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TWO NOVEL STEROLS FROM INHIBITED *CHLORELLA ELLIPSOIDEA**

L. G. DICKSON† and G. W. PATTERSON
Department of Botany, University of Maryland College Park, MD 20742, U.S.A.

and

C. F. COHEN and S. R. DUTKY
Insect Physiology Laboratory, Entomology Research Division, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705, U.S.A.

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Abstract—The biosynthesis of the normal Δ^5-sterols of *Chlorella ellipsoidea* was completely inhibited by AY-9944 at a concentration of 4 ppm in the medium. From AY-9944-inhibited *Chlorella ellipsoidea*, two sterols previously unidentified from biological material, namely, (24*S*)-5α-ergosta-8,14-dien-3β-ol and (24*S*)-5α-stigmasta-8,14-dien-3β-ol were isolated. They represented 26 and 43%, respectively, of the total sterols isolated from treated cultures. If these sterols are normal intermediates in the biosynthetic sequence for sterols in this alga, then AY-9944 appears to inhibit the reduction of the 14(15) double bond. The syntheses of both sterols are described.

INTRODUCTION

THE HYPOCHOLESTEROLEMIC drug, *trans*-1,4-bis-(2-chlorobenzylaminomethyl)cyclohexane dihydrochloride (AY-9944) is known to inhibit the biosynthesis of cholesterol in animals by preventing the reduction of 7-dehydrocholesterol to cholesterol.[1-3] Since AY-9944 has been regarded as a quite specific Δ^7-reductase inhibitor and since the effect of AY-9944 on plant sterol biosynthesis is unknown, it was of interest to determine the effect of AY-9944 on the biosynthesis of Δ^5-sterols in a unicellular green alga, *Chlorella ellipsoidea*. If Δ^5-sterols are synthesized via the pathway expected for plants, and if AY-9944 is a Δ^7-reductase inhibitor in algae, we would predict an altered sterol composition in favor of the $\Delta^{5,7}$-sterol intermediates. This paper describes the isolation and identification of the two major sterols from AY-9944-treated *Chlorella ellipsoidea* and their subsequent synthesis.

RESULTS AND DISCUSSION

Sterols were extracted with $CHCl_3$–CH_3OH(2:1) from freeze-dried cells of normal *C. ellipsoidea* and those grown in the presence of 4 ppm AY-9944. This concentration of inhibitor gave approx. 50% inhibition of growth. Total sterol of treated and control cultures

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† Present address: Department of Biology, Walla Walla College, College Place, WA 99324, U.S.A.

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was approximately 0·3% of the dry wt. Sterols of control cultures were identified as those naturally occurring in *C. ellipsoidea*: Δ^5-ergostenol, poriferasterol and clionasterol.[4]

The initial gas chromatograms of the sterols isolated from AY-9944-treated cultures of *C. ellipsoidea* exhibited two minor peaks and two major peaks. The relative retention times (RRT's) of these peaks were not only completely unlike those of control sterols, but matched none of our authentic standards. Alumina column chromatography allowed a complete separation of minor and major peaks. The two minor peaks were eluted together in the 40% ether in hexane fraction, while the two major peaks were eluted together in the 70 and 90%-ether in hexane fraction. The acetate derivatives of the material from the 70 and 90%-ether in hexane fraction was separated into a monounsaturated and diunsaturated sterol fraction by chromatography on a $AgNO_3$-impregnated silica gel column. The diunsaturated sterol fraction showed by GLC analyses two major sterols that comprised 26 and 43%, respectively, of the total sterols from AY-9944-treated cell of *C. ellipsoidea*. The remainder of the discussion will deal with the identification of these sterols. The identity of the remaining sterols that appeared with the major sterol peaks and the minor peaks isolated from AY-9944-treated *C. ellipsoidea* will be the subject of a subsequent paper.

