TABLE I

STEROID DIMETHYLHYDRAZONES

Sterol[a]	Yield, %	M.p.,[b] °C.	Solvent[c]	Formula	Analyses[d] Calcd. C	Calcd. H	Calcd. N	Found C	Found H	Found N
Pregnenolone	90	166	M	$C_{23}H_{38}N_2O$	77.04	10.68	7.81	76.92	10.74	7.92
Progesterone (bis)	93	148	E	$C_{25}H_{42}N_4$	75.32	10.62	14.06	75.23	10.60	14.52
Testosterone	93	179	M	$C_{21}H_{34}N_2O$	76.31	10.37	8.48	76.03	10.69	8.76
17-α-Methyltestosterone	96	190	M	$C_{22}H_{36}N_2O$	76.69	10.53	8.13	76.60	10.63	8.31
Ethisterone	92	206	M	$C_{23}H_{34}N_2O$	77.92	9.67	7.90	77.62	9.60	8.07
cis-Testosterone[e]	92	200	E	$C_{21}H_{34}N_2O$	76.31	10.37	8.48	75.83	10.35	8.28
Estrone	60	174	E	$C_{20}H_{28}N_2O$	76.88	9.03	8.97	77.12	9.07	8.88
Cortisone (bis)	96	218	M	$C_{25}H_{40}N_4O_3$	67.53	9.07	12.60	67.34	8.99	13.11
Dihydrotestosterone	87	180	M	$C_{21}H_{36}N_2O$	75.85	10.91	8.43	75.08	10.6	8.69
19-Nortestosterone	96	171	M	$C_{20}H_{32}N_2O$	75.90	10.19	8.85	75.33	10.11	8.52
Methyl dehydrocholate[f] (mono)	73	158	C	$C_{25}H_{36}N_2O_4$			6.54			6.23

[a] Commercial materials were used as received. [b] All melting points are with decomposition. [c] Solvent for recrystallization: M, aq. methanol; E, aq. ethanol; C, cyclohexane. [d] Analyses by Micro Tech Laboratories, Skokie, Illinois. [e] Methanol solution used in preparation. [f] Prepared as described in ref. 5.

by Dr. G. R. McKinney of the Mead Johnson Company.

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The Microbiolgoical Preparation of 17-Deoxytriamcinolone

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The recognition that glucocorticoid activity may be enhanced by the addition of certain functional groups to steroids has motivated the preparation of biologically active compounds that lack one or more of the structural features of the hydrocortisone molecule. An early example is provided by 9α-fluorocorticosterone acetate, which, though lacking the 17α-hydroxy group, displayed a glucocorticoid activity greater than that of hydrocortisone.[1] However, this compound also has powerful sodium retaining properties.[1] It is known that 16α-hydroxylation of 9α-fluorohydrocortisone and 9α-fluoroprednisolone yields derivatives that are lacking in sodium retention properties and yet retain considerable glucocorticoid activity.[2] It was of interest to ascertain whether the analogous 17-deoxysteroids would possess similar biological properties.

Fermentation of 9α-fluorocorticosterone (I) with *Streptomyces roseochromogenes* (ATCC 3347) yielded a product that was identified as 9α-fluoro-11β,16α,21-trihydroxypregn-4-ene-3,20-dione (II). This product was ultraviolet-absorbing and displayed a positive blue tetrazolium reaction, indicating the continued presence of a Δ^4-3-ketone and the α-ketolic side chain, in agreement with infrared absorption data. Elemental analysis supported the presence of one additional hydroxyl group. Appearance of the typical 415 mμ-absorbing chromogen after reaction of II with the Porter–Silber reagent[3] strongly suggested the presence of a hydroxyl group at C-16 or C-17. The rate of formation of the 415 mμ-absorbing chromogen favored C-16 as the hydroxylated position.[4] The ready formation of a diacetate and the separation of II from 9α-fluorohydrocortisone by paper chromatography further confirmed that the hydroxyl was located at C-16. Assignment of the configuration as 16α is based on the known capacity of *Streptomyces roseochromogenes* to 16α-hydroxylate steroids,[5] and on optical rotation data (Table I).

