# ANTIBIOTICS PRODUCED BY *STREPTOMYCES*. VIII*

## A NEW POLYENIC ANTIBIOTIC, OLEFICIN, EXHIBITING ANTIBACTERIAL ACTIVITY

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A new polyenic antibiotic has been isolated from a strain A-461, related to *Streptomyces parvulus* which is described in this paper. Oleficin was obtained as a dark red powder by methanolic extraction of the filtered mycelium, followed by evaporation of the solvent and re-extraction into ethyl acetate, evaporation, precipitation with petroleum ether and chromatography on alumina. Its ultraviolet and visible spectra suggest the possible presence in its molecule of two chromophores, one of which is a polyene-like. It is active against Gram-positive bacteria and has no effect upon the growth of fungi and yeasts.

During the past years an extensive literature has accumulated on the production of polyenic macrolide antibiotics (nystatin, trichomycin, candicidin, *etc.*) by *Streptomyces* species. These substances were highly active against pathogenic yeasts and yeast-like fungi, but practically inactive against bacteria. Limocrocin[1] is an acidic substance produced by *S. limosus*. Its structure with the presence of a chain of seven conjugated olefinic double bonds not in a macrolide ring was established in 1969. Its antimicrobial action is not described. The isolation of an octaene-type antibiotic ochramycin[2] was reported in 1967. This substance was found to be active against Gram-positive bacteria, but not against Gram-negative bacteria; its antifungal activity is very slight.

The polyenic antibiotic isolated by us and named oleficin has activity against Gram-positive bacteria but no effect on fungi or yeasts. The antibiotic seems to be similar in some respects to limocrocin and ochramycin, but the compounds are not identical.

### Description of the strain A-461

The strain A-461 was isolated from a soil sample. Its condensed description is given below:

Spore chain morphology: Section spirales. Spores form numerous closed spirals with 2～8 turns. This morphology is seen on yeast-malt agar, oatmeal agar, salts-starch agar and glycerol-arparagine agar. Spore surface: Smooth (Fig. 1).

* Previous paper: J. BÉRDY, J. ZSADÁNYI, M. HALÁSZ, I. HORVÁTH and K. MAGYAR: Antibiotics produced by *Streptomyces*. VII. Cytotetrin, a new antitumor antibiotic. J. Antibiotics 24: 209～214, 1971.

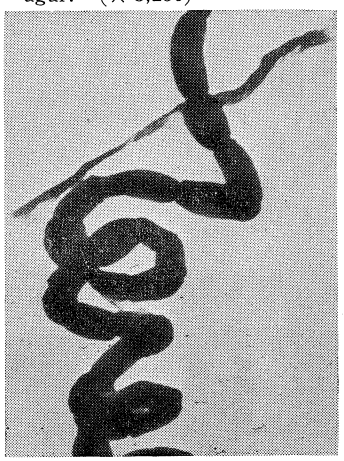
Color of colony: Aerial mass color in the gray color-series on yeast-malt agar, oatmeal agar, salts-starch agar and glycerol-asparagine agar.

Reverse side of colony: No distinctive pigments on salts-starch agar; Yellow-brown plus red on yeast-malt agar, oatmeal agar and glycerol-asparagine agar.

Color in medium: Melanoid pigments not formed in peptone-yeast-iron agar and tyrosine agar. No pigment found in medium in yeast-malt agar and oatmeal agar.

The cultural characteristics on various media are the following: (The codes in parentheses denote, in relation to the aerial mycelium, the number-letter code of that tabs of the TRESNER-BACKUS[3] color wheels which most nearly characterize the spore mass color. The substrate mycelium color was identified in terms of the PRAUSER color scale).

Fig. 1. Strain A-461. Smooth spores; electron micrograph form 14 day culture on oatmal agar. (× 3,250)



1. Yeast extract-malt extract agar: Aerial mycelium abundant, powdery, gray (3 ig). Substrate mycelium wrinkled, red (05-s-r). No soluble pigment.

2. Oatmeal agar: Aerial mycelium abundant, powdery, gray (3 ig). Growth wrinkled, yellowish red (Coo 2 a). No soluble pigment.

3. Glycerol-asparagine agar: Aerial mycelium abundant, gray (e). Substrate mycelium yellowish red (Oc 2 r). Soluble pigment yellowish.

