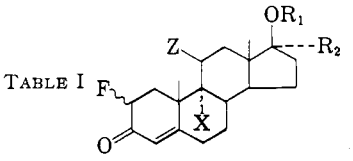
TABLE I



	R_1	R_2	X	Z	M.p., °C.	Crystn. solvent	U.v. λ_{max}, (in EtOH)	I.r., cm.$^{-1}$ 3-C=O (ν)	Analyses, % Calcd. C	Calcd. H	Calcd. F	Found C	Found H	Found F	Yield, %
I	H	H	H	H	159.5–161	EtOH[a]	242	1690	74.47	8.88	6.20	74.54	9.12	5.91	70
II	CH_3CO–	H	H	H	182–183	EtOH–H_2O	..	1687	72.38	8.39	5.45	72.12	8.40	5.42	..
III	CH_3CH_2CO–	H	H	H	146.5–147	EtOH–H_2O	..	1688	72.90	8.62	5.24	72.49	8.94	5.32	..
IV	H	CH_3	H	H	174–174.5	EtOH[a]	242	1683	74.96	9.12	5.93	75.17	9.53	6.10	42
V	H	CH_3	H	β-OH	217–220	Acetone–petr. ether	243	1690	71.39	8.69	5.65	71.79	8.51	5.6[b]	60
VI	H	CH_3	Δ 9:11		182–182.5	EtOAc	240	1698	75.44	8.55	5.97	75.27	8.95	5.96	53
VII	H	CH_3	F	β-OH	288 d.[c]	EtOH–EtOAc	..	1680	67.77	7.96	10.72	68.08	8.29	9.67	8[d]

[a] Compound crystallized with bound solvent, driven off at 105° *in vacuo*. [b] Analysis by Huffman Micro-analytical Laboratories, Wheatridge, Colo. [c] Variable decomposition point, not indicative of purity. [d] Yield from VI *via* epoxide: 63.5%.

furan and methylene chloride[12] to give 2.0 g. of the 2,9α-difluoro compound VII melting at *ca.* 210–217° with decomposition, and having an infrared spectrum identical with that of the product prepared by the direct route. Recrystallization from a mixture of ethanol and ethyl acetate, in which it dissolved sparingly, brought the sample to a comparable degree of purity—m.p. 238–240° dec.

2-Fluoroprogesterone.—The mixture of isomeric alcohols (0.02 mole) obtained by reduction with lithium aluminum hydride of the 3-pyrrolidinyl enamine of progesterone,[5] and then removal of the pyrrolidine group,[13] was fluorinated with perchloryl fluoride *via* its glyoxalate, and the crude product was oxidized in 67 ml. of methylene chloride solution by stirring overnight at room temperature with a solution of 4 g. of sodium dichromate dihydrate and 5.4 ml. of sulfuric acid in 33 ml. of water. The oxidation products were chromatographed on Florisil; 2-fluoroprogesterone (2.05 g.) was eluted by 10% acetone in Skellysolve B. The substance melted at 187–191° and was analyzed; λ_{max} 242 mμ (*E* 15,475), $[\alpha]D$ + 220° ($CHCl_3$). Carbonyl bands appeared in the infrared spectrum at 1700 (C-20) and 1690 cm.$^{-1}$ (C-3).

Anal. Calcd. for $C_{21}H_{29}FO_3$: C, 75.87; H, 8.79; F, 5.71. Found: C, 75.98; H, 9.25; F, 5.24.

Acknowledgment.—We are indebted to Mr. John D. Highstrete for technical assistance in many of the preparations described.

KALAMAZOO, MICH.

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[CONTRIBUTION FROM THE CHEMICAL PROCESS IMPROVEMENT DEPARTMENT, LEDERLE LABORATORIES DIVISION, AMERICAN CYANAMID CO.]

16α-Hydroxy Steroids. IV.[1] Microbiological Reduction of Triamcinolone

BY LELAND L. SMITH, JOHN J. GARBARINI, JOSEPH J. GOODMAN, MICHAEL MARX AND HAROLD MENDELSOHN

RECEIVED AUGUST 20, 1959

Microbiological reduction of triamcinolone and of 9α-fluoro-16α-hydroxyhydrocortisone with *Corynebacterium simplex* or with *Bacterium cyclo-oxydans* yields the respective 20β-dihydro derivative. Further reduction of triamcinolone by these microorganisms results in the loss of the C-1,2 double bond as well as reduction of the C-20 ketone. 20β-Dihydrotriamcinolone forms a 16α,20β,21-triacetate, a 20β,21-acetonide and a 16α,17α;20β,21-bis-acetonide.

The several reported syntheses of triamcinolone (9α-fluoro-11β,16α,17α,21-tetrahydroxy-1,4-pregnadiene-3,20-dione) (II) involve microbiological dehydrogenation of an appropriate Δ^4-3-ketosteroid[2,3] with the organisms *Corynebacterium simplex*,[4] *Bacterium cyclo-oxydans*,[5] *Mycobacterium rhodochorus*,[3] *Nocardia corallina*,[2] etc. As a competing reaction we find that the 20-carbonyl group of triamcinolone and its Δ^4-3-ketone precursors is reduced by *C. simplex* and *B. cyclo-oxydans*, yielding the 20β-dihydro derivative III. The combination of C-1,2 dehydrogenation and 20-carbonyl reduction has been noted in other cases.[4-6]

Reduction of triamcinolone by either organism was manifested by the presence of a single very polar, non-reducing component (III) on papergrams of fermentation broth extracts and of crude steroid preparations isolated from fermentation broths. While possessing the common character-

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istics of $\Delta^{1,4}$-3-ketosteroids of this series (characteristic polarographic reduction behavior,[7,8] sulfuric acid spectra,[9] infrared spectra[7,10] and specific color test[11]) the polar steroid III did not absorb in the infrared regions as expected for a saturated carbonyl group. Acetylation produced a triacetate $C_{27}H_{35}O_9F$, IV, and treatment with acetone–perchloric acid produced a bis-acetonide V. These several points implied the structure 9α-fluoro-11β,16α,-17α,20ξ,21-pentahydroxy-1,4-pregnadien-3-one (20-dihydrotriamcinolone) for the polar steroid III. Chromatographic examination of mother liquor fractions, etc., did not afford any evidence for the presence of the 20-epimer of III.

