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IB-96212, a Novel Cytotoxic Macrolide Produced by a Marine *Micromonospora*

I. Taxonomy, Fermentation, Isolation and Biological Activities

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A novel bioactive macrolide, IB-96212 has been isolated from the fermentation broth of a marine actinomycete, L-25-ES25-008. The strain belongs to the genus *Micromonospora*. The macrolide showed a very strong cytotoxic activity against P-388, and lower but significant activity against A-549, HT-29, and MEL-28 cell lines. We describe the isolation, taxonomy and fermentation of the producing strain as well as the isolation of IB-96212.

A marine *Micromonospora* has been isolated from homogenates of a sponge. The strain has been found to produce a fermentation broth with cytotoxic activity. A new macrolide responsible for this activity has been isolated from this fermentation broth. We describe in this paper the taxonomical characterization of the strain, its fermentation and the isolation of the active compound, together with the study of the *in vitro* activities. The structural elucidation is presented in an accompanying paper[1].

Materials and Methods

Microorganisms

Strain L-25-ES25-008 was isolated from a sponge collected at the Indian Ocean near the coast of Mozambique. It has been deposited in the Colección Española de Cultivos Tipo (CECT) under the accesion code CECT 3333. All the other bacterial strains used in this study were obtained from different culture collections.

Taxonomic Studies

Micro-morphological studies were carried out with a light microscope and a scanning electron microscope from the cultures grown at 28°C for 14 days on chitine agar medium[2] and ATCC's 172 agar medium[3]. The isomer of diaminopimelic acid from the cell wall was determined by the method of HASEGAWA *et al.*[4]. Carbon source requirements were studied by the method of SHIRLING and GOTTLIEB[5]. Fatty acids profile was obtained by the method of VAN DER AUWERA *et al.*[6]. Whole cell sugar pattern was obtained by the method of GUERRANT and MOSS[7].

Fermentation

IB-96212 was produced in 2 liter Erlenmeyer flasks containing 250 ml of fermentation medium. Inoculum was grown in a medium consisting of glucose 0.5%, soluble starch 2%, beef extract 0.3%, yeast extract 0.5%, tryptone 0.5%, calcium carbonate 0.4%, sodium chloride 0.4%, sodium sulfate 0.1%, potassium chloride 0.05%, magnesium chloride 0.2%, and monobasic potassium phosphate 0.05%, distilled water to one liter, and the pH was adjusted to 7 prior to sterilization at 121°C for 20 minutes. Seed preparation was a sequence of two steps, the first in 50 ml Erlenmeyer flasks containing 10 ml of inoculum medium and the second in 250 ml Erlenmeyer flasks containing 40 ml of the same medium. Both flasks were incubated at 28°C on a rotary shaker at 250 rpm for 48 hours. The fermentation medium consisted of glucose 0.5%, soluble starch 2%, soybean meal 1.5%, yeast extract

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0.5%, tryptone 0.2%, calcium carbonate 0.4%, sodium chloride 0.2%, and monobasic potassium phosphate 0.05%, distilled water to one liter, and the pH was adjusted to 7 prior to sterilization. The fermentor was inoculated with 12.5 ml of culture from the 250 ml flasks. The culture was grown in shake flasks with rotary agitation at 250 rpm and the flasks were incubated for 96 hours at 28°C.

Analysis of Fermentation Samples

The growth was monitored by packed cell volume (PCV) measurement. The amount of IB-96212 in the mycelium was quantified by HPLC (C18 reversed phase radial pack cartridge; mobile phase: methanol - water - trifluoroacetic acid (90 : 10 : 0.1); flow rate: 2 ml min^{-1}; detection: 360 nm). The sample for the HPLC assay was prepared as follows: 30 ml of the culture broth was sampled and filtered through a sintered glass. The retenate was extracted with 50 ml of ethyl acetate at room temperature. The extract was centrifuged and aliquots of 20 μl were injected.

