sium sulfate, filtered and the filtrate allowed to stand over nitrogen at −10° for 2 days. By filtering this mixture in the cold it was possible to remove all traces of triphenylphosphonium oxide.[14] Finally, the filtrate was evaporated under reduced pressure leaving a yellowish-brown viscous liquid; 0.9880 g. The ultraviolet spectrum of this crude product showed the maxima: $E^{1\%}_{1cm}$ (298 mμ) 153, (284 mμ) 146, (272 mμ) 132, (262 mμ) 138, (228 mμ) 140. This crude product was taken up in petroleum ether then adsorbed on alumina (Act. III) and eluted successively in nitrogen with 100-cc. portions of solvents according to the scheme: petroleum ether–benzene, 99:1, 49:1, 19:1, 9:1, 4:1, 1:1 and finally benzene. Each of these fractions was examined spectroscopically. The chromatographic elutions of petroleum ether–benzene, 19:1, 9:1 and 4:1 were combined and the solvent removed leaving a white amorphous solid, 0.2305 g. (23% yield). This solid had a m.p. of 193° dec. with softening at 160°. The ultraviolet spectrum had a principal maximum at 265 mμ with an ε 20,200 (ether). To purify this solid further, it was taken up in a mixture of petroleum ether–benzene 4:1 and flushed in nitrogen through a column of 3.0 g. of alumina. The solvent was evaporated and the white amorphous solid subjected to a high vacuum at 40–50° for 48 hours, $[\alpha]^{25}D$ −10.6, $[M]^{25}D$ −5520 (chloroform).

Anal. Calcd. for $C_{37}H_{60}O$: C, 85.32, H, 11.61. Found: C, 84.22; H, 11.55.

The principal prominent ultraviolet maximum of this homolog occurred at 267 mμ with an ε value of 31,200 (ether). Other maxima of much lower intensity occurred at 260, 264, 273 and 276 mμ, respectively. Like crystalline, vitamin D_3, this homolog gives with antimony trichloride, in chloroform a pink coloration which shows a prominent maximum at 514 mμ, $E^{1\%}_{1cm}$ 200.[15] The infrared absorption spectrum of this homolog is recorded in Fig. 1. When this homolog was tested biologically on rachitic rats it was found to be nearly as active as the crystalline vitamin D_2.

2,1′-*trans* Isomer of 1-Cholestanylidene-2-(5′-methoxy-2′-methylene-1′-cyclohexylidene)-ethane (VIII).—The 2,1′-*trans*-dienone III, m.p. 214–215°, was treated with triphenylphosphinemethylene (VI) exactly under the same conditions and concentrations as the 2,1′-*cis*-dienone IV, and after chromatographic treatment of the product formed, the eluent petroleum ether–benzene fractions 99:1 and 49:1 which had similar ultraviolet absorption maxima were combined and the solvent removed to give 0.2738 g. (27% yield) of a colorless gum, ε (272 mμ) 26,400 (ether). The gum was redissolved in 100 cc. of petroleum ether–benzene 49:1 mixture and flushed once in nitrogen through 3 g. of alumina. The solvent was then removed and the residue subjected to a vacuum at 40–50° for 48 hours and analyzed: $[\alpha]^{25}D$ −39.0°, $[M]^{25}D$ −20,400 (chloroform).

Anal. Calcd. for $C_{37}H_{60}O$: C, 85.32; H, 11.61. Found: C, 83.85; H, 11.34.

The principal prominent ultraviolet maximum of this homolog occurred at 272 mμ with an ε value of 34,700 (ether). Other maxima of much lower intensity occurred at 262, 265 and 277 mμ, respectively. With antimony trichloride in chloroform this homolog also gave a pink color which had an absorption maximum at 512 mμ, $E^{1\%}_{1cm}$ 159.[15] The infrared absorption spectrum is shown in Fig. 1. Like the *cis* homolog this was found to be highly unstable to heat, light and air.