Reduction of triamcinolone 16α,21-diacetate (IIa) with sodium borohydride in methanol at 0° according to the technique of Norymberski and Woods,[12] followed by acetylation, afforded the same triacetate IV as was formed from 20-dihydrotriamcinolone prepared microbiologically. Hydrolysis of the chemically produced triacetate IV gave the pentahydroxy ketone III identical in all respects with the fermentation product III, thus establishing the formulation of III as 20ξ-dihydrotriamcinolone.

Attempts to use the microörganism *C. simplex* for the preparation of 20-dihydrotriamcinolone on a larger scale led to a further product of reduction, 9α-fluoro-11β,16α,17α,20ξ,21-pentahydroxy-4-pregnen-3-one(VI). Reduction of the C-1,2 double bond and of the C-20 carbonyl group has been reported with another organism, *Streptomyces hydrogenans*, acting on prednisone,[13] and reduction of the C-1,2 double bond of prednisolone in mammalian systems has been reported.[14]

The same 20-dihydro Δ^4-3-ketone VI may be prepared by subjecting 9α-fluoro-11β,16α,17α,21-tetrahydroxy-4-pregnene-3,20-dione (I) to the anaerobic action of *C. simplex*. The reversibility of the A-ring dehydrogenation and some kinetic relationships between triamcinolone, its 1,2-dihydro analog I, and their respective 20-dihydro derivatives III and VI will be discussed more fully elsewhere.[15]

Acetylation of the 20-dihydro Δ^4-3-ketone derivative VI yielded a triacetate VII identical to the triacetate prepared by sodium borohydride reduction of 16α,21-diacetoxy-9α-fluoro-11β,17α-dihydroxy-4-pregnene-3,20-dione and subsequent acetylation.[16]

Hydrolysis of the triacetate VII (prepared chemically) with base yielded the pentahydroxy Δ^4-3 ketone VI identical with the microbiologically produced steroid.

20-Dihydrotriamcinolone III forms a bis-acetonide V when treated with acetone and perchloric acid; however acetone–hydrochloric acid causes the formation of a mono-acetonide IX. Extended exposure to this reagent does, however, yield a mixture of mono- and bis-acetonides. The mono-acetonide IX is the 20,21-acetonide, as a monoacetate X is formed from IX by mild acetylation using acetic anhydride–pyridine. The alternate formulation as a 16α,17α-mono-acetonide (or as a 17α,-20β-acetonide) would require that a 20,21-diacetate (or 16α,21-diacetate) be formed under such conditions.

Infrared absorption spectra in KBr disk of the monoacetonide IX do not resemble spectra of triamcinolone (or other 16α,17α-diol) 16α,17α-acetonide as much as do spectra of the 16α,17α;20,21-bis-acetonide V. In particular a prominent band occurs in the 11.63–11.67 μ region for a series of known 16α,17α-acetonides of this type, such absorption being limited to a very minor band in the spectra of the 20,21-acetonides IX and X.[17]

An attempted Oppenauer oxidation using cyclohexane of 20-dihydrotriamcinolone gave a non-reducing reaction product, recognized as a 20ξ,21-cyclohexylidenedioxy derivative analogous to the 20ξ,21-acetonide IX on the basis of infrared absorption spectra and papergram mobility. Similar ketal formation has been reported by Tschesche and Lipp[18] in an attempted Oppenauer oxidation of digifolein.

The configuration of the 20-carbon atom requires some comment. From most previous experience with sodium borohydride reductions of 20-ketosteroids, stereospecific reduction occurs to yield the 20β-hydroxyl derivative.[12,19] Similarly, microbiological reduction of the C-20 carbonyl group of steroids of this series by a variety of microörganisms[4-6,13,20,21] has resulted in formation of the 20β-isomer. These assigned configurations have been based in their entirety on molecular rotational changes attendant on acetylation of the C-20 hydroxyl group, according to the conventions suggested by the Fieser's and Sarett.[22]

Reduction of triamcinolone microbiologically or chemically results in a single 20-dihydro product. This specificity is to be expected from the several literature references cited with other steroid 20-ketones. By analogy the 20β-hydroxyl orientation is suggested.

Examination of the molecular rotational increments for acetylation of the dihydro derivatives III and VI indicate a negative contribution (Table I).

