IONOMYCIN, A NEW POLYETHER ANTIBIOTIC

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Ionomycin, a new polyether antibiotic with a high affinity for calcium ions, is obtained in pure form from fermentation broths of *Streptomyces conglobatus* sp. nov. TREJO by solvent extraction. It is unique amongst known polyether antibiotics in that it has a UV absorption maximum at 300 nm, thereby distinguishing it from other antibiotics of its class. The Ca salt has the molecular formula $C_{41}H_{70}O_9Ca$. Ionomycin is a narrow spectrum antibiotic being active against Gram-positive bacteria.

Polyether antibiotics constitute a large and growing class of antibiotics, with more than 30 having been recognized since the first members of this class, X-206, X-464, X-537A and nigericin, were reported in 1951.[1,2] Although the antibacterial activity of polyethers is directed primarily toward Gram-positive bacteria *in vitro*, they have not as yet been accorded any niche in human therapy, mainly because of a high degree of toxicity when administered parenterally. However, they are of economic importance in the poultry industry, being used for the oral treatment of coccidiosis, a disease whose control is estimated to have cost approximately $100 million worldwide in 1976. Other potential uses for polyethers are currently being explored in a number of different laboratories. This and other aspects of the biology and the chemistry of polyethers have been ably reviewed in a recent article by WESTLEY.[3]

In this report, we wish to describe the production, isolation and several biological and chemical characteristics of ionomycin, a new polyether antibiotic. The producing organism, *Streptomyces conglobatus* sp. nov. TREJO is also described.

Taxonomy

The key characteristics of *Streptomyces conglobatus* sp. nov. TREJO are:

Morphology: The sporophores are in compact spirals forming ball-like clusters along the axial hyphae. The spores are hairy.

Cultural characteristics: Sporulation is sparse and powdery after 10 days of growth on the standard international streptomyces project medium ISP-3 (oatmeal agar)[4]. On ISP medium-2 (yeast extract-malt extract agar), a salt and pepper appearance is noted because of areas of dense sporulation (gray) and of nonsporulation (white). On the inorganic salts-starch agar medium ISP-4, an abundant, gray aerial mycelium is produced. No soluble pigments are produced in any of the media used. Examination of the colony reverse shows either no color or a faint greenish-gray tinge (ISP-3) to a faint yellowish-orange tinge (ISP-2). No melanoid pigments are produced on tyrosine agar or on other protein media.

A summary of the key characteristics and of the carbohydrate utilization pattern is shown in Table 1. A culture of *Streptomyces conglobatus* sp. nov. TREJO has been deposited in the American

Type Culture Collection under the accession number ATCC 31005.

Table 1. Characteristics of *Streptomyces conglobatus* sp. nov. TREJO ATCC 31005

Spore color series	Gray
Morphology group	Spira
Spore surface	Hairy
Reverse color	Light yellow-light green
Melanin production	—
Carbohydrate utilization pattern	
Glucose	+
Mannitol	—
Inositol	—
d-Xylose	+
Arabinose	+
Rhamnose	—
Fructose	+
Raffinose	+
Sucrose	—

Production

Streptomyces conglobatus is maintained by storage in a mechanical freezer at −90°C. When needed, working stock cultures were grown on tomato paste-oatmeal agar slants, made by the addition of a volume of boiling water containing tomato paste (2%) and oatmeal (2%) to an equal volume of boiling water containing 2% agar. To inoculate media for seed cultures, the growth from well sporulated slants was suspended in 0.01% sodium lauryl sulfate solution and used to inoculate the seed medium. This medium consisted of the following: Toasted Nutrisoy Flour (Archer Daniels Midland Co., Minneapolis, Min.), 15.0 g; Hi-starch (Illinois Cereal Mills, Kankakee, Illinois), 15.0 g; glucose, 50.0 g; $CoCl_2 \cdot 6H_2O$, 0.005 g; $CaCO_3$, 10.0 g; and distilled water to 1,000 ml. The seed flasks were incubated at 25°C for 96 hours on a rotary shaker (280 rpm, with a 2-inch throw). Fermentation flasks with a 5% (v/v) inoculum were incubated for 5~7 days using similar conditions in a medium consisting of: extracted soybean meal, 30 g; glucose, 50 g; $CaCO_3$, 7 g; and distilled water to 1,000 ml.

Larger scale fermentations were conducted in 100-gallon, stainless steel fermentation vessels containing 250 liters of medium. For these operating conditions, the medium consisted of: extracted soybean meal, 30 g; cerelose, 55 g; $CaCO_3$, 7 g; Ucon LB625 (Union Carbide, New York), 6.5 ml and tap water to 1,000 ml. During the course of the 144-hour incubation period, air was supplied to the growing culture at the rate of 2.3 cubic feet per minute with agitation at 220 rpm.

Antibiotic production, and isolation, were followed by conventional two-fold broth dilution assays and by paper-disc, agar diffusion assays with *Staphylococcus aureus* FDA 209P. Thin-layer chromatography on silica gel was also employed to monitor the fermentation and isolation stages. The developing solvent was 4% methanol in chloroform, and detection was by bioautography on *S. aureus* FDA 209P. In this system, ionomycin has an Rf value of approximately 0.35.

