IZUMENOLIDE—A NOVEL β-LACTAMASE INHIBITOR PRODUCED BY *MICROMONOSPORA*

I. DETECTION, ISOLATION AND CHARACTERIZATION

WEN-CHIH LIU, GAIL ASTLE, JERRY SCOTT WELLS, Jr., WILLIAM H. TREJO, PACIFICO A. PRINCIPE, MARLENE L. RATHNUM, WILLIAM L. PARKER, OCTAVIAN R. KOCY and RICHARD B. SYKES*

The Squibb Institute for Medical Research, Princeton, New Jersey 08540, U.S.A.

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Micromonospora chalcea subsp. *izumensis* produces a novel β-lactamase inhibitor izumenolide (EM4615). Isolation of izumenolide was performed by extraction into butanol under acidic conditions and then back extraction into water at neutrality. The compound was precipitated from the aqueous phase by the addition of calcium or barium salts. Further purification was achieved by distribution in BuOH - 1 N NaOH. Izumenolide is a macrolide containing sulfate ester groups.

The discovery of clavulanic acid[1] as the first naturally occurring well documented β-lactamase inhibitor, stimulated the search for other such inhibitors of natural origin. Recently a number of β-lactamase inhibitors have been described, all belonging to the same chemical family of carbopenems and containing reactive β-lactam rings. These are, the olivanic acids[2,3,4], epithienamycins[5,6] and PS-5[7]. This paper describes the detection, isolation and characterization of a novel β-lactamase inhibitor. The biological properties of this compound are described in the accompanying paper[8].

Screening of Soil Actinomycetes

Cultures of aerobic actinomycetes were isolated from soil samples by stamping air dried soils onto a selective culture medium of the following composition: 1% soluble starch, 0.03% casein, 0.2% KNO_3, 0.2% NaCl, 0.2% K_2HPO_4, 0.005% $MgSO_4 \cdot 7H_2O$, 0.002% $CaCO_3$, 0.001% $FeSO_4 \cdot 7H_2O$ and 2% agar (pH 7.0). Immediately before use, actidione (Calbiochem) was added to the medium to give a final concentration of 100 μg/ml. After 7~10 days incubation at 25°C, colonies were picked and transferred to agar slants containing 0.1% beef extract, 0.1% yeast extract, 0.2% NZ amine A, 1% glucose and 1.5% agar (pH 7.3). Submerged culture was carried out at 28°C for 5 days in 250-ml flasks containing 50 ml of liquid medium having the following compositions:

Medium A: 0.4% yeast extract, 1.0% malt extract and 0.4% dextrose (pH 7.3)

Medium B: 0.3% beef extract, 0.5% tryptone, 0.5% yeast extract, 0.24% soluble starch, 0.1% dextrose

Selective Screening for β-Lactamase Inhibitors

Fermentation samples (50 μl) were added to 11-mm cellulose discs (Whatman), which, after drying were placed on agar plates (3.5% BBL seed agar +0.5% NaCl) containing a crude sonicate of TEM-2 β-lactamase. The enzyme was added to give a final concentration of 1 unit/ml in the agar—one

unit being defined as the amount of β-lactamase hydrolyzing 1 μmole of chromogenic cephalosporin/minute at 30°C. Plates were incubated at 37°C for 3 hours and after removing the discs, flooded with a solution (500 μg/ml) of a chromogenic cephalosporin in 0.05 M phosphate buffer. β-Lactamase inhibiting substances appear as yellow halos against a red background.

Taxonomy of the Producing Strain

The izumenolide producing strain SC 11,133 was isolated from a soil sample collected from the site of an old gold mine, south of Johannesburg S.A. and shows the following characteristics.

Morphology

The organism forms no true aerial mycelium and spores are borne singly at the ends of simple sporophores appearing in monopodially arranged clusters.

Cultural Characteristics

Cultural characteristics of SC 11,133 are based on observations made at 14 and 21 days on media listed in Table 1. Color designations and color chip numbers are taken from the Color Harmony Manual (Container Corp. of America, 1958) and the ISCC-NBS method of designating colors and color names (National Bureau of Standards Circ. 533, 1955). All plates are incubated at 28°C.

