Phytochemistry, 1977, Vol. 16, pp. 359–362, Pergamon Press, Printed in England.

BIOSYNTHESIS OF THE OOGONIOLS, STEROIDAL SEX HORMONES OF *ACHLYA*: THE ROLE OF FUCOSTEROL

TREVOR C. MCMORRIS and ROBERT H. WHITE

Department of Chemistry, University of California, San Diego, CA 92093, U.S.A.

(*Revised received* 20 *September* 1976)

Key Word Index—*Achlya heterosexualis*; *A. ambisexualis*; water mould; sex hormone; oogoniol; fucosterol; sitosterol; clionasterol.

Abstract—Fucosterol-[3-^{3}H] was converted to the oogoniols, sex hormones of *Achlya*, in *A. heterosexualis*. A similar conversion occurred in *A. ambisexualis* provided antheridiol was added.

The water mould *Achlya* which inhabits fresh water lakes and rivers is unique among plant genera because it is the only one whose sexual reproduction has been shown to be controlled by steroid hormones [1]. Female strains of *Achlya* secrete the sterol antheridiol which induces the formation of antheridial branches in male strains. These branches develop into the male sex organs (antheridia). Antheridiol also stimulates the male strains to secrete other sterols, the oogoniols (hormone B), which in turn induce the formation of oogonial branches and oogonia (female sex organs) in the female [2]. In most species of *Achlya*, antheridia and oogonia can also be formed on a single thallus. However, in species such as *A. bisexualis* and *A. ambisexualis* this occurs infrequently [3].

Biosynthetic studies have shown that fucosterol, the major sterol of *A. bisexualis*, is a precursor of antheridiol [4]. The structures of the oogoniols (Scheme 1) indicate that they might be formed from sitosterol or its 24 epimer, clionasterol, by hydroxylation at carbons 11, 15 and 26, oxidation at 7 and esterification of the 3-hydroxyl. In this paper we report the first part of a study aimed at defining the intermediates in the late stages of the biosynthesis of the oogoniols.

RESULTS AND DISCUSSION

Of a number of strains of *Achlya* which were examined, the hermaphroditic strain B-14 of *A. heterosexualis* Whiffen-Barksdale proved to be most suitable for the biosynthetic experiments. This strain secretes relatively large amounts of the oogoniols and does not require exogenous antheridiol for initiation of the sexual process [5,6]. Repeated attempts to produce the oogoniols with the male strain *A. ambisexualis* E 87 were unsuccessful. Barksdale and Lasure have obtained similar results [5]. We have confirmed that strain E 87 produces oogoniols only when grown together with *A. ambisexualis* 734 (a female strain) or when stimulated by the addition of antheridiol.

