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INHIBITORS OF HUMAN PLACENTAL C_{19} AND C_{21} 3β-HYDROXYSTEROID DEHYDROGENASES

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SUMMARY

The effect of several natural and synthetic steroids on the activity of Δ^5,3β-hydroxysteroid dehydrogenase in homogenates of human placenta has been measured by a method which determines the conversion of labelled dehydroepiandrosterone to androstenedione, testosterone, estradiol-17β, and estrone and of labelled pregnenolone to progesterone and 5α-pregnane-3,20-dione. The method utilizes thin-layer chromatography systems and radio-gas–liquid chromatography which separate each steroidal product from each substrate. Enzymatic activity can be determined rapidly and efficiently in multiple samples of very small amounts of tissue. The present report demonstrates that nucleophilic substituents on, adjacent to, or at some distance from the site on the steroid molecule catalyzed by the enzyme may increase the inhibitory capacity of the parent steroid or confer inhibitory capacity to an inactive parent steroid. Selective inhibition of the conversion of pregnenolone by several steroids demonstrates substrate specificity of the C_{19}- and C_{21}-3β-hydroxysteroid dehydrogenases. The most potent of these selective inhibitors are, in descending order of inhibitory potency: 2α-bromo-17β-hydroxy-5α-androstane-3-one-17β-acetate; 3β,17α-dihydroxy-5-pregnene-3,20-dione-16α-nitrile; 3β-hydroxy-5α-pregnane-20-one-16α-nitrile; and 2α-bromo-5α-androstane-3,17-dione. The most potent inhibitors of both enzymes are 2α-cyano-4,4-dimethyl-2,3α-tetrahydrofuran-2-spiro-17,5-androstene-3-one and 6,16β-dimethyl-3β-hydroxy-5-pregnene-16α-nitrile. The usual form of cyanoketone (2α-cyano-17β-hydroxy-4,4,17α-trimethyl-5-androstene-3-one) does not inhibit either enzyme.

INTRODUCTION

Two substrate analogs (2α-cyano-4,4,17α-trimethyl-17β-hydroxyandrost-5-en-3-one and 17β-hydroxy-4,4,17α-trimethylandrost-5-ene-(2,3d)-isoxazole) are both

Abbreviations: dehydroepiandrosterone, 3β-hydroxy-5-androsten-3-one; epiandrosterone, 3β-hydroxy-5α-androstan-3-one; 16α-cyano-pregnenolone, 16α-cyano-3β-hydroxy-5-pregnen-20-one.

stoichiometric inhibitors of Δ^5,3β-hydroxysteroid dehydrogenase and Δ^{5-4},3-ketosteroid isomerase in rat adrenals and gonads[1-4]. These analogs stoichiometrically bind to the enzyme's active site, titrate activity of the enzyme, cannot be removed from the active site by dilution, and because they structurally resemble the substrate, have a very high degree of lock and key specificity of action. Marked specificity of action *in vivo* has been demonstrated with the isoxazole analog labelled with ^{14}C in the C-2 position of the isoxazole ring[5]. These analogs have been called stoichiometric or active-site directed pseudo-irreversible inhibitors[4]. Since the inhibitory activity of these two analogs is due to the nucleophilic nitrile substitution adjacent to the site on the steroid nucleus catalyzed by the enzyme, we suggested that inhibitors of other steroidogenic enzymes may be found with nucleophilic substitutions at other sites on the steroid nucleus[4].

In this report we have performed screening experiments to determine whether the conversion of labelled dehydroepiandrosterone or pregnenolone by homogenates of human steroidogenic enzymes, *i.e.*, the Δ^5,3β-hydroxysteroid dehydrogenase system from human placenta, may be affected by these or other synthetic steroids selected as potential inhibitors by virtue of nucleophilic substituents at various sites on the steroid nucleus.

MATERIALS AND METHODS

The incubation consisted of 100 μl of homogenate of the fetal surface of the human placenta frozen shortly after delivery (125 mg/tissue per ml of phosphate buffer, pH 7.4), 150 μg of NAD^+, either [7-^{3}H]dehydroepiandrosterone (5.5 μM) or [7-^{3}H]pregnenolone (200 ng/200 000 cpm) (5.0 μM) in 5 μl of dimethylsulfoxide vehicle in a total volume of 130 μl. To each incubation was added either 5 μg of the inhibitor in 5 μl of vehicle or vehicle alone. The incubations with dehydroepiandrosterone as substrate also contained 2.5 units of glucose-6-phosphate dehydrogenase, 2.5 μg of glucose 6-phosphate and 40 μg of NADH. The mixture was incubated for 1 h at 37 °C. The reaction was stopped by the addition of 0.4 ml of ethanol–acetone (1:1, v/v) and after centrifugation, the labelled products formed were separated by thin-layer chromatography using Gelman Type SA chromatograms. The percent conversion of [7-^{3}H]dehydroepiandrosterone into the ^{3}H-labelled products; androstenedione, testosterone, estradiol-17β, and estrone and the conversion of [7-^{3}H]pregnenolone to progesterone and 5α-pregnane-3,20-dione was then determined as described below.

These conditions of incubation were chosen as optimum after kinetic studies had shown that the enzyme was saturated with substrate and that the production of androstenedione, testosterone, estradiol-17β and estrone from dehydroepiandrosterone and that of progesterone and 5α-pregnane-3,20-dione from pregnenolone was proportional to time of incubation as well as to the amount of homogenate.