Physiological Characteristics

The physiological characteristics of the strain are summarized in Table 2. Carbohydrate utilization was determined in the basal medium of AYERS, RUPP and JOHNSON[9], consisting of 0.1% $(NH_4)_2HPO_4$, 0.02% KCl, 0.02% $MgSO_4 \cdot 7H_2O$, 0.02% yeast extract and 1.5% (v/v) of 0.04% (w/v) bromocresol purple. Test carbohydrates were added to a final concentration of 1% except for glycerol which was added at 2%. The carbohydrate utilization pattern of SC 11,133 is summarized in Table 3.

Table 1. Cultural characteristics on various media.

Media	Incubation	
	14 days	21 days
Micromonospora maintenance agar	Vegetative mycelium dusty orange (4 lc); ISCC-NBS (m.o. 53) No soluble pigment	Vegetative mycelium overlaid with dry black mass of spores
Tomato-paste oatmeal agar	Mycelium dusty orange (4 lc)	Dry black mass of spores
Dextrose-yeast extract agar	Mycelium dusty orange	Sporulation appearing as blackish flecks on the vegetative mycelium
Sodium caseinate-tyrosine agar	No melanoid pigment produced	No melanoid pigment

Table 2. Physiological characteristics of *Micromonospora* sp. SC 11,133.

Proteolysis	Milk plate: Positive clearing Gelatin plate (FRAZIER method): Positive clearing
Starch hydrolysis	Positive
pH Range	No growth at pH 5.5. Growth from pH 6 to 8 on *Micromonospora* maintenance agar buffered at 0.5 pH unit intervals from 5.5 to 8.0.
Temperature range	Optimal growth between 30~37°C. No growth above 45°C.
NaCl tolerance	No growth above 1.5% NaCl. A medium was used containing 1% yeast extract, 2% soluble starch, 1.5% agar, distilled water and the appropriate concentration of NaCl ranging 1.5~7%.

Utilizing the methodology of LECHEVALIER and LECHEVALIER[10], hydrolysates of purified cell walls were shown to contain Meso-dap, glycine, xylose and arabinose. Based on its characteristic morphology and type II cell-wall pattern, the organism has been assigned to the genus *Micromonospora*.

Strain SC 11,133 shows greatest resemblance to *Micromonospora chalcea*[11] and *Micromonospora chalcea* subsp. *izumensis*[12]. In an unpublished numerical taxonomy study (TREJO, 1979), SC 11,133 had similarity coefficients of 80% to a type strain of *M. chalcea* and 92% to a type strain of *M. chalcea* subsp. *izumensis*. The similarity of the two reference cultures was 84%.

In view of the clustering with these reference strains, SC 11,133 is considered to be a strain of *Micromonospora chalcea* subsp. *izumensis* and has been deposited in the American Type Culture Collection under the accession number ATCC 31395.

Table 3. Carbohydrate utilization of *Micromonospora* sp. SC 11,133.

Carbohydrate	Growth
Basal medium	−
Glucose (Positive control)	+
Glycerol	−
Mannitol	−
Inositol	−
d-Xylose	+
l-Arabinose	+
Rhamnose	−
Raffinose	+
Melibiose	+
Sucrose	+
Fructose	+
Lactose	+

Fermentation

Seed Medium		Production Medium	
1.5% Nutrisoy flour	0.0005% $CoCl_2 \cdot 6H_2O$	0.4% Yeast extract	0.4% Dextrose
1.5% Soluble starch	1% $CaCO_3$	1.0% Malt extract	0.03% $FeSO_4 \cdot 7H_2O$
5% Glucose			

A 38-liter stainless steel vessel containing 30 liters of seed medium was inoculated with 1,500 ml of a seed culture grown in 500-ml Erlenmeyer flasks on a rotary shaker for 96 hours at 28°C. The 38-liter vessel was operated at 28°C for 96 hours using an agitation rate of 220 rpm and an air flow of 30 liters/min. A 12.5-liter portion of seed culture was used to inoculate a 380-liters stainless steel fermentor containing 250 liters of the production medium. The fermentor was operated for 6 days at 25°C using an agitation rate of 155 rpm and an air flow of 10 cfm. The production of izumenolide was followed by a disc plate assay using TEM-2 β-lactamase and the chromogenic cephalosporin.