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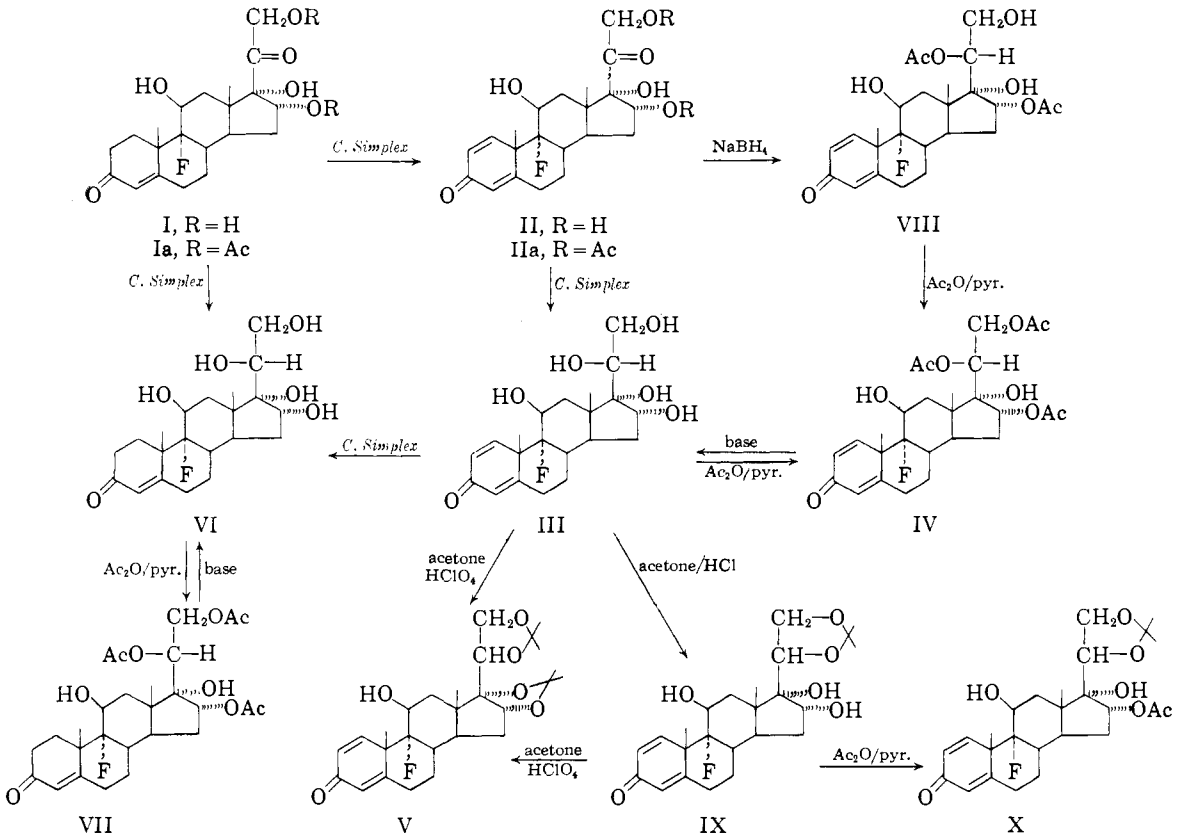

The increment is anticipated as being positive in sign for 20β-hydroxy steroids, negative in sign (or more negative than for the 20β-epimer) for 20α-hydroxy steroids.[22] The extension of these rotational conventions into the area of 16α,17α,20,21-tetrahydroxy steroids may well be untenable, since there is at present insufficient information regarding the rotational increments of the individual 16α- or 20-hydroxyl groups in the series on acetylation, or of

TABLE I

MOLECULAR ROTATIONAL DATA FOR 20-DIHYDROTRIAMCINOLONE DERIVATIVES

Steroid	[α]D	Solvent	[M]D	Δ[M]D
20-Dihydrotriamcinolone	+ 15.1°	Methanol	+ 60°	
20-Dihydrotriamcinolone 16α,20β,21-triacetate	− 20.0	Methanol	−105	−165°
	− 2.4	Chloroform	− 13	
20-Dihydrotriamcinolone diacetate	+101.8	Methanol	+490	+430
	+ 80.3	Chloroform	+385	
1,2,20-Tetrahydrotriamcinolone (dihydrate)	+ 49.5	Methanol	+216	
1,2,20-Tetrahydrotriamcinolone 16α,20β,21-triacetate	+ 40.0	Chloroform	+210	− 6
Triamcinolone 16α,21-diacetate	+ 22	Chloroform	+105	
	+ 63	Methanol	+301	
Triamcinolone	+ 67.1	Methanol	+264	
1,2-Dihydrotriamcinolone 16α,21-diacetate	+ 70	Chloroform	+336	
1,2-Dihydrotriamcinolone	+108	Methanol	+429	

vicinal effects of the 16α,17α-diol systems. Some rotational anomalies ascribed to vicinal interaction of the 17α-hydroxyl with the 16α-substituent have been mentioned by Allen and Bernstein[23] in connection with 16α,17α,21-trihydroxy-20-ketones.

An attempt to employ the correlations of band position in the infrared with configuration at C-20[24] was unrewarding. Although the triamcinolone derivatives absorb in the regions of interest, no useful argument could be developed.

The 20β-configuration is assigned to III tentatively on the basis of mode of formation (microbiological and chemical).

If the reduction product of triamcinolone 16α,21-diacetate obtained with sodium borohydride is isolated without further acetylation, a dihydro diacetate VIII is recovered. On acetylation the dihydro diacetate is converted to the dihydro-16α,-20β,21-triacetate IV. Initial formulation of VIII as the 20β-dihydro-16α,21-diacetate was not supported by oxidative experiments or optical rotation (Table I). Using the sodium bismuthate–trichloroacetic acid conditions of Edwards and Kellie (designed for cleaving vicinal diols)[25] the dihydro diacetate VIII was recovered unaltered. No alteration product could be detected by papergram at any time in the reaction mixture or in fractions obtained during isolation. In view of the recognized potential of acetyl groups to migrate in certain sodium borohydride reductions,[26] it was deemed unlikely that VIII was indeed the 16α,21-diacetate, but rather may be the isomeric 16α,20β-diacetate. This likelihood was not explored further.

Acknowledgments.—The authors are grateful for aid in obtaining and interpreting infrared data from W. Fulmor, ultraviolet spectra from W. H.

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Muller, and papergram behavior from T. Foell. Microanalytical data was obtained from L. Brancone.

