

HYDROHEPTIN: A WATER-SOLUBLE POLYENE MACROLIDE

I. TAXONOMY, FERMENTATION AND ISOLATION

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A soil isolate of *Streptomyces*, which has been deposited in the culture collection of the Waksman Institute of Microbiology, Rutgers University as IMRU 3962, produces a new heptaene macrolide antifungal antibiotic, hydroheptin. The producing microorganism, which co-produces the antibiotic, chartreusin, has been identified as a strain of *Streptomyces chartreusis*. Fermentation and bioassay procedures were developed for the production and analysis of hydroheptin and chartreusin. Isolation and purification procedures based on solvent extraction and precipitation of an organic acid yielded a relatively pure product of hydroheptin.

The polyene macrolides represent a large class of antifungal antibiotics which over the past several decades have been isolated primarily from cultures of *Streptomyces* species. Many of these antibiotics are poorly defined and consist of mixtures of closely-related components. All naturally-occurring polyene macrolides reported to date are insoluble in water and most organic solvents with the exception of the more polar aqueous alcohols, dimethyl sulfoxide, dimethyl formamide, glacial acetic acid, etc. The new heptaene macrolide antibiotic, hydroheptin, isolated in our laboratories from a *Streptomyces* isolate, exhibits water solubility at neutrality. This report describes the taxonomic studies with the producing microorganism, fermentation, conditions, bioassay, isolation and purification of the antibiotic.

Materials and Methods

Microorganisms

The producing culture is an isolate from soil obtained from the State of Kansas, U.S.A. and has been identified as a strain of *Streptomyces chartreusis*, and deposited in the culture collection of the Waksman Institute of Microbiology, Rutgers University as IMRU 3962. Primary and working stocks of the organism were grown on seed medium. At the end of the log phase of growth, samples were aseptically transferred to screw cap 2-ml glass vials. Primary stocks were maintained in the gas phase of a liquid nitrogen refrigerator¹⁾ whereas working stock cultures, initially frozen in liquid nitrogen, were stored at -17°C . Due to the loss of viability of cultures stored at 5°C , the frozen-culture method was adopted for the maintenance of working stocks.

Stock cultures of *Streptomyces chartreusis* strains ATCC 14922, ATCC 23336, NRRL B-2199, NRRL B-8150, NRRL B-8151, and NRRL B-8152 were also maintained as described above.

Taxonomic Characterization

The methods of the International Streptomyces Project²⁾ were used for the characterization of the microorganism. Studies determining the morphological, physiological and color characteristics of the

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microorganism were carried out. Electron microscopic spore studies at $16,000\times$ were carried out with the hydroheptin-producing culture and comparative strains of microorganisms, grown on malt-yeast extract agar medium.

Culture Media

The initial screening medium for antibiotic production by the soil *Streptomyces* isolate, IMRU 3962, consisted of 3% sucrose (edible grade), 1.5% cotton seed meal (CSM, Pharmamedia, Traders Protein Division, Traders Oil Mill Co., Fort Worth, Texas), and 0.5% yeast extract (YE, Difco Laboratories, Detroit, Mich.). The stock culture and seed media employed contained 4% sucrose, 2% CSM and 0.16% YE which was buffered with 0.2 M KH_2PO_4 adjusted to pH 5.8 with KOH. Frozen stock cultures, thawed, were used as inocula for the seed medium. After incubation at 28°C for 2~3 days the seed medium culture served as an inoculum (2%, v/v) for the fermentation production medium, consisting of 4% sucrose, 2% CSM, 0.16% YE, 0.03% $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$, 0.01% $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ and 0.01% $\text{MnSO}_4\cdot \text{H}_2\text{O}$, buffered with 0.2 M KH_2PO_4 at pH 5.8. The production medium is designated as "hydroheptin medium". Distilled water was used in the preparation of both media. Agar (2%) was employed in the preparation of solid media.

