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IB-00208, a New Cytotoxic Polycyclic Xanthone Produced by a Marine-derived *Actinomadura*

I. Isolation of the Strain, Taxonomy and Biological Activites

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A new compound, IB-00208, has been isolated from the fermentation broth of an actinomycete isolated from a marine environment. The strain was identified as *Actinomadura* sp. by its chemical and phylogenetic characteristics. The compound shows cytotoxic activity on tumor cell lines and bactericidal activity against Gram-positive bacteria.

Natural products are an important source of novel structures with biological activities. In our search of new antitumor compounds, some marine environments have been screened as a source of actinomycetes. The northern coast of Spain has been explored in order to isolate actinomycetes that could produce new cytotoxic compounds. Several marine invertebrates and algae were collected by scuba diving and the microbes associated to them were isolated by incubating a homogenate of the macroorganisms on standard isolation media. The isolated strains were fermented and the broths were tested for antiproliferative activity on tumor cell lines. Several known compounds were identified in this project (staurosporin, bafilomycin B, antimycin, dinactin, nonactin, ikaguramycin, and venturicidin A) but a strain produced a new structure coded IB-00208 which was isolated from its mycelial cake. This novel compound IB-00208 shows a potent cytotoxic and antibiotic activity. We describe here the isolation, classification and fermentation of the producing strain. We also show the study of the *in vitro* activities. The isolation of IB-00208 (Fig. 1) and its structural elucidation are presented in an accompanying paper.[1]

Materials and Methods

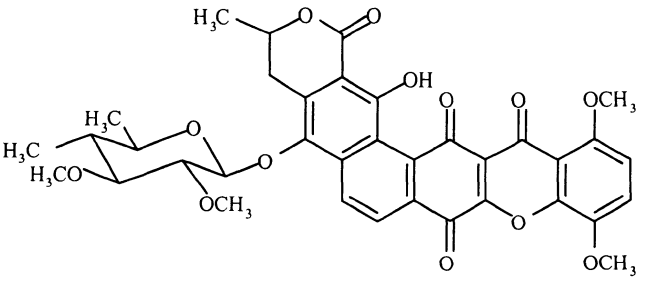
Microorganisms

Strain BL-42-PO13-046 was isolated from the northern coast of Spain. It has been deposited in the Colección Española de Cultivos Tipo (CECT) under the accession code CECT 5318. All the other bacterial strains used in this study were obtained from different culture collections.

Isolation

All the manipulations were carried out in aseptic

Fig. 1. Structure of IB-00208.



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conditions. BL-42-PO13-046 was isolated from a homogenate of a polychaete by dilution-plate methods using ATCC's 172 medium agar supplemented with cycloheximide (0.7 mM) and nalidixic acid (0.2 mM). The plates were incubated at 28°C for three weeks and colonies were picked and isolated onto agar plates for taxonomy and fermentation studies.

