NOVEL POLYETHER ANTIBIOTICS X-14868A, B, C, AND D PRODUCED BY A NOCARDIA

DISCOVERY, FERMENTATION, BIOLOGICAL AS WELL AS IONOPHORE PROPERTIES AND TAXONOMY OF THE PRODUCING CULTURE

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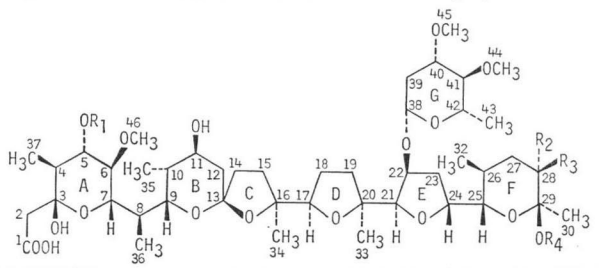
A hitherto undescribed Nocardia culture has been isolated from an Australian soil sample and found to produce novel polyether antibiotics X-14868A, X-14868B, X-14868C and X-14868D.

Polyether antibiotics are a large class of microbial secondary metabolites generally produced by soil actinomycetes of the genus Streptomyces[1]. Intensive search for new polyether antibiotics has in recent years revealed that other genera of streptomycetes also produce these antibiotics. Laidlomycin is produced by *Streptoverticillum eurocidium*[2]. Cationomycin[3], antibiotics CP-47,433 and CP-47,434[4] as well as CP-51,532[5] are produced by strains of Actinomadura. Antibiotic CP-44,161 was isolated from the fermentation broth of a Dactylosporangium culture[6]. In this paper, we report the discovery of a novel polyether antibiotic complex produced by a hitherto undescribed Nocardia, strain X-14868.

Nocardia X-14868 produces a complex of antibiotics with X-14868A the major component. A second component, X-14868B, originally thought to be elaborated directly by the producing organism, was later found to be an artifact chemically derived from X-14868A during the isolation work-up process. Other minor components that have been isolated from the culture broth are antibiotic X-14868C and X-14868D. The structure of each of these antibiotics shown in Fig. 1 was determined by X-ray crystallographic analysis[7]. This report is concerned with the taxonomy of the antibiotic producing

Fig. 1. The structures of antibiotic X-14868A, B, C and D.



	R_1	R_2	R_3	R_4
X-14868A	Me^{47}	H	Me^{31}	H
X-14868B	Me^{47}	H	Me^{31}	Me^{43}
X-14868C	H	H	Me^{31}	H
X-14868D	Me^{47}	Me^{31}	H	H

Table 1. Cultural characteristics of strain X-14868.

Agar medium	Amount of growth and aerial mycelium	Color of aerial mycelium[a]	Color of reverse substrate mycelium[a]
Yeast extract - malt extract (ISP-2)[b]	Moderate to abundant growth; some aerial mycelium in isolated edges; leathery growth	*b* (oyster white)	2 *ie* (light mustard tan)
Oatmeal (ISP-3)[b]	Moderate growth; moderate aerial mycelium	*b* (oyster white)	3 *dc* (natural)
Inorganic salts - starch (ISP-4)[b]	Sparse growth;	*b* (oyster white)	2 *dc* (natural, string)
Glycerol - asparagine (ISP-5)[b]	Poor growth; nearly no aerial mycelium; substrate mycelium	*b* (oyster white)	2 *ge* (covert tan)
Yeast extract[c]	Abundant growth; sparse aerial mycelium; leathery	*b* (oyster white)	3 *ge* (beige)
Glucose - yeast[d] extract - peptone	Moderate growth; sparse aerial mycelium at edge; leathery, brown soluble pigment	*b* (oyster white) at edge	3 *ge* (beige)
Glucose - asparagine[e]	Moderate growth; some aerial mycelium; leathery	2 *dc* (natural, string)	3 *ge* (beige)
Sucrose - nitrate[f]	Sparse growth; moderate aerial mycelium	*b* (oyster white)	translucent *c* (light gray)
Czapek - Dox (BBL)	Poor growth; moderate aerial mycelium	*b* (oyster white)	translucent *c* (light gray)
Nutrient (BBL)	Moderate growth; sparse aerial mycelium; leathery	*b* (oyster white)	2 *dc* (natural, string)
Potato - dextrose (Difco)	Moderate growth; some aerial mycelium at edges; leathery	3 *dc* (natural)	2 *ie* (light mustard tan)
Starch - nitrate[g]	Sparse growth; moderate aerial mycelium	*b* (oyster white)	translucent *c* (light gray)
BENNETT'S[h]	Abundant growth; sparse to moderate aerial mycelium; leathery	*b* (oyster white)	3 *ge* (beige)
Glucose - peptone[i]	Moderate growth; some aerial mycelium; soft leathery	*b* (oyster white)	3 *ge* (beige)
Glycerol - nitrate[j]	Poor growth; very sparse aerial mycelium; substrate mycelium flat and embedded in agar	*b* (oyster white)	translucent *c* (light gray)

