MICROBIAL PRODUCTS. IV[1]

X-14847, A NEW AMINOGLYCOSIDE FROM *MICROMONOSPORA ECHINOSPORA*

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(Received for publication August 4, 1980)

Micromonospora echinospora strain X-14847 produces gentamicin A as the major antibiotic together with a new aminoglycoside, termed X-14847, and identified as a 2-amino-2-deoxy-α-D-glucopyranosyl *myo*-inositol. This report describes the taxonomy of the culture, fermentation conditions, the isolation and the identification of X-14847.

Aminoglycoside-producing *Micromonospora* generally elaborate these compounds as complex mixtures. The culture described in this paper attracted our attention by apparently elaborating only one aminoglycoside antibiotic which we identified as gentamicin A. The required isolation work, however, revealed a second component, X-14847, whose antibiotic activity could only be demonstrated after purification.

Isolation of Strain X-14847

Strain X-14847 was isolated from a soil sample collected on a farm near Singleton, Australia and was deposited at the Northern Regional Research Center, Peoria, Illinois (NRRL B-12180). It can be grown and maintained in standard media for actinomycetes.

The methods for the phenotypic characterization of the culture were the following: Cultural characters (amount of growth and sporulation, pigmentation) are described after growth for 2 weeks on solid media such as those recommended by the International *Streptomyces* Project[2] (Difco ISP media, Difco Laboratories, Detroit, Michigan), CZAPEK-DOX (BBL, Cockeysville, Maryland), and CZAPEK-DOX broth with the addition of 1% soluble starch.

Growth on potato plugs was tested according to LUEDEMANN[3]. Casein hydrolysis was assayed by the method of GORDON and SMITH[4], and gelatin hydrolysis was assayed according to SKERMAN[5], but substituting Difco *Actinomyces* broth for meat infusion. Starch hydrolysis was tested on Difco *Actinomyces* broth supplemented with 0.25% soluble starch and 2% agar. Carbon utilization was tested with the basal media of SHIRLING and GOTTLIEB[2] and of LUEDEMANN[2], and a modification of LUEDEMANN's medium containing 0.01% yeast extract instead of 0.1%. Nitrate reduction was tested in the *Micromonospora* medium of LUEDEMANN[3], and sodium chloride tolerance was determined by the method of GORDON and SMITH[4]. The inoculum for the various media consisted of homogenized and washed suspensions of cells cultivated in ISP-1 broth for 48~96 hours at 28°C. All cultures were incubated at 28°C, but the carbon assimilation tests were performed both at 28° and 37°C.

Taxonomy

Macroscopic characters: Strain X-14847 produces a brick-red to eggplant-colored mycelium and an ebony-brown spore mass. The type of vegetative growth, sporulation and pigmentation in various media are given in Table 1. Soluble pigments are not usually produced, although a faint diffusible red-

Table 1. Cultural characteristics of strain X-14847.

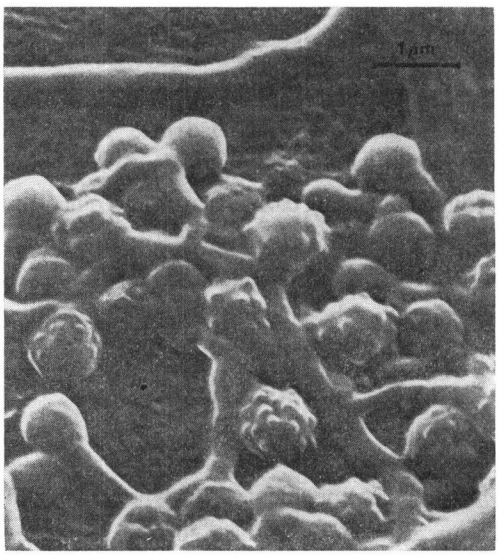
Agar medium	Amount of growth degree of sporulation	Color of spore mass[a]	Color of reverse substrate mycelium[a]
Yeast malt extract (ISP 2)[b]	moderate to abundant growth; well sporulated	8 pn (ebony brown) and some 5 cb (no name) at edge	8 pn (ebony brown) and 3 dc (natural)
Oatmeal (ISP 3)	moderate growth; well sporulated	8 pn (ebony brown); 6 1g (dark redwood) at edge	8 pn (ebony brown)
Inorganic salts starch (ISP 4)[b]	moderate growth; well sporulated	8 pn (ebony brown); 6 ng (brick red); + 5 1e (rust tan)	8 pn (ebony brown) and 6 ng (brick red)
Glycerol asparagine (ISP 5)[b]	poor growth; poor sporulation	6 ng (brick red)	6 ng (brick red)
CZAPEK-DOX[c]	moderate growth; moderate sporulation	10 nl (eggplant); 5 1e (rust tan); + 6 ng (brick red)	10 nl (eggplant); 5 1e (rust tan)
CZAPEK-DOX[c] + 1 % starch	moderate to abundant growth; moderate sporulation	7½ ni (mauve wine); 5 ng (brick red)	6 ng (brick red)