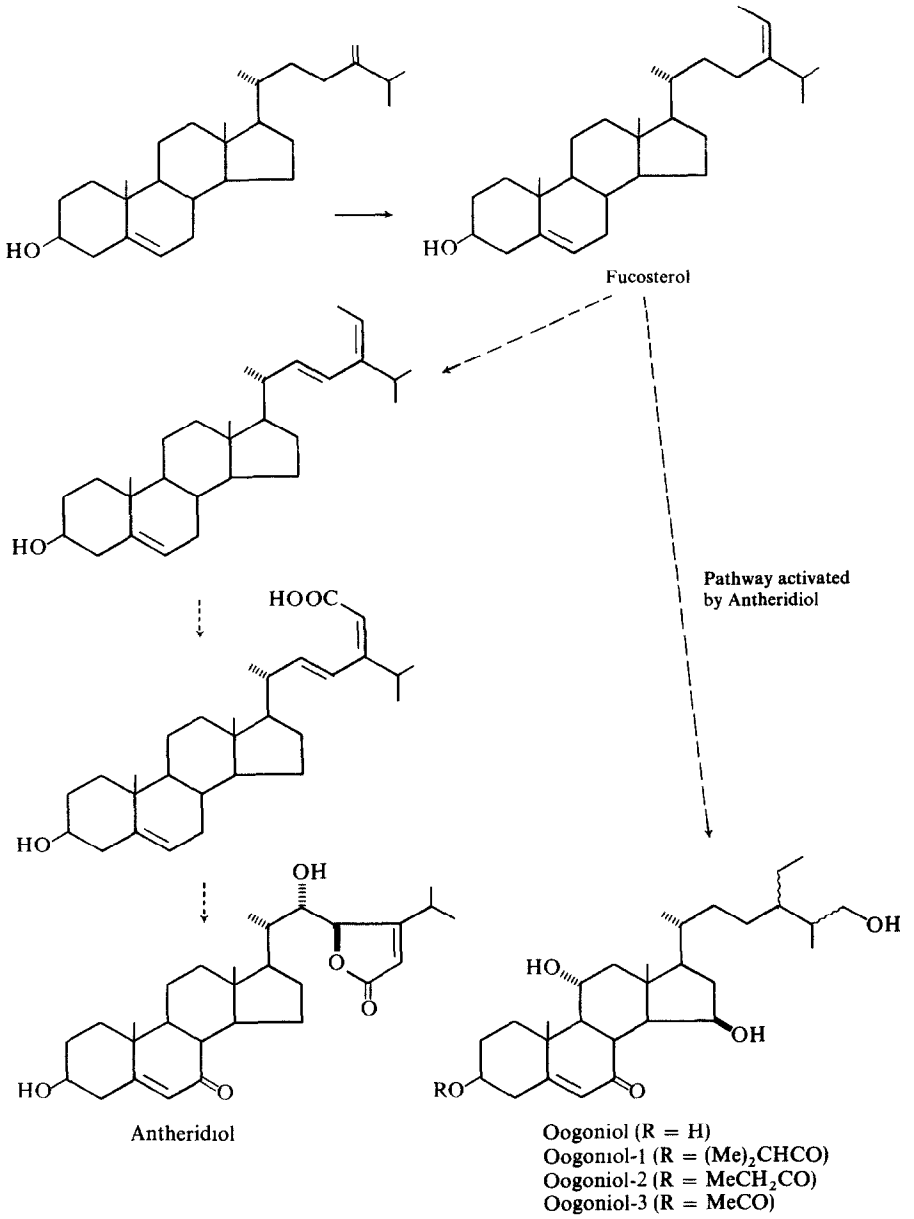
When *A. heterosexualis* was grown in the presence of sitosterol-[22, 23-^{3}H] (sp. act. 4.7×10^{10} cpm/mg), oogoniol-2 was isolated which, after purification by TLC and GLC (of the silyl ether), possessed slight radioactivity (sp. act. 2.1×10^4 cpm/mg). A more polar sterol isolated in this experiment was found to be identical to the sterol obtained by hydrolysis of the ester group of oogoniol-1 or -2. The sterol had slightly higher biological activity than oogoniol-1 [1]. It appears to be a natural product and has been named oogoniol. The specific radioactivity of oogoniol was the same as that of oogoniol-2.

In view of the rather low incorporation of radioactivity into the oogoniols, a feeding experiment was next carried out with clionasterol-[22, 23-^{3}H] as substrate. A comparison experiment with sitosterol-[22, 23-^{3}H] was also run at the same time. The sterols were prepared by tritiation of poriferasterol and stigmasterol, respectively, and had similar specific radioactivities. In addition to polar metabolites in the culture liquid, nonpolar sterols in the mycelium were also examined. The results of the feeding experiments are given in Tables 1 and 2. They show that clionasterol as well as sitosterol can be metabolized to oogoniol to about the same degree. Since natural oogoniol is probably not a mixture of C-24 epimers, these results imply that the stereochemistry at C-24 is altered in the conversion of one or other of the substrates.

Evidence as to the nature of the conversion comes from the sterols in the mycelium. The major sterol in the mycelium of *A. heterosexualis* was found to be fucosterol. Recovered sitosterol and clionoasterol had specific radio-

Table 1. Sterols isolated after incubation of *A. heterosexualis* with clionasterol [22, 23-^{3}H] for three days at 27° (5 l. of culture liquid)

	Wt (mg)	Specific activity (cpm/mg)	Total radioactivity (%)
Initial substrate added	1.14	3.65×10^5	100
Fucosterol	17.7	1.8×10^3	0.75
24-Methylene cholesterol	1.73	6.9×10^3	0.29
Cholesterol	1.05	3.03×10^4	0.78
Clionasterol	0.189	3.5×10^5	16.0
Oogoniols	0.50	3.2×10^2	0.037



Scheme 1. Proposed routes for the biosynthesis of steroid hormones in *Achlya*.