4. Inorganic salts-starch agar: Aerial mycelium abundant, gray (3 ig). Substrate mycelium yellow (C 5 a). Soluble pigment slight brown coloration.

5. Peptone-yeast extract iron agar: No aerial mycelium. Substrate mycelium abundant, light yellow. Soluble pigment light yellow.

6. Tyrosine agar: Aerial mycelium thin, powdery, grayish. Growth colorless to yellow. No soluble pigment.

7. Potato agar: Aerial mycelium abundant, powdery gray. Growth reddish-orange. Soluble pigment brown.

8. Cellulose agar: No growth.

9. Peptone-yeast extract-glucose agar: Aerial mycelium abundant, powdery, ash-gray. Substrate mycelium wrinkled, dark brown. Soluble pigment grayish brown.

10. Glycine-agar: Aerial mycelium powdery, gray. Growth wrinkled, reddish brown. Soluble pigment light gray.

11. Arginine-agar: Aerial mycelium powdery, grayish. Growth reddish-brown. Soluble pigment light yellow.

12. Sodium nitrate agar: No aerial mycelium. Growth restricted, yellowish. Nitrate reduction: positive.

13. Tryptophane agar: No aerial mycelium. Growth abundant, yellow. No soluble pigment.

14. Glucose-ammonium sulfate agar: Aerial mycelium poorly developed, grayish. Growth abundant, wrinkled, reddish-yellow. No soluble pigment.

15. Glycerol-ammonium sulfate agar: No aerial mycelium. Growth abundant, wrinkled, reddish yellow. No soluble pigment.

Utilization of carbon sources on PRIDHAM-GOTTLIEB[4] basal medium: D-glucose, D-mannose and glycerol are utilized for growth. No growth or only trace of growth on L-arabinose, sucrose, D-mannitol, D-fructose, *i*-inositol. Utilization of D-xylose, raffinose and rhamnose is doubtful.

Systematically the strain A-461 is significantly different from *S. orinoci* producing ochramycin and *S. limosus* producing limocrocin.

According to the classification system of HÜTTER[5], strain A-461 relates to the species *S. parvulus* W. et G. HÜTTER united, in his system, all streptomycete species which possess spiral sporophores, smooth spores, cinereus (gray) aerial mycelium and produce melanoid pigments, into a single "aggregate (polytypic) species" (*S. parvulus*). Among the type cultures which are belonging, in the HÜTTER's system, to *S. parvulus*, A-461 resembles most closely to *A. griseoruber* INA N 6974/54, but differs from *S. parvulus* IMR4 3677.

On the base of the HÜTTER's classification scheme our strain A-461 relates to *S. parvulus*.

## Production, Isolation and Properties of Oleficin

Shaking cultures were fermented by 260 rpm with 10 cm in amplitude and 100 ml of medium in 500-ml flasks. The strain was cultured for 70 hours. Tank fermentation was carried out in a 140-liter stainless steel vessel, which contained 100 liters of medium; 100 ml of 36-hour shaking culture was inoculated. The vessel was stirred at 260 rpm and 100 liters of sterile air was pressed per minute. Palm oil was used as antifoam.

Oleficin was determined by disc-method using *Bacillus subtilis* as a test organism, on peptone agar medium, at pH 6.5.

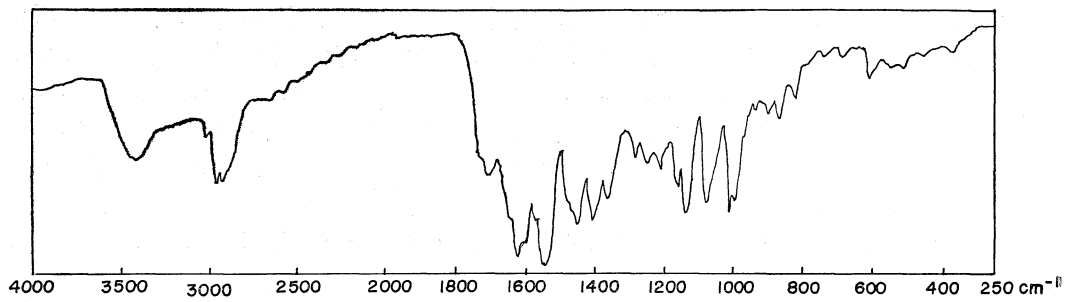
When the strain A-461 was shake-cultured at 28°C in a medium containing 3.0 % soybean meal, 2.0 % corn steep liquor, 0.1 % casamino acid, 0.3 % NaCl, 0.5 % $CaCO_3$, 3.5 % glucose (the pH was adjusted to 3.0 before sterilization), the culture filtrate showed 140~180 mcg/ml oleficin concentration after 72 hours.

