X-14766A, A HALOGEN CONTAINING POLYETHER ANTIBIOTIC PRODUCED BY *STREPTOMYCES MALACHITOFUSCUS* SUBSP. *DOWNEYI* ATCC 31547

DISCOVERY, FERMENTATION, BIOLOGICAL PROPERTIES AND TAXONOMY OF THE PRODUCING CULTURE

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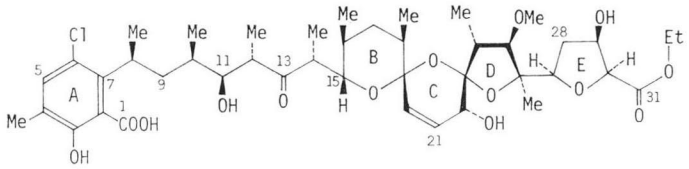
X-14766A is a novel, chlorine containing polyether antibiotic produced by *Streptomyces malachitofuscus* subsp. *downeyi*. The antibiotic is active *in vitro* against Gram-positive bacteria and is capable of complexing and transporting monovalent as well as divalent metal cations.

As part of our search for new antibiotics from soil microorganisms, a *Streptomyces* strain was found to produce a chlorine containing antibiotic which we designated X-14766A (Fig. 1). This report presents the taxonomy of the producing culture, fermentation condition for the antibiotic production, and biological as well as ionophore properties of X-14766A. The isolation and characterization of the antibiotic are described in the accompanying paper[1].

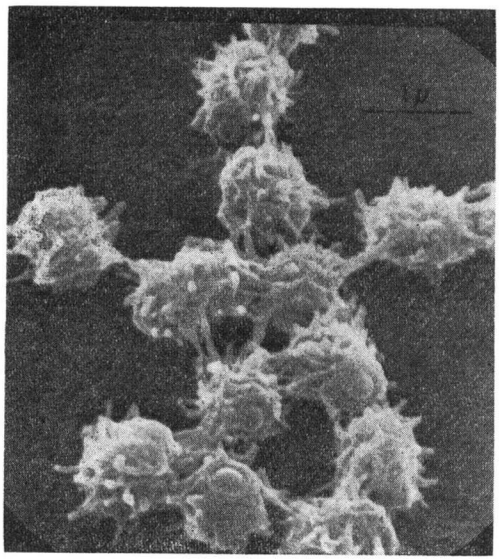
Fig. 1. The structure of X-14766A.



Taxonomy of the Producing Organism

Streptomyces sp. X-14766 was isolated from a sand sample collected from Playa Blanca, Mexico in 1976. The culture has been deposited at the Northern Regional Research Laboratory, Peoria, Illinois and the American Type Culture Collection, Rockville, Maryland, where it has been assigned the numbers NRRL 11335 and ATCC 31547 respectively. The culture resembles the type strain of *Streptomyces malachitofuscus* (ATCC 25471) in phenotypic properties. It produces a substrate mycelium which does not fragment into spores, and an aerial mycelium forming spiral chain of spores with 15~20 spores per chain. Spores are spiny and range in size from 1.2×1.0 μm to 1.1×0.73 μm (Fig. 2). The cell wall contains the LL-isomer of diaminopimelic acid which, together with the above characteristics, places this organism in the genus *Streptomyces*[2]. The growth characteristics of strain X-14766 (ATCC 31547) are summarized in Table 1. The morphological and physiological properties

Fig. 2. Spiny spores of *Streptomyces* sp. X-14766 after 14 days of incubation on ISP-2 agar at 28°C.
Spores were treated with glutaraldehyde, then ethanol, and subjected to critical point drying. Scanning electron micrograph. 45° angle.



of the strain in comparison with those of the type strain of *S. malachitofuscus* ATCC 25471 are presented in Table 2.

A comparison of the properties of strain X-14766 with *S. malachitofuscus* ATCC 25471 indicates the close resemblance of these two strains. However, in view of the difference in spore morphology (spiny, instead of spiny/hairy, spores for ATCC 31547) and antibiotic production between the two cultures, we proposed the new designation *S. malachitofuscus* subsp. *downeyi* for the strain X-14766 after ARTHUR DOWNEY who isolated the organism.