When tested on rachitic rats this homolog was found to be very much less biologically active than either the 2,1′-*cis* homolog or crystalline vitamin D_2.

In the original chromatography of both isomers was eluted with petroleum ether a colorless semi-crystalline solid, m.p. 48.5–50°, $[\alpha]^{25}D$ + 7.44°, $[M]^{25}D$ + 38.0 (chloroform).

Anal. Calcd. for $C_{37}H_{60}O$: C, 85.32; H, 11.61. Found: C, 85.44; H, 12.47.

The ultraviolet spectrum of this product was abnormal; it showed maxima with ε(234 mμ) 13,300, ε(240 mμ) 13,600, ε(246 mμ) 13,900, ε(250 mμ) 13,500, ε(256 mμ) 11,400. The infrared spectrum (10% in chloroform) showed the following bands, respectively, for the groups: $=CH_2$, 3100(r), 1648(s), 885(s) cm.$^{-1}$; $R_2C=CHR$, 1630(r), 1598(vw), 1300(w), 860(r) cm.$^{-1}$; $-OCH_3$, 1315(vw), 1260(m), 1182-(w), 1157(w), 1143(w), 1134(w), 1115(r), 1090(m), 1020(r), 1024(m), 1008(r) cm.$^{-1}$; $-C_6H_{10}$, 973(r), 960(w), 943(w), 930(r), 922(w), 902(r) cm.$^{-1}$. With antimony trichloride in chloroform this fraction gave only a slight pink coloration with an $E^{1\%}_{1cm}$ (510 mμ) 38.4. Microhydrogenation in ethyl acetate using palladium-on-charcoal absorbed 3.0 mole-equivalents of hydrogen showing the presence of 3 double bonds.

In spite of the abnormal ultraviolet spectrum exhibited by this fraction it was found to be as active biologically as the 2,1′-*cis*-homolog when tested on rachitic rats.

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(14) Care must be taken to remove all traces of triphenylphosphonium oxide since its ultraviolet absorption spectrum shows a maximum at 266 mμ with an $E^{1\%}_{1cm}$ of 77 and could easily be mistaken for the vitamin D maximum at 265 mμ.

(15) Since this homolog is highly sensitive to air oxidation and no special precautions were taken to exclude air during measurements the value given is low.

[CONTRIBUTION FROM THE FISHERIES RESEARCH BOARD OF CANADA, CHEMISTRY SECTION OF THE TECHNOLOGICAL STATION AT VANCOUVER]

Marine Sterols. V. Isolation of 7,24(28)-Ergostadien-3β-ol from Starfish[1]

BY U. H. M. FAGERLUND AND D. R. IDLER

RECEIVED JULY 24, 1958

A new sterol, 7,24(28)-ergostadien-3β-ol, has been isolated by a chromatographic separation of the azoyl esters prepared from the sterols of the starfish *Pisaster ochraceus*. Starfish sterols contained 19% of this sterol.

The sterols of starfish are generally conceded to be entirely of the C_7-unsaturated type and 7-cholestenol,[2–4] previously found in mammalian skin,[5] recently has been isolated. Hitodesterol has been shown to be identical with α-spinasterol (7,22-stigmastadienol).[6–8] 7-Stigmastenol, a recog-

(1) Presented at the Portland Meeting of the American Chemical Society, June, 1958.

