF ANTIBIOTICS pp. 696 — 701

New Dithiolopyrrolone Antibiotics from *Saccharothrix* sp. SA 233

I. Taxonomy, Fermentation, Isolation and Biological Activities

LYNDA LAMARI[a,b], ABDELGHANI ZITOUNI[d,a], HADJIRA BOUDJELLA[a,b], BOUBEKEUR BADJI[a,b], NASSERDINE SABAOU[a,b,*], AHMED LEBRIHI[d], GÉRARD LEFEBVRE[c], ELISABETH SEGUIN[e] and FRANÇOIS TILLEQUIN[f]

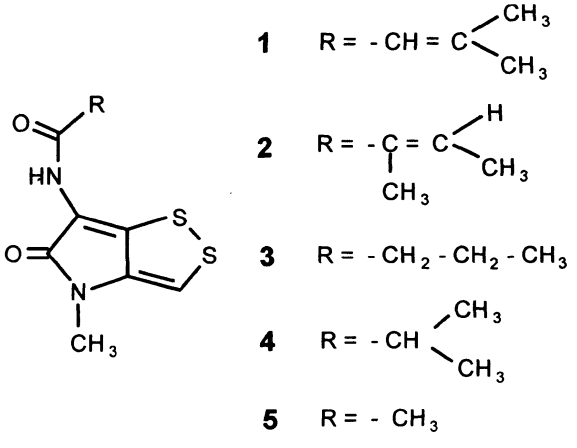
[a] Laboratoire de Recherche sur les Produits Bioactifs et la Valorisation de la Biomasse, Ecole Normale Supérieure de Kouba, B.P. 92, 16 050 Vieux-Kouba, Alger, Algérie
[b] Centre de Recherche Scientifique et Technique sur les Régions Arides, Front de l'Oued, B.P. 1682, 07 000 Biskra, Algérie
[c] Ecole Nationale Supérieure d'Agronomie et des Industries Alimentaires, INPL, 2 avenue de la Forêt de Haye, F-54 000 Nancy, France
[d] Ecole Nationale Supérieure d'Agronomie de Toulouse, INPT, Laboratoire de Génie Chimique, UMR 5503 (CNRS/INPT/UPS), 1, avenue de l'Agrobiopôle, B.P. 107, F-31 326 Castanet-Tolosan Cedex, France
[e] Laboratoire de Pharmacognosie de l'Université de Rouen-Haute Normandie, Faculté de Pharmacie, 22, Boulevard Gambetta, F-76 183 Rouen Cedex 1, France
[f] Laboratoire de Pharmacognosie de l'Université René Descartes, UMR/CNRS n° 8638, Faculté des Sciences Pharmaceutiques et Biologiques, 4, Avenue de l'Observatoire, F-75 006 Paris, France

(Received for publication April 30, 2002)

Three new natural antibacterial and antifungal dithiolopyrrolone antibiotics were isolated along with the known iso-butyropyrrothine and thiolutine from the fermentation broth of an actinomycete strain which was isolated from a saharian palm grove soil collected at Adrar, south Algeria. The strain was identified as *Saccharothrix* sp. The three new antibiotics exhibited broad antimicrobial activity against Gram-positive bacteria, yeasts and fungi *in vitro*.

In recent years, the increasing prevalence of infectious diseases resistant to chemotherapy has caused an urgent need for new antibiotics. In this context, rare *Actinomycetales* appear as a promising source of new antibacterial and antifungal compounds. We are currently involved in a screening for the isolation of new antibiotic agents from rare microorganisms presents in the soils of the palm groves of southern Algeria, which are characterized by a high diversity of actinomycetes.[1,2] As part of this program we discovered three novel antibiotics belonging to the pyrrothine group designated as PSA (**1**), PSB (**2**) and PSC_2 (**3**) along with the known iso-butyropyrrothine (**4**) and thiolutin (**5**) (Fig. 1), from the culture broth of *Saccharothrix* strain SA 233. This paper deals with the taxonomy of the producing strain, fermentation, isolation and biological activities of the compounds. Physico-chemical properties and structure elucidation of the antibiotics are described in the accompanying paper.[3]

Fig. 1. Dithiolopyrrolone antibiotics from *Saccharothrix* sp. SA 233.