Fermentation

The vegetative inoculum of X-14766A producing culture was developed by transferring spores of *S. malachitofuscus* subsp. *downeyi* ATCC 31547 to a 500-ml Erlenmeyer flask containing 100 ml of an inoculum medium with the following composition (in g/liter): Tomato pomace 5.0, distillers soluble 5.0, O.M. peptone (Oscar Meyer & Co., Madison, Wis.) 5.0, debittered dried yeast 5.0, cornstarch 20.0, $CaCO_3$ 1.0, and K_2HPO_4 1.0. The pH of the medium was adjusted to 7.0 before autoclaving. The inoculated flask was incubated for 72 hours at 28°C on a rotary shaker operating at 250 rpm. Three ml (3%, v/v) of the resulting vegetative growth were used to inoculate a 500-ml Erlenmeyer flask containing 100 ml of the following medium (in g/liter): Glucose 10.0, molasses (Black strap) 20.0, HySoy T (Hunko Sheffield Chemical Co., Lyndhurst, N. J.) 5.0, and

Table 1. Cultural characteristics of *Streptomyces malachitofuscus* subsp. *downeyi* ATCC 31547.

Agar medium	Amount of growth; Degree of sporulation	Spore mass color[a]	Color of reverse-substrate mycelium[a]
Yeast malt extract (ISP-2)[b]	Abundant growth; well sporulated	2*ih* (dark covert gray) mostly; 2*fe* (covert gray) at edge	2*ge* (covert tan) mostly; 2*li* (covert brown)
Oatmeal (ISP-3)[b]	Abundant growth; well sporulated	3*fe* (silver gray)	2*ec* (biscuit)
Inorganic salts starch (ISP-4)[b]	Moderate growth; well sporulated; hydrolyzes starch	2*fe* (covert gray); edges of *b* (oyster white)	2*gc* (bamboo), trace of 1½*ie* (light olive)
Glycerol asparagine (ISP-5)[b]	Moderate growth; moderate sporulation	2*dc* (natural string)	2*gc* (bamboo)
CZAPEK-DOX[c]	Moderate growth; sparse sporulation	2*dc* (natural string)	2*ie* (light mustard tan)

[a] Color determinations were made after two weeks of incubation at 28°C. Color scheme used was that taken from the Color Harmony Manual, 4th ed., 1958, Container Corporation of America, Chicago.
[b] Media recommended by the International *Streptomyces* Project[3].
[c] CZAPEK-DOX broth (BBL) to which 1.5% agar was added.

Table 2. Comparison of morphological and physiological characteristics of strain ATCC 31547 and ATCC 25471.

Test[a]	ATCC 31547 (X-14766)	*S. malachitofuscus* ATCC 25471
ISP-6 darkening	+	+
Melanin, ISP-7	+, variable	−
Casein hydrolysis[b]	+	+
Gelatin hydrolysis[c]	±	+
Starch hydrolysis[d]	+	+
NaCl (%) tolerance[b]	<5	7
Growth range temp. (°C)	28~45	≥10~45
ISP-1 darkening	+	+
Reverse-side pigment	none	none
Soluble pigment	none	none
Antibiotic production	X-14766A	anti-bacterial
Nitrate reduction[b]	−	−
Hygroscopic property	+, variable	−
Spore chain form/# spores per chain	spira/15~20	RA to spira/15~25
Spore surface	spiny	spiny/hairy[f]
Carbon utilization tests[e]		
D-Glucose	++	++
D-Xylose	+	+(+)
L-Arabinose	++	++
L-Rhamnose	++	++
D-Fructose	++	++
D-Galactose	+(+)	++
Raffinose	−	−
D-Mannitol	++	++
i-Inositol	++	++
Salicin	±	+
Sucrose	++	+(+)
Cellulose	−	−

[a] All tests were run at 28°C.
[b] Test was done by method recommended in reference (4).
[c] See reference (5) for the test.
[d] Determination was made, after growth on agar of Actinomyces broth (Difco) with 0.25 % soluble starch, by flooding the plates with iodine-KI solution.
[e] −, Negative response; ±, doubtful response; +, more growth than on carbon control but less than on glucose; +(+), growth almost equal to glucose; ++, positive response equal to glucose. Tests performed on carbon utilization agar (Bacto ISP-9) containing 1.0 % of the indicated carbon source.
[f] Data obtained from reference (6).

$CaCO_3$ 2.0. The pH of the medium was adjusted to 7.2 (NaOH) before autoclaving. The fermentation was carried out for 5 days at 28°C on a rotary at 250 rpm. Time course of a typical X-14766A fermentation in shake flask is shown in Table 3.

For tank fermentation, 6 liters of inoculum growth prepared as described above was used to start the fermentation in a 100-gallon fermentor containing 60 gallons of medium with the following composition (in g/liter): Cerelose 10.0, molasses (Black strap) 20.0, HySoy T (Hunko Sheffield

Table 3. Fermentation production of X-14766A in shake flask.