Experimental[27]

9α-Fluoro-11β,16α,20β,21-pentahydroxy-4-pregnen-3-one. (VI).—To a growing culture of *Corynebacterium simplex* in a medium[5] consisting of commercial dextrose, 20 g./l., yeast extract, 5 g./l., tryptone, 5 g./l., peptone, 5 g./l., calcium carbonate, 2.5 g./l. (26°, on a rotary shaker) was added a solution of triamcinolone in dimethylformamide, so that the final concentration of steroid was 500 μg./ml., and the final volume of dimethylformamide was *ca.* 1% of the total. After 24 hours of shaking (aerated) the shaker was stopped and the fermentation allowed to continue for 6 hours without aeration. Polarographic analysis of broth extracts indicated *ca.* 320 μg./ml. of Δ^4-3-ketone and *ca.* 80 μg./ml. of $\Delta^{1,4}$-3-ketone. Papergram examination indicated a single component (detected with isonicotinic acid hydrazide[11] only) with R_f 0.13 (*vs.* triamcinolone, R_f 0.42, system II), negative to tetrazolium blue.

The steroid was extracted from the broth with two equal volume portions of ethyl acetate, the combined extracts were evaporated and the residue crystallized from acetone, yielding 212 mg. of crystalline VI, homogeneous on papergrams. After several recrystallizations from acetone, aqueous acetone, aqueous 2-propanol, etc., VI dihydrate was obtained, m. 132–145°, resolidifying 170–185° and remelting 227–231° dec.; $[\alpha]^{22}D$ +49.5°; λ_{max} 239 mμ (ε 15,000); $\lambda_{max}^{H_2SO_4}$ ($E^{1\%}_{1cm.}$) 2 hours, 283 mμ (346), 20 hours, 283 mμ (360), 370 mμ (36); λ_{max}^{KBr} 2.91, 3.39, 6.00, 9.39, 9.55, 11.25 μ etc. Paper chromatographic mobility in system I: 15 cm./16 hr., in system II[28] R_f 0.13 (17.5 cm./16 hours); negative to tetrazolium blue, yellow fluorescence with isonicotinic acid hydrazide reagent.

Anal. Calcd. for $C_{21}H_{31}O_6F\cdot 2H_2O$: C, 58.05; H, 8.12; F, 4.37; water, 8.29. Found: C, 58.26; H, 8.17; F, 4.22; water (KF), 9.93.

The sample was identified as 9α-fluoro-11β,16α,17α,20β,-21-pentahydroxy-4-pregnen-3-one (previously prepared by Bernstein and Lenhard[16]) by comparison of infrared spectra and papergram behavior.

16α,20β,21-Triacetoxy-9α-fluoro-11β,17α-dihydroxy-4-pregnen-3-one (VII).—Twenty milligrams of VI was dissolved in 3 drops of pyridine to which 3 drops of acetic anhydride was added. After standing overnight methanol was added and the mixture was evaporated repeatedly with addition of methanol until the odor of pyridine could not be detected. The residue, m. 271–274°, was identified as impure VI triacetate by infrared spectra and papergram mobilities (compared with an authentic sample prepared by Bernstein and Lenhard[16]). Recrystallization from ethanol yielded pure VII, m. 274–278° dec. with presoftening from 266°. Recrystallization of the VII sample of Bernstein and Lenhard (prepared by reduction with sodium borohydride and acetylation) from ethanol gave a Kofler melting point of 274–277° dec. with presoftening from 265°. Infrared spectra of the two pure samples were identical, and papergram comparisons gave identical mobilities and color tests; $\lambda_{max}^{H_2SO_4}$ ($E^{1\%}_{1cm.}$), 2 hr., 281 mμ (295), 20 hr., 281 mμ (286); λ_{max}^{KBr} 2.80, 2.91, 3.38, 5.72, 6.00, 6.14, 6.94, 7.25, 8.01, 9.50, 11.20, 11.33 μ, etc.; papergram mobility, system V, R_f 0.16; system IV, R_f 0.84; system II, R_f 0.95; negative to tetrazolium blue, typical yellow fluorescence with isonicotinic acid hydrazide.

9α-Fluoro-11β,16α,20β,21-pentahydroxy-1,4-pregnadien-3-one (III). (A) **Microbiological Reduction.**—The microbiological conversion of I to triamcinolone is accomplished in aerobic fermentation using *C. simplex* and the conditions described by either Nobile[4] or Kroll, *et al.*,[5] for the microbiological dehydrogenation of other steroids. At the termination of the fermentation, at which time no further dehydrogenation occurs, the broth is extracted with methyl isobutyl ketone, and the extracts are evaporated. The crude crystalline triamcinolone so obtained is contaminated with small amounts of very polar steroid, as witnessed by partition chromatographic analyses.

A typical harvest fermentation producing triamcinolone assayed 200 μg./ml. of $\Delta^{1,4}$-3-ketone and 18 μg./ml. of Δ^4-3-ketone polarographically. The crystalline triamcinolone obtained by extraction and concentration assayed *ca.* 684 μg./mg. as triamcinolone, 79 μg./mg. as a very polar component, and *ca.* 880 μg. mg. total steroid (partition column chromatography and polarographic assay). The polar component was isolated by partition chromatography as follows:

Twenty-five grams of crude triamcinolone containing the polar component was partitioned on a 9-inch column charged with 9.0 kg. of Celite diatomaceous earth moistened with the lower phase of the solvent mixture dioxane–cyclohexane–water (5:2:1). The column was developed with the upper phase of the solvent mixture at a rate of 8 l./20 min., one hold-back volume (h.b.v.) was 15 l. At 3.7 h.b.v. triamcinolone was eluted, and at 6.4 h.b.v. the polar component was eluted. The appropriate fractions were concentrated *in vacuo* at 24–26° to *ca.* 50 ml. Addition of ethyl acetate and chloroform caused a precipitate of solids, 280 mg. (containing 203 mg. of spectrophotometric activity as triamcinolone), which had greatly reduced infrared absorption at 5.80 μ. The sample was characterized on papergrams as an ultraviolet absorbing spot at R_f 0.10 (system II), negative to tetrazolium blue, negative to the dilute ethanolic isonicotinic acid hydrazide reagent but positive to the strong methanolic reagent.[11] From the mother liquors was recovered an additional 27.8 mg. of material (24 mg. spectrophotometric activity). Crystallization of the solids from ethyl acetate–ethanol–petroleum ether, followed by several recrystallizations from ethanol–toluene and from ethanol yield pure 20β-dihydrotriamcinolone, m. 262–264° dec.; λ_{max} 237 mμ (ε 14,800); $\lambda_{max}^{H_2SO_4}$ ($E^{1\%}_{1cm.}$), 2 hr., 259 mμ (320); 309 mμ (165), 20 hr., 259 mμ (332), 308 mμ (172), 350 mμ (25, inflection), 470 mμ (28); λ_{max}^{KBr} 2.88, 2.95, 3.37, 3.43, 6.01, 6.23, 9.40, 9.65, 11.11, 11.21 μ, etc.; papergram mobility, R_f 0.08 (system I), R_f 0.10 (system II), color tests as described above for the crude preparation.

(B) **Chemical Reduction.**—Five grams of triamcinolone 16α,21-diacetate was dissolved in 125 ml. of dry methanol, cooled to 0–2°, and 0.6 g. of sodium borohydride was added. Portions of the reaction mixture were withdrawn at convenient intervals and spotted onto filter paper, dried momentarily, and sprayed with alkaline tetrazolium blue reagent. After 55 minutes the test was negative, at which time the reaction mixture was diluted with 125 ml. of water containing 1.5 ml. of glacial acetic acid. After concentration *in vacuo* to *ca.* 75 ml. the solution was extracted with ethyl acetate, the extracts washed with saturated sodium bicarbonate solution, with brine and with water, then dried over anhydrous magnesium sulfate prior to concentration to dryness. The residue was dissolved in 13 ml. of dry pyridine to which was added 3.3 ml. of acetic anhydride. After standing overnight at room temperature 150 ml. of methanol was added, and the mixture was evaporated to dryness in vacuum. Additional methanol was added, and evaporation continued, etc., until the odor of pyridine could not be detected. The residue was dissolved in 21 ml. of methanol, cooled in Dry Ice for 45 minutes and filtered. The crystals were washed with methanol and with petroleum ether, yielding 2.8 g., λ_{max} 239 mμ (ε 14,300). Evaporation of the mother liquors afforded an additional 1.2 g. of material with λ_{max} 239 mμ (ε 14,400).

The crude triacetate was recrystallized from ethanol containing 2.5% of chloroform by cooling to −10°, yielding crystals, 3.0 g., m. 283–285° dec. with presoftening from 268°. This material is suitable for alkaline hydrolysis to form the free alcohol III.

A solution of 1.0 g. of the triacetate IV in 75 ml. of dry methanol was purged of air with nitrogen, and a similarly purged solution of 145 mg. of sodium metal in 30 ml. of methanol was added. The solution was stirred under nitrogen for 15 minutes, quenched with 6 ml. of glacial acetic acid, and evaporated to dryness *in vacuo*. The residue was washed with water, yielding 0.7 g. of crude III, m. 257–260° dec., homogeneous on papergrams. The product was dissolved in 12 ml. of hot methanol and petroleum ether (b.p. 30–60°) was added to turbidity. After 12 hours at

(27) Melting points were taken on a Kofler block unless noted otherwise. Optical rotations were made on *ca.* 0.5% solutions in methanol; other solvents are specifically mentioned. Ultraviolet absorption measurements in ethanol and in concentrated sulfuric acid were recorded with the Cary recording spectrophotometer model 11S. Infrared spectra were obtained on KBr disks using the Perkin-Elmer model 21 double beam instrument. Paper chromatography was conducted using methods already described.[28]

(28) L. L. Smith, T. Foell, R. DeMaio and M. Halwer, *J. Am. Pharm. Assoc.*, **48**, 528 (1959).

room temperature the crystals were filtered, 0.3 g., λ_{max} 239 mμ (ϵ 15,000), m. 263–265° dec. From the evaporated filtrates an additional 0.4 g. of product was obtained which on recrystallization yielded 0.2 g. of crystals, m. 262–264° dec., λ_{max} 239 mμ (ϵ 14,300), which was combined with the first crop of III for purification. Recrystallization from methanol–petroleum ether and from methanol several times yielded an analytical sample, m. 262–263.5° dec.; $[\alpha]^{22}D$ +15.1°; λ_{max} 238 mμ (ϵ 14,600); $\lambda_{max}^{H_2SO_4}$ ($E^{1\%}_{1\,cm.}$) 2 hr., 259 mμ (326), 310 mμ (163); polarographic half-wave potential, −1.04 v. (triamcinolone −1.04 v.),[29] with the same diffusion current as triamcinolone; papergram mobility the same as III described in (A) above; λ_{max}^{KBr} 2.95, 3.40, 6.01, 6.19, 6.21, 9.40, 11.20 μ, etc.

Anal. Calcd. for $C_{21}H_{29}O_6F$: C, 63.62; H, 7.37; F, 4.79. Found: C, 63.63; H, 7.49; F, 4.56.

Reacetylation of III produced by hydrolysis afforded III triacetate identical with IV prepared in other ways.

Recrystallization from ethanol of the analytical sample III originally prepared from methanol yielded a polymorph, m. 263.5–264.5° dec.; with altered infrared absorption spectra, different from III prepared from methanol, but identical with III isolated from fermentation sources (see (A) above) and crystallized from ethanol. Both polymorphs had identical papergram behavior.

Anal. Calcd. for $C_{21}H_{29}O_6F$: C, 63.62; H, 7.37. Found: 63.55; H, 7.76.