Substrates

Substrates used were: [7-^{3}H]dehydroepiandrosterone (10.5 mCi/mole) and [7-^{3}H]pregnenolone (10.5 mCi/mole). The following commercially-prepared ^{14}C-labelled steroids were used for reverse isotope dilution or recrystallization studies: 4-[4-^{14}C]androsten-3,17-dione (48 mCi/mole), [4-^{14}C]testosterone (45.5 mCi/mole),

[4-^{14}C]estradiol 17β (58.2 mCi/mole), [4-^{14}C]estrone (45.2 mCi/mole), and [4-^{14}C]-progesterone (52.8 mCi/mole). The commercially-prepared labelled steroids were obtained from New England Nuclear Corporation (Boston, Mass.). Labelled steroids were found to be 98% radiochemically and chemically pure and the unlabelled compounds were found to have no contaminants by thin-layer chromatography and gas–liquid chromatography prior to use.

Inhibitors

Compound Nos 5, 6, 7, 8, 34, 40, 46, 49, 51, 56, 65, 80, 81, 82–84, 103, 104, 141–143, 166 and 167 were purchased from Steraloids (Pawling, N.Y.). Nos 52, 53, 62, 66, 91, 92, 94 were the kind gifts of Dr F. Neumann, Schering, A/G, Berlin; 42, 69, 156 and 157 of Dr W. Lynn Hunt, Searle Laboratories, Chicago, Ill., 24, 29, 33, and 158 of Dr Glen W. Arth, Merck, Sharp and Dohme, Rahway, New Jersey, and the rest of Dr John Babcock, Upjohn Company, Kalamazoo, Mich.

Thin-layer chromatography

The products formed from dehydroepiandrosterone were separated on thin-layer chromatographic plates developed three times in isopropyl ether–chloroform–hexane (70:20:10, v/v/v) (Solvent A). The products formed from pregnenolone were separated by thin-layer chromatography in benzene–acetone (90:10, v/v) followed by chloroform–acetone (96:4, v/v) (Solvent B).

Unlabelled steroid standards were added to the incubates prior to spotting on thin-layer plates and were visualized with anisaldehyde reagent and heat. These standards included for Solvent A: dehydroepiandrosterone, androstenedione, testosterone, estradiol-17β and estrone. For Solvent B: pregnenolone, 17-hydroxypregnenolone, progesterone and 5α-pregnane-3,20-dione. The steroid areas were marked and traced upon tissue paper, and the entire channel was cut into 0.5-cm segments. Each segment was immersed in toluene scintillation media containing 4 g PPO and 100 mg (*p*-bis-[1-methylstyryl]-benzene) per l and counted in a Packard Tri-Carb 3375 Liquid Scintillation Spectrometer. The instrument gave an absolute counting efficiency of 53% for ^{3}H and 89.5% for ^{14}C. Counting time was adjusted to give a standard deviation of no more than ± 7.5%. Recovery of the radioactivity added to the incubation medium was between 84 and 93%.

Identity of label peaks

Chloroform extracts of pooled supernatant remaining from incubations were streaked across a propyleneglycol-saturated thin-layer plate and developed twice in Solvent A or Solvent B. Label was determined in a 0.5 cm wide strip of this channel. A separate channel of standards was run along side and treated with anisaldehyde reagent and heat. Ethanolic eluates of the centers of individual label peaks on Solvent A and on Solvent B were prepared and unlabelled standards were added to each eluate. Aliquots of each of these eluates were then used for studies of the identity of the label peaks by derivative formation and radio gas–liquid chromatography, reverse isotope dilution, or recrystallization to constant specific activity.

Radio gas–liquid chromatography

The eluates were either used directly or after evaporation of the ethanol under N_2, and converted to trimethylsilyl ethers by reacting the residue with a 1:1 mixture of chloroform and Regisil™ (bis-[trimethylstyryl]trifluoracetamide) containing 1% trimethylchlorosilane for 30 min at 56 °C. Separation and identification were achieved on a 6 ft × 0.25 inch diameter stainless steel column packed with 3% OV-210 on Supelcoport 80–100 mesh (Supelco, Inc.) in a Packard 409 gas chromatograph equipped with a Model 776 automatic solid sampler. Carrier gas was helium at a flow of 130 ml/min. Temperature was programmed at 190 °C for 5 min, 0.5 °C/min to 250 °C for 15 min. The effluent was split 4 to 1, 1/5 passing through a flame ionization detector and 4/5 passing through a Packard Model 894 radioactivity monitor. Average recovery of standard [1,2-$^3H_2$]testosterone, not corrected for the 4 to 1 split, determined at various times during the experimental period averaged 81.4% (range 75–85%). Quantitation was performed by comparative areas of label peaks to those of labelled standards.

Reverse isotope dilution and recrystallization studies

To each of the remaining eluates of the label peaks was added about 40 000 cpm of the ^{14}C-labelled standard according to the preliminary identification of the peak. Half of each eluate was spotted on thin-layer plates and developed in Solvent C (chloroform–ethanol; 99.25:0.75, v/v); Solvent D (chloroform–acetone; 96:4, v/v) and Solvent E (hexane–isopropyl ether–chloroform; 70:20:10, v/v/v) and the $^3H/^{14}C$ ratio determined as described above. To the remaining halves was added 50 mg of authentic standard which were recrystallized to constant ratio of $^3H/^{14}C$. The peaks identified as 5α-pregnane-3,17-dione and estrone were recrystallized without the addition of ^{14}C-labelled standard.