Day	pH	Potency (μg/ml)
1	7.1	—
2	7.0	7
3	6.85	28
4	7.2	56
5	7.45	64

Fermentation was carried out as described in the text. Antibiotic potency in broth was estimated by an assay against *Staphylococcus aureus* ATCC 6538P using an agar diffusion method.

Table 4. *In vitro* antimicrobial activity of antibiotic X-14766A.

Organism	MIC (μg/ml)*
Pseudomonas aeruginosa ATCC 8705	>100
Proteus vulgaris ATCC 6380	>100
Escherichia coli ATCC 27856	>100
Klebsiella pneumoniae ATCC 27858	>100
Serratia marcescens ATCC 27857	>100
Serratia sp. ATCC 93	>100
Acinetobacter calcoaceticus ATCC 10153	>100
Staphylococcus aureus ATCC 6538P	0.01
Sarcina lutea ATCC 9341	0.03
Bacillus megaterium ATCC 8011	0.03
Bacillus sp. E ATCC 27859	0.006
Bacillus subtilis NRRL 558	0.05
Bacillus sp. TA ATCC 27860	0.03
Mycobacterium phlei ATCC 355	0.09
Streptomyces cellulosae ATCC 3313	0.09
Bacteroides fragilis ATCC 12290	0.19
Clostridium histolyticum 503-86	0.01
Clostridium septicum 503-34	0.01
Paecilomyces varioti ATCC 26820	>100
Penicillium digitatum ATCC 26821	>100
Candida albicans NRRL 477	>100
Saccharomyces cerevisiae ATCC 4226	>100

* Lowest two-fold dilution giving a zone of inhibition in an agar diffusion assay.

Table 5. Displacement of $^{86}Rb^+$ and $^{45}Ca^{2+}$ from X-14766A-cation complexes by other cations.

Displacing cation	Cation radius (Å)	$^{86}Rb^+$ or $^{45}Ca^{2+}$ remaining in complex (%)	
		$^{86}Rb^+$	$^{45}Ca^{2+}$
none	—	68	70
Mg^{2+}	0.82	67	55
Ca^{2+}	1.18	68	40
Sr^{2+}	1.12	70	37
Ba^{2+}	1.34	69	5
Li^+	0.68	68	64
Na^+	0.97	17	0.4
K^+	1.33	10	0.3
Rb^+	1.47	33	1
Cs^+	1.67	52	6

The cation selectivity sequence of X-14766A was determined by the method described previously.[12] Two μ moles of X-14766A (in 2 ml of 70 % toluene-30 % *n*-butanol) were equilibrated by shaking for 2 hours at 20°C with 2 μ moles of $^{86}RbCl$ or 1 μ mole of $^{45}CaCl_2$ (in 1 ml of dimethyl glycine-tetramethyl-ammonium hydroxide buffer, 25 mM, pH 10.0). Complex formation was measured by liquid scintillation counting of both phases (see Fig. 3).

Displacement of labelled cation from the ionophore complexes was determined by the addition of unlabelled cations (2 μ moles of monovalent or 1 μ mole of divalent metal chloride).

Chemical Co.)5.0, $CaCO_3$ 2.0, and SAG 4130 silicon antifoam (Union Carbide) 0.1. pH of the medium was adjusted to 7.2 (NaOH) before sterilization. The tank was stirred at an impeller speed of 280 rpm and aerated at 3 cubic feet per minute. The fermentation was harvested after 5 days of incubation at 28°C.

Biological and Ionophorous Properties

Like other polyether antibiotics, X-14766A is primarily active against Gram-positive bacteria and some anaerobes (Table 4). In addition, it exhibits *in vitro* activity against several strains of *Treponema hyodysenteriae*, a causal agent of swine dysentery (MIC: 0.02~0.04 μg/ml), and is active as an anti-malarial agent with an ED_{50} of 2.5 mg/kg against *Plasmodium bergei* in mice. The LD_{50} for X-14766A in mice is 350 mg/kg, p.o. and 5.75 mg/kg, i.p. The antibiotic is also effective in stimulating the production of propionic acid in an *in vitro* rumen fermentation[7].