* Corresponding author: sabaou@yahoo.fr

Materials and Methods

Micro-organism

Strain SA 233 was isolated from a saharian soil sample collected at a palm grove in Adrar, Algeria by a dilution agar plating method using Humic-vitamins B-agar[4] supplemented with streptomycin sulphate (10 μg/ml) and actidione (50 μg/ml).

Taxonomic Studies

Morphological, cultural and physiological characteristics of the strain SA 233 were examined according to the methods described by GOODFELLOW[5], SHIRLING and GOTTLIEB[6] and WAKSMAN.[7] Color of aerial and substrate mycelia were determined with the ISCC-NBS centroid color charts (U. S. National Bureau of Standard, 1976). Cultural characteristics were observed on various media at 30°C for 14~21 days. Detailed observation of mycelial morphologies was performed with the use of scanning electron microscope (Cambridge Stereoscan, model 240). Chemical composition of the cell was analyzed by the methods of BECKER *et al.*[8] and LECHEVALIER and LECHEVALIER.[9] Phospholipids and mycolic acids were analyzed by using the procedures of MINNIKIN *et al.*[10,11]

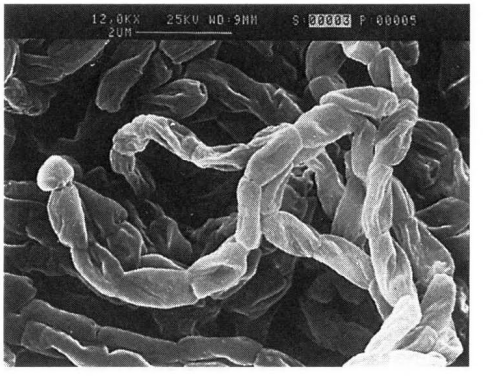
Fermentation

A mature slant culture of the strain SA 233 was inoculated into 250-ml Erlenmeyer flasks, each containing 50 ml of seed medium consisting of glucose 0.4%, malt extract 1% and yeast extract 0.4% (pH 7.2 before sterilization). The culture was incubated on a rotary shaker (250 rpm) at 28°C for 2 days. The seed culture (50 ml) of the strain was transferred into a 2.5-liter fermentor containing 1.5 liters of the same medium. The fermentation was carried out at 28°C for 7 days under aeration of 3 liters/minute and agitation at 375 rpm. The fermentation procedure was repeated to obtain a total of 12 liters of culture broth.

Analytical Procedure

The culture broth (12 liters) was centrifuged and the supernatant was extracted twice with 12 liters of CH_2Cl_2. The extract was evaporated *in vacuo* to dryness. The yellow residue was washed with *n*-hexane, partially purified on preparative silica gel 60 plates and eluted by a mixture of EtOAc and MeOH (100 : 15). The purification steps and the content of the compounds was monitored by reverse phase HPLC under the following conditions: Uptisphere UP15WOD C_{18} column (300×7.8 mm i.d., Interchim); mobile phase MeOH - H_2O (50 : 50); flow rate, 2 ml/minute; detection, UV (220 nm).

Photo 1. Scanning electron micrograph of *Saccharothrix* sp. SA 233 grown on ISP No. 2 for 7 days at 30°C.



Antimicrobial Activities

The minimum inhibitory concentrations (MIC) of the antibiotics were determined by a conventional agar dilution method using nutrient - 1% glucose agar for bacteria and Sabouraud's medium for fungi and yeasts. The antimicrobial activity was observed after 24~48 hours incubation at 37°C for bacteria and 48~72 hours incubation at 28°C for fungi and yeasts.

Results and Discussion

Taxonomic Characterization of the Producing Strain

Morphological Characteristics

The strain SA 233 produced well-developed and branched substrate mycelium that showed no or little fragmentation on agar media and in broth media. Aerial mycelium were divided irregularly into long, straight, flexuous, open loops and hooks spore chains which had 10 to 50 or more spores per chains. The spores were non motile, 0.7~0.8×1~2 μm in size. Scanning electron micrograph of the spores exhibited smooth surfaces (Photo 1). No endospores, sporangia, sclerotia or synnemata were formed.

Cultural and Physiological Characteristics

The cultural characteristics of strain SA 233 on some agar media are shown in Table 1. The aerial mycelium grew abundantly on Bennett agar medium, yeast extract - malt

Table 1. Cultural characteristics of strain SA 233.