Taxonomic and Phylogenetic Studies

Light microscopy and scanning electron microscopy (SEM) were performed on cultures grown on chitin agar and ATCC's 172 agar medium after incubation at 28°C during 21~40 days. Petri dishes containing the media were covered aseptically with cellophane and inoculated with the organism. After incubation, the cellophane with the colonies was carefully removed and washed for 30 minutes hour at room temperature in phosphate-buffered saline (NaCl 0.8%, KCl 0.02%, KH_2PO_4 0.012%, Na_2HPO_4 0.091%). The cellophane was then transferred to a solution of 2.5% glutaraldehyde for 30 minutes at room temperature and washed for 30 minutes in H_2O. For SEM studies, samples were additionally fixed in a saturated atmosphere of osmium tetroxide for 30 minutes at 20°C, dried at room temperature and coated with a 2 nm layer of gold. The diaminopimelic acid composition was performed as described by HASEGAWA *et al.*[2]. The fatty acids profile was obtained by the method of VAN DER AUWERA *et al.*[3]. Fatty acid methyl esters were identified either by co-elution with standards or by mass spectrum analysis using a mass detector HP 5973 and the NIST library of spectra. Carbon utilization was studied by the methods described by SHIRLING and GOTTLIEB[4]. The whole cell sugar composition was performed by analyzing the aldonitrilic derivatives or the sugars through gas-liquid chromatography following the protocols of GUERRANT and MOSS[5]. Salt resistance experiments were performed by incubation of the strain in ATCC's 172 agar medium containing increasing NaCl concentrations ranging from 0 to 10%. The DNA of BL-42-PO13-042 was obtained after treating the cell wall by the procedure described by HASEGAWA[6] with modifications. The strain was grown for 7 days in ATCC's 172 liquid medium at 28°C but supplemented with mannitol at 5%. The cells were recovered by centrifugation, washed with lysis buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8), resuspended in the same buffer and incubated with lysozyme (5 mg/ml) at 37°C until a decrease in turbidity was evident. The DNA was purified following established procedures (MARMUR[7]). The 16S rDNA was amplified using the 27f and the 1492r primers described by LANE[8]. The amplified DNA segment was purified by electrophoresis and partially sequenced at the CSIC facility, Madrid, Spain. A search of the most related sequences was performed using the BLASTN algorithm (ALTSCHUL *et al.*[9]). The phylogenetic analysis was performed using the neighbor joining algorithm of SAITOU and NEI[10] as implemented in the Phylip package (FELSENSTEIN[11]).

Fermentation

The product isolated from the fermentation broth of strain BL-42-PO13-046, was coded IB-00208. It 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 6.8 prior to sterilization at 121°C for 20 minutes. Seed preparation was a sequence of two steps, the first in 50 ml-capacity Erlenmeyer flasks containing 10 ml of inoculum medium and the second in 250 ml-capacity Erlenmeyer flasks containing 40 ml of the same medium. Both flasks were incubated at 28°C on a rotatory shaker at 250 rpm for 48 hours. The first inoculum was seeded with three plugs of a solid culture of BL-42-PO13-046. The second inoculum was seeded with 12.5 ml of the first inoculum broth. The fermentation medium consisted of glucose 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 adjusted to 7 prior to sterilization. The fermentation flasks were inoculated with 12.5 ml of culture from the 250 ml flasks. The culture was grown in 2-liter shake flasks with rotary agitation at 250 rpm and the flasks were incubated for 96 hours at 28°C.

Analysis of Fermentation Samples

The analysis of the fermentation samples was performed by HPLC analysis as described by CASTRO-RODRÍGUEZ *et al.*[1].

Evaluation of Biological Activity

The antimicrobial activities of IB-00208 were determined by dilution techniques measuring cell growth after 12 hours of pre-incubation 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 35°C.

After incubation with the compound, cytostatic effects were differentiated from cytotoxic effects by the ability of the cells to re-grow when transferred to fresh Mueller-Hinton medium without the compound.

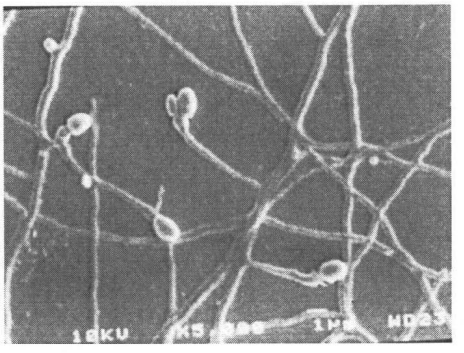
The cytotoxic activities of IB-00208 were determined *in vitro* in cell cultures of P-388 (ATCC CCL 46), A-549 (ATCC CCL 185), HT-29 (ATCC HTB-38), and SK-MEL-28 (ATCC HTB-72) following the procedure of BERGERON *et al.*[12]. P-388 cells were seeded into 16 mm wells at 1×10^4 per well in 1 ml aliquots of MEME SFCS containing serial dilutions of IB-00208. At the same time, A-549 and HT-29 cells were seeded in the same conditions but at a concentration of 2×10^4. A separate set of cultures without drug was 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.