Fermentation Procedures

For shake flask fermentations, baffled flasks, 300 ml capacity, with indentations 6~7 mm deep were used (Bellco Glass, Inc., Vineland, N.J.). A medium volume of 50 ml per flask was employed. The flasks were placed on a model V rotary shaker (New Brunswick Scientific Co., New Brunswick, N.J.) which describes a 1-inch circle. Inocula for shake flask experiments were prepared as described above. For 50-liter fermentors, the working stock was first cultured in 300-ml flasks and then transferred into 2-liter baffled flasks, containing 500 ml of the seed medium. One liter of inoculum was used to inoculate 25 liters of production medium. The agitation speed on the rotary shaker was 245 rpm which gives an oxygen absorption rate of 3.2 mmoles of oxygen/liter/min (as determined by sulfite oxidation rate measurement). All fermentations were carried out at 28°C .

For pilot plant production a 1,200-liter fermentor with a 22-inch diameter, 3-blade anchor-type impeller was used. The fermentor was charged with 500 liters of hydroheptin production medium to which 0.5% ethanol was added before sterilization. It had been observed that the addition of ethanol increases hydroheptin yields. Operating conditions of the fermentor were as follows: 150 rpm agitation speed, 170 liters/min air flow, and $28^\circ \pm 1^\circ\text{C}$ temperature. The 5% inoculum for the 1,200-liter fermentor was grown in a 40-liter seed tank for 48 hours, employing the seed medium.

Thin-layer Chromatography

Silica gel G plates, 250 mm thickness (Analtech, Inc., Newark, Del.) were used. In the examination of antibiotic production in screening medium the lower phase of the solvent system, chloroform - methanol - 0.02 M borate buffer, pH 8.2 (2: 2: 1, v/v/v) was initially employed for development at 30°C . The antibiotic components were detected under ultraviolet light. Later TLC analyses of hydroheptin were carried out with the lower phase of the solvent system, chloroform - methanol - 2% aqueous ammonium hydroxide (2: 2: 1, v/v/v).

Quantitative Assays of Chartreusin and Hydroheptin

It was found that hydroheptin is soluble in water at pH 4 and above, and insoluble in chloroform while chartreusin is insoluble in water and soluble in chloroform. An assay procedure was developed based on the observed differences in the solubility of the antibiotics in water and chloroform (Fig. 1). The procedure involved the use of the solvent system consisting of chloroform - methanol - water (6: 3: 5, v/v/v). This two-phase solvent system gives 6 parts lower phase and 8 parts upper phase. With a fermentation mixture of chartreusin and hydroheptin the lower phase of this solvent system extracts chartreusin while the upper phase extracts hydroheptin. The separation factor with this solvent system enables complete fractionation of both antibiotics.

The antibiotics in solution were assayed spectrophotometrically by measuring their optical densities (O.D.) using a Cary recording spectrophotometer, model 14 M (Applied Physics Corp., Pasadena, California): chartreusin was measured at 400 nm and hydroheptin at 380 nm. The concentration of

Fig. 1. Schematic diagram of the antibiotic assay method.

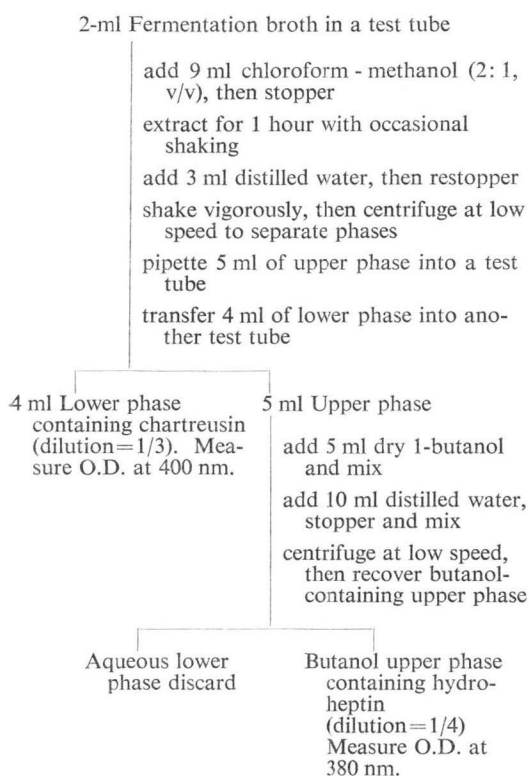
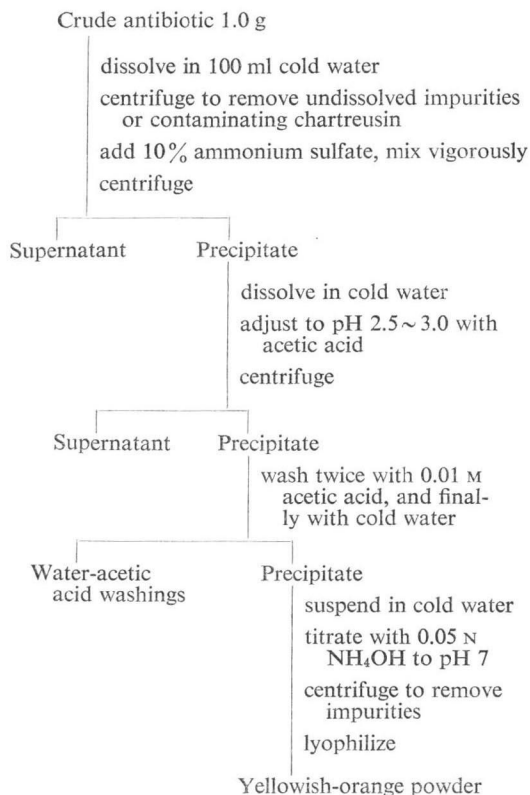