Medium	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigment
Tryptone-yeast extract agar (ISP No 1)	Good	None	Moderate yellowish brown (77)	Moderate yellowish brown (77)
Yeast extract-malt extract agar (ISP No 2)	Good	Abundant Pale orange yellow (73)	Strong yellow (84)	Dark yellow (88)
Oatmeal agar (ISP No 3)	Moderate	Poor Yellowish white (92)	Vivid yellow (82)	Vivid yellow (82)
Inorganic salts-starch agar (ISP No 4)	Poor	Poor Yellowish white (92)	Yellowish white (92)	None
Glycerol-asparagine agar (ISP No 5)	Good	Good Pale orange yellow (73)	Light orange yellow (70)	None
Peptone-yeast extract-iron agar (ISP No 6)	Good	None	Moderate yellowish brown (77)	None
Tyrosine agar (ISP No 7)	Good	Good Pale orange yellow (73)	Dark orange yellow (72)	None
Bennett agar	Good	Good Yellowish gray (93)	Dark yellowish brown (78)	Dark yellowish brown (78)
Nutrient agar	Moderate	None	Moderate yellowish brown (77)	None

extract agar, glycerol-asparagine agar and tyrosine agar. The color of the aerial mycelium was yellow orange, while that of the reverse side was vivid yellow, orange yellow or yellowish brown. A bright yellow soluble pigment was produced, but no melanoid pigments were observed. The physiological properties of the strain SA 233 are summarized in Table 2. The strain utilized few carbohydrates for growth (D-fructose, galactose, D-glucose, glycerol and trehalose) and was resistant to lysozyme (0.05%). Permissive temperatures for growth ranged from 20 to 45°C, with the optimal temperature at 30°C.

Chemotaxonomic Characteristics

The cell-wall hydrolysates contained *meso*-diaminopimelic acid but not glycine (cell-wall type III). The whole-cell sugar pattern consisted in rhamnose and galactose (cell sugar pattern E)[12] in addition to glucose, mannose and ribose. The diagnostic phospholipid detected was phosphatidyl-ethanolamine (phospholipids type PII). Mycolic acids were not present.

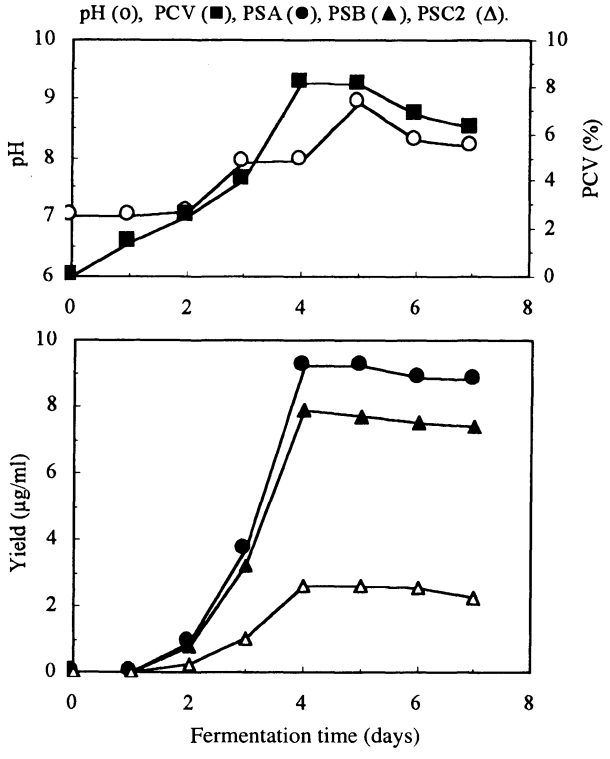
Classification

Based on the morphological and chemical characteristics described above, strain SA 233 is considered to belong to the genus *Saccharothrix*[13]. The comparison with the nearest species, *S. australiensis*,[13] showed differences in the cultural characteristics (color of aerial and substrate mycelia and color of diffusible pigments) and in the utilization of cellobiose, dextrin, mannose, sorbitol, erythritol, sodium citrate and sodium propionate.

Table 2. Physiological characteristics of strain SA 233.