[a] No spores were found in the aerial mycelium on any of the media examined after 4 weeks of incubation. The color scheme used was Color Harmony Manual, 4th ed. 1958 (Container Corporation of America, Chicago).

[b] Media recommended by the International Streptomyces Project.

[c] Yeast extract 1.0%, glucose 1.0%, agar 1.5%, pH 6.8.

[d] Glucose 0.3%, yeast extract 0.5%, peptone 0.5%, $CaCO_3$ 0.75%, agar 1.5%, pH 7.0.

[e] Glucose 1.0%, asparagine 0.05%, K_2HPO_4 0.05%, agar 1.5%, pH 6.8.

[f] Sucrose 1.0%, $NaNO_3$ 0.2%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, KCl 0.05%, agar 1.5%.

[g] Starch 1%, $NaNO_3$ 0.2%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, KCl 0.05%, agar 1.5%.

[h] Yeast extract 0.1%, beef extract 0.1%, N-Z Amine A (enzymatic casein hydrolysate, Humko-Sheffield Chemical Co., Lyndhurst, N.J.) 0.2%, glucose 1.0%, agar 1.8%, pH 7.3.

[i] Glucose 0.5%, Bacto peptone 0.5%, yeast extract 0.5%, agar 1.75%.

[j] Glycerol 1%, $NaNO_3$ 0.2%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, KCl 0.05%, agar 1.5%.

culture, fermentation conditions for the production of the antibiotics, and the biological and ionophore properties of antibiotics X-14868A and X-14868B.

Taxonomy of the Producing Organism

The streptomycete culture producing antibiotic X-14868A and other minor antibiotic components was isolated from a beach sand sample collected in Colloroy, Australia. The culture was designated X-14868 and has been deposited at the American Type Culture Collection, Rockville, Maryland, where it has been assigned the number ATCC 31585. Strain X-14868 has the following morphological, physiological and chemical characteristics.

Microscopic and Macroscopic Examination

Strain X-14868 cultivated on the standard ISP media (Difco) produces a substrate mycelium which fragments after several days allowing extensive mycelial development. It produces an aerial mycelium consisting of rope-like tufts but no spores were found. Table 1 summarizes the amount of growth, aerial mass color, color of reverse substrate mycelium and presence of any soluble pigment produced by strain X-14868 on various solid media after 4 weeks of incubation at 28°C.

Physiological Characteristics

The carbon utilization and other metabolic characteristics of strain X-14868 are shown in Tables 2 and 3. Strain X-14868 hydrolyzed gelatin and casein but not starch. The culture was resistant to penicillin in an agar diffusion test (10 units benzylpenicillin in a *ca.* 6 mm disk), and when tested according to the method of GORDON[8], was resistant to lysozyme (0.005%) as well. The strain completely peptonized litmus milk (Difco) and no melanin production was detected on ISP-6 or ISP-7 medium.

Table 2. Carbon utilization on ISP-9 medium by strain X-14868.

Carbon source	Response[a]
No carbon control	−
D-Glucose	++
D-Xylose	−
L-Arabinose	−
L-Rhamnose	−
D-Fructose	±
D-Galactose	+
Raffinose	−
D-Mannitol	−
i-Inositol	−
Salicin	±
Sucrose	−
Cellulose	−

[a] −, Negative response at 28°C after one month incubation.
±, Doubtful response.
+, More growth than no carbon control but less than on glucose.
++, Positive response, equal to the amount of growth on glucose.

Table 3. Physiological characteristics of X-14868.