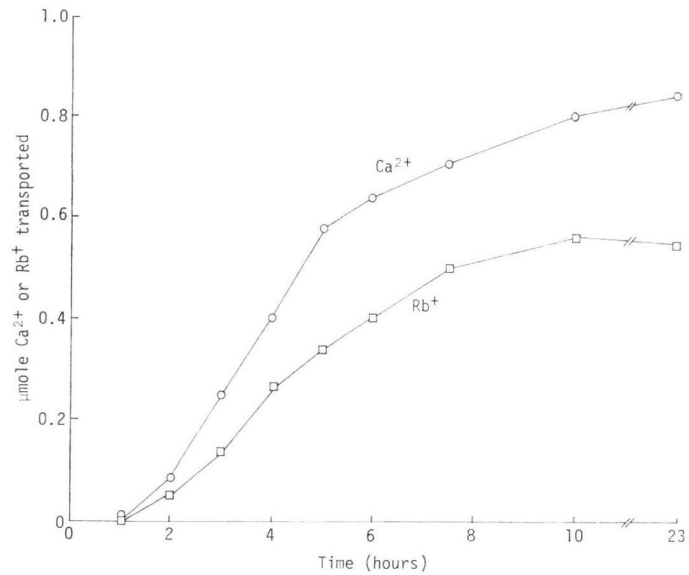
Table 6. Cation selectivity sequence of X-14766A and other ionophorous antibiotics.

Ionophore	Cation selectivity sequence
X-14766A	$K^+ > Na^+ > Rb^+ > Cs^+ \gg Li^+$ $Ba^{2+} \gg Sr^{2+}$, $Ca^{2+} > Mg^{2+}$ $K^+ > Na^+ > Rb^+ > Cs^+$, $Ba^{2+} \gg Sr^{2+}$, $Ca^{2+} > Mg^{2+} > Li^+$
Lasalocid A	Rb^+, Cs^+, $K^+ > Na^+ > Li^+$ $Ba^{2+} \gg Sr^{2+} > Ca^{2+} > Mg^{2+}$ $Ba^{2+} \gg Rb^+$, Cs^+, $K^+ \gg Sr^{2+} > Ca^{2+} > Mg^{2+} > Na^+ > Li^+$
Lysocellin	$K^+ > Rb^+ > Cs^+ > Na^+ > Li^+$ $Ba^{2+} \gg Sr^{2+} > Mg^{2+}$, Ca^{2+} $Ba^{2+} \gg K^+ > Rb^+ > Cs^+ > Na^+ > Mg^{2+}$, $Ca^{2+} > Sr^{2+} > Li^+$
X-14547A	Rb^+, Cs^+, $K^+ > Li^+$, Na^+ Mg^{2+}, $Ca^{2+} > Sr^{2+} > Ba^{2+}$ Mg^{2+}, $Ca^{2+} > Sr^{2+} > Ba^{2+} > Rb^+$, Cs^+, $K^+ > Li^+$, Na^+

The cation selectivity sequence of lasalocid A, lysocellin and X-14547A was determined by the method described in Table 5.

Fig. 3. Time course of Ca^{2+} and Rb^+ transport by antibiotic X-14766A in a U-tube.

The U-tube system described by ASHTON and STEINRAUF[11] was employed for the assay. A glass U-tube was filled with 5 ml of a chloroform solution of antibiotic X-14766A (1×10^{-4} M). Two ml of an aqueous Tris-HCl buffer (20 mM, pH 9.5) containing 1 mM [^{45}Ca] calcium chloride or [^{86}Rb] rubidium chloride was placed in one arm of the U-tube, and an equal volume of the same buffer solution with unlabelled calcium or rubidium chloride in the other arm. The reaction was started by the addition of the respective labelled metal chloride, and the chloroform phase separating the two aqueous phases was then gently stirred with a magnetic stirrer. The rate of appearances of radioactive calcium or rubidium in the label-free side was determined by counting samples (50 μl) taken from both aqueous phases with 10 ml Aquasol (New England Nuclear, Boston, Mass.) in an intertechnique liquid scintillation spectrometer.



As an ionophore, X-14766A is capable of extracting monovalent and divalent metal cations from an aqueous solution into a non-miscible organic solvent[8]. The cation selectivity sequence of X-14766A as determined by an ion-competition experiment in a two-phase system (results shown in Table 5) is compared with that of other divalent cation ionophores, lasalocid A[8], antibiotic X-14547A[9] and lysocellin[10] in Table 6. X-14766A is also capable of transporting Ca^{2+} and Rb^+ across a solvent barrier ($CHCl_3$) from one aqueous phase to another as demonstrated in an experiment using a U-tube

system of ASHTON and STEINRAUF[11] (Fig. 3). These observations indicate that X-14766A is a broad cation selective ionophore. However, unlike the other divalent cation-binding monocarboxylic polyethers, the antibiotic binds most tightly with monovalent cations such as potassium; although it transports divalent cation (calcium) at a faster rate than monovalent cation (rubidium) across the chloroform barrier.

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