[a] Colors according to the Color Harmony Manual, 4th ed., 1958 (Container Corporation of America, Chicago).
[b] Difco media recommended by the International *Streptomyces* Project[2].
[c] CZAPEK-DOX broth (BBL) to which 1.5 % agar was added.

dish pigment may be observed in some media. The strain grows well on potato plugs with added calcium carbonate, but poorly without.

Microscopic characters: The organism grows in the form of a branching substrate mycelium which does not disintegrate with age, and spores are produced singly on short side branches. The spores are knobby (echinulate, but with dull, rounded spikes) spheres, ranging in size from 0.8 μm to 1.0 μm in diameter (Fig. 1).

Fig. 1. Knobby spores of strain X-14847 grown on ISP 2 agar after 2-weeks incubation.
Preparation was fixed with glutaraldehyde and dried at the critical point. Scanning electron microphotograph.



Cell wall composition[6]: The cell wall contains *meso*-diaminopimelic acid. This fact, and the morphological and colonial characteristics, indicate that culture X-14847 is a member of the genus *Micromonospora*.

Physiological characters: Strain X-14847 reduces nitrate to nitrite only slightly. It is capable of hydrolyzing gelatin, casein and starch. The results of the carbon assimilation tests are given in Table 2. Additional physiological criteria of diagnostic importance are given in Table 3.

Taxonomic conclusions: A comparison of the properties of *Micromonospora* X-14847 with those of various species described in the literature[7,8] suggests similarities of our strain to both *M. echinospora* and *M. purpurea*. In the opinion of LUEDEMANN and BRODSKY[9], variants of *M. purpurea* with echinulate spores should be assign-

Table 2. Carbon utilization[a] by strain X-14847 on various media.

Carbon source	ISP 9[2] at 37°C	Luedmann's medium[3]		0.1 % yeast extract, 0.1 % $CaCO_3$, and 1.5 % agar[b]	
		28°C	37°C	28°C	37°C
Control	−	+	+	−	−
D-Glucose	−	++	++	++	++
D-Xylose	+	++	+(+)	++	++
L-Arabinose	+	++	+	+	+
L-Rhamnose	++	++	+	± ~ −	−
D-Fructose	−	++	++	+(+)	+(+)
D-Galactose	−	+	++	+(+)	++
Raffinose	−	+	+	−	−
D-Mannitol	−	+	+	−	−
myo-Inositol	−	+	+	−	−
Salicin	−	−	−	−	−
Sucrose	−	++	+(+)	+(+)	++
Cellulose	−	−	−	− ~ ±	±
Maltose	+	++	++	+	++
Glycerol	−	+	+	−	−
Melizitose	−	+	+	−	−
Dulcitol	−	+	+	−	−
Melibiose	−	+	+	−	−
D-Arabinose	−	+	+(+)	+	++
Ribose	−	±	+	−	−

[a] −: No growth; ±: poor growth; +: growth more abundant than on control plate without carbon source, but less than maximum growth with best carbon source on the same medium; +(+): growth almost as good as with best carbon source; ++: abundant growth. Due to their relative value, the same symbols do not necessarily have the same meaning for different basal media or conditions of incubation.

[b] Growth obtained on this medium at 28°C was generally poor, even with the best carbon source.

Table 3. Metabolic and macroscopic characteristics of strain X-14847.

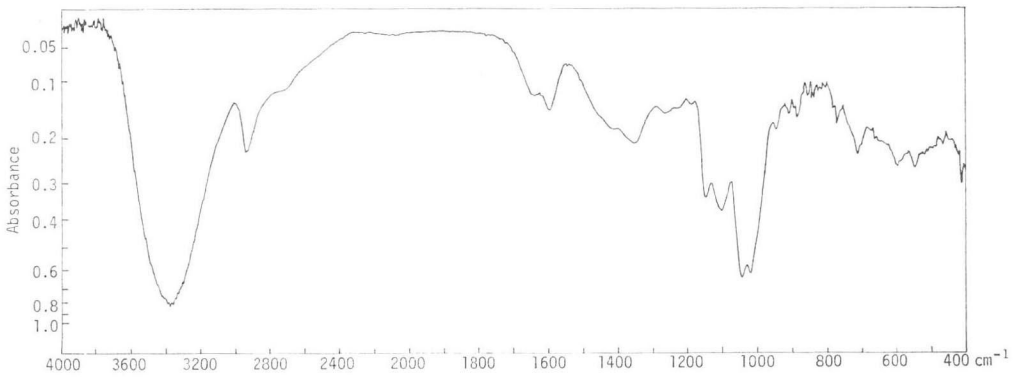
Test	Result
ISP 6, darkening	−
Melanin, ISP 7	+
Casein hydrolysis	+
Gelatin hydrolysis	+
Starch hydrolysis	+
NaCl (%) tolerance	≦3
Reverse-side pigment	brick-red to eggplant
Soluble pigment	reddish
Nitrate reduction	+ slight