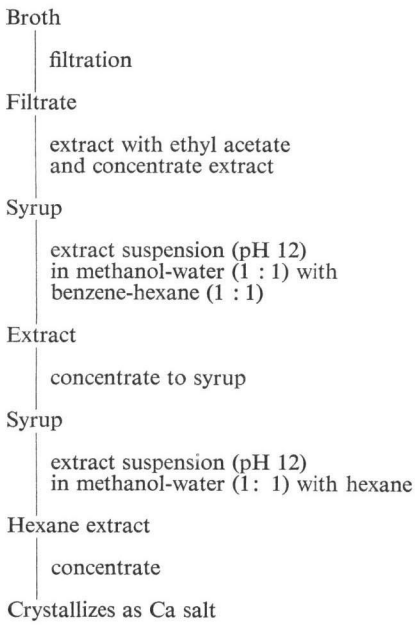
Isolation

The isolation scheme for ionomycin from a 250-liter fermentation is shown in Fig. 1. After removal of the mycelium by filtration, the filtrate, 200 liters, at pH 7.5, was extracted twice with 0.5 volume each of ethyl acetate. The organic phase was concentrated *in vacuo* to a thick syrup that was then diluted by the addition of 400 ml of methanol. After adjustment of the pH to 12 by the addition of 5 N sodium hydroxide, the suspension was further diluted by the addition of an equal volume of water. The diluted suspension was extracted repeatedly (five times) with 500 ml of a mixture of equal parts of benzene and hexane. The extracts were combined and concentrated *in vacuo* to a thick syrup. The

syrup was dissolved in 200 ml of methanol and the pH again adjusted to 12 by the addition of sodium hydroxide. An equal volume of water was added to the alkaline methanolic solution and the antibiotic was extracted four times with 200-ml aliquots of hexane each time. The hexane layers were combined and concentrated *in vacuo* to a small volume. Ionomycin crystallized readily from the concentrate as rectangular plates. Approximately 10 g of crystalline ionomycin as the calcium salt were obtained after recrystallization from acetone-hexane.

To prepare the free acid from the calcium salt, a solution of ionomycin in hexane was acidified with hydrochloric acid. Excess acid was removed by washing the solution with water. The free acid of ionomycin, as a colorless oil, was recovered by removal of the hexane *in vacuo*.

Fig. 1. Isolation and purification of ionomycin



Physical and Chemical Properties

The calcium salt of ionomycin is a colorless crystalline material that melts at 205~206°C. Like other polyether antibiotics, it is soluble in hexane, benzene, chloroform and acetone, is slightly soluble in methanol and ethanol, and is insoluble in water, dilute acid and base. Elemental analysis gave the following values: C, 66.00, H, 9.17, Ca, 5.77%. A molecular weight of 740 was obtained by the RAST technique, and a value of 746.4705 by high resolution mass spectroscopy. From these values, the formula was calculated to be $C_{41}H_{70}O_9Ca$. The calculated analysis for $C_{41}H_{70}O_9Ca$ is C, 66.00, H, 9.42, Ca, 5.37% and the calculated molecular weight is 746.4645. The optical rotation was measured in methanol: $[\alpha]_D^{23}+33°$ (c 0.4).