Statistics

± represents one standard deviation.

RESULTS

Separation of products

Solvent A separates all of the androgens from the estrogens and Solvent B separates progesterone and 5α-pregnane-3,17-dione from pregnenolone (Table I). Radio gas–liquid chromatography separates the trimethylsilyl ethers of the androgens, estrogens and pregnanes (Table I). Radio gas–liquid chromatographic analysis shows that each of the label peaks is not contaminated by any of the other compounds. These findings are confirmed by the reverse isotope dilution and recrystallization studies (Table I).

Δ^5-Androstene derivatives

Dehydroepiandrosterone (compound No. 1) inhibits the conversion of dehydroepiandrosterone to estrogens and more effectively that of pregnenolone to progestrone (Table II). The 3β-sulfate (10) and 3β-acetate of dehydroepiandrosterone (11) inhibits both of these enzymatic reactions but to a lesser degree. Changing the 17-ketone to the 17β-alcohol (4) reduces the degree of inhibition of each substrate

TABLE I

SEPARATION AND IDENTIFICATION OF PRODUCTS

Abbreviations: R_t, mobility relative to testosterone; R_{rt}, retention time relative to testosterone; D, dehydroepiandrosterone; A, androstenedione; T, testosterone; E^1, estrone; E^2, estradiol-17β, P, pregnenolone; Pro, progesterone; 5αPro, 5α-pregnane-3,20-dione.

Substrate	*System*		*Metabolites*				
			D	*A*	*T*	E^2	E^1
Dehydroepiandrosterone	Solvent A (cm)		9.5	7.0	5.5	11.5	14.0
	R_t		1.73	1.27	1.00	2.09	2.55
	Products (%)		1.6	45.2	8.7	38.9	5.8
	Radio gas–liquid chromatography						
	Parent compound	(min)	11.2	21.1	17.4	9.2	12.2
	Trimethylsilyl	R_{rt}	0.60	1.21	1.00	0.53	0.66
	ethers	(min)	8.6	21.1	13.4	5.2	7.3
	Products (%)		0.7	51.9	9.7	33.5	5.0
	Reverse isotope	Solvent B		1.59	0.58	0.85	0.22
	dilution	Solvent C		1.70	0.59	0.82	0.20
		Solvent D		1.59	0.58	0.89	0.22
	Recrystallization	$^3H/^{14}C$ I		1.92	0.60	0.650	241 cpm/mg
		II		1.95	0.61	0.577	243
		III		1.97	0.59	0.584	232
Pregnenolone				*P*	*Pro*	*5αPro*	
	Solvent B (cm)			8.5	13.5	15.0	
	R_t			1.3	1.60	2.00	
	Products (%)			2.1	88.2	8.6	
	Radio gas–liquid chromatography						
	Parent compound	(min)		12.5	23.8	20.5	
	Trimethylsilyl	R_{rt}		6.68	1.36	1.18	
	ethers	(min)		5.0	23.8	20.5	
		R_{rt}		0.47	1.78	1.53	
	Products (%)			3.2	88.0	8.0	
	Reverse isotope	Solvent A $^3H/^{14}C$			0.384		
	dilution	Solvent C			0.415		
		Solvent D			0.367		
	Recrystallization	$^3H/^{14}C$ I			0.674	71 cpm/mg	
		II			0.648	71	
		III			0.671	69	

produced by dehydroepiandrosterone. Compounds 2 (Δ^{16}-17-cyano-dehydroepiandrosterone) and 3 (17β-hydroxy-17α-cyano-dehydroepiandrosterone) are inhibitors of both enzymes with about 1/2 to 1/4 of the potency of dehydroepiandrosterone. When the C-17 cyano group is replaced by an iodine atom (5) or amino group (6), the inhibition is markedly reduced. Substitution of dehydroepiandrosterone with a 16α-hydroxyl group (8) similarly markedly reduces the inhibitory capacity of dehydroepiandrosterone. Δ^{16},17-cyano-dehydroepiandrosterone-3β-acetate (13) is also a strong inhibitor of these two enzymes with about the same effectiveness of inhibition of the conversion of dehydroepiandrosterone as is the parent compound, and half the inhibitory potency with the C_{21} substrate. The 3β-acetate (14) of 17β-hydroxy-17-cyano-dehydroepiandrosterone is a considerably less effective inhibitor

TABLE II

EFFECT OF Δ^5-ANDROSTENE DERIVATIVES ON HUMAN PLACENTAL CONVERSION OF DEHYDROEPIANDROSTERONE AND PREGNENOLONE

Abbreviations: D, dehydroepiandrosterone; A, androstenedione; T, testosterone; E^1, estrone; E^2, estradiol-17β; I_{50}, concentration required to inhibit 50%; P, pregnenolone; Pro, progesterone; 5αPro, 5α-pregnane-3,20-dione (Tables II–IX).