TABLE I

MOLECULAR ROTATION DATA OF SOME C-16 HYDROXYLATED STEROIDS

Steroid	M_D	Solvent	ΔM_D (16α-OH-H)
Progesterone[a]	+629°	$CHCl_3$	
16α-Hydroxyprogesterone[a]	+519°	$CHCl_3$	−110°
16β-Hydroxyprogesterone[a]	+635°	$CHCl_3$	+6°
9α-Fluorocorticosterone (I)	+675°	MeOH	
9α-Fluoro-16α-hydroxy-corticosterone (II)	+483°	MeOH	−192°

[a] See ref. 6.

Fermentation of II with *Nocardia corallina* (ATCC 999) produced 9α-fluoro-11β,16α,21-trihydroxypregna-1,4-diene-3,20-dione (IV) which was isolated from the fermentation mash. Compound IV was ultraviolet absorbing, reduced blue tetrazolium, and gave a positive reaction with the Porter–Silber reagent. The infrared spectrum of IV is in accord with the assignment of a 1,4-diene-3-one system. The bathochromic shift observed in the ultraviolet in proceding from II to IV, together with the characteristic reaction of IV for a 1,4-diene-3-one with isonicotinic acid hydrazide,[7] and phthalic acid *p*-phenylenediamine[8] provide further support for the assigned structure.

(1) J. Fried, J. E. Herz, E. F. Sabo, A. Borman, F. M. Singer, and P. Numerof, *J. Am. Chem. Soc.*, **77**, 1068 (1955).

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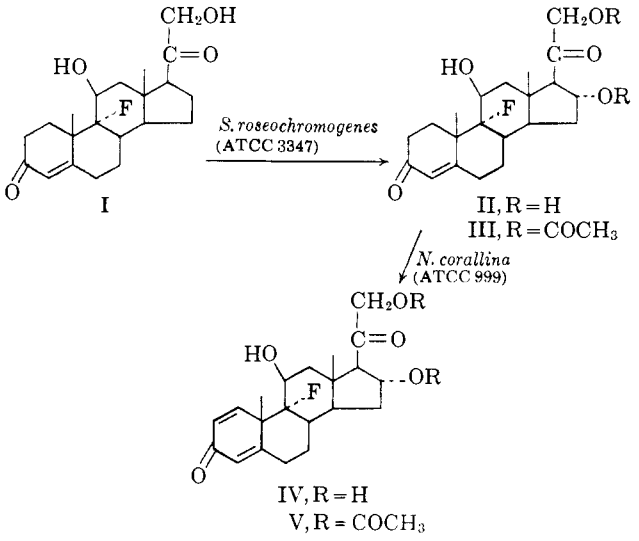
(8) Unreported results of R. H. Blank indicate that $\Delta^{1,4}$-3-ketosteroids produce a yellow color much more slowly than Δ^4-3-ketosteroids when treated with the *p*-phenylenediamine–phthalic acid reagent [A. Bodanszky and J. Kollonitsch, *Nature*, **175**, 729 (1955)].

In the combination liver glycogen and thymus involution assays,[9] 17-deoxytriamcinolone (IV) was, respectively, 3.3 (95% confidence limits: 2.7–4.0) and 1.4 (1.2–1.6) times as active as hydrocortisone. Compounds II and IV caused excretion of sodium, potassium, and water in saline-loaded adrenolectomized male rats.[10] Thus, it appears that the presence of a 16α-hydroxyl group is capable of reversing the sodium retaining property of the 9α-fluorine atom even in 17-deoxysteroids. The presence of a 16α-hydroxyl group in 9α-H-17-deoxysteroids also alters sodium metabolism by either decreasing sodium retention[11] or effecting sodium excretion.[12] Other substituents at C-16 have also been reported to reduce the sodium-retaining property of a 9α-fluoro-17-deoxysteroid.[13]



Experimental

General.—Melting points were determined on a Fisher–Johns block and are corrected. Infrared spectra were determined in KBr disks with a Perkin-Elmer spectrophotometer (Model 21). Ultraviolet data were determined in methanol solution with a Cary recording spectrophotometer. Polarimetric data were obtained in methanol solution in a 1-dm. semimicro tube. All evaporations were carried out *in vacuo*. The solvent system employed for paper chromatography consisted of benzene–acetic acid–*p*-dioxane–water in the volume ratio 4:1:1:2.