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nized plant sterol,[9] has also been found in starfish.[10-11] Indeed the sterols of starfish have probably been more thoroughly investigated than have those of any other marine invertebrate. A review is scheduled to appear on the sterols of marine invertebrates, including those of starfish.[12]

Previous reports in the present series have described the isolation, characterization and partial synthesis of 24-methylenecholesterol and 24-dehydrocholesterol.[13-16] The former sterol has been suggested to lie on a biochemical pathway common to sterols of the cholesterol (C_{27}) and ergosterol (C_{28}) type. The structure of the latter sterol suggests that the side-chain double bond of lanosterol has remained unchanged while the ring double bond has migrated from the 8(9) to the 5-position. The structural similarity of the new starfish sterol to 24-methylenecholesterol is apparent. If sterols with a C_7-double bond represent a transition between lanosterol and cholesterol, then the present sterol is of considerable interest because it not only has the C_7-double bond but also the 24-methylene group. The recently reported isolation of 4α-methyl-7,24(28)-stigmastadien-3β-ol from grapefruit[17] suggests a link between the triterpenes and fucosterol in the C_{29}-series. By analogy the new starfish sterol would represent the next intermediate in the transition from triterpenes to 24-methylenecholesterol in the C_{28}-series. Further evidence that the C_7-double bond can be introduced prior to the removal of the 4-methyl group is afforded by the isolation of 4α-methyl-7-cholesten-3β-ol from rat feces[18] and a cactus.[19]

The sterols of marine invertebrates are of particular interest because they offer an isolation–characterization approach to the problem of sterol biogenesis and metabolism. The studies of these organisms are clearly showing that at least in most animals the evolutionary trend is toward cholesterol as the major or sole sterol component of most tissues and organs of the organism. These lower forms of animal life provide a means of studying sterols which thus far have been difficult or impossible to obtain in detectable amounts from more advanced forms of life such as the more common experimental animals.

These studies are also directed toward the attainment of a more solid foundation for the comparative biochemistry of marine invertebrates. The results of the investigations on marine sterols have been previously discussed in relation to the evolutionary development of mollusca and crustacea.[14,16]

The sterol reported in this paper was isolated by chromatography of the steryl azoates (*p*-phenylazobenzoyl esters), prepared from the sterols of the starfish *Pisaster ochraceus*, on silicic acid–Celite. The isolation of formaldehyde in good yield, following ozonolysis of the sterol, established the presence of a terminal methylene group and this was confirmed by the presence of a strong band at 890 cm.$^{-1}$ and a band of medium strength at 1640 cm.$^{-1}$ in the infrared spectra. The sterol and certain of its derivatives have molecular rotations (Table I) consistent with those of a C_7-double bond.[20] The terminal methylene grouping was established to be in the 24(28)-position employing acid rearrangement followed by ozonolysis (Fig.1) as previously described.[21] A terminal methylene group at this position is known to not greatly influence the optical rotation of the sterol molecule. The modified Liebermann–Burchard reaction confirmed the presence of the double bond in the 7-position.[5] It has previously been reported that the C_7-double bond takes up 2 moles of perbenzoic acid, under the conditions employed, and as expected the new sterol consumes a total of 3 moles of oxygen.[5] Catalytic reduction of the sterol with Adams catalyst in glacial acetic acid resulted in the formation of a $\Delta^{8(14)}$-sterol and the uptake of one mole of hydrogen. The 8(14) double bond is readily characterized by means of the modified Liebermann–Burchard reaction and the "reduction" product of zone 2 sterol was thus established to have an 8(14)-double bond.[5] The sterol was confirmed to be in the C_{28}-series by its molecular weight which was determined from the saponification equivalent of the acetate under carefully controlled conditions.

TABLE I

PROPERTIES OF ZONE 2

	M.p., °C.	$[\alpha]_D$	M_D
Sterol	131	+ 6.4°	+23.9°
Acetate	140	+ 6.0	+28.2
Benzoate	160	+11.9	+58.0

Experimental[22]

Preparation of Crude Sterols.—The sterol content was determined on 755 g. of macerated starfish. After moisture, 76.8%, had been determined on an aliquot, the material was extracted by shaking with 3 batches of acetone. The oil, 4.49 g. (0.60%), was treated with acetone to remove phospholipids, 1.00 g. (22.3%), and the residue yielded 1.01 g. (22.5%) of non-saponifiable material. When this was chromatographed on an alumina column, 328 mg. (32.5%) of crude sterol was obtained. In one experiment the gonads and digestive organs of mixed male and female animals were determined separately and were found to contain 88.4% of the total sterols.