TABLE 1. RRT'S* OF STEROL ACETATES FROM *Chlorella ellipsoidea* AND SYNTHETIC $\Delta^{8(9),14}$ STEROL ACETATES

Sterol acetates	GLC systems QF-1†	Hi-Eff-8-BP‡	PMPE§	SE-30‖
5α-Ergosta-8(9),14-dien-3β-ol (isolated)	1·24	1·44	1·38	1·32
5α-Ergosta-8(9),14-dien-3β-ol (synthetic)	1·25	1·44	1·38	1·33
5α-(24*S*)-Stigmasta-8(9)-dien-3β-ol (isolated)	1·48	1·74	1·65	1·66
5α-(24*S*)-Stigmasta-8(9)-dien-3β-ol (synthetic)	1·48	1·74	1·64	1·65

* Relative to cholesterol acetate.
† Column 1·8 m × 3·4 mm i.d., 1% QF-1 on 100–120 mesh Gas Chrom Q, 25 p.s.i., 231°.
‡ Column 1·8 m × 3·4 mm i.d., 3% Hi-Eff-8-BP on 100–120 mesh Gas Chrom Q, 25 p.s.i., 238°.
§ Column 1·8 m × 3·4 mm i.d., 2% PMPE on 100–120 mesh Gas Chrom Q, 20 p.s.i., 250°.
‖ Column 1·8 m × 3·4 mm i.d., 3% SE-30 on 100–120 mesh Gas Chrom Q, 20 p.s.i., 244°.

Though these compounds could not be further separated by TLC or column chromatography, they were well separated by GLC. GC–MS of the free sterols gave, for the first peak, a molecular ion at *m/e* 398, with additional prominent peaks at *m/e* 383, 365 and 271, indicating a loss of CH_3, $CH_3 + H_2O$, and C_9H_{19} (side chain), respectively. The GC–MS of the second sterol contained a molecular ion peak at *m/e* 412, with other prominent peaks at *m/e* 397, 379 and 271 (loss of CH_3, $CH_3 + H_2O$ and $C_{10}H_{21}$, respectively). These data indicate that the sterols have a saturated side chain and perhaps differ from each other only in that the sterol with a MW of 398 has a methyl group at C-24 while that with a MW of 412 has an ethyl group at this position. The MS of these sterols below *m/e* 280 were identical, and were also identical to that of authentic 5α-cholesta-8(9), 14-dien-3β-ol below *m/e* 280.

A quantitative UV analysis on the mixture of the two sterols gave an ϵ value of 18 000 with a λ_{max} in MeOH at 251 nm which is characteristic of a heteroannular conjugated diene

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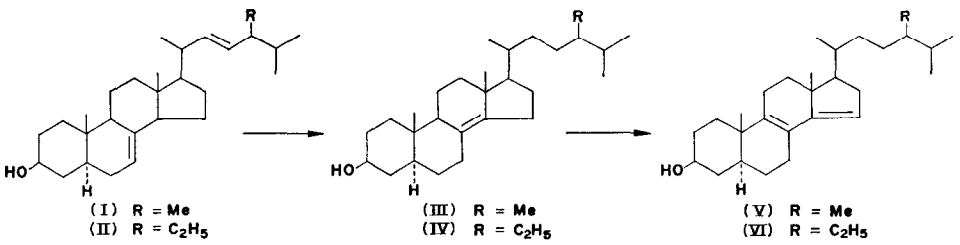
system and is in agreement with spectra obtained with $\Delta^{8,14}$-sterols.[5-7] The strong absorption bands in the IR spectrum at 3050 and 795 cm^{-1} also indicate a conjugated diene system.[8] The optical rotation of the acetate mixture $[\alpha]_D$ $-30°$ was also close to that expected for a mixture of these $\Delta^{8,14}$-sterol acetates (see Experimental).

The physical properties, spectral data, and the agreement in their observed and calculated[9] RRT's on several GLC systems indicate that the sterols are (24*S*)-5α-ergosta-8,14-dien-3β-ol (V) and (24*S*)-5α-stigmasta-8,14-dien-3β-ol (VI). These sterols were assigned the 24*S* configuration solely because the other sterols isolated from this plant were established to have the 24*S* configuration. Identities were confirmed by direct comparison of the IR, UV, MS and GLC behavior (Table 1) with authentic samples of V and VI prepared (see Experimental) according to sequence of reactions as shown in Scheme 1.



SCHEME 1. SYNTHESIS OF (24*S*)-5α-ERGOSTA-8,14-DIEN-3β-OL (V) AND (24*S*)-5α-STIGMASTA-8,14-DIEN-3β-OL (VI).

The sterols V and VI have not previously been identified from natural sources, although 5α-cholesta-8,14-dien-3β-ol has been shown to be a precursor in the biosynthesis of cholesterol in rat liver homogenate.[10] The accumulation of V and VI suggest that these sterols may be precursors in the biosynthesis of the Δ^5-sterols of *Chlorella ellipsoidea* and that AY-9944 is preventing the reduction of the Δ^{14}-bond of the $\Delta^{8,14}$-sterols. AY-9944 has been considered to be a specific inhibitor of Δ^7-reductase enzyme in animals and as such prevents the biosynthetic conversion of 7-dehydrocholesterol to cholesterol.[1-3] Interestingly, in animals and *C. ellipsoidea*, the end results are similar in that there is an accumulation of sterol(s) with a conjugated diene system.