16α,20β-Diacetoxy-9α-fluoro-11β,17α,21-trihydroxy-1,4-pregnadien-3-one (VIII).—One gram of triamcinolone 16α,21-diacetate (IIa) was dissolved in 25 ml. of dry methanol and reduced with sodium borohydride in the same manner as described above for the preparation of IV. Instead of acetylating the crude reduced steroid to form IV, the residue obtained from the ethyl acetate extracts was dried well and induced to crystallize from acetone (one week at room temperature), yielding needles, 880 mg., homogeneous on papergrams (R_f in system IV, 0.57 *vs.* R_f of triamcinolone diacetate 0.86), negative to tetrazolium blue. Recrystallization from acetone and from acetone–petroleum ether yielded the pure diacetate, m. 253–256.5° dec.; $[\alpha]^{22}D$ +101.8°, +80.3° (chloroform); λ_{max} 239 mμ (ϵ 14,780); λ_{max}^{KBr} 2.91, 3.43, 5.74, 6.02, 6.20, 6.24, 7.29, 8.04, 9.36, 11.2 μ, etc.

Anal. Calcd. for $C_{25}H_{33}O_8F$: C, 62.49; H, 6.92; F, 3.95. Found: C, 62.61; H, 7.38; F, 3.99.

16α,20β,21-Triacetoxy-9α-fluoro-11β,17α-dihydroxy-1,4-pregnadien-3-one (IV). (A) Microbiological Source.—Fifteen milligrams of III isolated from fermentation sources was acetylated in the usual manner with 0.5 ml. of pyridine–0.2 ml. of acetic anhydride. After decomposition with methanol and evaporation the residue was crystallized from methanol–acetone, m. 288–300° dec., and recrystallized from ethanol, m. 305.0–307.0° dec. The infrared absorption spectra (in KBr disk) was superimposable on that of another sample of III prepared by chemical reduction according to section (B) below. In addition, spectra in sulfuric acid were identical with those listed under (B).

(B) **Chemical Reduction Source.**—One hundred milligrams of the 20-dihydrodiacetate VIII was dissolved in 0.75 ml. of pyridine and 0.20 ml. of acetic anhydride was added. After standing overnight at room temperature the mixture was diluted with methanol and then evaporated. More methanol was added and the evaporation repeated until the odor of pyridine could not be detected. The residue was crystallized from methanol, from methanol–acetone, and finally from acetone, yielding crystals, m. 305.0–307.0° dec.; $[\alpha]^{22}D$ −20.0°, −2.4° (chloroform); λ_{max} 238 mμ (ϵ 14,930); $\lambda_{max}^{H_2SO_4}$ ($E^{1\%}_{1\,cm.}$): 2 hr., 259 mμ (243), 309 mμ (111) (with λ_{min} 284 mμ), 20 hr., 259m μ (238), 308 mμ (114) (with λ_{min} 283 mμ); λ_{max}^{KBr} 2.85, 2.92, 3.38, 5.71, 5.98, 6.16, 7.25, 8.00, 9.45, 11.16 μ, etc.

Anal. Calcd. for $C_{27}H_{35}O_9F$: C, 62.05; H, 6.75; F, 3.64. Found: C, 62.08; H, 6.70; F, 3.44.

The three sources of IV, from fermentation III, from acetylation of isolated VIII (and from acetylation of non-isolated VIII), together with IV prepared from chemically reduced III, afforded identical triacetates according to all criteria of identity imposed.

(29) L. L. Smith and M. Halwer, *J. Am. Pharm. Assoc.*, **48**, 348 (1959).

9α-Fluoro-11β,16α,17α-dihydroxy-20β,21-isopropylidenedioxy-1,4-pregnadien-3-one (IX).—One gram of 20-dihydrotriamcinolone was slurried in 50 ml. of acetone containing 1 ml. of concentrated hydrochloric acid and placed on a rotary shaker for 30 minutes at room temperature. One hundred milliliters of acetone and 125 ml. of saturated aqueous sodium bicarbonate solution were added, the mixture shaken for 5 minutes, filtered and 250 ml. of water added to the filtrate. After concentration in vacuum to *ca.* 325 ml. the crystals formed were filtered and washed with 100 ml. of water. The acetonide was dried *in vacuo* at 50° for 20 hr., 800 mg., m. 278–280°, R_f (system V) 0.21, homogeneous, negative to tetrazolium blue, positive to isonicotinic acid hydrazide (strong) reagent. Recrystallization from acetone/water yielded an analytical sample, m. 276–277°; $[\alpha]^{22}D$ +28.8°, +40.2° (chloroform); λ_{max} 239 mμ (ϵ 14,850); $\lambda_{max}^{H_2SO_4}$ ($E^{1\%}_{1\,cm.}$): 2 hr., 258 mμ (273), 308 mμ (163), 20 hr., 259 mμ (302), 308 mμ (153) (with λ_{min} in each case at 285 mμ); λ_{max}^{KBr} 2.95, 3.43, 6.02, 6.17, 6.22, 7.28, 7.31, 9.38, 11.21 μ, etc.; papergram mobility, R_f (system V) 0.21.

Anal. Calcd. for $C_{24}H_{33}O_6F$: C, 66.03; H, 7.62; F, 4.35. Found: C, 65.87, 65.84; H, 7.74, 7.72; F, 4.62.

When the reaction mixture was allowed to stand at room temperature for 20 hours the product obtained was a mixture of the mono-acetonide and the bis-acetonide, as determined by papergram analysis.