Isolation

The isolation of izumenolide from fermentations of *Micromonospora chalcea* subsp. *izumensis* is outlined in Fig. 1. Izumenolide is extractable into butanol under acidic conditions and then back extracted into water at neutrality. The inhibitor is precipitated by addition of barium or calcium salts and this constitutes a very convenient purification method. A little further purification can be achieved by distribution in BuOH - 1 N NaOH in which the inhibitor forms a third liquid phase of intermediate density. Material thus obtained contains one major and two minor components by thin-layer chromatography. Electrophoresis in formamide-containing buffers[13] gave a single inhibitory zone with a constant mobility of −123 Am units in the pH range of 3.3~9.3 showing that izumenolide is an acidic substance, as one would expect from its extraction behavior. Attempts at further purification by various chromatographic methods and counter-current distribution gave no substantial improvement. However, the purity was good enough to establish the structure of the major component and two minor variants. The ^{1}H-NMR and IR spectrum of izumenolide are shown in Figs. 2 and 3. The structure of izumenolide was determined by degradative and spectroscopic

Fig. 1. Isolation of izumenolide.

250-liter Fermentation of *Micromonospora chalcea*, SC 11,133

1) centrifuge
2) extract into butanol at pH 2
3) back extract into water at pH 7 (NaOH)
4) concentrate and freeze dry

Lyophilate

1) dissolve in water and precipitate with $Ca(NO_3)_2$
2) mix with dilute H_2SO_4 and extract into butanol
3) extract into water at pH 7 (NaOH)
4) concentrate
5) distribute between 1 N NaOH and butanol, separate middle phase and extract at pH 7 into butanol - propanol, 2: 1
6) concentrate organic extract

Izumenolide (Sodium salt)

Fig. 2. 100 MHz ^{1}H-NMR spectrum of izumenolide, Na salt, in DMSO-d_6.

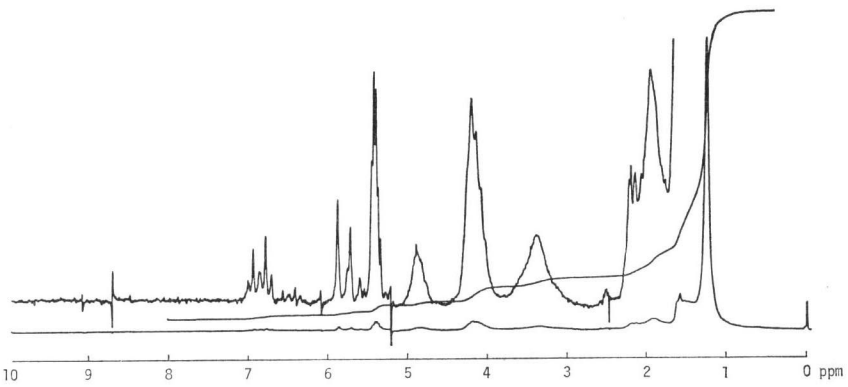
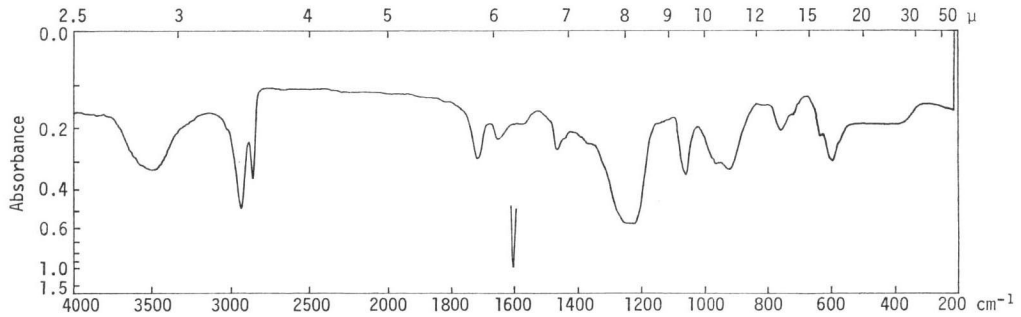


