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14α-METHYL-5α-ERGOST-8-EN-3β-OL AND 14α-METHYL-5α-ERGOSTA-8,24(28)-DIEN-3β-OL FROM TRIPARANOL-TREATED *CHLORELLA EMERSONII**

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Abstract—From triparanol-treated cultures of *Chlorella emersonii*, two new sterols, 14α-methyl-5α-ergost 8-en-3β-ol and 14α-methyl-5α-ergosta-8,24(28)-dien-3β-ol were isolated and identified. These two sterols represent 7 and 3 per cent, respectively, of the total sterols from treated cultures, though only the former was discernible in gas chromatograms of non-treated cultures. The triparanol-treated cultures also contained the naturally occurring sterols, Δ^7-ergostenol, Δ^7-chondrillastenol and chondrillasterol, but at concentrations less than that present in control cultures. The synthesis of 14α-methyl-5α-ergost-8-en-3β-ol is described.

INTRODUCTION

TRIPARANOL (MER-29), a hypocholesterolemic agent in experimental animals,[1,2] has been shown to block the conversion of desmosterol (24-dehydrocholesterol) to cholesterol in vertebrates.[3] Hence, it prevents the reduction of the 24(25) double bond, the terminal step in *de novo* biosynthesis of cholesterol. Triparanol has also been shown to block the conversion of β-sitosterol to cholesterol in an insect[4] and a nematode,[5] and to bring about an accumulation of desmosterol. Sterol biosynthesis in plants is thought to proceed similarly to that in animals except that in plants alkylation of the 24(25) double bond usually occurs, rather than reduction. Because of this basic difference in sterol biosynthesis of animals and plants it was of interest to determine the effect of triparanol on sterol biosynthesis in the unicellular green alga, *Chlorella emersonii*.

RESULTS AND DISCUSSION

A gas chromatogram of the sterol mixture obtained from triparanol-treated cultures of *Chlorella emersonii* exhibited several minor peaks in addition to the three major commonly

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occurring sterols, Δ^7-ergostenol, chondrillasterol, and Δ^7-chondrillastenol of non-treated cultures of *Chlorella emersonii*.[6,7] These three sterols represented 14, 47, and 10%, respectively of the total sterols, the relative quantities of sterols present in non-treated cultures were: 15% Δ^7-ergostenol, 76% chondrillasterol, and 7% Δ^7-chondrillastenol. The total sterol content was 0·29% and 0·16% of the dry weight for non-treated and triparanol-treated cultures, respectively.

Acetylation of the sterol mixture, followed by repeated chromatography on a silver nitrate-impregnated silica gel column permitted the isolation and separation of the fastest moving minor components. The three fastest moving minor components were eluted with the 20%—and 30% benzene in hexane with the most prevalent minor component being eluted first. The natural sterols of *Chlorella* were eluted primarily in the 50% benzene in hexane fraction. The mass spectrum of the most prevalent minor component (free sterol m.p. 136°, $[\alpha]_D + 50°$) had a strong molecular ion peak at *m/e* 414 ($C_{29}H_{50}O$) with prominent peaks at *m/e* 399 (base peak), 381, and 287 indicating the loss of CH_3, $CH_3 + H_2O$, and C_9H_{19} (side chain), respectively. The IR spectrum showed hydroxyl absorption and no indication of a double bond. However, on the basis of the molecular weight and spectral data the best possible structure for this compound is a compound that has four rings and a tetrasubstituted double bond, which is most likely at the $\Delta^8(^9)$ position. The specific rotation is within the range of specific rotation values of $\Delta^8(^9)$-sterols.[8] Since the fragmentation pattern of the C_{29}-sterol indicated that there was only one additional methyl group in the side chain than there is in a C_{27}-sterol, then the other carbon most likely as a methyl substituent would be in the sterol nucleus. The sterol when tested with Liebermann–Burchard reagent gave a yellow color that is highly indicative of 14α-methyl sterols.[9]

The physical properties and spectral data indicate that the sterol is 14α-methyl-5α-ergost-8-en-3β-ol (VIIb). Identity was confirmed by a direct comparison of the IR, NMR, and mass spectra and the GLC behaviour (Table 1) with an authentic sample of VIIb

TABLE 1. RELATIVE RETENTION TIMES OF TRIPARANOL-TREATED *Chlorella emersonii* 14α-METHYL STEROL ACETATES AND SYNTHETIC 14α-METHYL-5α-ERGOST-8-EN-3β-YL ACETATE