Fig. 2. Hydroheptin purification procedure.



chartreusin was calculated on the basis of an observed $E_{1\text{cm}}^{1\%}$ 230 for pure chartreusin, and hydroheptin on the assumed basis of a theoretical $E_{1\text{cm}}^{1\%}$ 1,000 for a pure non-aromatic, heptaene macrolide.

Thus, chartreusin and hydroheptin concentrations were calculated as follows:

$$\text{Chartreusin } (\mu\text{g/ml}) = \text{O.D. } 400 \text{ nm} \times \text{dilution factor} \times 43.48$$

$$\text{Hydroheptin } (\mu\text{g/ml}) = \text{O.D. } 380 \text{ nm} \times \text{dilution factor} \times 10$$

Isolation Procedures

For the extraction of hydroheptin from the fermentation broth from a 1,200-liter fermentor, the whole broth was adjusted to pH 7~8 by the addition of 0.5% NaOH, followed by the recovery of the supernatant after passage of the broth through a centrifuge (Sharples, Model 16, Pennwalt Corp., Warmister, Pa.). The supernatant was then extracted with a one-third volume of 1-butanol followed by concentration of the butanol extract to a volume of approximately 3 liters in a vacuum evaporator (Turba Film Evaporator, Rodney-Hunt Machine Co., Orange, Mass.). The dark concentrate was reduced to an oily consistency in a Rinco vacuum evaporator. Two washings with diethyl ether produced a yellow-green precipitate consisting of crude hydroheptin.

Hydroheptin Purification

Crude hydroheptin obtained by extraction procedures with fermentation broths was further purified by precipitation with ammonium sulfate and at acid pH. Being uniquely water-soluble at neutrality, hydroheptin was found to be precipitable at acidic pH or by the addition of ammonium sulfate. Taking advantage of these properties, the purification scheme presented in Fig. 2 was employed to obtain hydroheptin of high purity. Precautions against light and thermal inactivation were taken.

Results and Discussion

Detection and Identification of Chartreusin

Production of what was believed to be a polyene macrolide was first detected in the screening medium after 4 days of shake-flask fermentation. A 1-butanol extract of the fermentation broth revealed ultraviolet-visible absorption maxima at 421, 399, and 378 nm, suggesting the possible presence of an octaene macrolide³⁾.

The infrared spectrum in KBr of a purified sample of the "octaene" showed that the product was not a polyene macrolide. However, the infrared spectrum resembled that of chartreusin as reported by LEACH *et al.*⁴⁾ The antimicrobial spectrum of the "octaene" isolate was also quite similar to that of chartreusin. Thin-layer chromatographic analysis revealed under ultraviolet light that the "octaene" had an identical Rf value with chartreusin, Rf 0.63. Thus, based on comparable infrared and ultraviolet-visible absorption spectra, and TLC characteristics, the product was identified as chartreusin.