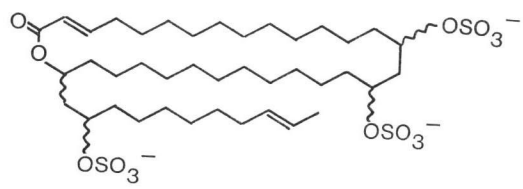
Fig. 3. Infrared spectrum of izumenolide in KBr.



methods[14] and is shown in Fig. 4. Izumenolide is a rather simple macrolide but is unusual in the fact that it has sulfate ester groups and is, to the best of our knowledge, the first macrolide reported that is thus adorned.

Izumenolide is the subject of U.S. Patent No. 4,205,068.

Fig. 4. Structure of izumenolide.



Experimental

Isolation and Purification of Izumenolide

Whole broth, 182 liters, from a fermentation of *Micromonospora chalcea* subsp. *izumensis* was centrifuged, giving 177 liters of clear supernate. This was cooled to 5~10°C, adjusted to pH 2.0 with H_2SO_4 and extracted with 110 liters of water-saturated butanol. The extract was mixed with 80 liters of water and adjusted to pH 7 with NaOH. The aqueous phase (75 liters) was concentrated *in vacuo* below 30°C to 3.4 liters and then freeze dried, giving 127 g of lyophilate. A solution of 20 g of the lyophilate in 400 ml of water was mixed with 300 ml of 1 M $Ca(NO_3)_2$. The resulting gummy precipitate was separated by centrifugation and mixed with 200 ml of butanol and 200 ml of 0.1 N H_2SO_4. The butanol phase was washed with 100 ml of 0.1 N H_2SO_4 and then extracted twice with 100 ml portions of water, adding NaOH to bring the pH to 7. The aqueous phase was concentrated *in vacuo*, giving 6.1 g of solid. A 3-g sample of this solid was stirred in a mixture of 60 ml of butanol and 30 ml of 1 N NaOH until solution was essentially complete. The mixture was centrifuged, giving three liquid phases, the upper and lower of which were inactive. The upper and lower phases were removed and the middle phase was simultaneously washed twice with 10 ml portions of butanol and 1 N NaOH. The middle phase was then stirred with 10 ml of saturated Na_2SO_4, 10 ml of water and 60 ml of butanol - propanol, 2: 1, and adjusted to pH 7 with 1 N H_2SO_4. The upper phase was removed and the lower two phases extracted with three 60 ml portions of butanol - propanol, 2: 1, adding water as necessary to maintain the volume of the lower phase. The combined organic extract was concentrated *in vacuo*. The residue was dissolved as much as possible in 60 ml of methanol and insoluble material removed by centrifugation. The supernate was mixed with acetonitrile and the solvents removed *in vacuo*, giving 2 g of izumenolide, sodium salt, as an amorphous, hygroscopic solid: UV λ_{max} (water) 214 nm ($E^{1\%}$ 95); $[\alpha]_D^{21}$ −8.5° (*c* 1, H_2O); TLC (Merck silica gel 60, eluting with 2-BuOH - HOAc - H_2O, 3: 1: 1, and detecting with phosphomolybdic acid-H_2SO_4) Rf 0.11 (tr), 0.14 (s) and 0.18 (w).

Anal. Calcd. for $C_{40}H_{71}O_{14}S_3Na_3$: C, 51.05; H, 7.60; S, 10.22; Na, 7.33
Found: C, 49.99; H, 7.71; S, 10.03; Na, 7.73

The ammonium salt of izumenolide was prepared by shaking a solution of the Na salt in water-saturated butanol with several portions of saturated aqueous $(NH_4)_2SO_4$. The butanol phase was concentrated and worked up as above, giving an amorphous, deliquescent solid: UV λ_{max} (water) 214 nm ($E^{1\%}$ 98).

Anal. Calcd. for $C_{40}H_{83}N_3O_{14}S_3$: C, 51.87; H, 9.03; N, 4.54; S, 10.38
Found: C, 50.54; H, 9.16; N, 4.79; S, 10.56

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