Results and Discussion

In the course of our screening project, 427 strains were fermented and their broths screened for the presence of compounds with cytotoxic activity. Ten of these strains were selected for further study because of their strong *in vitro* activity. The taxonomical characterization to the genus level was performed by comparing their fatty acids content against a database. Nine of these strains produced known compounds. Five strains belonged to the *Streptomyces* genus and produced staurosporin, bafilomycin B, antimycin, nonactin, and venturicidin. A *Micromonospora* strain produced ikaguramycin. A *Cellulomonas* strain produced both dinactin and nonactin. A non-classified actinomycete produced antimycin. Finally, a strain coded BL-42-PO13-046 produced a new structure. A more complete taxonomical study was carried out on this strain for its classification.

Strain BL-42-PO13-046 forms colonies with no aerial mycelium. The mycelium is well developed and extensively branched. The spores are smooth, non-motile and formed singly from the mycelium. (Fig. 2). The vegetative mycelium was light brown-white in most ISP media tested (Table 1). The secretion of soluble pigments was produce in ISP-2, ISP-6 and Bennet's agar. The strain showed a salt tolerance up to 5% when grown in solid media. The major

Fig. 2. Scanning electron micrograph of BL-42-PO13-046.



The strain was grown in chitin agar for 40 days at 28°C.

Table 1. Cultural characteristics of strain BL-42-PO13-046.

Medium	Growth	Substrate mycelium	Pigments
ISP-2	Good	Light brown	Brown
ISP-3	Good / Fair	White	None
ISP-4	Fair / None	White	None
ISP-5	Fair	White	None
ISP-6	Good	Brown	Brown
ISP-7	Good / Fair	White	None
Czapek's agar	Good	White	None
Bennet's agar	Good	Dark brown	Brown
ATCC 172	Good	Brown	Traces brown

Table 2. Physiological characteristics of strain BL-42-PO13-046.

Growth at 20-28°C:	+		
Soluble pigment production:	+		
NaCl resistance:	5%		
Major fatty acids	(Table 3)		
DAP isomer:	meso-		
Carbon source:		Nitrogen source:	
D - Glucose	+++	L- Glutamic acid	+/-
D - Mannitol	+/-	Ammonium	++
D - Fructose	-	L - Asparagine	+
D - Galactose	+	L - Valine	+/-
D - Xylose	+++	L - Histidine	-
L - Rammnose	+++	L - Arginine	+
Saccharose	-	L - Tyrosine	+/-
D-Raffinose	-	D - Glucosamine	+
Melibiose	-	L - Methionine	-
myo-Inositol	-		
Glycerol	-		
Ethanol	-		

++ = good; + = fair; +/- = doubtful; - = absent

diaminopimelic acid isomer was the *meso* form although traces of *LL* isomer could be detected in TLC analysis. The preferred carbon sources were glucose, rhammnose and xylose. Among the nitrogen sources tested, ammonium gave the best result on solid media, while asparagine, arginine and glucosamine gave a lower level of growth. Table 2 shows a summary of some physiological characteristics. The strain was identified as an *Actinomadura* sp. based on its fatty acids content and on the detection of madurose as a whole cell component. The most important fatty acids detected were i-16 : 0, 16 : 0, and *cis*-18 : 1. Table 3 shows the similarity of the fatty acids composition with that of other strains that belong to the *Actinomadura* genus. To corroborate the chemical identification with phylogenetic data, partial sequencing of 16S rDNA was made. The strain showed insensitivity to lysozyme so several additives were used in the growth medium to render the bacterial wall sensitive to enzymatic lysis. HASEGAWA *et al.*[13] and HASEGAWA[6] had devised a procedure for obtaining spheroplasts in lysozyme-resistant actinomycetes as *Micromonospora*. In our case, the addition of D-mannitol at 5% in the medium rendered our strain sensitive to lysozyme so we could obtain DNA by a gentle method. The partial sequence of the gene obtained after amplification (Fig. 3A) showed that the strain belonged to the phylogenetic cluster of *Actinomadura* (LECHEVALIER and LECHEVALIER[14]). Fig. 3B shows the phylogenetic tree obtained by comparison of the 16S rDNA partial sequence of the strain with the same homologous fragment of the five more related *Actinomadura* species, and using *Thermomonospora curvata* and *Streptomyces albus* as outsiders. The combination of chemical and phylogenetic data made unambiguous the ascription of this strain to the genus *Actinomadura* which is included phylogenetically in the Thermomonosporaceae family (STACKEBRANDT *et al.*[15]). This genus underwent a profound revision by KROPPENSTEDT *et al.*[16] based on the chemotaxonomical characters of the different species and now encompasses 28 validly published species that form a coherent group both chemically and phylogenetically although with a varied sporulation pattern.