Chromatography.—The azoyl esters of the crude sterol mixture were chromatographed on silicic acid–Celite columns with the use of 5.5:1 Skellysolve C–benzene developer as previously described.[13] Five zones developed. A column of double length (50 cm.) was necessary to completely separate zones 3 and 4 from each other. Starting with the

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uppermost zone the composition was: zone 1, 9.0%; zone 2, 19.2%; zone 3, 29.5%; zone 4, 39.8%; and zone 5, 2.5%.

Zone 2 Sterol and Derivatives.—The azoyl ester was crystallized from benzene–ethanol and hydrolyzed. The acetate was prepared and crystallized once from aq. ethanol. The acetate contained 0.3% of provitamin D as calculated from the ultraviolet spectrum. This was removed with maleic anhydride as previously described. After several crystallizations from aq. ethanol, the acetate melted at 140°C., $[\alpha]^{22}D$ + 6.4° (*c* 3.2 in $CHCl_3$).

Anal. Calcd. for $C_{30}H_{48}O_2$: C, 81.76; H, 10.98. Found: C, 81.80; H, 10.66.

The acetate was hydrolyzed and the free sterol crystallized from aq. ethanol, m.p. 131°, $[\alpha]^{22}D$ +6.0° (*c* 2.0 in $CHCl_3$).

Anal. Calcd. for $C_{28}H_{46}O$: C, 84.35; H, 11.63. Found: C, 84.28; H, 11.82.

The benzoate was crystallized from acetone, m.p. 160°, $[\alpha]^{22}D$ +11.9° (*c* 1.4 in $CHCl_3$); molecular rotational differences: 7,24-ergostadien-3β-ol, Δ^{Ac} +4, Δ^{Bz} + 34; Δ^7-stenols, Δ^{Ac} −15 ± 15, Δ^{Bz} +20 ± 14.[20]

Anal. Calcd. for $C_{34}H_{48}O_2$: C, 83.55; H, 9.90. Found: C, 83.64; H, 9.86.

Saponification Equivalent.—Zone 2 acetate, 257.766 mg., was saponified in 5.00 ml. of 0.04736 *N* alcoholic NaOH for 1 hour and the residual alkali was back-titrated with 17.95 ml. of 0.09968 *N* HCl. A correction factor, 0.9935, was employed which was derived from several determinations of the molecular weight of highly purified cholesteryl acetate.

Anal. Calcd. for $C_{30}H_{48}O_2$: mol. wt., 440.7. Found: mol. wt., 442.6.

Perbenzoic Acid Titration.—On standing for five days at −5° in an excess of perbenzoic acid in $CHCl_3$, 19.006 mg. of zone 2 sterol consumed 2.11 mg. of oxygen. The theoretical uptake for three atoms of oxygen is 2.07 mg.

Ozonolysis.—Zone 1 sterol (100 mg.) was suspended in 4 ml. of acetaldehyde-free acetic anhydride–acetic acid (4:1) and cooled in an ice-bath. Ozonated oxygen was passed through the suspension at a rate of 10 mg. ozone/l. of oxygen until all of the solid was dissolved. Water and zinc dust were then added and the mixture was heated in a water-bath in order to decompose the ozonide. The mixture was steam distilled into a 0.5% dimedone solution. The *p*H of the solution was adjusted to 5.8 and the precipitate collected.

The dimedone derivative weighed 52.8 mg. (71.8% of the theory for one methylene group), recrystallized m.p. 189°, mixed m.p. 190° with authentic formaldehyde dimedone.

Anal. Calcd. for $C_{17}H_{24}O_4$: C, 69.82; H, 8.27. Found: C, 69.91; H, 8.49.