Hydrolysis of		Utilization of	
Adenine	-	Acetate	+
Casein	+	Citrate	+
Esculin	+	Propionate	-
Gelatin	+	Adonitol	-
Hypoxanthine	-	L-Arabinose	-
Starch	-	Cellobiose	-
Testosterone	-	Erythrithol	-
Tyrosine	+	D-Glucose	+
Xanthine	-	D-Fructose	+
Nitrate reduction	+	Galactose	+
Tolerance to		Dextrin	-
Lysozyme (0.05%)	+	Glycerol	+
NaCl (5%)	-	Inositol	-
Growth at 45°C	+	D-Mannitol	-
Resistance to		D-Mannose	-
Gentamicine (5 μg/ml)	+	Lactose	-
Kanamycine 25 μg/ml)	-	Raffinose	-
Oxytetracyline (25 μg/ml)	+	L-Rhamnose	-
Streptomycine (10 μg/ml)	+	Sucrose	-
Vancomycin (5 μg/ml)	+	Trehalose	+
Production of melanoid pigments	-	Xylose	-

Fig. 2. Time course production of new dithiolopyrrolone antibiotics.



PCV: Packed cell volume.

Therefore, this strain was designated as *Saccharothrix* sp. SA 233. Detailed taxonomic study of strain SA 233 is now progress. Strain SA 233 has been deposited in the Agricultural Research Service Culture Collection, Peoria, U.S.A., with an accession number of NRRL B-24137.

Fermentation

A typical time course fermentation by *Saccharothrix* sp. SA 233 in a 1.5-liter fermentor is illustrated in Fig. 2. The production of the new compounds PSA (**1**), PSB (**2**) and PSC2 (**3**) began after 2 days of fermentation, and their concentrations reached levels of 9.2, 7.9 and 2.6 mg/liter respectively at day 4. The pH of the culture broth gradually increased from the beginning of fermentation, reached a maximum at 5 days and decreased after 6 days.

Isolation and Purification

The procedure of isolation of antibiotics is described in Scheme 1.

The culture filtrate obtained from the 4-day culture broth (12 liters) was extracted twice with an equal volume of CH_2Cl_2 and the CH_2Cl_2 layer was dehydrated with Na_2SO_4 and concentrated *in vacuo*. After washing with *n*-hexane, the crude extract was charged to preparative silica gel 60 (Merck) plates followed by elution with EtOAc-MeOH (100 : 15). Two active bands were obtained, the yellow (AJ) and the yellow-orange (PS) bands showing Rf values of 0.52 and 0.59, respectively. After elution with MeOH, crude AJ and crude PS were obtained and further purified by HPLC as described in Materials and Methods. AJ is composed by one component and was eluted at a retention time of 3.6 minutes. PS contains 4 components, PSA (**1**) (28 mg), PSB (**2**) (24 mg), PSC_2 (**3**) (8 mg) and PSC_1 (**4**) (4 mg) which were eluted at retention times of 11.0, 10.0, 7.1 and 6.5 minutes, respectively. The major component AJ (**5**) (88 mg) was identified as thiolutin, an antibiotic produced by many strains and species of *Streptomyces* such *S. albus*, *S. celluloflavus*, *S. kasugaensis*. *S. luteoreticuli*, *S. pimprina* and *S. thioluteus*[14,15]. PSC_1 (**4**) was identified as iso-butyropyrrothine, previously isolated from *S. pimprina* strain[16]. However, PSA (**1**), PSB (**2**) and PSC_2 (**3**) were found to be new derivatives. The known antibiotics thiolutin (**5**) and iso-butyropyrrothine (**4**), as well as the

Scheme 1. Isolation procedure of PSA (**1**), PSB (**2**), PSC_2 (**3**), PSC_1 (**4**) and AJ (**5**).