Test[a]	Result
ISP-1, darkening	−
ISP-6, darkening	−
ISP-7, melanin	−
Casein hydrolysis[b]	++
Gelatin hydrolysis[c]	++
Starch hydrolysis[d]	−
NaCl (%) tolerance[c]	5%
Temperature at which growth was observed	28 and 36°C
Reverse side pigment	None
Soluble pigment	brown on glucose - yeast extract - peptone
Penicillin (10 unit disk) sensitivity	−
Nitrate reduction[b]	++
Gram stain	+
Acid fast stain[b]	−
Catalase	+

[a] All tests were done at 28°C.
[b] Test was done by the method recommended in reference 8.
[c] See reference 12 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.

Cell Wall Analysis

The cell wall analysis of the isomer of diaminopimelic acid was performed by the method of BECKER *et al.*[9]. For sugar content of the cell wall, the method of LECHEVALIER and LECHEVALIER[10] was followed. These analyses revealed that the cell wall of X-14868 contains the *meso*-isomer of diaminopimelic acid as well as galactose and arabinose.

Based on these morphological and physiological characteristics as well as the chemical composition of its cell wall (cell wall type IV of the classification of Actinomycetales by LECHEVALIER and LECHEVALIER[10]), strain X-14868 is assigned to the genus Nocardia.

According to BERGEY's manual[11], strain X-14868 can be placed in the Nocardia morphological group III with abundant mycelial development due to delayed fragmentation. This is a vague classification based only on the acid-fast heterotrophic properties of the organism. Using this scheme, the closest species would be *Nocardia kuroishii*. However, the carbon utilization data and many physiological properties are inconsistent with this identity. Based on the available literature on Nocardia, there is no description of a species of Nocardia resembling X-14868. However, until a comparative study of several known Nocardia species with species X-14868 is done, a new species designation will not be proposed.

Fermentation

To prepare vegetative inoculum for the production of antibiotic X-14868A and its minor components, *Nocardia* sp. X-14868 (ATCC 31585) was grown in a 500-ml Erlenmeyer flask containing 100 ml of medium with the following composition (g/liter): Tomato pomace solid 5.0, distillers soluble 5.0, OM peptone (Oscar Meyer Co., Madison, Wis.) 5.0, debittered yeast 5.0, corn starch 20.0, $CaCO_3$ 1.0, K_2HPO_4 1.0. The pH of the medium was adjusted to 7.0 before sterilization. After 3 days of growth at 28°C on a rotary shaker (250 rpm), 250 ml of the culture broth were inoculated into a 14-liter jar fermentor containing 9 liters of medium with following composition (g/liter): Cerelose (technical grade glucose, C.P.C. International Co., Englewood Cliffs, N.J.) 45.0, Eclipse N starch (A.E. Staley Manufacturing Co., Decatur, I11.) 20.0, Soyalose 105 (Central Soya Co., Chemurgy Division, Chicago, I11.) 15.0, casein hydrolysate acid (General Biochemicals, Chagrin Falls, Ohio) 1.0, black strap molasses 3.0, $CaCO_3$ 2.5. The medium pH was adjusted to 7.1 before sterilization. The fermentation was carried out at 28°C with an aeration of one volume air per volume broth per minute and an agitation of 400 rpm. The potency of antibiotic in broth was estimated by bioassay using *Staphylococcus aureus* ATCC 6538P as test organism. The time course of X-14868 fermentation is shown in Fig. 2. The antibiotic production began about 2 days after inoculation and continued linearly with an estimated antibiotic yield of about 2,500 μg/ml after 8 days of fermentation.

Fig. 2. Time course of antibiotic X-14868A fermentation.

The fermentation was run in a 14-liter jar fermentor as described in the text.

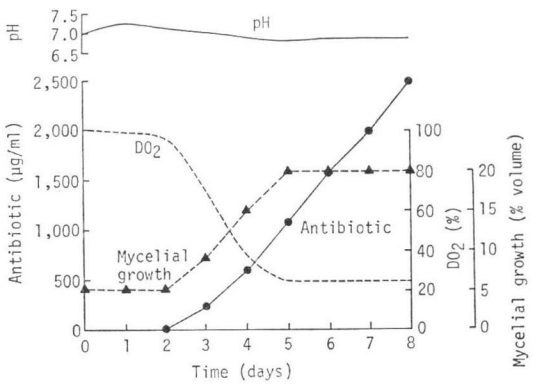


Table 4. *In vitro* antimicrobial activity of antibiotic X-14868A, X-14868B, X-14868C and X-14868D sodium salt.