The filtrate from the dimedone solution was steam distilled into a 2,4-dinitrophenylhydrazone solution, which

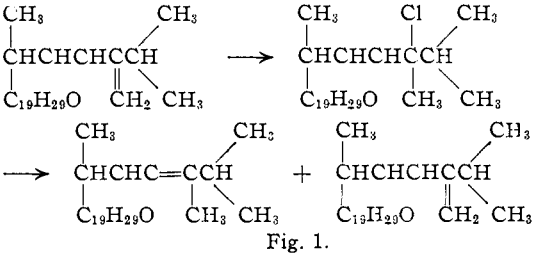

Fig. 1.

was then extracted with ethyl acetate. No hydrazone was obtained.

Rearrangement with HCl.—Zone 2 acetate (430 mg.) was dissolved in 5 ml. of $CHCl_3$ and HCl gas was led through the solution for 6 hours. The solvent was evaporated and the residue was boiled for 10 hours with 6 ml. of acetic anhydride. The solution was then diluted with water, the precipitate collected and chromatographed on an alumina column, from which 380 mg. of material was recovered.

The product from the column was suspended in 10 ml. of acetic anhydride–acetic acid (4:1) and ozonized as described above. The dimedone derivative weighed 71 mg. (28.3% calculated as acetate), m.p. 189° after crystallization, mixed m.p. 189° with authentic formaldehyde dimedone.

Anal. Calcd. for $C_{17}H_{24}O_4$: C, 69.82; H, 8.27. Found: C, 69.85; H, 8.89.

The remaining dimedone solution was steam distilled into a 2,4-dinitrophenylhydrazone solution. The solution was extracted with ethyl acetate and the evaporation residue chromatographed on a silicic acid–Celite column (2:1). Skellysolve C–benzene (2:1) moved a band which was eluted (66 mg. representing 28.7% calculated as acetate) and crystallized from aq. methanol, m.p. 123°, mixed m.p. 123° with authentic methyl isopropyl ketone.

Anal. Calcd. for $C_{11}H_{14}O_4N_4$: C, 49.62; H, 5.30. Calcd. for $C_{12}H_{16}O_4N_4$: C, 51.42; H, 5.75. Found: C, 49.68; H, 5.26.

The 2,4-dinitrophenylhydrazone of the ozonolysis product and of methyl isopropyl ketone had identical R_f values on a descending chromatogram run for 2.5 days in isopropyl alcohol–water (1:1) on Whatman No. 1 filter paper.[23] Ethyl isopropyl ketone, isovaleraldehyde, methyl isopropyl acetaldehyde and ethyl isopropyl acetaldehyde had lower R_f values.

Acknowledgment.—Mr. A. P. Ronald of our microanalytical section performed many of the analyses and recorded the infrared spectra.

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VANCOUVER 2, B. C., CANADA

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF CALIFORNIA]

The Biosynthesis of Ergosterol: Its Relationship to the Squalene Hypothesis[1,2]

BY WILLIAM G. DAUBEN, THOMAS W. HUTTON AND GEORGE A. BOSWELL[3]

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C^{14}-Ergosterol was biosynthesized from 1-C^{14}-acetate and the distribution of the labeled atoms studied. By conversion of ergosterol to progesterone it was shown that the distribution of label between the side-chain and the nucleus was that predicted on the basis of the squalene hypothesis. The specific carbons, C-3, C-4, C-11 and C-12 were obtained by degradation of appropriate precursors, and it was found that C-4, C-11 and C-12 were derived from the carboxyl of acetate, again as predicted by the squalene hypothesis. These results strongly support the concept of the utilization of the intact acyclic triterpene, squalene, in the biosynthesis of all steroids.

The squalene precursor[4] hypothesis for the mechanism of biosynthesis of steroids and tetracyclic triterpenes (C-30 steroids) has been widely investigated and the results obtained have substantiated

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