9α-Fluoro-11β,16α,21-trihydroxypregn-4-ene-3,20-dione (II).—Fifty 500-ml. erlenmeyer flasks, each containing 100 ml. of medium A,[14] were inoculated with 1 ml. of a 72 hr.-old inoculum of *Streptomyces roseochromogenes* (ATCC 3347). The flasks were placed on a reciprocating shaker (120 strokes/min.) at 28° for 16 hr. At this time 20 mg. of 9α-fluoro-11β,21-dihydroxypregn-4-ene-3,20-dione (I), dissolved in 1 ml. of methanol, was added to each flask. Incubation was continued as described above. The course of the fermentation was followed by paper chromatographic assays of ethyl acetate extracts of the fermentation mash. Quantitative paper chromatographic assay indicated a 68% yield of II (R_f 0.17) and only a trace of starting material (I, R_f 0.47) 20 hr. after introduction of steroid. The contents of all the flasks were then pooled and filtered. The filtrate was extracted three times with equal volumes of ethyl acetate. The mycelial pad was extracted with 500 ml. of ethyl acetate and filtered. All ethyl acetate extracts were pooled, washed with 4% (v./v.) of water, and dried with anhydrous sodium sulfate. Concentration of the solution yielded 632 mg. of a crystalline product which was chromatographed on a 200-g. diatomaceous silica (Celite 545, Johns Manville) column with the solvent system water–*p*-dioxane–cyclohexane in the volume ratio of 1:5:3.

From the major fraction at 6.6 column retention volumes, a partly crystalline residue was obtained on concentration. The residue was dissolved in 25 ml. of boiling acetone and, on cooling, a crystalline product was separated and dried, yielding 342 mg. of II. Recrystallization from acetone afforded 247 mg. of analytically pure II, m.p. 241.5–244.5°, $[\alpha]^{25}D$ +127°, λ_{max}^{MeOH} 237 mμ (ε 16,900); λ_{max}^{KBr} 2.95, 5.84, 5.98, 6.05, 9.33, 9.53 μ.

Anal. Calcd. for $C_{21}H_{29}FO_5$: C, 66.30; H, 7.68; F, 5.00. Found: C, 66.44; H, 8.06; F, 5.01.

Compound II reduced blue tetrazolium and developed the 415 mμ-absorbing chromogen more slowly than did 9α-fluorohydrocortisone, when treated with the Porter–Silber reagent.[4]

Time, min.	Extinction coefficient (415 mμ) II	9α-F-F
20	10,700	15,000
40	15,500	13,100
80	15,600	10,500

16α,21-Diacetoxy-9α-fluoro-11β-hydroxypregn-4-ene-3,20-dione (III).—II (49 mg.) was dissolved in 0.85 ml. of pyridine, and 0.4 ml. of acetic anhydride was added. The reaction mixture was allowed to stand at room temperature for about 18 hr., at which time a crude product was separated after the addition of 7 ml. of water. Recrystallization from acetone and carbon tetrachloride yielded 23 mg. of pure III, m.p. 161.5–162.5, $[\alpha]^{25}D$ +112° (acetone), λ_{max}^{MeOH} 237 mμ (ε 16,900); λ_{max}^{KBr} 2.81, 5.68, 5.75, 5.99, 6.10, 7.96 (plateau), 8.1 μ.

Anal. Calcd. for $C_{25}H_{33}FO_7$: C, 64.64; H, 7.16. Found: C, 64.51; H, 7.22.

9α-Fluoro-11β,16α,21-trihydroxypregna-1,4-diene-3,20-dione (IV).—Fifteen 500-ml. erlenmayer flasks, each containing 100 ml. of medium B,[15] were inoculated with 1 ml. of a 7 hr.-old inoculum of *Nocardia corallina* (ATCC 999). The flasks were placed on a reciprocating shaker (120 strokes/min.) at 28° for 16 hr. At this time 20 mg. of 9α-fluoro-11β,16α,21-trihydroxypregn-4-ene-3,20-dione (II), dissolved in 1 ml. of methanol, was added to each flask. The course of the fermentation was followed by partition column assays. Four hr. after the addition of steroid, assay indicated a 66% yield of IV. After 8 hr. no starting material remained so the contents of all flasks were pooled and extracted once with a mixture of 1500 ml. of ethyl acetate and 100 ml. of chloroform, and twice more with a mixture of 1500 ml. of ethyl acetate and 500 ml. of chloroform. The extracts were pooled and washed twice with 300 ml. of water, and then concentrated to a semicrystalline dry residue. The residue was washed with a small amount of acetone followed by a 1:1 mixture of acetone–ether. The dried crystalline residue yielded 199 mg. of crude IV. Recrystallization from hot acetone yielded 107 mg. of analytically pure IV, m.p. 254–256°, $[\alpha]^{25}D$ +92.1°, λ_{max}^{MeOH} 239 mμ (ε 15,100); λ_{max}^{HBr} 3.05, 5.84, 6.02, 6.18, 9.31, 9.40, 9.48, 11.11 μ.