The fact that this strain was isolated from a marine environment and that it can grow at salt concentrations of up to 5% indicate that BL-42-PO13-046 can live in marine

Table 3. Fatty acids of strain BL-42-PO13-046.

	i-14:0	14:0	15:0	i-16:0	16:1	16:0	17:1	17:0	i-18:1	i-18:0	Cis-18:1	18:0	10 methyl-18:0
BL-42-PO13-046	1.75	2.43	4.9	20.2	5.16	22.6	6.5	5.4	5.2	1.8	13.0	1.22	5.75
Acti. livida ATCC 33578	1.41	1.21	7.4	21.8	5.5	13.7	14.9	7.0	7.5	1.2	7.8	1.0	5.7
Acti. madurae NRRL-B 5390	1.0	2.5	6.1	11.5	5.56	21.2	10.9	7.9	3.0	1.6	16.5	1.1	5.3
Act. malachitica ATCC 27888	1.4	3.4	0.7	18.5	7.9	26.2	1.1	0.9	3.7	2.7	15.8	4.5	8.7
Acti. vinacea ATCC 33581	1.4	1.4	6.1	22.7	6.8	15.2	12.8	5.6	6.1	1.6	10.3	0.7	5.1
Acti. formosensis[1] ATCC 49059	1.3	1.2	2.8	22.5	3.4	17.8	6.9	4.6	6.8	1.8	10.8	2.4	5.5
*Strept. griseus** DSM 40236	15.1	0.8	0.9	21.2	5.1	6.4	<1	<1	1.1	<1	<1	<1	<1

Acti.: *Actinomadura*; *Strept. Streptomyces*. * i-15:0 (9.2%), a-15:0 (19.6%), i-17:0 (1.7%), and a-17:0 (3.25%) are important fatty acids for *Streptomyces* but are minor constituent in *Actinomadura*. [1]*Actinomadura formosensis* formerly *Thermomonospora formosensis*

Fig. 3. Phylogenetic studies of BL-42-PO13-046.

A

gggtccttttgggccggtgacaggtggtgcatggctgtcgtcagctcgtgtcgtgagatgttgggttaagtcccgc
ancgagcgcaaccctcgttccatgttgccagcacgttatggtggggactcatgggagaccgccggggtcaactcggaggaa
ggtggggatgacgtcaagtcatcatgcccctatgtcttgggctgcaaacatgctacaatggccggtacagagggctgcgata
ccgtgaggtggagcgaatcccttaaagccggtctcagttcggatcgaagtctgcaactcacttcgtgaagtcggagtcgctagt
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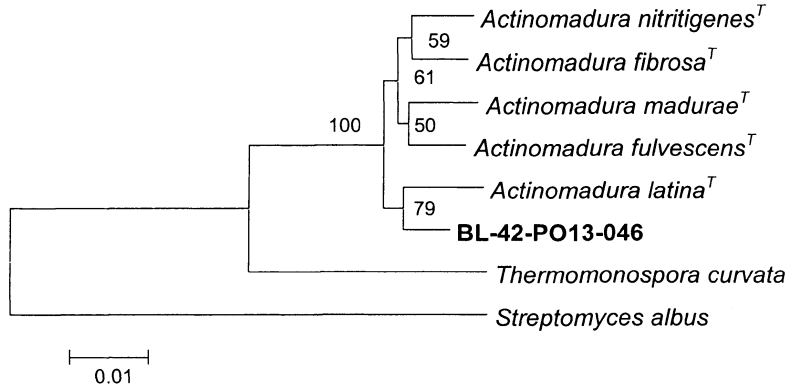
cccgaagc