		Minimal inhibitory concentration (μg/ml)*			
		X-14868A	X-14868B	X-14868C	X-14868D
Gram-negative rods	*Pseudomonas aeruginosa* 56 ATCC 8705	>1,000	>1,000	>1,000	>1,000
	Proteus vulgaris ATCC 6380	>1,000	>1,000	>1,000	>1,000
	Escherichia coli ATCC 27856	>1,000	>1,000	>1,000	>1,000
	Klebsiella pneumoniae ATCC 27858	>1,000	>1,000	>1,000	>1,000
	Serratia marcescens ATCC 27857	>1,000	>1,000	>1,000	>1,000
	Serratia sp. ATCC 93	>1,000	>1,000	>1,000	>1,000
	Acinetobacter calcoaceticus PCI3 ATCC 10153	>1,000	>1,000	>1,000	>1,000
Gram-positive cocci	*Streptococcus faecium* ATCC 8043	0.9	1.57	0.79	0.19
	Staphylococcus aureus ATCC 6538P	7.9	6.25	62.5	12.5
	Micrococcus luteus ATCC 9341	62.5	12.5	250	62.5
Gram-positive rods	*Bacillus megaterium* ATCC 8011	62.5	7.5	125	62.5
	Bacillus sp. E ATCC 27359	1.9	0.79	6.25	1.57
	Bacillus subtilis NRRL 558	15.7	25	250	62.5
	Bacillus sp. TA ATCC 27860	15.7	12.5	125	25
	Mycobacterium phlei ATCC 355	62.5	25	250	62.5
Gram-positive filament	*Streptomyces cellulosae* ATCC 3313	125	25	500	125
Molds	*Paecilomyces varioti* ATCC 25820	250	500	>1,000	>1,000
	Penicillium digitatum ATCC 26821	>1,000	1,000	>1,000	>1,000
Yeasts	*Candida albicans* NRRL 477	500	100	>1,000	>1,000
	Saccharomyces cerevisiae ATCC 4226	1,000	>1,000	>1,000	>1,000

* Lowest two-fold dilution still showing zone of inhibition by an agar well diffusion assay.

Table 5. Anticoccidial efficacy of antibiotic X-14868A and X-14868B against *Eimeria tenella* in 2-week-old chicks[a].

Treatment	Concentration in feed (ppm)	No. of birds	Weight gain (%)	Feed[b] conversion	% Mortality	ADI[c]
UUC[d]	0	30	100	1.68	0	0.0
IUC[e]	0	30	54	2.33	51	3.6
X-14868A	10	30	81	1.87	0	0.1
	7.5	30	89	1.75	0	0.0
	5	30	94	1.73	0	0.4
X-14868B	17.5	30	78	2.03	0	0.0
	15	30	85	1.97	0	0.1
	12.5	30	90	1.79	0	0.0

[a] Medicated 2 days before infection and for 9 consecutive days; 100,000 sporulated oocysts per bird: parameters recorded 7 days post infection. See reference 13 for experimental detail.

[b] Feed conversion=Average feed consumed/Average body weight gained.

[c] ADI: Average degree of infection: indicates the severity of pathological lesions; 0=normal; 1=slight; 2=moderate; 3=severe; 4=dead.

[d] UUC: Uninfected unmedicated control.

[e] IUC: Infected unmedicated control.

Biological and Ionophore Properties

The antimicrobial activity of antibiotics X-14868A, B, C, and D are shown in Table 4. They are mainly active against Gram-positive bacteria and exhibit no activity against Gram-negative bacteria and several fungi tested. Antibiotic X-14868A is also active against *Treponema hyodysenteriae*. The *in vitro* minimum inhibitory concentration against several strains of this organism was found to be 0.005 μg/ml. Antibiotics X-14868A and B are potent anticoccidial agents. The amount needed in feed to prevent chicken coccidiosis caused by *Eimeria tenella* is shown in Table 5. These data indicate that chicken coccidiosis can be effectively controlled by antibiotic X-14868A at about 7 parts per million (ppm) and by X-14868B at 15 ppm in feed. This is an order of magnitude more active than other known coccidiostats such as monensin (approved use level 98~121 ppm)[14], lasalocid (approved use level 75~125 ppm)[15] or salinomycin (60~100 ppm)[16].