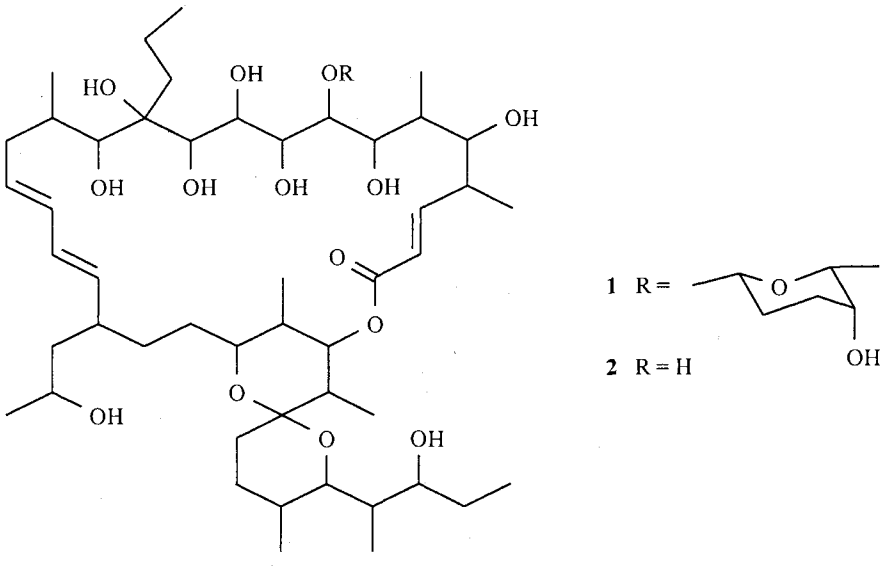
Analytical Procedure

Analytical TLC was done on pre-coated silica gel 60 F254 plates (0.2 mm thick, 20×20 cm, Merck) and the spots were detected under UV light (254 nm). Silica gel 60 (70~230 mesh, Merck) and Lichroprep RP-18 (40~63 μm, Merck) were used for column chromatography. HPLC analysis was performed using an analytical radial pack cartridge Resolve C18 (10 μ, Waters Chromatography), using as a mobile phase MeOH - H_2O 92 : 8, at a flow rate of 2.0 ml/minute and detection at 225 nm. The HPLC equipment was a Waters 991 with a photodiode-array detector and a Rheodyne injector.

Evaluation of Biological Activity

The antimicrobial activities of IB-96212 were determined by dilution techniques measuring cell growth after 24 hours of preincubation of target strains, *Escherichia coli* (ATCC 10536), *Klebsiella pneumoniae* (ATCC 29665), *Pseudomonas aeruginosa* (ATCC 10145), *Bacillus subtilis* (ATCC 6051), *Staphylococcus aureus* (ATCC 6538P), and *Micrococcus luteus* (ATCC 9341) with the antibiotic in Mueller-Hinton liquid medium at 37°C. The cytotoxic activities of IB-96212 were determined *in vitro* in cell cultures of P-388 (ATCC CCL 46), A-549 (ATCC CCL 185), HT-29 (ATCC HTB-38), and MEL-28 (ATCC HTB-72) following the procedure of BERGERON *et al.*[8]. P-388 cells were seeded into 16 mm wells at 1×10^4 per well in 1 ml aliquots of MEME SFCS containing serial dilutions of the macrolide. At the same time, A-549, HT-29, and MEL-28 cells were seeded in the same conditions but at a concentration of 2×10^4. A separate set of cultures without drug were seeded as control of growth to ensure that cells remained in exponential phase of growth. All determinations were carried out in duplicates. After three days of incubation at 37°C in 10% CO_2 atmosphere with 98% humidity, the IC_{50} was calculated by comparing the growth in wells with drug with the growth in control wells without the drug.

Fig. 1. Structure of macrolide IB-96212.