B



A. Partial sequence of 16S rDNA.

B. Phylogenetic tree of BL-42-PO13-046. Unrooted tree showing the phylogenetic relationship among the strain and the five more related sequences deposited in GenBank. *Thermomonospora curvata* and *Streptomyces albus* were included as outsiders. "Bootstrap" values are shown at the branches of the tree. Scale bar: accumulated changes per nucleotide.

habitats, but as the strain retains its ability to grow in the absence of salt it cannot be excluded a terrestrial life cycle. Because actinomycetes are more abundant in soil (GOODFELLOW and WILLIAMS[17]) and can acquire tolerance to higher NaCl concentrations (OKAZAKI and OKAMI[18]), it is not solved whether sporulating actinomycetes form part

of the autochthonous marine microbial community. Nonetheless, experimental evidence (MORAN *et al.*[19]) suggests that sporulating, non-motile actinomycetes such as *Streptomyces*, long considered to be terrestrial, can be present in marine environments in their mycelial, physiologically active, phase. In our case, the phylogenetical distance of BL-42-PO13-046 to the most related *Actinomadura* species with 16S rDNA sequence available (Fig. 3B) does not rule out this strain as being a new species. Whether our strain is autochthonous marine or secondarily adapted to the sea cannot be answered yet.

The fermentation parameters of BL-42-PO13-046 for the production of IB-00208 show that the level of the broth's activity increases in the first four days and then it is maintained for a period of time of 144 hours at which the pH of the medium reaches an alkaline value of around 8.5 (Fig. 4). The chemical stability of the compound (data not shown) suggests that the producing phase for the compound is simultaneous with the period of active growth.

IB-00208 shows a potent cytotoxic activity against several lines of tumor cells, both human and murine. Besides, this compound has a good antibiotic activity against Gram-positive organisms but poor activity against Gram-negative bacteria. This pattern of activity can be found in other kinds of antitumor compounds structurally related to IB-00208 as cervinomycins (OMURA *et al.*[20]) or citreamicins (MAIESE *et al.*[21]), or unrelated to IB-00208 as thiocolarine (ROMERO *et al.*[22]). The specificity in its bactericidal activity could be related to a different degree of overall permeability that the more complex structure of the Gram-negative cell wall could present when compared to the Gram-positive cell wall.

Fig. 4. Time course production of IB-00208.

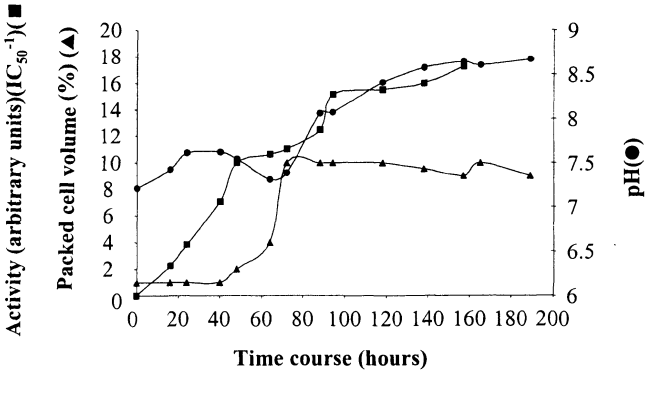


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Table 5. Cytotoxic activities of IB-00208.

Test line cells	MIC (nM)
P388D1	1
A-549	1
HT-29	1
SK-MEL-28	1

Table 4. Antimicrobial activities of IB-00208.

Test strains	MIC (nM)
Escherichia coli	> 150
Klebsiella pneumoniae	> 150
Pseudomonas aeruginosa	> 150
Staphylococcus aureus	1.4 (0.18)*
Bacillus subtilis	1.4 (0.18)*
Micrococcus luteus	0.09

*Cytostatic effect: cells retained viability and could grow after exposure with the compound when re-inoculated to fresh medium in the absence of IB-00208.

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