Tank fermentation, with a medium similar to that above except for 2.0 % soybean meal and 3.0 % glucose, produced 350 mcg/ml of the antibiotic after 72 hours at 28°C. The pH increased from 7.0 to 7.5. After 72 hours a considerable fragmentation (in value of about 60~70 %) was observed.

One hundred liters of the broth were filtered at pH 4. The filtrate was discarded and methanol added (twice 15 liters) to the mycelium, the mixture was stirred twice for 30 minutes at room temperature and the extracts filtered. The methanol was then removed *in vacuo* below 50°C, the residue was extracted twice with 5.0 liters ethyl acetate, dried over $Na_2SO_4$, filtered and concentrated to a small volume. The crude antibiotic was precipitated with petroleum ether, filtered and dried at 20°C. A yield of 15.80 g of a dark red-black powder was obtained, which assayed 800 mcg/mg (*B. subtilis*). Eight g of crude oleficin obtained was separated by column chromatography on 320 g of inactivated neutral alumina. The mixture propanol - ethyl acetate - 0.25 N aqueous ammonia (6 : 1 : 4) was used as eluent. The pooled biologically active fractions were evaporated at pH 5, the obtained aqueous suspension extracted with ethyl acetate at pH 4 and the extract was washed with water. After a procedure similar to the isolation of the crude product, the purified oleficin was isolated, yield 4.88 g, bioassay *B. subtilis* 900~1,000 mcg/mg.

Oleficin is obtained as a dark red-black solid. It gives $[\alpha]_D^{20}$ −113° (*c* 0.03, methanol) from the ORD curve. Oleficin is soluble in methanol, moderately soluble in

Fig. 2. Infrared absorption spectrum of oleficin.




acetone, ethyl acetate, benzene (yielding red solutions), slightly soluble in carbon tetrachloride, insoluble in petroleum ether and water. Its sodium salt formed with sodium bicarbonate, is moderately soluble in water (yellow-orange solutions).

The ultraviolet and visible spectra show absorption maxima at 262 and 420 nm in methanol (10 mcg/ml). The second maximum is broad, $E^{1\%}_{1cm}$ 950. In 0.1 N NaOH the latter is shifted to 415 nm, at pH 3 to 475 nm. In benzene the second maximum is shifted to 470 nm. In 0.4 N $Na_2CO_3$ solution oleficin shows maxima at 270 and at 420 nm.

A 100-tube countercurrent distribution experiment was run on oleficin using the solvent system pyridine - ethyl acetate - water (3.5 : 6.5 : 8.3). The only maximum at tube 59 was confirmed by the biological activity curve (being in good agreement with the theoretical curve) and by the extinction curve at 420 nm. The substances from tubes 56~62 were isolated after being united. The ultraviolet spectrum in 0.4 N $Na_2CO_3$ solution showed the same maximum (at 420 nm), in 0.01 N methanolic HCl solution the broad maximum was split in three peaks at 445, 475 and 485 nm.

The infrared spectrum (in KBr) is shown in Fig. 2.

In concentrated $H_2SO_4$ the blue color of oleficin changes after a few minutes. It gives positive CARR-PRICE reaction. After bromination in vapor phase[6] an about threefold increase in weight was obtained. Upon hydrogenation with palladium charcoal catalyst or bromination, the ultraviolet maximum at 425 nm disappeared. The above reactions suggest the possible presence of a polyene-like chromophore in the oleficin molecule beside an other chromophore represented by the ultraviolet maximum at 262 nm.