Control No.	Substituents on position 2	3	4	16	17	Products from dehydroepiandrosterone (%) D (0.7 ± 0.8)	A (27.2 ± 8.1)	T (9.9 ± 1.0)	E^1 (9.4 ± 4.1)	E^2 (50.5 ± 6.2)	I_{50} (μM)	Products from pregnenolone (%) P (1.0 ± 1.0)	Pro (90.7 ± 1.0)	5αPro (8.2 ± 1.1)	I_{50} (μM)
1	—	βOH	—	—	keto	56	28	7	1	4	47	96	3	1	9
2	—	βOH	—	Δ	C≡N	60	11	9	2	12	105	86	13	1	18
3	—	βOH	—	—	βOH, αC≡N	46	9	10	5	23	170	61	37	3	57
4	—	βOH	—	—	βOH	20	14	50	2	9	208	90	9	1	19
5	—	βOH	—	Δ	Iodo	0	20	14	11	54	—	23	71	6	261
6	—	βOH	—	—	NH_2	0	29	10	9	51	—	28	68	4	148
7	—	βOH	—	oxime	keto	4	46	3	4	43	—	19	77	3	—
8	—	βOH	—	αOH	keto	12	29	5	10	44	—	23	74	4	260
9	αC≡N	βOH	$(CH_3)_2$	—	βOH, αCH_3	0	37	9	7	43	—	3	89	9	—
10	αF	βOH	$(CH_3)_2$	—	βOH, αCH_3	0	36	7	8	49	—	3	89	8	—
11	—	βSO_4	—	—	keto	67	21	6	1	4	99	87	10	4	46
12	—	βCH_3OCO—	—	—	keto	67	20	6	1	5	44	85	11	4	12
13	—	βCH_3OCO–	—	Δ	C≡N	44	25	9	5	20	168	78	20	3	22
14	—	βCH_3OCO–	—	—	βOH, αC≡N	23	26	9	5	34	380	2	92	6	606
15	—	βCH_3OCO–	—	—	βC≡N	16	27	12	5	41	421	50	44	5	140
16	—	βCH_3OCO–	—	—	αC≡N	28	18	8	5	41	253	54	42	4	8
17	—	keto	—	—	keto	68	29	2	0	0	78	87	11	2	47
18	αC≡N	keto	$(CH_3)_2$	—	βOH, αCH_3	0	36	15	7	43	—	0	90	12	—
19	αC≡N	keto	$(CH_3)_2$	—	βOH	0	38	12	4	46	—	0	90	10	—
20	αF	keto	$(CH_3)_2$	—	βOH, αCH_3	0	32	5	7	56	—	2	88	9	—
21	(2,3d-isoxazole)		$(CH_3)_2$	—	βOH, αCH_3	1	33	8	8	40	—	10	78	12	—
22	HO—CH=	keto	$H_2C\Delta CH_2$*	—	βOH, αCH_3	0	54	5	3	38	—	30	60	10	192
23	αC≡N	keto	$H_2C\Delta CH_2$*	—	βOH, αCH_3	4	43	6	5	43	—	59	35	6	103
24	αC≡N	keto	$(CH_3)_2$	—	20 spiro**	75	14	4	0	7	2	92	7	1	2
25	—	βCl	—	—	keto	1	29	10	9	51	—	0	90	10	—

* 4,4′-Cyclopropyl.

** 2,3′α-Tetrahydrofuran-2′-spiro-17-.

TABLE III

EFFECT OF Δ^4-ANDROSTENE DERIVATIVES ON HUMAN PLACENTAL CONVERSION OF DEHYDROEPIANDROSTERONE AND PREGNENOLONE

								Products from dehydroepiandrosterone (%)						*Products from pregnenolone (%)*			
	Substituents on position							*D* (0.7 ± 0.8)	*A* (27.2 ± 8.1)	*T* (9.9 ± 1.0)	E^1 (9.4 ± 4.1)	E^2 (50.5 ± 6.2)	I_{50} (μM)	*P* (1.0 ± 1.0)	*Pro* (90.7 ± 1.0)	*5αPro* (8.2 ± 1.1)	I_{50} (μM)
Control No	*1*	*3*	*6*	*7*	*11*	*16*	*17*										
26	—	keto	—	—	—	—	βOH	30	19	45	0	2	62	90	8	2	40
27	—	keto	βOH	—	—	—	βOH	7	36	47	0	9	—	71	22	7	116
28	—	oxime	—	—	—	—	βOH	0	29	41	5	25	—	13	79	8	—
29	Δ	keto	—	—	—	—	βureide	3	39	8	12	39	—	0	94	6	—
30	—	keto	—	—	—	—	βOH, αethinyl	20	19	7	12	41	347	12	81	7	842
31	—	keto	—	—	βOH	—	βOH	13	9	22	7	48	—	56	35	7	130
32	—	βOH	—	—	—	—	βOH	55	12	25	0	8	57	93	6	1	23
33	—	keto	—	—	—	—	20spiro*	56	25	4	1	16	122	64	31	5	87
34	—	keto	—	—	—	αOH	βOH	8	26	7	11	46	—	3	88	9	—
35	—	keto	—	—	—	—	keto	68	21	6	0	1	57	92	7	2	30
36	—	keto	—	—	βOH	—	keto	49	16	4	3	26	144	64	30	6	101
37	—	keto	—	—	keto	—	keto	57	37	3	3	0	113	65	29	6	85
38	—	keto	—	αOH	—	—	keto	3	53	8	11	26	—	5	86	9	—

* 2′, 3′α-Tetrahydrofuran-2′-spiro-17 (4-androsten-3-one) or 20-spirox-4-en-3-one.

TABLE IV

EFFECT OF 5α-ANDROSTANE DERIVATIVES ON HUMAN PLACENTAL CONVERSION OF DEHYDROEPIANDROSTERONE AND PREGNENOLONE

Abbreviations: βCOCl, chloroformate; $N(CH_3)_2$, dimethylamine.