Sterol acetates	GLC Systems*			
	QF-1†	Hi-EFF-8-BP‡	PMPE§	SE-30‖
14α-Methyl-5α-ergost-8-en-3β-ol (synthetic)	1·38	1·23	1·20	1·34
14α-Methyl-5α-ergost-8-en-3β-ol (isolated)	1·38	1·23	1·20	1·34
14α-Methyl-5α-ergosta-8,24(28)-dien-3β-ol (isolated)	1·37	1·34	1·29	1·30
14α-Methyl-5α-ergost-8-en-3β-ol (from reduction of 14α-methyl-5α-ergosta-8,24(28)-dien-3β-ol)	1·38	1·23	1 20	1 34

* 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-8BP 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°.

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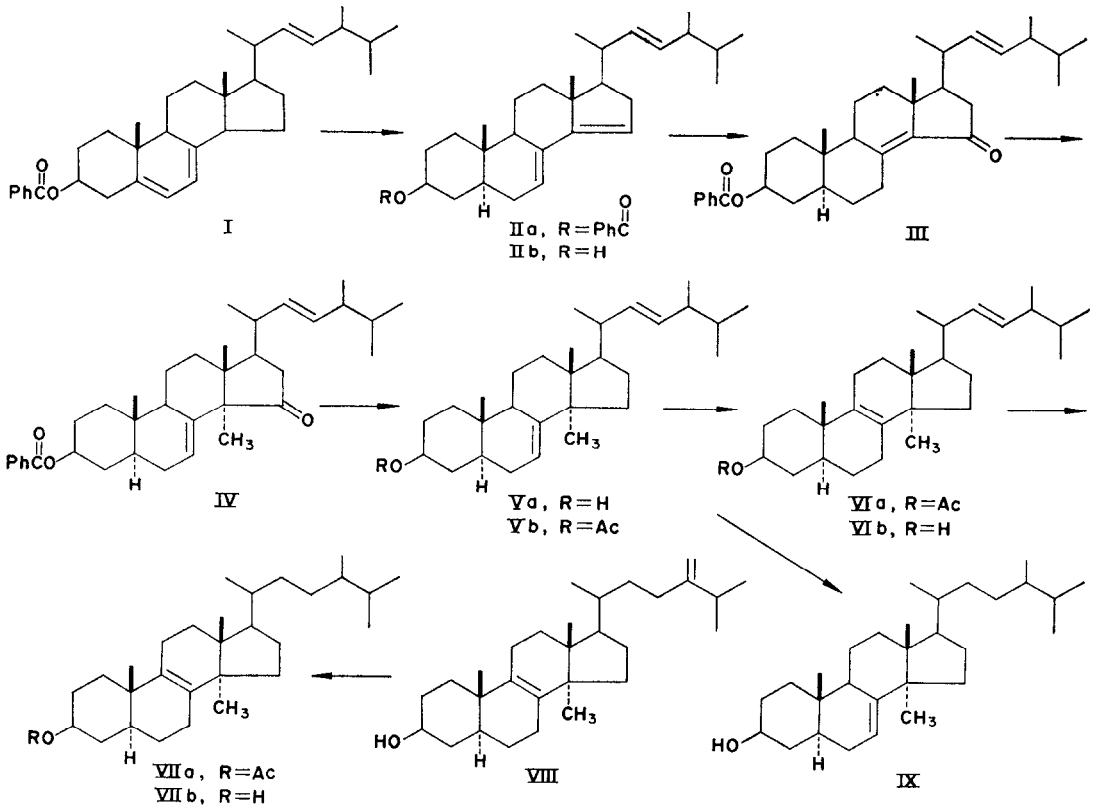

SCHEME 1. PREPARATION OF 14α-METHYL-5α-ERGOST-8-EN-3β-OL (VIIb).

prepared from ergosterol benzoate (1) according to the sequence of reactions as shown in Scheme 1. A detailed account of the synthesis is given in the Experimental along with the mass spectral data of compounds Va, VIb, VIIb, and IX.