The thin-layer chromatography data of purified oleficin are summarized in Table 1. In each system mentioned only one colored spot could be detected. Paper chromatographic experiments were carried out using the propanol - ethyl acetate - $NH_4OH$ system combined also with bioautography using *B. subtilis* as test organism on peptone agar medium at pH 6.5. The Rf value of the colored and biologically active spot was the same (0.84), and no other colored or active spots were present. The 100-tube counter-

Table 1. Thin-layer chromatography of oleficin on Kieselgel G

| Systems | Rf values |
|---|---|
| Propanol - ethyl acetate - 0.25 N $NH_4OH$ (6 : 1 : 4) | 0.60 |
| Butanol - acetic acid - water (4 : 1 : 5) | 0.79 |
| Ethanol - water - $NH_4OH$ (8 : 1 : 1) | 0.62 |
| Benzene - ethyl acetate (1 : 1) | 0.10 |

current distribution using the solvent system pyridine - ethyl acetate - water (3.5 : 6.5 : 8.3) yielded also only one biologically active substance, although some destruction of the antibiotic occurred.

Elementary analysis: C 65.20, H 7.55, N 2.72. $CH_3(C)$: 4.96 %.

The following formula can be calculated for oleficin: $C_{28}H_{39}NO_8$ (molecular weight 517).

The potentiometric titration curve in methanol with 0.02 N methanolic KOH solution shows that oleficin contains two different kinds of acidic groups. The first equivalent-point was found one third of the second one in KOH consumption, so the member of acidic functional groups in the molecule is presumably three. The equivalent weights were 540 and 200, respectively.

The molecular weight was determined in phenol with depression of the freezing point according to the method of BECKMANN. As oleficin contains different acidic groups (which dissociate), the molecular weight determined (180) was too low. We accepted practically the threefold molecular weight which is in accordance with the elementary analysis (The molecular weight of cinnamic acid in phenol was found also practically half of the real value).

The vapor phase bromination product of oleficin contains 65 % of bromine, corresponding with the uptake of 6 moles of bromine according to the above formula of the antibiotic. The preparation of different derivatives and degradation products is in progress.

The n.m.r. spectrum (60 Mc/sec.) in $CDCl_3$ solution shows the following signals: (a) aliphatic protons at 0.8~1.4 ($\delta$) ppm. (b) methyl protons attached to olefinic bonds at 1.8 ppm. (c) N-$CH_3$ or N-$CH_2$-protons at 3.0 ppm. (d) olefinic protons attached to functional groups and eventually aromatic protons at 6.4 and 7.3 ppm.

The proton intensities in the order above sohw the following ratios, 14 : 6 : 3 : 16.

Oleficin dissolved in 0.01 N methanolic hydrochloric acid at 0°C was decomposed after 24 hours. The aqueous solutions at pH 12 were stable for some days at 0°C.

The antibiotic is characterized also by a greater stability to light and air which differentiate it from the polyenic macrolide antibiotics. The antibacterial spectrum is shown in Table 2.

Table 2. *In vitro* antibacterial spectrum of oleficin

| | Minimal inhibitory concentrations in mcg/ml (24 hours) |
|---|---|
| *Pseudomonas pyocyanea* | >100 |
| *E. coli* $O_{111}$ | >100 |
| 〃 4R | >100 |
| 〃 6R | >100 |
| *Shigella sonnei* | >100 |
| *Staphylococcus* 53 | <0.4 |
| 〃 80/81 | <0.4 |
| 〃 1115 | <0.8 |
| 〃 Duncan | <0.4 |
| *Sarcina flava* | 0.8 |
| *B. subtilis* ATCC 6633 | <0.4 |
| *Str. faecalis* | 3.1 |

Medium: Nutrient Broth (Difco) + 1 % glucose, (pH 6.5).

Its activity against fungi and yeasts is negligible. The maximum concentration examined was 200 mcg/ml. The toxicity has been determined in mice by intravenous route: the $LD_{50}$ was found to be 40 mg/kg. Oleficin was effective against YOSHIDA subcutaneous sarcoma in mice.

## Discussion

The above experiments demonstrated that the new polyenic antibiotic oleficin, produced by strain A-461, is similar to antibiotics limocrocin and ochramycin in certain physical, chemical and microbiological properties, however among others the nitrogen analyses of the substances are different. On the basis of newer investigations oleficin shows similarity also to antibiotic streptolydigin[7] comparing the IR and n.m.r. spetra but numerous other properties are different. As a result oleficin can be differentiated from other antibiotics described in literature up to now.

Acknowledgement

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