									Products of dehydroepiandrosterone (%)						*Products from pregnenolone (%)*			
Control No.	*Substituents on position* 1	2	3	5	6	11	16	17	*D* (0.7 ± 0.8)	*A* (27.2 ± 8.1)	*T* (9.9 ± 1.0)	E^1 (9.4 ± 4.1)	E^2 (50.5 ± 6.2)	I_{50} (μM)	*P* (1.0 ± 1.0)	*Pro* (90.7 ± 1.0)	5α*Pro* (8.2 ± 1.1)	I_{50} (μM)
39	—	—	βOH	αH	—	—	—	keto	26	55	6	1	7	311	68	25	7	211
40	—	—	βCl	αH	—	—	—	keto	44	41	6	2	7	217	90	9	1	64
41	—	—	βCOCl*	αH	—	—	—	keto	33	49	6	3	9	192	84	14	2	82
42	—	Δ	C≡N	αH	—	—	—	keto	11	33	3	15	47	—	1	91	8	—
43	—	—	βOH	αH	—	—	—	βOH	66	24	34	3	25	—	14	65	16	—
44	—	—	βNH_2 αC≡N	αH	—	—	—	βOH	1	42	26	4	27	—	13	80	8	—
45	—	—	βOH	αBr	6, 9epoxy	—	—	benzoate	1	33	11	13	41	—	1	90	9	—
46	—	—	$\beta OCOCH_3$	αOH	αBr	—	—	benzoate	2	34	11	11	39	—	12	83	5	
47	—	—	αOH	αH	—	—	—	keto	1	54	4	9	33	—	7	83	11	—
48	—	—	αOH	αH	—	keto	—	keto	5	30	2	15	46	—	3	89	8	—
49	—	—	αOH	αH	—	OH	—	keto	6	25	4	15	48	—	3	90	8	—
50	—	—	αOH	αH	—	—		keto	17	21	7	9	46	—	3	88	9	—
51	—	—	αCOCl	αH	—	—	—	keto	6	41	6	7	37	—	3	89	8	—
52	—	2, 3 epoxy,	αC≡N	αH	—	—	—	$\beta OCOCH_3$	3	38	9	5	45	—	1	91	8	—
53	—	—	αN≡N	αH	—	—	—	βOH	2	27	12	13	45	—	4	87	9	—
54	—	—	αOH	αH	—	—	—	βOH	1	17	33	4	36	—	7	81	12	—
55	—	—	keto	αH	—	—	—	keto	1	81	6	1	6	—	44	42	14	200
56	—	αBr	keto	αH	—	—	—	keto	2	42	6	9	42	—	51	40	9	35
57	—	—	keto	αH	—	keto	—	keto	6	33	2	13	44	—	29	61	10	340
58	—	—	keto	αH	—	OH	—	keto	6	28	5	13	47	—	4	84	12	—
59	—	—	keto	αH	—	—		—	16	18	7	10	48	—	2	89	9	—
60	—	—	keto	αH	—	—	—	βOH	1	48	29	3	19	—	35	56	9	217
61	—	Δ	C≡N	αH	—	—	—	βOH	0	15	36	7	42	—	6	85	9	—
62	αC≡N	—	keto	αH	—	—	—	βOH	6	37	7	3	45	—	2	91	7	—
63	—	HO—CH=	keto	αH	—	—	—	$\beta OH\alpha CH_3$	89	6	2	0	1	21	90	6	5	5
64	—	—	—	αH	—	OH	—	$\beta N\text{-}(CH_3)_2$, αC≡N	1	35	3	13	45	—	1	89	10	—
65	—	αBr	keto	αH	—	—	—	$\beta OCOCH_3$	3	37	24	6	30	—	88	9	3	4
66	αC≡N	Δ	—	αH	—	—	—	$\beta OCOCH_3$	0	38	13	6	44	—	3	91	6	—
67	—	—	—	αH	—	—	—	αC≡N	2	15	9	12	56	—	3	84	7	—
68	—	—	keto	αH	—	—	—	20 spiro	1	46	20	5	28	—	1	90	9	—
69	—	—	keto	βC≡N	—	—	—	βOH	1	17	21	12	50	—	3	88	9	—

with both substrates. However, 17α-cyano-dehydroepiandrosterone-3β-acetate (16) is about as potent an inhibitor of pregnenolone conversion as is dehydroepiandrosterone but the effectiveness of inhibition with dehydroepiandrosterone as substrate is markedly reduced. The 17β-cyano derivative (15) is considerably less potent with each substrate. Cyanoketone (18) and derivatives (19, 20) do not affect conversion of either enzyme. However, when the 4,4-dimethyl group is converted to a cyclopropyl ring structure (23), the compound is an effective inhibitor of pregnenolone conversion. When a 20-spiro substitution (24) is made, the most potent inhibitor in the present study is obtained. The conversion of pregnenolone is selectively inhibited by 22 and 23.

Δ^4-Androstene derivatives

Testosterone (Compound 26) and androstenedione (35) are potent inhibitors of the conversion of both enzymes (Table III). 6β- (27) 11β- (31) and 16α-hydroxyl- (34) substitutions eliminate inhibitory capacity of testosterone for conversion of dehydroepiandrosterone markedly reduce inhibitory potency with pregnenolone. 17α-Ethinyl substitution (30) reduces inhibitory potency with either substrate. The Δ^1,17β-ureido form of testosterone (29) also does not inhibit either reaction. When the 17β-hydroxy group is converted to a 20-spiro compound (33), an inhibitor with about half the potency of testosterone is obtained. 11β-Hydroxyl- (36), or 11-keto- (37) substitution reduces the inhibitory capacity of androstenedione and 7α-hydroxy-substitution (38) eliminates it.