In addition to the 14α-methyl-5α-ergost-8-en-3β-yl acetate (VIIa), further elution of the silver nitrate-impregnated silica gel column yielded a mixture of two sterol acetates easily separated on GLC. Saponification of the acetates yielded a free sterol mixture which had very strong absorption bands at 1642 and 890 cm^{-1} indicating that both sterols contained a terminal methylene group. The faster moving sterol of this mixture had a RRT that differed from 14α-methyl-5α-ergost-8-en-3β-ol (VIIb), four GLC systems, by the same factor observed for 24-methylene cholesterol and campesterol, suggesting that this compound differed from VIIb by having a 24(28) double bond. The gas chromatography–mass spectrometry (GC–MS) of this sterol gave a molecular ion peak at *m/e* 412 ($C_{29}H_{48}O$) with other prominent peaks at *m/e* 397, 379, 369 and 313 indicating loss of CH_3, $CH_3 + H_2O$, $CH(CH_3)_2$, and $CH_3 + C_6H_{11}$ (the expected cleavage of the 22–23 bond accompanied by a hydrogen transfer), respectively. The small metastable peak at *m/e* 237·8 also supports the loss of the two groups ($CH_3 + C_6H_{11}$) in a concerted process. The loss of the fragment C_6H_{11} resulting from cleavage of the 22–23 bond places the terminal methylene group at C-24 and not at C-20 or C-25.

A catalytic reduction of the sterol mixture yielded two sterols with RRT higher than the non-reduced sterols. The GC–MS of the reduced, faster moving sterol now gave a molecular

peak at *m/e* 414 and its fragmentation pattern was identical to VIIb, thus establishing the identity of the second sterol as 14α-methyl-5α-ergosta-8,24(28)-dien-3β-ol (VIII).

The identity of the sterol accompanying VIII and present in greater concentrations than VIII and the other minor sterols present in triparanol-treated cultures of *Chlorella emersonii* will be the subject of a subsequent paper.

Naturally occurring 14α-methyl sterols in *Chlorella emersonii* represent an anomaly in the current accepted theory of sterol biosynthesis, since the elimination of both methyl groups at C-4 has preceded the elimination of the methyl substituent at C-14. The 14α-methyl sterols could be regarded as the end product of a defect in the normal scheme, but a more plausible explanation would be that they represent an intermediate stage in the biosynthesis of Δ^7-ergostenol, since small quantities of 14α-methyl-5α-ergost-8-en-3β-ol (VIIb) have also been identified in the sterol fraction from non-treated cultures of *Chlorella emersonii*.

EXPERIMENTAL

M.ps were taken on Kofler block and are corrected. Specific optical rotations were determined on approximately 1% solution in $CHCl_3$ at 23°. IR spectra were obtained with a Perkin-Elmer Model 221 prism-grating spectrophotometer and the UV spectra with a Bausch and Lomb spectrophotometer 505. NMR spectra were recorded at 60 Mc with a Varian A-60A NMR spectrometer using deuterated $CHCl_3$ as the solvent and TMS as an internal NMR standard. 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% SE-30. The ionization energy was 70 eV. Gas–liquid chromatographic (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 12-in recorder and A as a carrier gas. Column packings were prepared from 100–120 mesh Gas Chrom Q according to VandenHeuval.[10] The stationary phases used were: 1% QF-1, 3% Hi-EFF-8BP, and 3% SE-30, obtained from Applied Science Laboratories Inc., Pa., and 2% PMPE obtained from Varian Aerograph, Walnut Creek, Calif

Growth Conditions

Cells of *Chlorella emersonii* var. *emersonii* Shihira and Krauss, Maryland Culture Collection No. 2 were grown heterotrophically on basal inorganic medium containing 0·5% glucose for 10 days in 15 l. carboys. A constant flow of air from an oil-free compressor provided oxygen and served to keep the cells suspended. All cultures were grown as described except that triparanol succinate was added to the sterilized medium to give a final concentration of 5 ppm, in those cultures referred to as triparanol-treated cultures. The average algal yield in the control culture was 2·6 g/l. and in the triparanol-treated culture, 1 2 g/l. on a dry weight basis.

Sterol Extraction

Freeze-dried cells were extracted with $CHCl_3$–MeOH (2:1 v/v) in a Soxhlet apparatus and the lipid was saponified for 1 hr with 20% KOH in 80% ethanol, and the non-saponifiable lipids were removed with Et_2O in a liquid–liquid extraction apparatus.