ed to *M. echinospora*. It is evident that the spores of X-14847 are not truly echinulate, but in view of the general resemblance of our organism to the pictures published by Luedemann and Brodsky[9], we conclude that our strain belongs to the species *M. echinospora*.

Fermentation

Culture X-14847 was cultivated and maintained in a starch-casein agar slant (Soluble starch 1%, casein 0.1%, K_2HPO_4 0.05%, $MgSO_4$ 0.05%, agar 1.5%, pH adjusted to 7.4). The vegetative inoculum was prepared by transferring the spores from the agar slant into a 500-ml Erlenmeyer flask containing 100 ml of medium with the following composition (g/liter): Tomato pomace dried solids, 5.0 (Seabord Supply); distillers soluble, 5.0 (Brown and Forman); O. M. peptone, 5.0 (Oscar Meyers); corn starch, 20.0; $CaCO_3$, 1.0; and

Fig. 2. IR spectrum of X-14847 in KBr.



K_2HPO_4, 1.0. The pH was adjusted to 7.0 (NaOH) before sterilization. The culture was cultivated for 72 hours on a rotary shaker (250 rpm) at 28°C; 3% (v/v) of this vegetative growth was used to prepare a second stage vegetative inoculum in a 6-liter Erlenmeyer flask containing 2 liters of the medium described above. The flasks were incubated for 72 hours under the same condition. Four liters of the resulting broth were then used to start a 240-liter fermentation in a tank containing the same medium. The fermentation was carried out for 6 days at an aeration rate of 0.085 m^3/minute with an impeller speed of 280 rpm.

Isolation

The whole broth (240 liters) was adjusted to pH 2.0 (H_2SO_4), stirred (15 minutes) and filtered. The filtrate was readjusted to pH 7.5 (NH_4OH) and passed through a column of Amberlite IRC-50 (NH_4^+, 30 cm×90 cm) at a linear flow rate of 4 cm/minute. The column was washed with water and eluted with 3.0 M ammonium hydroxide. The activity was concentrated, filtered and freeze-dried to yield 29.6 g of solids.

The crude solids (23 g) were further purified by passage through a second column of Amberlite IRC-50 (NH_4^+, 10 cm×40 cm) which was washed with water and then eluted with 1.0 M ammonium hydroxide. The ammoniacal effluent was concentrated and freeze-dried. An aqueous solution of the resulting solids (8 g) was chromatographed on a column of Dowex 1-X4, 100~200 mesh (OH^-, 2.5 cm× 50 cm). Elution of the column with water[10] gave two biologically active bands as determined by bioassay with *Escherichia coli* on neomycin assay agar plates. Concentration and freeze-drying furnished 1.72 g from the first and 0.75 g of solids from the second band.

Identification of the major component as gentamicin A[11]: The first band represented the major antibiotic component which was identified as gentamicin A by tlc of the free base[10] and of the N-acetyl derivative[10]. The ^{13}C NMR spectrum was identical with that of gentamicin A[12].

Characterization of component X-14847: The minor component was rechromatographed on a column of Dowex 1-X4 and obtained as a homogeneous but amorphous, ninhydrin-positive, non-reducing, white powder with Rf 0.33 (tlc, chloroform - methanol - concd. ammonium hydroxide - water, 1: 4: 2: 1[10]), $[\alpha]+88.5°$ (c 0.24, water). The IR spectrum of X-14847 in KBr is shown in Fig. 2.

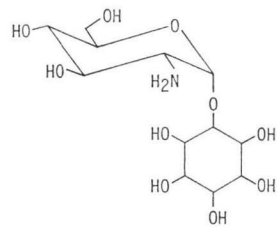
Anal. Calcd for $C_{12}H_{23}NO_{10}\cdot H_2O$ (359.33): C 40.11, H 7.01, N 3.90.
Found: C 40.08, H 6.68, N 4.24.