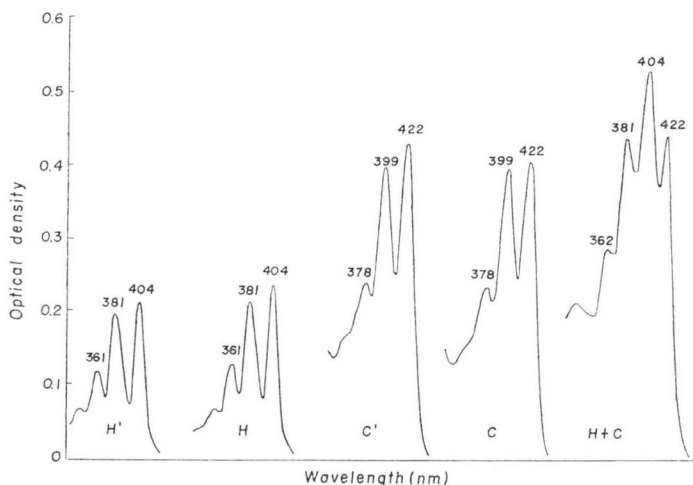
Detection of Hydroheptin

During the detection and isolation of chartreusin in the screening medium, thin-layer chromatography also revealed a minor component as a brown spot typical of a polyene macrolide (Rf 0.19) when examined under ultraviolet light. When the brown spots were scraped from two preparative TLC plates and the silica gel extracted with 1-butanol and with water, both extracts gave faint ultraviolet-visible absorption spectra with major absorption maxima at 404, 381 and 361 nm, characteristic of a heptaene macrolide.

Evaluation of the Quantitative Assay Procedure

To demonstrate the efficiency of the assay method that was developed, purified samples of hydroheptin and chartreusin were mixed and analyzed by this method. Fig. 3 shows the ultraviolet-visible absorption spectra of chartreusin and hydroheptin, individually as well as of the mixture of the two antibiotics. It can be seen that the separation of chartreusin and hydroheptin into the lower and upper

Fig. 3. Ultraviolet-visible spectra of standard hydroheptin (H), standard chartreusin (C), standard hydroheptin-chartreusin (H+C) mixture and hydroheptin fraction H' and chartreusin fraction C' after separation by the assay procedure.



phases of the solvent system, respectively, is complete. Hence, extraction of the chartreusin-hydroheptin mixture results in total separation and recovery of the two antibiotics. This indicates the reliability of the assay procedure.

Production of Hydroheptin

The screening medium employed in the isolation of *Streptomyces* culture IMRU 3962 produced only 2 $\mu\text{g}/\text{ml}$ of hydroheptin and 300 $\mu\text{g}/\text{ml}$ of chartreusin. As a result of medium development studies, a superior medium for the production of hydroheptin was developed. This was designated as "hydroheptin" medium. Production in shake flasks with this medium in a 3-day fermentation was 140 $\mu\text{g}/\text{ml}$ of hydroheptin and 35 $\mu\text{g}/\text{ml}$ of chartreusin.

From a 1,200-liter fermentor a batch produced with hydroheptin medium plus ethanol was harvested after 48 hours of fermentation with yields of 170 $\mu\text{g}/\text{ml}$ of hydroheptin and 80 $\mu\text{g}/\text{ml}$ of chartreusin. Seventy-one grams of crude hydroheptin ($E_{1\text{cm}}^{1\%}$ 330, in 1-butanol), yellow-green in color, was recovered from the 500 liters of fermentation broth by 1-butanol extraction of neutralized and centrifuged fermentation broth, vacuum concentration and ethyl ether precipitation.

Purification of Hydroheptin

Taking advantage of the unique water-solubility of hydroheptin at neutral pH with its precipitation by the addition of ammonium sulfate as well as the precipitation of hydroheptin at pH 2.5~3.0, a purification scheme as outlined in Fig. 2 was developed. From one gram of crude hydroheptin ($E_{1\text{cm}}^{1\%}$ 330, in 1-butanol) 270 mg of purified ammonium salt of hydroheptin ($E_{1\text{cm}}^{1\%}$ 980, in methanol) were recovered as a yellow-orange powder.

Hydroheptin Characterization

The water-solubility of hydroheptin, particularly at neutrality, is a unique property never seen with naturally-occurring polyene macrolide antibiotics. The exact physical-chemical description and characterization of hydroheptin as a new non-aromatic heptaene macrolide antifungal antibiotic is given in the accompanying paper⁵⁾. A review of the literature pertaining to the polyene macrolides clearly reveals their very limited solubility in water. With aqueous dispersion from solutions in organic solvents the polyene macrolides at best aggregate as colloidal micelles. In a few instances⁶⁻⁸⁾ there have been reports of increased water solubility of polyene macrolides by chemical modifications. Hydroheptin is indeed a novel polyene macrolide antifungal antibiotic, exhibiting excellent water solubility with molecular dispersion at neutral pH.