5α-Androstane derivatives

Epiandrosterone (39) is an inhibitor of conversion both substrates but to a much lesser degree than the Δ^5 unsaturated (41) steroid (Table IV). When a 3β-chloro (40) or 3β-chloroformyl (41) substitution is made for the hydroxyl group in epiandrosterone, a more potent inhibitor is obtained, particularly of the conversion of pregnenolone. However, the Δ^2,3- C=N, derivative (44) is completely inactive as an inhibitor.

5α-Androstane-3,17-dione (55) has about the same inhibitory capacity of the conversion of pregnenolone as does epiandrosterone and 11-keto (57) substitution reduces inhibitory potency. 2α-Bromodihydrotestosterone acetate (65) is a very potent selective inhibitor of pregnenolone conversion and 2-hydroxymethylene (63) is a potent inhibitor with each substrate.

Δ^4-Pregnene derivatives

21-Hydroxyprogesterone (76) and 20α-hydroxyprogesterone (77) are the only natural Δ^4-pregnenes that are inhibitory with each substrate (Table V). 20β-Hydroxyprogesterone (78) and 20β-cyano-3β-hydroxy-4-pregnene (79) affect conversion only of pregnenolone. 16α-Cyanoprogesterone (86) and 16α-cyano-1,4-pregnadien-20-one (87) inhibit conversion of both substrates more than 21-hydroxyprogesterone, but the substitution of a large acetal function at C-20 (88) eliminates inhibitory activity. Compounds 91 (cyproterone) and 92 (cyproterone acetate) effectively inhibit conversion of both substrates and 93 (medroxyprogesterone) is a weak inhibitor of con version of pregnenolone only.

Δ^5-Pregnene derivatives

Pregnenolone (95) inhibits only the conversion of pregnenolone (Table VI). A 17α-hydroxy group (96) or a Δ^{16}-20-oxime (100, 101) eliminates this inhibition. 16α-Cyanopregnenolone (101) is a somewhat more potent inhibitor of pregnenolone conversion than the parent compound, and also inhibits conversion of dehydroepiandrosterone as does the 3β-acetate of 16α-cyanopregnenolone (102). Changing the 16-substitution to 16-bromo- (103) or chloro- (104) removes the inhibition of conversion of dehydroepiandrosterone and makes a less effective inhibitor of pregnenolone conversion. When a double bond is introduced in the 16 position of 16α-cyanopregnenolone-3β-acetate (106), inhibition of conversion of dehydroepiandrosterone is eliminated and the inhibition of pregnenolone is reduced. 11β-Hydroxy substitution of 16α-cyanopregnenolone (107) eliminates inhibition of conversion of dehydroepiandrosterone and markedly reduces that of pregnenolone. However, introduction of a 17α-hydroxy group to 16α-cyanopregnenolone (108) increases inhibitory effectiveness of pregnenolone by 16α-cyanopregnenolone. When the 20-ketone is changed to an oxime (114) or methoxime (113) inhibitory potency of conversion of both substrates by 16α-cyanopregnenolone is markedly increased. However, a diketone at C-20 (109) reduces inhibitory potency and a larger acetal function at C-20 (119) eliminates inhibitory capacity. Introduction of a 6-methyl group (99) confers inhibitory capacity with each substrate to the inactive 3β,17α-hydroxypregnenolone (96) and increases inhibitory potency of 16α-cyanopregnenolone with each substrate markedly. Introduction of an additional 16β-methyl group (123) gives the second most potent inhibitor of conversion of each substrate in the present study. Compounds 129, 130, and 132 give selective inhibition of pregnenolone conversion.

5α-Pregnane derivatives

3β-Hydroxy-5α-pregnane-20-one (133) produces selective inhibition about half as effectively as the Δ^5-steroid conversion of pregnenolone (Table VII). Compounds 140 and 144 also give selective inhibition of pregnenolone conversion. 16α-Cyano substitution of Compound 144 increases the inhibitory potency of the parent compound (133) nearly 3-fold. Compound 143 gives very potent inhibition of conversion of pregnenolone and weaker inhibition of conversion of dehydroepiandrosterone.

5β-Pregnane derivatives

None of the five 5β-C_{21}-steroids (3-cyano-Δ^2,5β-pregnan-20-one, 3β-hydroxy-16α-cyano-5β-pregnane-11,20-dione-3β-acetate, and the 3β-hydroxy-, 3-keto-, or 3β-acetate of 16α-cyano-5β-pregnan-20-one) tested affect conversion of either substrate.

19-nor Androstane derivatives

19-nor testosterone (148) is a selective inhibitor of pregnenolone conversion as is the 17β-acetate (150) (Table VIII). A 17α-ethinyl derivative (149) also inhibits conversion of dehydroepiandrosterone.