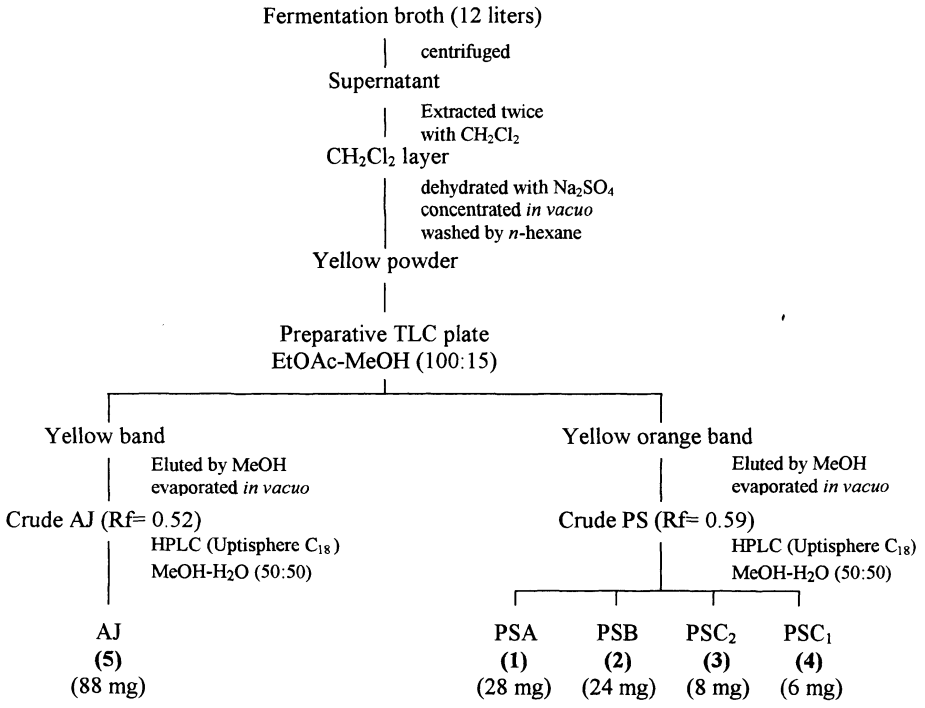


Table 3. Antimicrobial spectrum of antibiotics.

Test organisms	MIC (μg/ml)				
	PSA (1)	PSB (2)	PSC_2 (3)	PSC_1 (4)	AJ (5)
Bacillus coagulans CIP 6625	<0.2	0.5	1	3	<0.2
Bacillus subtilis ATCC 6633	1	4	5	20	2
Micrococcus luteus ATCC 9314	1	1	2	<0.2	<0.2
Staphylococcus aureus CIP 7625	50	40	40	75	20
Klebsiella pneumoniae CIP 82.91	1	5	1	5	1
Escherichia coli ATCC 10536	>100	>100	>100	>100	>100
Salmonella enterica CIP 81.3	>100	>100	>100	>100	>100
Pseudomonas aeruginosa CIP A22	>100	>100	>100	>100	>100
Pseudomonas syringae No 1882	>100	>100	100	100	100
Agrobacterium tumefaciens No 2410	>100	>100	100	100	100
Mucor ramannianus NRRL 1829	2	4	5	5	10
Penicillium sp.	20	10	20	30	20
Alternaria sp.	20	5	20	20	20
Fusarium oxysporum f.sp. *albedinis*	20	20	20	30	30
Fusarium oxysporum f.sp. *lini* Foln 3-5	20	20	40	40	40
Fusarium equiseti	10	20	10	20	10
Fusarium culmorum	4	5	10	20	10
Fusarium graminearum	4	10	10	10	10
Candida albicans IPA 200	20	20	20	30	20
Saccharomyces cerevisiae ATCC 4226	2	2	3	10	10

three novel compounds **1**~**3** are all *N*-acyl derivatives of 6 amino-4-methyl-1,2-dithiolo[4,3-b]pyrrol-5[4*H*]-one. This is the first report of secondary metabolites deriving from the dithiolopyrrolone basic skeleton in the genus *Saccharothrix*.

Antimicrobial Activity

The antimicrobial activity of the new antibiotics PSA (**1**), PSB (**2**) and PSC_2 (**3**) are shown in Table 3 in comparison with thiolutin (**5**) and iso-butyropyrrothine (**4**). The antibiotics showed a high activity against Gram-positive bacteria such as *Bacillus coagulans*, *Bacillus subtilis* and *Micrococcus luteus*, except for *Staphylococcus aureus* (MIC: 20~75 μg/ml). PSA (**1**) and PSB (**2**) showed a greater activity than thiolutin (**5**) and iso-butyropyrroyhine (**4**) against *Saccharomyces cerevisiae*, *Mucor ramanniamus* and the phytopathogenic fungi *Fusarium oxysporum* f. sp. *albedinis*, *F. o.* f. sp. *lini* and *F. culmorum*. All antibiotics had no or weak activity against Gram-negative bacteria except for *Klebsiella pneumoniae* which was strongly inhibited.