Co-production of Hydroheptin and Chartreusin by Various Strains of *S. chartreusis*

Seven strains of *S. chartreusis* were tested for antibiotic production in hydroheptin medium. Three strains in addition to *Streptomyces* isolate IMRU 3962 produced a heptaene which migrated the same as hydroheptin on TLC plates. Six strains produced chartreusin. Both hydroheptin and chartreusin were produced by the *S. chartreusis* type strain (ATCC 14922).

Table 1. Production of hydroheptin and chartreusin by several strains of *S. chartreusis*.

| Strains | Production of | |
|-------------|---------------|-------------|
| | Hydroheptin | Chartreusin |
| IMRU 3962 | + | + |
| ATCC 14922 | + | + |
| ATCC 23336 | - | - |
| NRRL B-2199 | + | + |
| NRRL B-8150 | + | + |
| NRRL B-8151 | - | + |
| NRRL B-8152 | - | + |

The results are summarized in Table 1.

Characterization of the Producing Organism

The observation that *Streptomyces* isolate IMRU 3962 produced a heptaene macrolide antifungal antibiotic as well as chartreusin immediately suggested a direct comparison with *Streptomyces chartreusis* known for its production of chartreusin. The *Streptomyces* isolate was thus compared with the type species, *Streptomyces chartreusis* ATCC 14922. Both had the following characteristics:

Spore chain morphology: Spore chains were arranged in loose spirals. The spore surfaces were spiny. This morphology was observed on yeast extract-malt extract agar, oatmeal agar, salts-starch agar, and glycerol-asparagine agar.

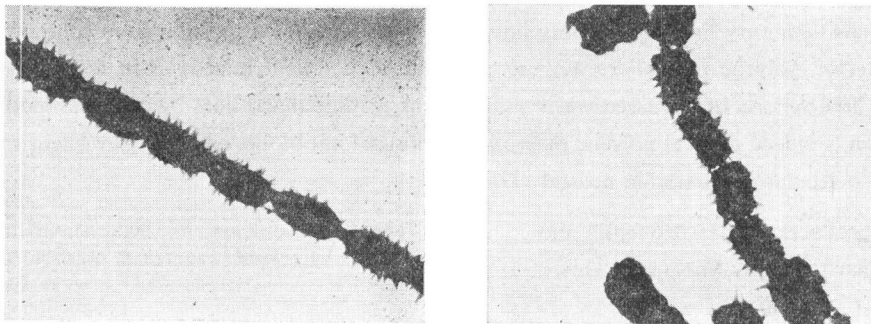
Color of colonies: Aerial mass color was in the blue color series on yeast-malt agar, oatmeal agar, salts-starch agar and glycerol-asparagine agar.

Color in medium: Melanoid pigment was formed on peptone-yeast iron agar and in Tryptone-yeast extract broth.

Carbon utilization: L-Arabinose, D-raffinose, D-xylose, D-mannitol, *D*-inositol, L-rhamnose, sucrose, D-fructose, and D-glucose were utilized for growth.

Based on its having properties similar to the type species, and on the fact that both cultures produce chartreusin and hydroheptin, *Streptomyces* isolate IMRU 3962 is considered to be a strain of *Streptomyces chartreusis*. Fig. 4 shows the electron micrograph (16,000 \times) of the spores of 14-day-old cultures of *Streptomyces* isolate IMRU 3962 and *Streptomyces chartreusis* ATCC 14922 grown on yeast extract-malt extract agar medium. Both isolates exhibited spiny spores. However, the spores of *Streptomyces chartreusis* ATCC 14922 appear relatively stouter and shorter with blunt ends than those produced by the *Streptomyces* isolate IMRU 3962, suggesting variation.

Fig. 4. Electron micrographs (16,000 \times) of spores of 14-day old cultures of *Streptomyces* isolate IMRU 3962 (A) and *Streptomyces chartreusis* ATCC 14922 (B) grown on yeast extract-malt extract agar medium.



A

B

Acknowledgements

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