Table 2. Sterols isolated after incubation of *A. heterosexualis* with sitosterol [22, 23-^{3}H] for 3 days at 27° (5 l. of culture liquid)

	Wt (mg)	Specific activity (cpm/mg)	Total radioactivity (%)
Initial substrate added	1.0	3.03×10^5	100
Fucosterol	13.9	0.9×10^3	0.43
24-Methylene cholesterol	2.16	2.07×10^3	0.15
Cholesterol	0.90	2.02×10^4	0.59
Sitosterol	0.138	3.05×10^5	13.8
Oogoniols	0.356	2.4×10^2	0.027

activities similar to those of the added substrates indicating that the mycelium contained no measurable amount of either sterol. There were small amounts of radioactivity in 24-methylene cholesterol, cholesterol and campesterol (or its 24 epimer) isolated from the mycelium. This suggests that sitosterol and clionasterol are converted, to a small extent, to fucosterol, 24-methylene cholesterol and cholesterol, perhaps by reversal of the established biosynthetic pathway from C_{27} to C_{29} sterols.

Since the level of radioactivity in oogoniol was about the same order of magnitude as that in fucosterol it seemed possible that the latter sterol might be a better substrate for feeding experiments than sitosterol or clionasterol. Fucosterol was therefore isolated from the brown alga *Macrocystis pyrifera* (a convient source) and oxidized to the Δ^5-3-ketone with pyridinium chlorochromate [7]. Reduction of the ketone with NaB^3H_4 gave back fucosterol with a sp. act. of 5.32×10^6 cpm/mg. *A. heterosexualis* was grown for three days in the presence

Table 3. Radioactivity of sterols isolated after incubation of *A. heterosexualis* with 4.8×10^{-3} mg 24-methylene cholesterol-[^{3}H] and 4.9×10^{-2} mg Fucosterol-[3-^{3}H] for 4 days at 27° (800 ml culture liquid in each experiment)

	24-Methylene cholesterol (1.8×10^8 cpm/mg)	Fucosterol (1.8×10^8 cpm/mg)
Cholesterol	$<1.5 \times 10^3$	$<5.5 \times 10^3$
24-Methylene cholesterol	5.0×10^4	$<3.7 \times 10^3$
Fucosterol	2.0×10^4	4.2×10^5
Oogoniol-2	1.6×10^4	2.1×10^5
Oogoniol	2.0×10^4	2.5×10^5

of this labelled fucosterol. Oogoniol was isolated with a specific activity of 0.725×10^6 cpm/mg which was 25% that of the recovered fucosterol (2.86×10^6 cpm/mg). Thus, the intermediacy of the latter in the biosynthetic pathway was demonstrated.

Although the fucosterol used for tritiation had been recrystallized repeatedly it still contained some 24-methylene cholesterol (5%) and cholesterol (1%) and these sterols were therefore tritiated also. Thus, the C_{28} and C_{27} sterols isolated from the mycelium had levels of radioactivity similar to that of the fucosterol. This raised the possibility that oogoniol might be derived from 24-methylene cholesterol by a pathway not involving fucosterol.

That possibility was ruled out by experiments with pure fucosterol and 24-methylene cholesterol (both purified by GLC). The results (Table 3) confirm the role of fucosterol and indicate that 24-methylene cholesterol was a good precursor of fucosterol, as expected. There is some uncertainty in the values obtained for cholesterol (and 24-methylene cholesterol in the fucosterol feeding experiment) because the minute amounts which were isolated gave counts only two to four times that of the background. However, the reverse pathway from C_{29} to C_{27} sterol appears to be weakly operative [8].

Similar results were obtained with the male strain *A. ambisexualis* E 87. When labelled fucosterol was added to the culture liquid it was gradually taken up by the mycelium over a period of several hours, but no oogoniol was produced unless antheridiol was added. In one experiment, the addition was made about 2 hours after that of the fucosterol. After a further hour, oogoniol could be detected in the culture liquid. Presumably the enzymes which catalyze the conversion of fucosterol to oogoniol are induced during this period [9]. There is evidence that protein synthesis is involved since addition of cycloheximide completely inhibits the production of the oogoniols. In the experiment with no cycloheximide, the amount of oogoniol increased steadily for about 6 hr then leveled off as illustrated in Fig. 1. Significantly, the radioactivity of the oogoniol also increased and in step with the increase of radioactive fucosterol in the mycelium. This shows clearly that the latter compound is metabolized to the hormone. The results of the biosynthetic experiments are summarized in Scheme 1 which also includes evidence recently found for antheridiol [4]. It is interesting that both male and female sex hormones are derived from fucosterol which is the major sterol of *Achlya* and of brown algae [10]. Fucosterol has also been found to be one of the most active sterols in promoting the formation of sexual oospores in the plant parasitic fungi *Phytopthora* and *Pythium*, close relatives of *Achlya* [11]. These pathogens apparently are unable to synthesize sterol and so they require exogenous fucosterol which may possibly be converted to the true hormones in a manner similar to that in *Achlya*.