9α-Fluoro-11β-hydroxy-16α,17α;20β,21-bis-isopropylidenedioxy-1,4-pregnadien-3-one (V). A. From III.—To a slurry of 200 mg. of III in 10 ml. of acetone was added 0.06 ml. of 70% perchloric acid, and the resulting solution was shaken for 55 minutes at room temperature. The acid was neutralized with saturated sodium bicarbonate solution (1.6 ml.), the mixture filtered, and the filtrate diluted with 7 ml. of water. After concentration *in vacuo* to *ca.* 7 ml. the crystals were filtered, washed with water and with petroleum ether, and then vacuum dried at 50°, 203 mg., homogeneous on papergrams. After recrystallizations from acetone–water, m. 290–291°; $[\alpha]^{22}D$ +61.5°, +67.0° (chloroform); λ_{max} 239 mμ (ϵ 14,800); papergram mobility, R_f (system V) 0.79, negative to tetrazolium blue, yellow fluorescence with isonicotinic acid hydrazide; $\lambda_{max}^{H_2SO_4}$ ($E^{1\%}_{1\,cm.}$): 2 hr., 258 mμ (262), 308 mμ (134), 20 hr., 258 mμ (258), 308 mμ (132), all with λ_{min} 285 mμ; λ_{max}^{KBr} 2.95, 3.38, 3.43, 6.01, 6.17, 6.21, 7.25, 7.31, 8.09, 9.43, 11.18, 11.27, 11.67 μ, etc.

Anal. Calcd. for $C_{27}H_{37}O_6F$: C, 68.04; H, 7.83; F, 3.99. Found: C, 68.01; H, 7.86; F, 4.12.

(B) **From IX.**—A solution of 100 mg. of IX in 5 ml. of acetone containing 0.03 ml. of 70% perchloric acid was held at room temperature for one hour, and then worked up according to (A) above, yielding 82 mg., m. 292–293.5°, R_f (system V) 0.78; infrared absorption spectra identical with that of V prepared under (A).

16α-Acetoxy-9α-fluoro-11β,17α-dihydroxy-20β,21-isopropylidenedioxy-1,4-pregnadien-3-one (X).—To a solution of 500 mg. of the 20β,21-acetonide IX in 3.0 ml. of pyridine was added 0.8 ml. of acetic anhydride. After standing overnight at room temperature the reaction mixture was worked up in the usual manner. The residue so obtained, 520 mg., m. 268–270°, was recrystallized from methanol–pyridine–water and from methanol, and yielded the pure acetonide acetate IX, m. 266–268°; $[\alpha]^{22}D$ −18.1°, −19.7° (chloroform); λ_{max} 238 mμ (ϵ 15,100); $\lambda_{max}^{H_2SO_4}$ ($E^{1\%}_{1\,cm.}$): 2 hr., 258 mμ (306), 308 mμ (143), 20 hr., 259 mμ (297), 307 mμ (135) (λ_{min} in each case, 285 mμ); papergram mobility, R_f (system V) 0.50, (system VI) 7.5 cm./16 hr.; negative to tetrazolium blue, yellow fluorescence with isonicotinic acid hydrazide (strong) reagent; λ_{max}^{KBr} 2.40, 3.40, 3.47, 5.78, 6.01, 6.15, 6.25, 7.30, 8.09, 9.50, 10.23, 11.20, 11.25 μ, etc.

Anal. Calcd. for $C_{26}H_{35}O_7F$: C, 65.25; H, 7.37; F, 3.97. Found: C, 65.68; H, 7.64; F, 3.91.

20β,21-Cyclohexylidenedioxy-9α-fluoro-11β,16α,17α-trihydroxy-1,4-pregnadien-3-one.—One gram of 20-dihydrotriamcinolone was suspended in 300 ml. of toluene and 200 ml. was distilled off. A solution of 1.0 g. of aluminum isopropoxide in 250 ml. of toluene was also distilled to dryness. The two solutions were mixed and 100 ml. of freshly distilled cyclohexanone was added with 100 ml. of toluene. After five hours of slow distillation a positive reaction to alkaline tetrazolium blue was noted, and the reaction mix-

ture was filtered hot and the filtrate concentrated to a gum *in vacuo*. The gum was crystallized from 60% aqueous isopropyl alcohol, yielding 680 mg. of the ketal, m.p. 214–215° (capillary); λ_{max} 239 mμ (ϵ 14,600); mobility R_f 0.45 (system V) compared with 20β-dihydrotriamcinolone at R_f 0 and with triamcinolone 16α,17α-acetonide at R_f 0.16; negative to tetrazolium blue reagent; λ_{max}^{KBr} 2.91, 3.41, 6.01, 6.17, 6.23, 6.91, 8.60, 9.06, 9.36, 9.45, 10.70, 11.24 μ, etc.

Anal. Calcd. for $C_{27}H_{37}O_6F$: C, 68.04; H, 7.83; F, 3.99. Found: C, 66.36; H, 7.99; F, 3.84.

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[CONTRIBUTION FROM THE INSTITUTE OF APPLIED MICROBIOLOGY, UNIVERSITY OF TOKYO]

Steroid Studies. XVI.[1] Isolation of 22-Dehydrocholesterol from *Hypnea japonica*

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RECEIVED JULY 27, 1959

22-Dehydrocholesterol was isolated from a species of red algae, *Hypnea japonica*.

Previous reports from these laboratories[3] and others[4] have indicated that only fucosterol (C_{29}-sterol) is generally to be isolated from various species of brown algae,[5] while more recently cholesterol (C_{27}-sterol), a characteristic animal sterol, was found to occur without exception in all fifteen species of red algae so far examined in these laboratories.[3b,6]

It would thus appear that fucosterol is the characteristic sterol of the brown algae and cholesterol is the common sterol not only in the animal kingdom but in red algae. It is, therefore, of considerable biogenetic interest to report in this paper the isolation of a cholesterol analog, 22-dehydrocholesterol, from a species of red algae, *Hypnea japonica* Tanaka.