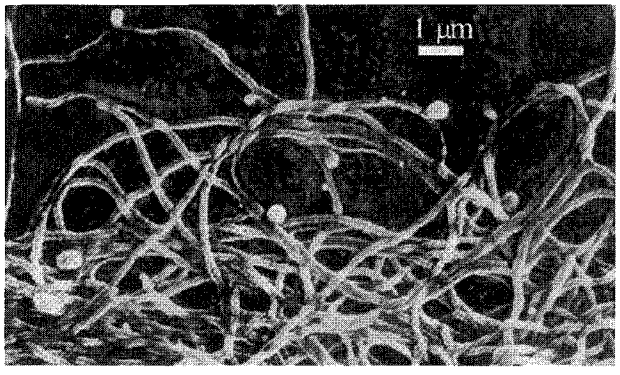


Results and Discussion

Strain L-25-ES25-008 forms colonies with no aerial mycelium. The mycelium is well-developed, and branched. The spores are nonmotile and are formed singly directly from the mycelium (Fig. 2). The vegetative mycelium was orange in most media, and mature sporulation showed a brown to brownish black in color (Table 1). No diffusible pigments were formed neither on solid or liquid media. The organism has its optimum growth temperature range between 25°C and 35°C. Besides, of all the carbon source tested, the strain grew on glucose, sucrose, xylose, and mannose, while growth on mannitol was doubtful and negative on raffinose, inositol, galactose, fructose, melibiose, ethanol, glycerol, and rhamnose. The isomer for the diaminopimelic acid found after whole-cell hydrolysis was the *meso*. The diagnostic sugars xylose and arabinose were found when whole-cell sugars were analyzed by gas chromatography, so this strain fits in the D group of LECHEVALIER and LECHEVALIER[9]. Other important sugars and polyalcohols detected by gas chromatography were ribose, arabitol mannose, galactose, glucose, *myo*-inositol, and mannosamine. Madurose was not detected as a cellular constituent. The fatty acid composition showed a quantitatively distinctive profile from those characteristic of several species belonging to the *Micromonospora* genus in terms of the concentration of several important fatty acids. *iso*-C15 (8.2% of the total fatty acids content), *iso*-C16 (35%), *iso*-C17:1 (3.2%), *anteiso*-C17 (3.4%), C17 (7.88%), and C17:1 (29.4%) are the predominant components. All these characteristics indicate that this strain is a *Micromonospora*, although it could not be identified as any of the type strains. The results are summarized in Table 2.

The macrolide IB-96212 is accumulated during fermentation in the mycelium and presents a maximum yield at around 120 hours. A typical time-course production

Fig. 2. Scanning electron micrograph of *Micromonospora* sp. L-25-ES25-008 grown in ATCC 172 medium for 21 days at 28°C.



Bar represents 1 μm.

Table 1. Cultural characteristics of strain L-25-ES25-008.

Medium	Growth	Substrate mycelium	Pigments
ISP-2	Good	Brown	None
ISP-3	Fair/Poor	Orange	None
ISP-4	Fair	Brown	None
ISP-5	Fair/Poor	Orange	None
ISP-6	Fair	Brown	None
ISP-7	Fair/Poor	Orange	None
Czapek's agar	Fair	Orange	None
Bennet's agar	Fair	Orange	None
ATCC 172	Good	Orange	None

of IB-96212 is shown in Fig. 3. The maximum compound's recovery is produced at the end of the growth phase.

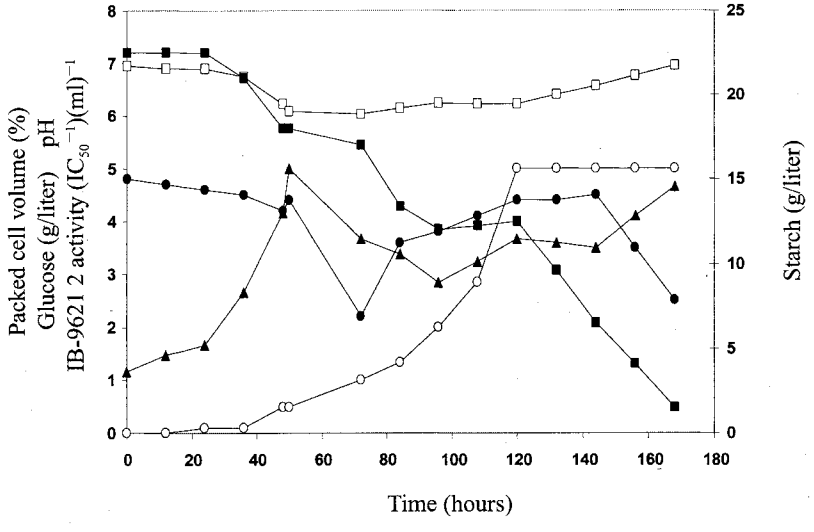
The isolation procedure of IB-96212 is summarized in Fig. 4. After completion of the cultivation, whole harvested broth (50 liters) was centrifuged to separate supernatant and cell mass. The cell mass was extracted twice with ethyl acetate (5 liters). After filtration, the extract was washed with water (5 liters) and concentrated under reduced pressure giving a brownish oily residue (9.6 g). The residue was dissolved in 1 liter of a mixture 10% aqueous NaCl-MeOH 1 : 1 and defatted by partitioning twice with 1 liter of *n*-hexane. The hydroalcoholic layer was extracted twice with 750 ml of $CHCl_3$ and the active $CHCl_3$ extracts concentrated to yield 1.9 g. The extract was chromatographed on silica gel by a VFC (Vacuum flash chromatography) system using *n*-hexane - EtOAc as eluting solvent. Fractions with cytotoxic activity (278 mg) were eluted with *n*-hexane - EtOAc 2 : 8~1 : 9. The final purification by C18 reversed phase chromatography afforded 200 mg of pure IB-96212 eluted with MeOH - H_2O 85 : 15. The purity of each preparation was confirmed by TLC visualized with vanillin in conc. H_2SO_4, by analytical HPLC with photo-diode array detector and by cytotoxic activity against P-388 cells.