Estratriene derivatives

Selective inhibition of conversion of pregnenolone is produced by several estratriene compounds: 154, 158, 159, 161, 162, and 165 (Table IX). The most potent of these are 161 (2,4-dibromo) and 162 (4-bromo-estradiol-17β). A 17-cyano sub

TABLE V

EFFECT OF Δ^5-PREGNENE DERIVATIVES ON HUMAN PLACENTAL CONVERSION OF DEHYDROEPIANDROSTERONE AND PREGNENOLONE

	Substituents on position										*Products from dehydroepiandrosterone (%)*						*Products from pregnenolone (%)*			
Control No.	*1*	*2*	*3*	*6*	*7*	*11*	*16*	*17*	*20*	*21*	*D (0.7 ±0.8)*	*A (27.2 ±8.1)*	*T (9.9 ±1.0)*	*E^1 (9.4 ±4.1)*	*E^2 (50.5 ±6.2)*	*I_{50} (μM)*	*P (1.0 ±1.0)*	*Pro (90.7 ±1.0)*	*5αPro (8.2 ±1.1)*	*I_{50} (μM)*
71	—	—	keto	—	—	—	—	—	keto	—	1	39	7	10	41	—	8	40	10	—
72	—	—	keto	—	—	—	—	αOH	keto	—	10	44	8	4	36	—	17	75	8	—
73	—	—	keto	—	—	αOH	—	—	keto	—	6	30	8	12	45	—	4	75	9	—
74	—	—	keto	—	—	βOH	—	—	keto	—	3	23	7	9	55	—	9	82	9	—
75	—	—	keto	—	—	βOH	—	—	keto	OH	8	22	7	12	48	—	4	87	10	—
76	—	—	keto	—	—	—	—	—	keto	OH	46	28	2	1	22	163	46	44	9	163
77	—	—	keto	—	—	—	—	—	αOH	—	15	32	13	6	34	263	70	5	5	111
78	—	—	keto	—	—	—	—	—	βOH	—	1	32	8	14	46	—	63	31	5	128
79	—	—	βOH	—	—	—	—	—	βC≡N	—	2	6	8	11	74	—	57	41	2	101
80	—	—	keto	—	—	βOH	—	αOH	keto	OH	2	23	10	12	52	—	3	88	9	—
81	—	—	keto	—	—	βOH	—	αOH	keto	—	4	25	10	10	49	—	5	86	9	—
82	—	—	keto	—	—	—	αCH_3	—	keto	—	1	42	7	6	44	—	1	51	8	—
83	—	—	keto	—	—	—	βCH_3	—	keto	—	2	33	12	7	46	—	2	88	10	—
84	—	—	keto	—	—	—	αOH	—	keto	—	2	32	11	6	49	—	12	78	10	—
85	—	—	keto	—	—	—	Δ	—	oxime	—	1	38	11	11	37	—	1	89	10	—
86	—	—	keto	—	—	—	αC≡N	—	keto	—	28	41	6	4	20	232	70	26	4	105
87	—	—	keto		—	—	αC≡N	—	keto	—	34	33	9	5	30	111	66	30	4	53
88	—	—	keto	—	—	—	αC≡N	—	 **	—	0	39	9	6	45	—	5	87	9	—
89	—	—	keto	—	αC≡N	—	—	αC≡N	keto	—	1	28	9	11	51	—	2	89	9	—
90	—	—	keto	—	αSH	—	αCSNOCOCH$_3$*	—	keto	—	1	26	8	10	55	—	1	91	8	—
91	1,2α=CH$_2$		keto	Cl	—	—	—	αOH	keto	—	67	13	3	2	15	81	80	15	5	49
92	1,2α=CH$_2$		keto	Cl	—	—	—	αOCOCH$_3$	keto	—	48	18	4	3	26	102	74	22	4	60
93	—	—	keto	CH	—	—	—	αOCOCH$_3$	keto	—	3	39	7	6	45	—	27	66	7	201
94	—	—	keto	βF	—	—	αCH_3	—	keto	mesyl-oxy	1	41	7	6	36	—	0	91	9	—

* CSNOCOCH$_3$, carbothioamide acetate.