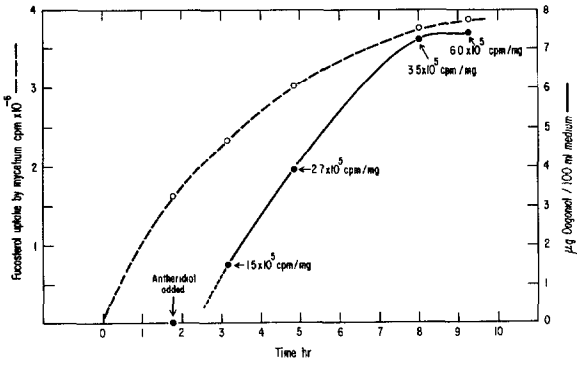


Fig. 1. Induction of oogoniol synthesis by antheridiol in *Achlya ambisexualis* ♂.

EXPERIMENTAL

Sitosterol-[22, 23-^{3}H] and clionasterol [22, 23-^{3}H] were prepared by hydrogenation of stigmasterol acetate and poriferasterol acetate respectively, in ethyl acetate with 5% palladium on carbon. Tritium was generated by reaction of tritiated H_2O and $NaBH_4$. Reduction of the sterol acetates was monitored by GLC. The Δ^5-steroids were freed from saturated steroids, also formed in the reduction, by TLC on Si gel plates (0.25 mm) previously dipped in a sol of 22 ml H_2O, 20 g $AgNO_3$, 20 ml EtOH, and 22 mg rhodamin 6G and dried at 120° for 30 min. The solvent system for development was petrol –$CHCl_3$– HOAc (75:25:0.5). The labelled sterol acetates were hydrolyzed to the free sterols (dil K_2CO_3 in MeOH) prior to feeding. Fucosterol was isolated from *Macrocystis pyrifera*. Recrystallized material (150 mg) in CH_2Cl_2 (10 ml) was added to a suspension of pyridinium chlorochromate (126 mg) in CH_2Cl_2 (10 ml) and the mixture stirred at 0° for 3 hr. Et_2O was added and the mixture filtered through a column of Si gel giving 92 mg of stigmasta-5, 24(28)-dien-3-one. This compound (18 mg) dissolved in 0.7 ml EtOH (95%) was treated with NAB^3H_4 (2 mg, sp. act. 205 mCi mmol, New England Nuclear). After 2 hr solvent was removed in a stream of N_2 and dil HCl added to the residue. Insoluble sterol was collected and purified by TLC to give 9 mg fucosterol-[3-^{3}H]. It was diluted with a known wt unlabelled sterol and recrystallized. There was no loss in specific activity. GLC showed the fucosterol sample to contain 4.85% 24-methylene cholesterol and 1.02% cholesterol. The 3 sterols were readily separated by prep GLC and all had the same specific activity of 1.8×10^8 cpm/mg. *Achlya heterosexualis* B-14 and *A. ambisexualis* E 87, American type culture collection No. 16939 and 11400 were maintained on agar slants of Barksdale's medium A [6]. They were used to inoculate medium A in petri dishes (90 mm in diam, each containing 25 ml). After growth for 2 days at 20°, the mycelium in each dish was cut into 4 mm squares and distributed equally in 4 flasks each containing 50 ml of medium A diluted 10 × with H_2O. Sporulation was induced by shaking the flasks for 24 hr at 100 rpm at 24°. The resulting spores were used as inoculum as described below. Radioactive sterols were dissolved in the minimal vol. of EtOH containing 2% Tween-80 and transferred asceptically to the production medium [6]. Spores were then added and the medium maintained at 27° while being aerated with sterile air

