Production of Hormone B by Achlya heterosexuualis

ALMA W. BARKSDALE and LINDA L. LASURE
The New York Botanical Garden, Bronx, New York 10458

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Sexual morphogenesis in Achlya, a filamentous water mold, is initiated by two hormones. Hormone A, or antheridiol, is a C-29 steroid. Four stereoisomers of antheridiol have been synthesized. The natural one (antheridiol 22S 23R) and its 7-deoxy 7-dihydro form, when added to an aerated culture of hermaphroditic Achlya heterosexuualis, stimulated this mold to secrete twice as much hormone B as the untreated control. An unnatural stereoisomer (antheridiol 22R 23S) and fucosterol, however, did not stimulate strain 8-6 in the same way. Methods by which hormone B can be produced in sufficient yield for isolation and characterization are described.

The formation of sex organs in Achlya is initiated by two substances, hormones A and B. The compound having hormone A activity has been isolated (6) and characterized as a C-29 steroid (1). Hormone A has been named antheridiol because it brings about the formation of branches on which antheridia are delimited (6). Hormone B, which initiates the formation of branches on which oogonia are delimited, is secreted by certain sterile strains only when stimulated by exogenously supplied antheridiol (3, 8). Certain hermaphroditic strains secrete hormone B even when not stimulated by exogenous antheridiol (3). Strain 8-6 of A. heterosexuualis, as will be shown here, secretes hormone B with or without the addition of antheridiol.

The methods by which hormone B can be produced on a pilot-plant scale are described in this paper.

MATERIALS AND METHODS
Strains and laboratory media. Strain 8-6 of A. heterosexuualis was chosen for the present study because it secretes little antheridiol while secreting as much hormone B as any known hermaphroditic strain (3).

The strain was inoculated from agar slants onto petri dishes (90 mm in diameter) containing 25 ml of a medium composed of the following: glucose, 2,400 mg; Edamin (hydrolyzed lactalbumin from Sheffield Chemical Co., Norwich, Conn.), 400 mg; tris(hydroxymethyl)aminomethane, 100 mg; calcium glycerophosphate, 80 mg; KCl (2 M), 0.5 ml; MgSO₄·7H₂O (0.5 M), 1.0 ml; metal mix no. 4 (2 mg/ml; ref 2), 0.5 ml; distilled water, 1,000 ml; agar (Difco), 15 g; and HCl to a pH of 6.8. The strain was grown on this medium for 3 days at 25 C. Mycelium in each dish was cut into 0.5-inch squares and transferred to a 2-liter Erlenmeyer flask containing 400 ml of sporulation medium. The sporulation medium consisted of: sodium glutamate, 50 mg; tris(hydroxymethyl)aminomethane, 100 mg; L-methionine (1,500 mg in 90 ml of water plus 10 ml of concentrated HCl), 0.1 ml; hydroxyethyltetramine triacetic acid, (10 mg/ml), 0.1 ml; CaCl₂·2H₂O (0.5 M), 0.1 ml; MgSO₄·7H₂O (0.5 M), 0.1 ml; metal mix no. 4, 0.1 ml; KCl (2 M), 0.1 ml; KH₂PO₄ (1 M), 0.1 ml; distilled water, 1,000 ml; and HCl to a pH of 6.5.

Sporulation was induced by shaking the flasks overnight on a rotary shaker at 20 C and 100 rpm.

Production medium. Medium was prepared in 20-liter glass carboys and sterilized by autoclaving for 90 min. Each carboy contained a Kimax glass sparger tube (4 mm in diameter, terminal bulb 10 mm in diameter with six 1-mm holes), held in place by a plug of cotton wool, and 10 liters of the following medium: glucose, 2,400 mg; sodium glutamate, 330 mg; tris(hydroxymethyl)aminomethane, 330 mg; L-methionine (15 mg/ml), 0.66 ml; hydroxyethyltetramine triacetic acid (10 mg/ml), 0.66 ml; CaCl₂·2H₂O (0.5 M), 0.66 ml; MgSO₄·7H₂O (0.5 M), 0.66 ml; metal mix no. 4 (2 mg/ml), 0.66 ml; KCl (2 M), 0.66 ml; KH₂PO₄ (1 M), 0.66 ml; distilled water, 1,000 ml; and HCl to a pH of 6.8.

Contents of two sporulation flasks were combined to obtain a suspension of zoospores (800 ml of 2 x 10⁴ zoospores per ml), and this suspension was used to inoculate each carboy.

The carboys, at the time of inoculation, were placed in a 27 C water bath (6-post New Brunswick fermentor apparatus), and the sparger tube of each carboy was connected to an airway which supplied sterile air at the rate of 10 liters per min.