The ionophore properties of antibiotics X-14868A and B were studied. The affinity of these antibiotics to monovalent and divalent metal cations was examined by a cation competition experiment using a two-phase system described in Table 6. The results show that antibiotic X-14868A has the highest affinity for potassium ion and that X-14868B binds most tightly with the divalent cation, magnesium. Based on these results, the cation selectivity sequence of these antibiotics can be summarized below:

X-14868A: $K^+ > Rb^+ > Na^+ > Li^+ > Cs^+ \gg Mg^{2+}, Ca^{2+}, Ba^{2+}, Sr^{2+}$

X-14868B: $Mg^{2+} > Ca^{2+} > Ba^{2+}, Sr^{2+} > Li^+ > K^+ > Rb^+, Na^+ > Cs^+$

A time course of cation transport by these antibiotics in a U-tube system[13] is shown in Fig. 3. X-14868A transports the monovalent cation rubidium effectively in this system, but the transport of divalent cation calcium is much less efficient. On the other hand, X-14868B transports both rubidium and calcium with about equal efficiency but at a slower rate compared with that of antibiotic X-14868A.

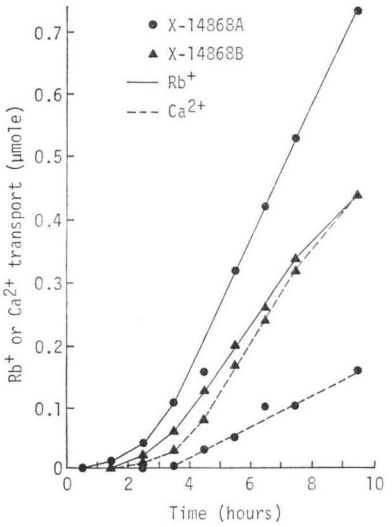
Table 6. Displacement of $^{86}Rb^+$ and $^{45}Ca^{2+}$ from X-14868A and X-14868B-cation complexes by other cations.

Displacing cations	Cation radius (Å)	^{86}Rb or ^{45}Ca remaining on complex (%)			
		X-14868A		X-14868B	
		$^{86}Rb^+$	$^{45}Ca^{2+}$	$^{86}Rb^+$	$^{45}Ca^{2+}$
none	—	86	72	38	79
Ca^{2+}	0.82	84	44	11	49
Mg^{2+}	1.18	83	44	8	43
Sr^{2+}	1.12	85	57	15	59
Ba^{2+}	1.34	83	52	14	60
Li^+	0.68	54	11	23	67
Na^+	0.97	52	6	29	74
K^+	1.33	32	1	25	72
Rb^+	1.47	44	4	28	74
Cs^+	1.67	67	20	31	74

The cation selectivity sequence of X-14868A and X-14868B was determined by the method described previously[17]. Two μmole of antibiotic (in 2 ml of 70% toluene - 30% *n*-butanol) were equilibrated by shaking for 2 hours at 20°C with 2 μmole of ^{86}RbCl or 1 μmole of $^{45}CaCl_2$ (in 1 ml of dimethyl glycine-tetramethylammonium 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 μmole of monovalent or 1 μmole of divalent metal chloride).

Fig. 3. Time course of Ca^{2+} and Rb^{+} transport by antibiotic X-14868A and X-14868B in a U-tube.



The U-tube system described by ASHTON and STEINRAUF[18] was employed for the assay. A glass U-tube was filled with 5 ml of a chloroform solution of antibiotic (1×10^{-4} M). Two ml of an aqueous tris-HCl buffer (20 mM, pH 9.5) containing 1 mM $^{45}CaCl_2$ or $^{86}RbCl$ 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 appearance 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.

It is interesting to note the striking difference in the cation binding and transporting properties of antibiotics X-14868A and B in view of the fact that these antibiotics differ only by a single methyl group (Fig. 1). It should be noted, however, that this slight change in structure leads to a marked conformational change which, in turn, alters the cation binding property of the antibiotic. The peculiar cation binding property of antibiotic X-14868B is also reflected in the unusual 2: 1 stoichiometry found for the X-14868B: Na complex[7].

Acknowledgment

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