Acknowledgments

This work was supported in part by the "Agence Nationale pour le Développement de la Recherche Universitaire" (The Ministry of Education and Scientific Research) which is gratefully acknowledged.

References

1) SABAOU, N.; H. HACENE, A. BENNADJI, H. BENNADJI & N. BOUNAGA: Distribution qualitative et quantitative des actinomycètes dans les horizons de sol de surface et profonds d'une palmeraie algérienne. Can. J. Microbiol. 38: 357~360, 1992
2) HACENE, H.; N. SABAOU, N. BOUNAGA & G. LEFEBVRE: Screening for non-polyenic antifungal antibiotics produced by rare *Actinomycetales*. Microbios 79: 81~85, 1994
3) LAMARI, L.; A. ZITOUNI, T. DOB, N. SABAOU, A. LEBRIHI, P. GERMAIN, E. SEGUIN & F. TILLEQUIN: New dithiolopyrrolone antibiotics from *Saccharothrix* sp. SA 233. II. Physicochemical properties and structure elucidation. J. Antibiotics 55: 702~706, 2002
4) HAYAKAWA, M. & H. NONOMURA: Humic acid-vitamin agar, a new medium for the selective isolation of soil actinomycetes. J. Ferment. Technol. 65: 501~509, 1984
5) GOODFELLOW, M.: Numerical taxonomy of some nocardioform bacteria. J. Gen. Microbiol. 69: 33~90, 1971
6) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
7) WAKSMAN, S. A.: Classification, identification and descriptions of genera and species. *In* The Actinomycetes, Vol. II, The Williams & Wilkins Co., Baltimore, 1961
8) BECKER, B.; M. P. LECHEVALIER, R. E. GORDON & H. A. LECHEVALIER: Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole-cell hydrolysates. Appl. Microbiol. 12: 421~423, 1964
9) LECHEVALIER, M. P. & H. A. LECHEVALIER: Composition of whole-cell hydrolysates as a criterion in the classification of aerobic actinomycetes. *In* The *Actinomycetales*, *Ed.*, H. PRAUSER, pp. 311~316, Fisher Verlag, Jena, 1970
10) MINNIKIN, D. E.; P. V. PATEL, L. ALSHAMAONY & M. GOODFELLOW: Polar lipid composition in the classification of *Nocardia* and related bacteria. Int. J. Syst. Bacteriol. 27: 104~107, 1977
11) MINNIKIN, D. E.; I. G. HUTCHINSON, A. B. CALDICOTT & M. GOODFELLOW: Thin-layer chromatography of methanolysates of mycolic acid-containing bacteria. J. Chromatography 188: 221~233, 1980
12) KROPPENSTEDT, R. M.: The genus *Nocardiopsis*. *In* The Procaryotes. *Ed.*, A. BALOWS *et al.*, pp. 1139~1156, Springer Verlag, Berlin, 1992
13) LABEDA, D. P.; R. T. TESTA, M. P. LECHEVALIER & H. A. LECHEVALIER: *Saccharothrix*: new genus of the *Actinomycetales* related to *Nocardiopsis*. Int. J. Syst. Bacteriol. 34: 426~431, 1984
14) YAMAGISHI, S., Y. KOYAMA, Y. FUKAKUSA, N. KYOMURA, J. I. OHISHI, N. HAMAMISHI & T. ARAI: On the metabolites of *Streptomyces luteoreticuli* KATOH et ARAI. I. Isolation of the metabolites. Yakugaku Zasshi 91: 351~357, 1971
15) FURUMAI, T.; K. TAKEDA & M. OKANISHI: Function of plasmids in the production of aureothricin. I. Elimination of plasmids and alteration of phenotypes caused by protoplast regeneration in *Streptomyces kasugaensis*. J. Antibiotics 35: 1367~1373, 1982
16) BHATE, D. S.; R. K. HULYALKAR & S. K. MENON: Isolation of iso-butyropyrrothine along with thiolutin and aureothricin from a *Streptomyces* sp. Experimentia 16: 504~505, 1960

Dedication

This work is in honour and memory of Professor GÉRARD LEFEBVRE (Institut National Polytechnique de Lorraine, ENSAIA, Nancy, France) who was actively involved in this research.