MS: m/z(%) 341(<1), 162 (7.5, 2-amino-2-deoxy-glucopyranosylium cation), 59 (100).

Hydrolysis of X-14847 in 6 N hydrochloric acid at 100°C for 12 hours liberated one ninhydrin positive degradation product which was adsorbed on Dowex 50-X4 and could be eluted with 1 M hydrochloric acid from the resin. The residue obtained upon evaporation of the acidic eluate was recrystallized from aqueous ethanol and identified as 2-amino-2-deoxy-D-glucose. The second hydrolysis product was not adsorbed on Dowex 50. It was obtained as colorless needles with mp 225°C upon evaporation of the aqueous column washings and recrystallization of the residue and was identified as *myo*-inositol.

X-14847 consumed 6 equivalents of periodate. The N-acetyl derivative of X-14847 was prepared from the free base (200 mg) by suspending it in methanol (10 ml) and shaking the mixture with acetic anhydride (2 ml) overnight. Precipitation of the product was completed by the addition of diethyl ether yielding a white powder (^{1}H NMR in D_2O: δ 2.51, s, 3H, CH_3CON; 3.60~4.74, m, 12H; 5.58, H-l, $J_{1,2}$=3 Hz) which consumed 5 equivalents of periodate without liberation of formaldehyde so that the amino sugar must be present in the pyranosyl form.

The ^{1}H NMR spectrum of X-14847 exhibits the anomeric proton at δ 5.68 with $J_{1,2}$=3.5 Hz, typical of a synclinal dihedral angle. The glucosidic linkage is therefore of the α-D-configuration and the structure of X-14847 can be represented as shown.



Structure of X-14847

Component X-14847 exhibited only slight antibacterial activity *in vitro* with minimum inhibitory concentrations of 0.2~1.0 mg/ml when tested on neomycin agar (BBL) against several sensitive Gram-positive bacteria.

Acknowledgment

We thank Mrs. F. WEISS for the isolation of the microorganism, Dr. D. N. BULL for fermentations, Dr. P. A. MILLER for useful discussions, and Mr. A. DOWNEY for capable technical assistance.

References

1) Previous paper in this series: MAEHR, H.; J. SMALLHEER, M. CHIN, N. PALLERONI, F. WEISS & C-M. LIU: Microbial products. III. *Epi*-deoxynegamycin from a *Micromonospora*. J. Antibiotics 32: 531~532, 1979
2) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. System. Bacteriol. 16: 313~340, 1966
3) LUEDEMANN, G.: *Micromonospora purpureochromogenes* (WAKSMAN and CURTIS 1916) comb. nov. (Subjective synonym: *Micromonospora fusca* JENSEN 1932). Int. J. System. Bacteriol. 21: 240~247, 1971
4) GORDON, R. E. & M. M. SMITH: Rapidly growing, acid-fast bacteria. J. Bacteriol. 66: 41~48, 1953
5) SKERMAN, V. B. D.: A Guide to Identification of the Genera of Bacteria. The Williams and Wilkins Co., Baltimore, 1967
6) BECKER, B.; M. P. LECHEVALIER, R. E. GORDON & H. A. LECHEVALIER: Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole-cell hydrolysates. Appl. Microbiol. 12: 421~423, 1964
7) BUCHANAN, R. E. & N. E. GIBBONS (*ed.*): BERGEY's Manual of Determinative Bacteriology. 8th ed., pp. 846~855, The Williams and Wilkins Co., Baltimore, 1974
8) LUEDEMANN, G.: *Micromonospora* taxonomy. *In* D. PERLMAN (*ed.*) Advances in Applied Microbiology. Vol. II. pp. 101~103. Academic Press Inc., N. Y., 1970
9) LUEDEMANN, G. & B. BRODSKY: Taxonomy of gentamicin-producing *Micromonospora*. Antimicr. Agents & Chemoth.-1963: 116~124, 1964

10) MAEHR, H. & C. P. SCHAFFNER: The separation and differentiation of the gentamicin complex. J. Chromatogr 30: 572~578, 1967
11) MAEHR, H. & C. P. SCHAFFNER: Chemistry of the gentamicins. II. Stereochemistry and synthesis of gentosamine. Total structure of gentamicin A. J. Amer. Chem. Soc. 92: 1697~1700, 1970
12) NAGABUSHAN, T. L.; W. N. TURNER, P. J. L. DANIELS & J. B. MORTON: The gentamicin antibiotics. 7. Structures of the gentamicin antibiotics A_1, A_3, and A_4. J. Org. Chem. 40: 2830~2834, 1975