After inoculation, the carboys were incubated for 88 h. The mycelium was then removed on a Buchner filter and discarded. The culture filtrate was assayed for hormone activity after freezing to destroy mycelial strands.

Biological assays. Antheridiol was assayed with strain E87 of A. ambisexualis (8), whereas hormone B was measured with strain 734 of A. ambisexualis (2, 3). One milligram of antheridiol (SR) assayed 6 x 10⁹.
dilution units (6). The weight of 1 U of pure hormone B is not known.

**Preparation of steroids.** The steroids tested for their ability to stimulate the production of hormone B are natural products of *Achlya* with the exception of deoxy-dihydro antheridiol SR and three unnatural stereoisomers, which had been previously synthesized (4, 5). Fucosterol, a major steroid in *A. bisexualis* (7), was recently isolated from kelp by T. C. McMorris (unpublished data).

The test compounds were dissolved in methanol and were added to the carboy at various concentrations in 100 ml of sterile water 66 h after inoculation. An untreated carboy in each batch served as a control.

**RESULTS AND DISCUSSION**

Only the natural stereoisomer, antheridiol (SR), and its deoxy-dihydro form stimulated strain 8-6 of *A. heterosexualis* to secrete more hormone B than the untreated control (Table 1). An unnatural stereoisomer, antheridiol (RS), failed to stimulate at the concentrations tested. Fucosterol either inhibited secretion of hormone B or repressed the formation of oogonial initials by strain 734 in the presence of hormone B.

Fucosterol, which lacks hormone A activity, and antheridiol RS, which is about 1,000 times less active than antheridiol SR, had hormone activity less than or at most equal to that of hormone A of the control. The culture filtrate to which deoxy-dihydro antheridiol had been added, however, showed more hormone A activity than the filtrate containing antheridiol SR.

![Figure 1. Increase in dry weight of mycelium and production of hormones in relation to time in aerated carboy.](image)

**TABLE 1. Effect of C-29 steroids on the production of sex hormones by strain 8-6 of *A. heterosexualis***

| Compound added | Amount added (µg/liter) | Hormone activity |                  |                   |
|----------------|-------------------------|------------------|------------------|------------------|
|                |                         | Hormone A (U/ml)*| Hormone B (U/ml)*|
|                |                         | - cpd | + cpd | - cpd | + cpd |
| Antheridiol 22S 23R<sup>c</sup> | 1 | <10 | 100 | 10 | 10 |
|                | 5 | <10 | 100 | 10 | 20 |
|                | 10 | <10 | 100 | 10 | 20 |
|                | 20 | <10 | 100 | 10 | 20 |
|                | 40 | <10 | 1,000 | 20 | 40 |
|                | 80 | <10 | 1,000 | 10 | 5 |
| 7-Deoxy-7-dihydroantheridiol 22S 23R<sup>c</sup> | 10 | <10 | 100 | 10 | 20 |
|                | 20 | <10 | 1,000 | 10 | 10 |
|                | 40 | <10 | 1,000 | 10 | 20 |
|                | 80 | <10 | 10,000 | 10 | 20 |
| Antheridiol 22R 23S<sup>c</sup> | 10 | <10 | <10 | 10 | 5 |
|                | 20 | <10 | <10 | 10 | 5 |
|                | 40 | <10 | <10 | 10 | 5 |
| Fucosterol | 10 | <10 | <10 | 20 | <1 |
|                | 20 | <10 | <10 | 20 | <1 |
|                | 40 | <10 | <10 | 20 | <1 |
|                | 80 | <10 | <10 | 20 | <1 |

* Dilution units.
* cpd, Compound.
* Synthetic.
Antheridiol SR is known to be taken up by the mold (3). Whether increased activity of the deoxy-dihydro compound, compared to antheridiol SR, is due to stimulation of antheridiol SR secretion by the former or to failure of the latter to be taken up has not been determined.

In carboy-grown cultures, antheridiol and hormone B were detectable about 46 to 48 h after inoculation, and they reached a maximal level when the oogonial initials were formed and growth had ended (Fig. 1). Oogonial and antheridial branches appeared between 56 and 60 h. The oogonial initials became surrounded with antheridial branches, and after the antheridia and oogonia were delimited, oospores were cleaved.

The importance of the absolute stereochemistry at carbons 22 and 23 in antheridiol is apparent from Table 1. Both the natural stereoisomer, antheridiol SR and its deoxy-dihydro compound, stimulated the mold to secrete twice as much hormone B as the untreated control, whereas the stereoisomer, antheridiol RS, did not have this effect. These results suggest that hormone B may be derived from antheridiol SR. This conversion could be tested if radioactive antheridiol SR were available.

Assuming that hormone B is as active as hormone A, then hormone B is produced by A. heterosexualis 8-6 in an amount that is 0.001 that of the antheridiol secreted by A. bisexualis T5 (6).

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