Fig. 2. 100 MHz NMR spectrum of ionomycin in $CDCl_3$

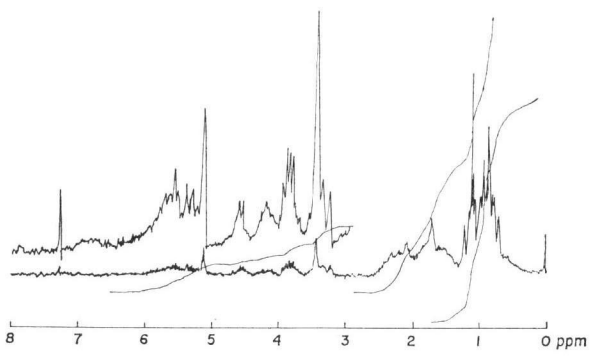


Fig. 3. UV spectra of ionomycin in methanol

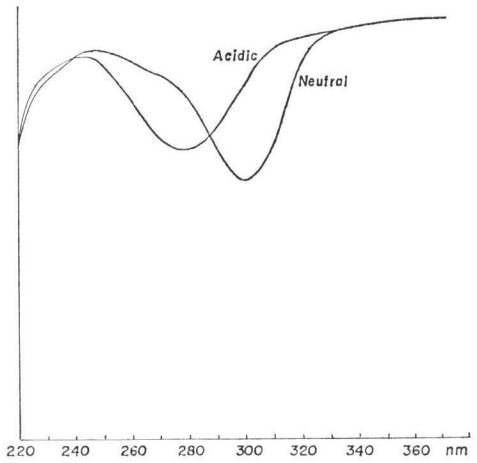


Fig. 4. IR spectrum of the calcium salt of ionomycin in KBr

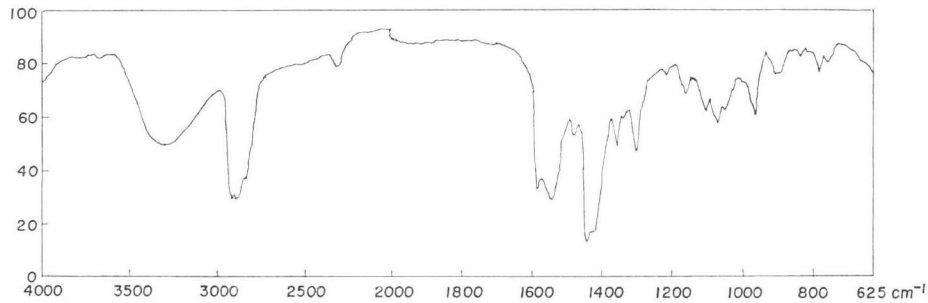
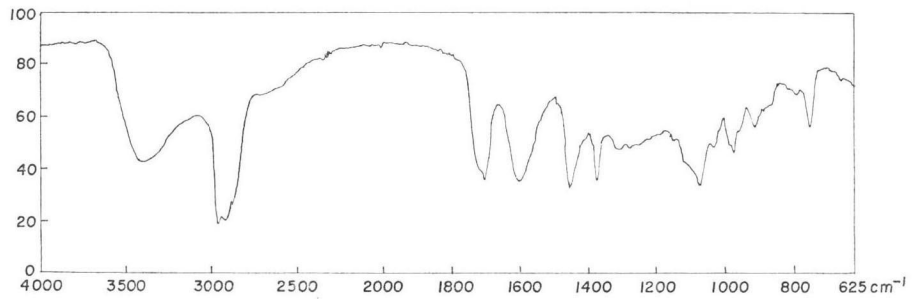


Fig. 5. IR spectrum of the free acid of ionomycin in KBr



The proton nmr spectrum is shown in Fig. 2. The ultraviolet light absorption spectrum of the calcium salt, in methanol, has a maximum at 300 nm ($E^{1\%}_{1cm}$ 290); that of the free acid is at 280 nm ($E^{1\%}_{1cm}$ 250) (Fig. 3). The infrared absorption spectrum of the calcium salt is shown in Fig. 4. The dominant peaks are: 3260, 2860, 1540, 1420, 1355, 1300, 1068, 963, 890, and 780 cm^{-1}. Those of the free acid (Fig. 5) are: 3400, 2960, 1705, 1600, 1455, 1378, 1075, 973, 915 and 757 cm^{-1}.

Table 2. Antimicrobial activity of ionomycin calcium salt

Organism	MIC (μg/ml)*
Staphylococcus aureus FAD 209P	1.6
Streptococcus pyogenes C 203	0.8
Bacillus subtilis ATCC 6633	2.8
Micrococcus luteus ATCC 9341	0.6
Diplococcus pneumoniae ATCC 6303	6.3
Corynebacterium diphtheriae ATCC 19401	18.7
Clostridium tetanomorphum SC 3103	0.6
Escherichia coli ATCC 10536	>100
Klebsiella pneumoniae SC 8411	>100
Pseudomonas aeruginosa SC 8329	>100
Salmonella schottmuelleri SC 3850	>100
Candida albicans SC 5314	>100

* Minimum inhibitory concentrations were determined by two-fold broth dilution assay.

Biological Properties

Ionomycin, like other polyether antibiotics, is active primarily against Gram-positive bacteria

Table 3. Effect of inoculum density upon the minimum inhibitory concentration of ionomycin vs. *Staphylococcus aureus* FDA 209P

Inoculum density	MIC (μg/ml)
10^7	3.1
10^6	1.6
10^5	1.2
10^4	0.8
10^3	0.6

(Table 2), with no demonstrable activity against Gram-negative bacteria. No cross-resistance was observed with a number of other antibiotics, *i.e.*, aminoglycosides, macrolides, fusidic acid, thiostrepton, penicillin, actinomycin, chloramphenicol, *etc.* A decrease in activity was observed with increasing inoculum levels of *S. aureus* FDA 209P (Table 3). The acute toxicity of ionomycin, LD_{50}, administered subcutaneously to mice is 28 mg/kg.

Discussion

Ionomycin is distinguishable from all known polyether antibiotics by its ultraviolet absorption maximum at 300 nm (Ca salt). Also of interest is the apparent avidity of the antibiotic for calcium. When isolated, the antibiotic was in the form of the calcium salt, even though sodium hydroxide was used for pH adjustment in the course of isolation. This is in agreement with the report of LIU,[5] that ionomycin is a divalent anion polyether, capable of extracting divalent cations, *e.g.*, calcium, from aqueous into organic solutions, but not capable of doing so with the monovalent cation, rubidium. The structure of ionomycin will be reported elsewhere.

Acknowledgement

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