The macrolide only showed antibiotic activity against *Micrococcus luteus* out of the six strains tested for that activity (Table 3). Besides, the compound presents very strong cytotoxic effect against P-388, and a good level of cytotoxicity against A-549, HT-29, and MEL-28 cell lines (Table 4). The activity on P-388 is about four orders of magnitude higher than on the other cell lines tested.

Table 2. Physiological characteristics of strain L-25-ES25-008.

Temperature:	
Growth rate:	15-50°C
Melanoid pigment production:	-
Salt (NaCl) resistance:	5%
Major fatty acids:	i-16:0 and 17:1
Characteristic sugars:	Arabinose and xylose
Diaminopimelic acid isomer:	Meso
Utilization of:	
D-Glucose:	+
D-Mannitol:	+/-
D-Fructose:	+/-
Raffinose:	-
myo-Inositol:	-
Saccharose:	++
Melibiose:	-
Rammnose:	-
Xylose:	++
Galactose:	-
Mannose:	+/-

Fig. 3. Time course production of macrolide IB-96212.



Cellular growth as packed (▲). Production of IB-96212 measured as cytotoxic activity per ml of broth (○). Concentration of starch in the medium (■). Concentration of glucose in the medium (●). pH of the medium (□).

Fig. 4. Isolation procedure of macrolide IB-96212.

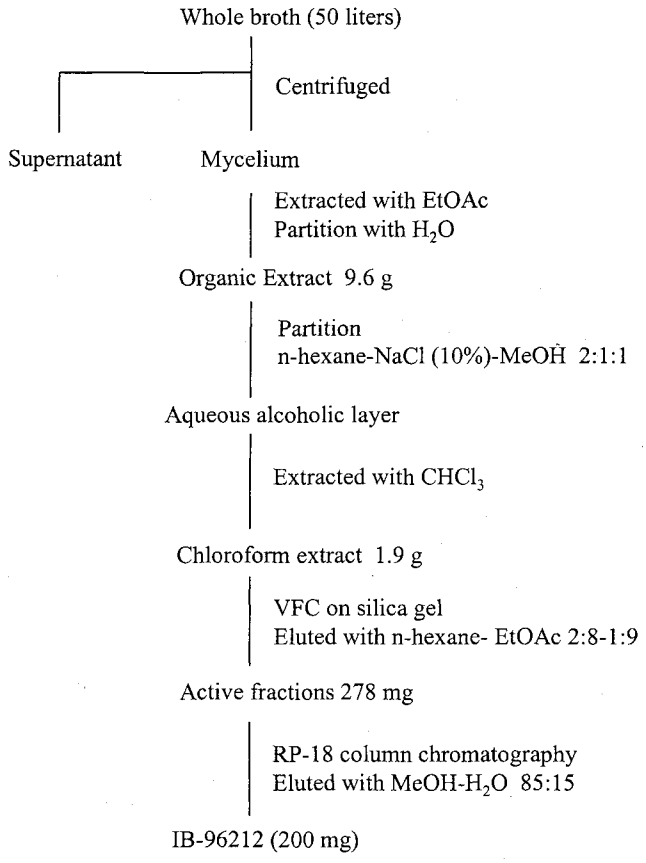


Table 3. Antimicrobial activities of macrolide IB-96212.

Test strains	MIC($\mu g.ml^{-1}$)
Escherichia coli	>100
Klebsiella pneumoniae	>100
Pseudomonas aeruginosa	>100
Staphylococcus aureus	100
Bacillus subtilis	100
Micrococcus luteus	0.4

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Table 4. Cytotoxic activities of macrolide IB-96212.

Activities are expressed as IC_{50} in $\mu g \cdot ml^{-1}$.

	Cell lines			
Compound	P-388	A-549	HT-29	MEL-28
IB-96212	0.0001	1	1	1
Aglycone	0.001	1	1.2	1
cis-platin	2.5	2.5	5	2.5
adriamicin	0.02	0.002	0.05	0.02
taxol	0.2	0.002	0.002	0.002
etopoxide	0.1	0.1	1	0.5