at 500 ml/min for each litre of medium. After 3–5 days growth, mycelium was separated from the culture liquid by filtration then air-dried and extracted with CH_2Cl_2–MeOH (1:1 500 ml). The extract was saponified with 5% KOH in refluxing MeOH (200 ml) for 8 hr. H_2O (400 ml) was added and the sterols extracted with hexane (3 × 200 ml). They were purified by TLC on Si gel with $CHCl_3$. For the sitosterol and chonasterol feedings, the purified sterol mixture was acetylated then separated by TLC $AgNO_3$ Si gel developed with petrol–$CHCl_3$–HOAc(75. 25.1). Final purification of the sterol acetates was achieved by prep GLC with a column of 3% OV-17 on Gas chromosorb Q at 290° with FID. For the fucosterol and 24-methylene cholesterol feedings, the sterols were purified directly by GLC. The culture liquid was extracted with CH_2Cl_2 to isolate the oogoniols which were purified by TLC with EtOAc–petrol (2.1) as solvent. Fluorescent bands (in UV light) corresponding to oogoniol-1, -2 and -3 were obtained. A fourth, more polar, fluorescent band was found to be identical to the sterol (oogoniol) obtained by hydrolysis of the ester group of oogoniol-1 or -2. The latter sterols were further purified by two dimensional TLC with first $CHCl_3$–MeOH (94:6), then EtOAc while oogoniol was purified by TLC with $CHCl_3$–MeOH (4:1) followed by Me_2CO. In the experiment with *A. ambisexualis*, 5 l. of culture liquid were inoculated with spores and incubated for 3 days before addition of fucosterol (0.55 mg, sp. act. 1.8×10^8 cpm/mg). Culture liquid (+ mycelium) (1 l.) was then transferred to another flask and antheridiol was added to both flasks to give a conc of 10 ng/ml. Cycloheximide (1 mg) was added to the flask containing 1 l. of liquid in order to test its effect on the induction of oogoniol synthesis. Aliquots of culture liquid (800 ml) with mycelium were taken at intervals from the other flask and processed to isolate both polar and nonpolar sterols as described above. Quantitation and identification of sterols from the mycelium were made by GLC. Identification of the oogoniols was confirmed by UV, TLC and MS. In addition, they were silylated and subjected to GLC. Quantitation was based on UV absorption (λ_{max} 235 nm, ε 14 300 for oogoniol-2). Radioactivity was determined by scintillation counting. Each sterol sample was dissolved in 15 ml toluene containing 2,5-diphenyloxazole (4 g/l.) and POPOP (50 mg/l.). Bioassays for hormone B were performed with *Achlya ambisexualis* 734. Cultures were grown for two days at 27° on medium A in petri dishes. Square plugs (3 mm) of the cultures were used to inoculate 2 ml of assay medium in a 4 cm petri dish at 23°. After 24 hr, methanolic solns of the fractions to be assayed were added. Activity was measured by the number of oogonia produced after 48 hr at 23°.

Acknowledgements—We thank Prof. G. W. Patterson for the sample of poriferasterol and Katherine Forbes and Marie Liu for technical assistance. This investigation was supported by N.I.H. Grants GM 21350, RR 08135 and RR-708.

REFERENCES

1. McMorris, T. C., Seshadri, R., Weihe, G. R., Arsenault, G. P and Barksdale, A. W. (1975) *J. Am. Chem. Soc.* **97**, 2544.
2. Raper, J. R. (1939) *Am. J. Botany* **26**, 639.
3. Barksdale, A. W. (1965) *Mycologia* **57**, 493.
4. Popplestone, C. R. and Unrau, A. M. (1974) *Can. J. Chem.* **52**, 462.
5. Barksdale, A. W. and Lasure, L. L. (1973) *Bull. Torrey Bot. Club* **100**, 199.
6. Barksdale, A. W. and Lasure, L. L. (1974) *J. Appl. Microbiol.* **28**, 544.
7. Corey, E. J. and Suggs, J. W. (1975) *Tetrahedron Letters* **31**, 2647.
8. Svoboda, J. A., Thompson, M. J. and Robbins, W. E. (1971) *Nature, New Biol.* **230**, 57; Pettler, P. J., Lockley, W. J. S., Rees, H. W. and Goodwin, T. W. (1974) *J.C.S. Chem. Commun.* 844.
9. Silver, J. C. and Horgen, P. A. (1974) *Nature* **249**, 252.
10. Ikekawa, N., Morisaki, N., Tsuda, K. and Yoshida, T. (1968) *Steroids* **12**, 41.
11. Gooday, G. W. (1974) *Ann. Rev. Biochem.* **43**, 35.