Anal. Calcd. for $C_{21}H_{27}FO_5$: C, 66.64; H, 7.19; F, 5.02. Found: C, 66.82; H, 7.45; F, 4.78.

On paper chromatograms, IV migrated with R_f 0.06 reduced blue tetrazolium and behaved as a $\Delta^{1,4}$-3-ketosteroid when treated with isonicotinic acid hydrazide[7] or *p*-phenylenediamine phthalic acid.[8]

16α,21-Diacetoxy-9α-fluoro-11β-hydroxypregna-1,4-diene-3,20-dione (V).—IV (80 mg.) was dissolved in 2 ml. of pyridine and 0.5 ml. of acetic anhydride and allowed to stand for 3 hr. at

(9) I. Ringler, S. Mauer, and E. Heyder, *Proc. Soc. Exptl. Biol. Med.*, **107**, 451 (1961).

(10) Compound II was tested at 500 μg., and compound IV in graded doses from 50–1600 μg., in the manner reported by I. Ringler, L. Bortle, E. Heyder, A. Monteforte, J. Perrine, and E. Ross, *ibid.*, **102**, 628 (1959).

(11) 16α-Hydroxy-11-deoxycorticosterone was found to be inactive in the sodium retention assay [H. Hirschmann, F. B. Hirschmann, and G. L. Farrel, *J. Am. Chem. Soc.*, **75**, 4862 (1953)].

(12) 3β,5α,6β,16β-20α-Pentahydroxypregnane has been classed as an aldosterone inhibitor [T. Nakao, K. Hiraga, T. Saito, and Y. Muragawa, *Jikeikai Med. J.*, **6**, 1 (1959)]], and 3β,16α-dihydroxyallopregnan-20-one has been reported to act under certain biological conditions as a sodium-excreting factor [R. Neher, P. Desaulles, E. Vischer, P. Wieland, and A. Wettstein, *Helv. Chim. Acta*, **41**, 1667 (1958)].

(13) A. S. Hoffman, H. M. Kissmann, and M. J. Weiss, *J. Med. Pharm. Chem.*, **5**, 962 (1962).

(14) Medium A consisted of soybean oil meal, 0.22%; corn steep liquor, 0.3%; yeast extract, 0.25%; glucose, 1%; ammonium diacid phosphate, 0.3%; and calcium carbonate, 0.25%. The pH was adjusted to 7.0 with sodium hydroxide.

(15) Medium B consisted of cerelose, 1%; beef extract, 0.4%; yeast extract, 0.1%; peptone, 0.4%; sodium chloride, 0.25%. The pH was adjusted to 7.0 with sodium hydroxide.

room temperature. Addition of water afforded a crude product, which, after drying and recrystallization 4 times from acetone–petroleum ether (30–70° fraction), yielded 13 mg. of pure V, m.p. 195–196°, $[\alpha]^{25}D$ +81.5° (acetone), λ_{max}^{MeOH} 239 mμ (ε 17,000); λ_{max}^{KBr} 2.84, 5.74, 5.98, 6.12, 6.18, and a plateau at 8.00–8.12 μ.

Anal. Calcd. for $C_{25}H_{31}FO_7$: C, 64.92; H, 6.76. Found: C, 65.14; H, 7.02.

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Peptinogan, a Polypeptide Moiety of Actinogan with Antitumor Properties

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Recently, we reported some of the biological and physicochemical properties of actinogan,[1] a glycoprotein derived from an actinomyces culture. During an investigation of the structure of actinogan we separated the polypeptide and the carbohydrate moieties by treatment with trichloroacetic acid.[2] The polypeptide has been named peptinogan.