The sterol reported in this paper was isolated as a non-saponifiable matter from the extract of the dried and powdered alga and showed m.p. 134–135.5°, $\alpha^{29}D$ −56.3°, on purification by successive chromatography of its acetate and of the free sterol on alumina.

The homogeneity of the sterol was confirmed by the appearance of a single orange colored zone on a silicic acid–Celite column when the *p*-phenylazobenzoyl ester was subjected to chromatography according to the method of Idler, *et al.*;[7] the melting point and optical rotation value of the sterol regenerated from the azoyl ester thus purified remained unchanged.

The sterol formed a monoacetate, m.p. 127–128°, and a monobenzoate; the latter melted at 145–146° to milky white, showing a characteristic fluorescence, and clears at 167–170° with sudden disappearance of a play of colors.

The steryl acetate afforded on treatment with bromine a tetrabromide, m.p. 186–187° dec., and on hydrogenation over platinum oxide in acetic acid at 1 atm. pressure gave a saturated acetate, m.p. 109–110°, which was identical with cholestanyl acetate by mixed melting point and infrared spectra. The sterol must be therefore a doubly unsaturated cholestanol.

Oppenauer oxidation of the sterol gave a stenone, m.p. 69–70°, absorbing at 240.5 mμ (ϵ 18.000) in the ultraviolet region, and on treatment with ozone the steryl acetate furnished isovaleraldehyde, whose 2,4-dinitrophenylhydrazone, m.p. 121–123°, was identified with an authentic sample by mixed melting point and infrared spectra.[8] Two ethylenic linkages were thus shown to be at C_5 and C_{22}, and this was also supported by the infrared absorption bands at 798, 840 (Δ^5) and 970 cm.$^{-1}$ (Δ^{22}).[9]

From these results the sterol in *Hypnea japonica* was clearly shown to be 22-dehydrocholesterol, already synthesized by Bergmann and Dusza who expected its natural occurrence on biogenetic grounds.[10] The physical properties of synthetic and natural sterols and some of their derivatives are in good agreement as shown in Table I.

Experimental[11]

Extraction and Purification of 22-Dehydrocholesterol.—Dried, powdered *Hypnea japonica* (8.5 kg.) was extracted twice with 40 l. of boiling benzene for 25 hours. The dark brown oil (44 g.) obtained after removal of the solvent was saponified with a mixture of methanol (50 ml.), benzene (10 ml.) and 40% aqueous sodium hydroxide solution (20 ml.) by refluxing for 2.5 hours. The cooled reaction mixture was diluted with 150 ml. of methanol and 100 ml. of water and then extracted four times with benzene. The combined extract was washed twice with 40% aqueous methanol solu-

(1) Preceding paper, E. Ohki, *Chem. Pharm. Bull.* (*Tokyo*), in press.

(2) Takamine Laboratory, Sankyo Co., Ltd., Shinagawa, Tokyo, Japan.

(3) (a) K. Tsuda, R. Hayatsu, Y. Kishida and S. Akagi, THIS JOURNAL, **80**, 921 (1958); (b) K. Tsuda, S. Akagi, Y. Kishida, R. Hayatsu and K. Sakai, *Chem. Pharm. Bull.* (*Tokyo*), **6**, 724 (1958); (c) R. Hayatsu, *Pharm. Bull.* (*Tokyo*), **5**, 452 (1957).

(4) I. M. Heilbron, R. F. Phipers and H. R. Wright, *Nature*, **133**, 419 (1934); *J. Chem. Soc.*, 1572 (1934); D. H. Coffey, I. M. Heilbron, F. S. Spring and H. R. Wright, *ibid.*, 1205 (1935); I. M. Heilbron, E. G. Parry and R. F. Phipers, *Biochem. J.*, **29**, 1376 (1935); D. H. Coffey, I. M. Heilbron and F. S. Spring, *J. Chem. Soc.*, 738 (1936); P. W. Carter, I. M. Heilbron and B. Lythgoe, *Proc. Roy. Soc.* (*London*), **B128**, 82 (1939); H. B. MacPhillamy, THIS JOURNAL, **64**, 1732 (1942); D. H. Hey, J. Honeyman, W. G. Peal, *J. Chem. Soc.*, 2881 (1950); W. Bergmann and M. Klosty, THIS JOURNAL, **73**, 2935 (1951).

(5) We have also found that a new sterol, sargasterol, 20-isofucosterol[3] occurred concomitantly with fucosterol in two species of brown algae (*Sargassum ringgoldianum* and *Eisenia bicyclis*).

(6) K. Tsuda, S. Akagi and Y. Kishida, *Science*, **126**, 927 (1957); K. Tsuda, S. Akagi and Y. Kishida, *Pharm. Bull.* (*Tokyo*), **6**, 101 (1958).

(7) D. R. Idler, S. W. Nicksic, D. R. Johnson, V. W. Meloche, H. A. Schuette and C. A. Baumann, THIS JOURNAL, **75**, 1712 (1953).

(8) We have noticed that α-methylisovaleraldehyde 2,4-dinitrophenylhydrazone (m.p. 120–121°) obtained on ozonolysis of ergosteryl acetate showed only slight depression (*ca.* 1°) of melting point on admixture with isovaleraldehyde 2,4-dinitrophenylhydrazone; however, the infrared spectra were different in the finger-print region, the former exhibiting bands at 829, 834 and 980 cm.$^{-1}$ and the latter at 833, 840 and 960 cm.$^{-1}$.

(9) A. R. H. Cole, "Progress in the Chemistry of Organic Natural Products," Vol. XIII, Springer-Verlag, Wien, 1956, p. 41.

(10) W. Bergmann and J. P. Dusza, *J. Org. Chem.*, **23**, 1245 (1958).

(11) All melting points are uncorrected. The optical rotations are for chloroform solutions unless otherwise noted.