Digitonin Precipitation of Sterols

Sterols were precipitated with 1% digitonin in 60% EtOH according to Windaus.[11] After separation by centrifugation and decantation, the digitonide was split with Me_2SO and the sterols were extracted into *n*-hexane.[12]

Sterol Separation

The digitonin precipitated sterols were chromatographed on a Woelm Grade II alumina column (100 g) and eluted with 200-ml vol. of an increasing concentration of Et_2O in *n*-hexane. Fractions containing 0-, 10-, 20-, 30-, 40-, 50-, 70-, and 90% Et_2O in n-hexane and 100% Et_2O ether were collected. Isolated sterols, discussed in this paper, were eluted in the 40–90% fractions. The unknown sterols, as their acetates, were further separated from each other and from the acetates of the known sterols of *Chlorella emersonii* by

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$AgNO_3$-silica gel columns (30-mm glass), using the method of Vroman and Cohen.[13] Fractions containing 0-, 10-, 20-, 30-, 40-, and 50% benzene in hexane and 100% benzene were collected.

5α-Ergosta-7,14,22-trien-3β-yl benzoate (IIA). 10 g of ergosterol benzoate (I, m.p. 164–165°) in 300 ml of $CHCl_3$ was treated with dry HCl gas at −30° for 1 5 hr.[14] The solution was poured at once, into an excess of an ice cold $NaHCO_3$. The solution was extracted with Et_2O and the Et_2O washed with H_2O, dried (Na_2SO_4) and concentrated to dryness in vacuum. Crystallization of the residue from ethyl acetate-EtOH gave 4·6 g of IIa, m.p. 157–159°, $[\alpha]_D$ −169°; lit.[14] m.p. 158–159°, $[\alpha]_D$ −187°.

Alkaline hydrolysis of 100 mg of IIa followed by crystallization from ethyl acetate-MeOH gave 58 mg of IIb m.p. 139–141°, $[\alpha]_D$ −234°, λ_{max} 244 nm in MeOH ϵ 14,800; lit.[14] m.p. 139°, $[\alpha]_D$ −238°.

3β-Hydroxy-5α-ergosta-8(14),22-dien-15-one 3-benzoate (III). A solution of 1·54 g of *m*-chloroperbenzoic acid in 60 ml of dry Et_2O was added over a 15 min period to a solution of 3·8 g of IIa in 275 ml of dry Et_2O at 0°.[15] The mixture was kept at 0° for 18 hr. The solution was washed with cold, 2 N $NaOH_2$, HO_2, dried (Na_2SO_4) and concentrated to dryness in vacuum. The residue in 300 ml MeOH, 100 ml of $CHCl_3$ and 20 ml of conc. HCl was refluxed for 15 min. The solution was concentrated to about one-third its volume in vacuum, diluted (H_2O) and extracted with Et_2O. The ethereal phase was washed with 2% $NaHCO_3$, H_2O, and dried (Na_2SO_4) and evaporated to dryness in vacuum, to give 3·9 g of oil. The oil was chromatographed over 90 g of hexane-washed Unisil and the following 100-ml fractions were collected and monitored by TLC: 1–4, hexane–benzene (3:1); 5–8, hexane–benzene (1:1); 9–12, benzene–hexane (3:1); and then stripped with benzene. Fraction 6–12 on recrystallization from acetone-MeOH gave 2·7 g of III, m.p. 175–177°, $[\alpha]_D$ +69°, $\nu_{max}^{CCl_4}$ 1715 cm^{-1} (benzoate), 1625 cm^{-1} (ketone) λ_{max} 230 and 250 nm, EtOH, ϵ 19,400 and 17,700 respectively; lit.[16] m.p. 175°, $[\alpha]_D$ +75° λ_{max} 230 and 259 nm (ϵ 21,100 and 16,800 respectively).

3β-Hydroxy-14α-methyl-5α-ergosta-7,22-dien-15-one 3-benzoate (IV). To a freshly prepared solution of K *t*-butoxide, obtained by the reaction of 10 g K in 225 ml of *t*-BuOH, was added 2·7 g of III.[16] After stirring at room temp. for 5 min, 25 ml of MeI was added in one portion and stirring was continued for 2 hr. H_2O was then added and the product was extracted with Et_2O. The Et_2O was washed (H_2O), dried (Na_2SO_4) and evaporated to dryness in vacuum. An IR analysis of the residue showed some —OH and benzoate absorption. The product was rebenzoylated and crystallized from acetone–methanol to give 1·0 g of IV as rectangular plates, m.p. 157–159°, $[\alpha]_D$ +36°; lit.[16] m.p. 158–159°, $[\alpha]_D$ +36°. Chromatography of the mother liquor over Unisil and crystallization of the desired product only gave an additional 255 mg of IV, m.p. 158–159°.