At the level of 5 ppm, AY-9944 completely inhibited the growth of *C. ellipsoidea*. At 4 ppm, *ca.* 50% inhibition of growth was recorded, although the total sterol concentration is unchanged in the treated-cells from that of normal cells. However, at this level, it gives essentially complete inhibition of the formation of the normal Δ^5-sterols of *Chlorella ellipsoidea*. The identification of the other sterols isolated from AY-9944-treated *C. ellipsoidea* should present further insight into the metabolic pathway of sterols in this alga providing that AY-9944 is not bringing about a pathway that is different than that of normal cells.

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EXPERIMENTAL

M.ps were taken on Kofler block and are corrected. Specific optical rotations were determined on *ca.* 1% solution in $CHCl_3$ at 23°. MS were recorded on an LKB Model 9000 GLC-MS (LKB-Produkter AB, Stockholm). The compounds were introduced directly into the ion chamber or into the ion chamber through the GLC column, and the GLC system used was 0·75% SE30. The ioniziaton energy was 70 eV. GLC analyses were made on a Barber Colman Model 10 chromatograph and a Glowall Chromalab, Model A-110, chromatograph equipped with an argon ionization detector, a Honeywell 25 cm recorder and A as a carrier gas. Column packings were prepared from 100 to 120 mesh Gas Chrom Q according to Vanden-Heuval[11] as previously described.[12]

Culture conditions. Control cultures of *Chlorella ellipsoidea* Gerneck, Indiana Culture Collection No. 247, were grown heterotrophically in 15-l carboys on basal inorganic medium containing 0·5 glucose. A constant air flow from an oil-free compressor provided oxygen and kept the cells in suspension. Treated cultures were grown as the controls, except that upon inoculation, 4 ppm AY-9944 was added to the sterile medium.

Sterol extraction and separation. Sterols extracted from freeze-dried cells were partially purified by precipitation with digitonin as previously described by Doyle *et al.*[13] The sterols were chromatographed on a Woelm Grade II alumina column (50 g). 200 ml fractions containing 0, 10, 20, 30, 40, 50, 70 and 90% Et_2O in *n*-hexane and 100% Et_2O were collected. The 2 minor peaks (10% of total sterol) were eluted primarily in the 40% Et_2O fraction, while the 2 major peaks (90% of total sterol) were eluted together in the 70 and 90% Et_2O fractions. The 4 unknown sterols comprising the 2 major peaks were further separated from one another, as the acetate derivatives, using $AgNO_3$-silica gel column chromatography as described by Vroman and Cohen[14] but modified by using a mixture of 1 part Silica Gel G and 1 part Anasil B impregnated with 20% $AgNO_3$. Also, elutions were made with increasing concentrations of Et_2O in *n*-hexane (3, 4, 5, 7, 10, 15 and 90%) and 100% Et_2O. Two sterol fractions were obtained. The first was eluted in the 5% Et_2O in *n*-hexane and consisted of 2 sterols comprising approximately 20% of the total sterol. The second fraction was eluted in the 90- and 100% Et_2O fractions and likewise consisted of 2 sterols. These are the major sterol components, comprising about 69% of the total sterol, and it is with these sterols that this paper is concerned.

5α-Ergosta-7,22-dien-3β-ol (I). 10 g of freshly purified ergosterol, 250 ml of dioxane and 10 g of freshly prepared Raney Nickel Catalyst was shaken with H_2 at room temp. and atmospheric pressure for 5 hr.[15] The catalyst was removed by filtration and the solution was concentrated to dryness in vacuum. Two recrystallizations of the residue from acetone–MeOH gave 8·4 g of I, m.p. 172–173°, $[\alpha]_D$ −12°, $\nu_{max}^{CS_2}$ 3610 cm^{-1} (hydroxyl), 3020, 3040, 1660 cm^{-1} double bonds, 968 cm^{-1} (Δ^{22}-bond).