The various biological properties of actinogan, such as pyrogenicity, inhibition of rodent tumors, protection against bacterial infections, enhancement of horse serum sensitization in mice, and tissue culture inhibition were found to be associated with peptinogan. This polypeptide may be preferable to the carbohydrate-containing compound as a potential antitumor agent because of improved stability and solubility and a more favorable ratio of toxic to effective dose. The carbohydrate fraction was inactive against Sarcoma 180 as well as against Staphylococcus infections in mice.

Experimental

Preparation and Purification.—A 2% aqueous solution of actinogan was treated with an equal volume of 50% trichloroacetic acid. The precipitate was suspended in water and dialyzed. The nondialyzable supension was then centrifuged, and the supernatant liquid containing the peptinogan was freeze-dried. The yield was about 10% based on the weight of actinogan; the product did not give a Molisch test and, on acid hydrolysis and chromatography of the hydrolyzate on thin-layer plates,[3] it appeared to contain at least 10 amino acids. The supernatant liquid from the trichloroacetic acid treatment, on dilution with an equal volume of ethanol, yielded a nitrogen-free carbohydrate, 57% of the weight of actinogan.

Peptinogan was further purified by chromatography on sulfoethyl cellulose[4] in the hydrogen form. A solution of 400 mg. of the polypeptide in 100 ml. of water was placed on a column of 8 g. of the exchanger; after washing with water the active material was eluted with a pH 8 buffer solution, 0.1 *M* with respect to sodium chloride and 0.05 *M* to sodium phosphate. Dialysis and freeze-drying of the fractions showing absorption at 270 mμ afforded 150 mg. of the polypeptide which inhibited S180 in mice at 2 μg. per mouse per day.

Finally this material was subjected to continuous electrophoresis, employing 600 v. in a Beckman CP apparatus and a pH 6.5 buffer consisting of 5% pyridine and 0.2% acetic acid in water. The peptide under these conditions behaved as a cation. The most active material gave a positive response against S 180 and against Carcinoma 755 in mice at 0.2 μg./day per animal.

Chemical and Physical Properties.—Peptinogan has a molecular weight of 15,000 ±20%, as determined from ultracentrifuge data. It is excluded on Sephadex G75[5] and retarded on G100.

Anal. Found: C, 50.0; H, 6.82; N, 14.8, 15.1.

It decomposes at about 240° without melting. It is soluble in water to the extent of 35 mg./ml. and insoluble in common organic solvents including glacial acetic acid, dimethylformamide, and dioxane. It is precipitated from aqueous solution with 60% ethanol or 0.3 *M* ammonium sulfate. On electrophoresis in phosphate buffer up to pH 8.2 and in sodium tetraborate–sodium hydroxide buffer from pH 8.4 to 9.0 the polypeptide behaved as a cation. A single band was obtained. At higher pH values it moved by endoosmosis in the same position as neutral yellow (Apolon®).[6] The polypeptide was detected by spraying with hypochlorite and starch–iodide.[7]

The ultraviolet spectrum in water shows one peak at 270 mμ with an absorptivity of 1.68. The infrared spectrum has bands at 2.8, 3.2, 3.4, 5.9, 6.2, 6.3, and 6.7 μ.; $[\alpha]D$ −53.7° (*c* 1, water).

The peptinogan with the greatest antitumor activity contained no Molisch-positive material. The Pauly, Sakaguchi, and diacetyl tests were positive. It was resistant to trypsin, peptidase, and carboxypeptidase but was made reactive to trypsin and peptidase by prior treatment with urea.

The percentages of amino acids were found by an analytical ion-exchange technique[8] and are listed in Table I.

TABLE I

	g./100 g.		g./100 g.		g./100 g.
Aspartic acid	11.38	½ Cystine	1.74	Lysine	13.23
Threonine	8.12	Valine	8.03	Histidine	0.64
Serine	5.01	Methionine	1.29	Arginine	6.53
Glutamic acid	10.17	Isoleucine	5.66		
Proline	6.50	Leucine	7.89		
Glycine	7.12	Tyrosine	3.32		
Alanine	7.85	Phenylalanine	3.04		

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(5) Pharmacia Corp., Upsala, Sweden.

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(7) R. H. Mazur, B. W. Ellis, and P. S. Cammarata, *J. Biol. Chem.*, **237**, 619 (1961).

(8) Determined by Analytica Corporation, New York 16, New York.