14α-Methyl-5α-ergosta-7,22-dien-3β-ol (Va). To 40 ml of triethylene glycol was added 0·4 g Na and the solution was heated to 180° and then $NH_2.NH_2$ (*ca.* 4 ml) was added until the solution began to reflux at 180°.[15] The solution was cooled to about 100° and then 1·1 g of 3β-benzoxy-14α-methyl-5α-ergosta-7,22-dien-15-one (IV) was added and the temperature was again raised to reflux while being stirred vigorously. After 8 hr of reflux the temperature was raised to 210° by allowing some $NH_2.NH_2$ to distill from the solution and the solution was kept at 210° for 18 hr. The mixture was cooled and poured into cold 3 N HCl. The resultant precipitate was collected, washed (H_2O) and dried to give 825 mg of crude Va. The compound was chromatographed over 30 g of hexane-washed neutral alumina (Woelm, activity grade II) and the following 100 ml fractions were collected: 1–2, hexane–benzene (3:1); 3–4, benzene; 5–7 benzene–ether (3:1). Fractions 5–7 were combined and crystallized from MeOH to give 640 mg of Va m.p. 149–151°, $[\alpha]_D$ −9°, $\nu_{max}^{CS_2}$ 3610 cm^{-1} (hydroxyl), 3035, 1655 cm^{-1} (double bonds), 968 cm^{-1} (Δ^{22}-bond), lit.[16] m.p. 149–150°, $[\alpha]_D$ −15°; mass spectrum *m/e* (rel. intensity) (M^+ 412)(10), 397(30), 379(5), 313(7), 287(20), 285(11), 271(12), 269(18), 257(5), 255(5), 245(8), 227(9), 219(5), 213(7), 201(7), 187(5), 175(9), 173(6), 161(11), 159(10), 147(9), 145(11), 133(11), 125(63), 109(16), 107(24), 105(18), 95(22), 93(16), 91(16), 83(22), 81(27), 79(15), 69(100), 67(17), 57(20), 55(51), 43(22), 41(22) with metastables at 382·5, 362, 292, 252·2, 236·8, 225, 210, 196·5, 184·5, 89, 77 and 38 2. Acetylation of 600 mg of Va with acetic anhydride-pyridine at 65° for 2 hr and crystallization from acetone-MeOH yielded 600 mg of Vb, m.p. 117–119°, $[\alpha]_D$ −15°.

14α-Methyl-5α-ergosta-8,22-dien-3β-yl acetate (VIa). A solution of 500 mg of 14α-methyl-ergosta-7,22-dien-3β-yl acetate (Vb) in 25 ml of HOAC was treated with HCl gas at room temp. for 2 hr. The product by GLC analysis on an SE-30 column, was a 44:56 mixture of 14α-methyl-ergosta-8,22-dien-3β-yl acetate (VIa) and 14α-methyl-ergosta-7,22-dien-3β-yl acetate (Vb), respectively. (When the isomerization was done in $CHCl_3$ with the 3-benzoate derivative of Va, m.p. 157–159°, $[\alpha]_D$ −5°, a 44:56 mixture of the Δ^8 and Δ^7 compounds, respectively was also obtained). These difficult to separate compounds were purified by preparative TLC. The mixture was streaked on a large TLC plate (20 × 40 cm) coated with silica gel G, impregnated with Rhodamine-6 G, and allowed to develop in a solvent system of hexane–acetone (99:1) for 21 hr. By wedging open the lid of the chromatographic tank to permit the solvent vapors to

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escape, the solvent moved continuously up the plate during development. The broad band from 4–6 cm wide that showed up under UV light was divided into three or four equal zones, scraped from the plate, and the compounds eluted with acetone. The zones were analysed by GLC, and the upper zone contained about 87% of the Δ^8-compound. (The point at which the $\Delta^{8,22}$- and $\Delta^{7,22}$-compounds were in about equal concentration showed up under short UV wavelength light as a dark blue zone whereas the remainder appeared as a yellow band.) The lower zone contained about 95% of the $\Delta^{7,22}$-compound, while the middle half of the band consisted of a mixture of $\Delta^{7,22}$-and $\Delta^{8,22}$-compounds The $\Delta^{8,22}$-compound of about 87% purity was collected from several preparative plates and further purified by preparative TLC. Recrystallization of the upper zone from acetone-MeOH yielded 100 mg of VIa as spears, m.p. 124–125°, $[\alpha]_D$ +26°.