(24S)-*5α-Ergost-8(14)-en-3β-ol* (III). A mixture of 7 g of I, 200 ml of cyclohexane, 200 ml HOAc, and 700 mg of PtO_2 was shaken with H_2 at room temp. and atmospheric pressure for 5 hr (one molecular equivalent had been consumed within 2 hr and the uptake of H_2 had ceased). The catalyst was removed by filtration and the solution was concentrated to dryness in vacuum. The residue recrystallized from acetone–MeOH gave 6·2 g of III, m.p. 133–134°, $[\alpha]_D$ +11°, $\nu_{max}^{CS_2}$ 3610 cm^{-1} (hydroxyl) and showed no absorption band in the 3000, 1660 and 968 cm^{-1} regions thus indicating no olefinic protons; lit[16] m.p. 130–133°. The compound by GLC analysis on an SE30 column showed only one peak.

(24S)-*5α-Stigmast-8(14)-en-3β-ol* (IV). Treatment of a mixture of 135 mg of chondrillasterol (II), m.p. 169–170° $[\alpha]_D$ ±0 obtained from *Chlorella emersonii* var. *emersonii* Shihira and Krauss, 10 ml of cyclohexane, 10 ml of HOAc and 25 mg of PtO_2 as in the preparation of III gave after workup and recrystallization from acetone–MeOH 80 mg of IV, m.p. 112–114°, $[\alpha]_D$ +9, $\nu_{max}^{CS_2}$ 3610 cm^{-1} (hydroxyl), also showed no absorption bands in the 3000, 1660 and 968 cm^{-1} region, thus indicating no olefinic proton.

(24S)-*5α-Ergosta-8,14-dien-3β-ol* (V). A mixture of 4 g of III, 4 g of SeO_2 and 800 ml of 75% EtOH was refluxed for 1 hr and the selenium removed by filtration.[16] The filtrate was cooled and the precipitate collected to give 2·17 g of material m.p. 122–125°. It was acetylated with Ac_2O–pyridine. The acetate derivative by TLC, developed in benzene–hexane (1:1), showed two zones on an $AgNO_3$-impregnated silica gel plate. The material was then chromatographed over 60 g of a hexane-washed 20% $AgNO_3$-silica gel column and eluted with 100 ml of each of the following fractions: hexane, hexane + 1% benzene, and each succeeding fraction being increased in concentration with benzene by 1% for a total of 20 fractions. The fractions were monitored by TLC and fractions 3–8 showed the acetate of III. Fractions 10–17 that contained the desired compound were combined and recrystallized from EtOH to give 975 mg of (24*S*)-5α-ergosta-8,14-dien-3β-yl acetate, m.p. 136–138°, $[\alpha]_D$ −39°, lit[16] m.p. 136–137°, $[\alpha]_D$ −34.° Saponification of 795 mg of the acetate with 10% MeOH–KOH and its recrystallization from EtOH yielded 540 mg of (24*S*)-5α-ergosta-8,14-dien-

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3β-ol (V), m.p. 143–145°, $[\alpha]_D$ −24°, $\nu_{max}^{CS_2}$ 3610 cm^{-1} (hydroxyl), 3050, 1620 and strong band at 795 cm^{-1} indicating the conjugated double bond. λ_{max} 251 nm in MeOH, ε 16 500; lit.[16] m.p. 139·5–141°, $[\alpha]_D$ −17·5°. Analyses of the free sterol on several different GLC columns showed only one peak and it exhibited RRT's identical to V isolated from AY-9944-treated *Chlorella*. Results with the acetates were similar (Table 1). Their GC–MS were also identical.

(24S)-5α-*Stigmasta*-8,14-*dien*-3β-*ol* (VI). The treatment of 67 mg of (24*S*)-5α-stigmast-8(14)-en-3β-ol with 67 mg of SeO_2 in 15 ml of 75% EtOH at reflux temp. and worked up as in the preparation of V gave 30 mg of (24*S*)-5α-stigmasta-8,14-dien-3β-yl acetate m.p. 110–112° $[\alpha]_D$ −17°. The saponification of the acetate and its crystallization from EtOH gave 23 mg of VI m.p. 136–138° $[\alpha]_D$ −21°, $\nu_{max}^{CS_2}$ 3610 cm^{-1} (hydroxyl) 3050, 1620 and strong band at 795 cm^{-1} (conjugated double bond) λ_{max} 251 nm in MeOH, ε 18 600. Analyses of the free sterol on several different GLC columns showed only one peak and it exhibited RRT's identical to those of the major sterol isolated from AY-9944-treated *Chlorella*. Results with the acetates were similar (Table 1). Their GC-MS were also identical.

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