14α-*Methyl*-5α-*ergosta*-8,22-*dien*-3β-*ol* (*VIb*). The saponification of 30 mg of 14α-methyl-5α-ergosta-8,22-dien-3β-yl acetate (VIa) with 2% MeOH–KOH solution gave after crystallization from acetone-MeOH 22 mg of VIb, m.p. 144–146°, $[\alpha]_D$ +46°, mass spectrum *m/e* (rel. intensity) (M^+ 412) (20), 397(33), 379(5), 287(6), 286(8), 271(14), 255(9), 231(8), 219(5), 213(5), 201(5), 187(5), 175(5), 161(10), 159(8), 147(7), 145(9), 133(7), 125(88), 119(11), 109(14), 107(14), 105(13), 95(15), 93(12), 91(12), 83(25), 81(19), 79(9), 69(100), 67(12), 57(29), 55(41), 43(23), 41(15) with metastables at *m/e* 382·8, 362, 292, 252 5, 236·5, 210·5, 195·6, 184·5, 89, 77·2 and 38·2.

14α-*Methyl*-5α-*ergost*-8-*en*-3β-*yl acetate* (*VIIa*). A mixture of 60 mg of VIa, 7 ml of ethyl acetate, and 20 mg of Pt_2O was shaken with H_2 at room temp. and atmospheric pressure for 4 hr. (One molecular equivalent of H_2 had been consumed within 1 hr and the uptake of H_2 ceased.) The catalyst was removed by filtration and the solution was concentrated to dryness in vacuum. A recrystallization of the residue from acetone–methanol gave 54 mg of VIIa, m.p. 110–111°, $[\alpha]_D$ +41°. Analyses on several different GLC columns showed only one peak and it exhibited RRT identical to the acetate of the most prevalent minor sterol isolated from triparanol-treated *Chlorella* (Table 1). Their mass spectra were also identical.

14α-*Methyl*-5α-*ergost*-8-*en*-3β-*ol* (*VIIb*). The saponification of 50 mg of VIIa with 2% MeOH–KOH solution gave after crystallization from dilute MeOH 35 mg of 14α-*Methyl*-5α-*ergost*-8-*en*-3β-*ol* (*VIIb*) m.p. 138–140°, $[\alpha]_D$ +55°, mass spectrum *m/e* (rel. intensity) (M^+ 414)(15), 399(100), 381(11), 287(6), 245(6), 231(10), 219(10), 201(6), 187(8), 161(11), 159(9), 147(9), 145(9), 133(8), 119(13), 107(6), 105(13), 97(18), 95(22), 93(13), 83(16), 81(14), 71(18), 69(23), 57(22), 55(32), 43(37), 41(16) with large metastables at *m/e* 385 and 364; small metastables at 238·2, 210·5, 198·7, 196·5, 184·6, 182·5, 170·8 and 89.

14α-*Methyl*-5α-*ergost*-7-*en*-3β-*ol* (*IX*). The hydrogenation of 50 mg of 14α-methyl-5α-ergosta-7,22-dien-3β-ol (Va) in ethyl acetate with Pt_2O. yielded after crystallization from dilute MeOH 40 mg of IX, m.p. 141–143°, $[\alpha]_D$ +3°, mass spectrum *m/e* (rel. intensity) (M^+ 414)(15), 399(100), 381(36), 297(7), 287(10), 273(10), 260(10), 259(10), 245(21), 242(11), 231(8), 227(22), 219(16), 201(13), 187(8), 161(12), 159(12), 147(13), 145(14), 135(13), 133(17), 121(14), 119(19), 109(15), 107(30), 105(22), 97(17), 95(30), 93(20), 91(17), 83(17), 81(24), 97(15), 69(30), 57(26), 55(39), 43(50), 41(25) with metastables at 385, 363 5, 280, 225, 213, 210·5, 184·5 and 89.

Hydrogenation of the second sterol (*VIII*) *from* Chlorella emersonii. The hydrogenation of a 3 mg sample of the second sterol (RRT 1·34 on 3% Hi-EFF-8BP) of about 40% purity, in ethyl acetate with Pt_2O at room temp. and pressure yielded a product that had a RRT identical to that of 14α-methyl-5α-ergost-8-en-3β-ol (VIIb) on four GLC systems (Table 1). Its IR spectrum no longer showed absorption bands typical of a terminal methylene group and its mass spectrum was identical to VIIb.

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