**  : 2,2-dimethyltrimethylene acetal.

TABLE VI

EFFECT OF Δ^5-PREGNENE DERIVATIVES ON HUMAN PLACENTAL CONVERSION OF DEHYDROEPIANDROSTERONE AND PREGNENOLONE

Control No.	Substituents on position 2	3	4	6	7	11	16	17	20	Products from dehydroepiandrosterone (%) D (0.7 ±0.8)	A (27.2 ±8.1)	T (9.9 ±1.0)	E^1 (9.4 ±4.1)	E^2 (50.5 ±6.2)	I_{50} (μM)	Products from pregnenolone (%) P (1.1 ±1.0)	Pro (90.7 ±1.0)	5αPro (8.2 ±1.1)	I_{50} (μM)
95	—	βOH	—	—	—	—	—	—	keto	2	43	8	9	38	—	73	23	5	45
96	—	βOH	—	—	—	—	—	αOH	keto	3	45	9	8	36	—	10	85	5	—
97	—	βOH	—	—	—	—	Δ	—	oxime	2	48	6	4	39	—	8	86	6	—
98	—	βOH	—	CH_3	—	—	Δ	—	keto	1	29	9	9	52	—	48	47	5	240
99	—	βOH	—	CH_3	—	—	—	αOH	keto	23	34	9	5	29	351	70	26	4	85
100	—	β-$OCOCH_3$	—	—	—	—	Δ	—	oxime	0	37	8	7	47	—	2	92	7	—
101	—	βOH	—	—	—	—	αC≡N		keto	55	12	11	4	26	182	88	11	0	37
102	—	β-$OCOCH_3$	—	—	—	—	αC≡N	—	keto	29	15	9	6	35	217	76	23	1	85
103	—	βOH	—	—	—	—	αBr	—	keto	1	35	1	9	42	—	28	69	3	109
104	—	β-$OCOCH_3$	—	—	—	—	αCl	—	keto	2	41	8	9	40	—	20	77	4	161
105	—	β-$OCOCH_3$	—	—	—	—	βC≡N	—	keto	0	33	8	11	46	—	7	88	5	—
106	—	β-$OCOCH_3$	—	—	—	—	ΔC≡N	—	keto	4	33	10	8	46	—	46	52	2	130
107	—	βOH	—	—	—	αOH	αC≡N	—	keto	4	30	7	9	50	—	20	76	4	73
108	—	βOH	—	—	—	—	αC≡N	αOH	keto	4	29	9	11	48	—	73	26	2	17
109	—		—	—	—	keto	αC≡N	—		1	3	8	12	56	—	1	91	9	—
110	—		—	—	—	keto	αC≡N	—	keto	1	31	10	13	46	—	0	91	9	—
111	—	βOH	—	—	—	—	αC≡N	—		28	25	6	4	37	254	76	22	2	69
112	—	βOH	—	—	keto	—	αC≡N	—	keto	1	19	7	12	60	—	2	93	5	—
113	—	βOH	—	—	—	—	αC≡N	—	methoxime	35	20	8	5	32	180	84	14	2	24
114	—	βOH	—	—	—	—	αC≡N	—	oxime	85	6	7	1	11	75	94	6	0	10
115	—	βNH_4SO_4	—	—	—	—	αC≡N	—	keto	3	19	6	14	57	—	49	49	2	180
116	—	βAdamant.*	—	—	—	—	αC≡N	—	keto	2	18	6	14	59	—	1	90	9	—
117	—	βHept.*	—	—	—	—	αC≡N	—	keto	12	19	7	13	54	—	2	89	9	—
118	—	βNaHemi.*	—	—	—	—	αC≡N	—	keto	1	28	10	9	54	—	9	86	5	—
119	—	βOH	—	—	—	—	αC≡N	—		1	28	10	8	54	—	9	86	5	—
120	—	βPO_4	—	—	—	—	αC≡N	—	keto	1	30	9	12	48	—	6	88	6	—
121	—	$Na+\beta PO_4$	—	—	—	—	αC≡N	—	keto	2	30	11	8	50	—	7	86	7	—
122	—	βOH	—	CH_3	—	—	αC≡N	—	keto	33	18	9	4	35	62	75	21	4	12
123	—	βOH	—	CH_3	—	—	βCH_3, αC≡N	—	keto	84	5	4	0	7	29	96	4	0	2
124	—	βOH	—	CH_3	—	—	βCH_3, αC≡N	iso*	keto	18	27	7	8	40	181	83	15	2	73
125	—	keto	$(CH_3)_2$	—	—	—	αC≡N	—	keto	0	29	8	8	55	—	2	92	7	—
126	αC=N	keto	$(CH_3)_2$	—	—	—	αC≡N	—		1	28	9	10	52	—	2	90	9	—
127	αC=N	keto	$(CH_3)_2$	—	—	—	αC≡N	—	keto	4	29	7	8	51	—	4	85	11	—

128	2,3d-isoxazole	—	—	—	—	αC≡N	—	keto	0	41	8	6	45	—	3	87	10	—	
129	—	βOH-3Coo-4'H*	—	—	—	16, 17d-isox.		keto	5	31	11	8	46	—	74	24	2	68	
130	—	βOH-3Coo-K⁺-4'H*	—	—	—	16, 17d-isox.		keto	5	32	10	9	49	—	34	62	4	127	
131	—	3.....5α	βOCOCH$_3$	—	—	αC≡N	—	keto	0	29	11	8	53	—	3	88	9	—	
132	—	β-OCOCH$_3$	—	—	—	—	αC≡N	17-20 sulfite ester	8	34	3	7	48	—	33	62	5	125	

* (2', 2'-dimethylpropane-1', 3'-diol) ketal;  2, 2-dimethyltrimethylene acetal; β-adamant., 3(1'adamantyl carboxylate); Hept., heptanoate; NaHemi., sodium hemisuccinate; iso., 17α-pregnene; 3Coo-4'H, 4'H, 4'H-pregnen-3'carboxylic acid (potassium salt); 3.....5α' 3α, 5α-cyclopregnane.

TABLE VII

EFFECT OF 5α-PREGNANE DERIVATIVES ON HUMAN PLACENTAL CONVERSION OF DEHYDROEPIANDROSTERONE AND PREGNENOLONE

Control No.	*Substituents on position*							*Products from dehydroepiandrosterone (%)*						*Products from pregnenolone (%)*			
	3	*4*	*11*	*16*	*17*	*20*	*21*	*D (0.7 ± 0.8)*	*A (27.2 ± 8.1)*	*T (9.9 ± 1.0)*	*E¹ (9.4 ± 4.1)*	*E² (50.5 ± 6.2)*	*I_{50} (μM)*	*P (1.0 ± 1.0)*	*Pro (90.7 ± 1.0)*	*5αPro (8.2 ± 1.1)*	*I_{50} (μM)*
133	βOH	—	—	—	—	keto	—	0	15	9	11	64	—	50	46	4	110
134	αOH	—	—	—	—	keto	—	3	12	10	11	64	—	5	87	8	—
135	keto	—	—	—	—	keto	—	0	9	10	12	68	—	7	81	11	—
136	αOH	—	—	—	—	αOH	—	5	12	6	14	62	—	7	86	7	—
137	βOH	—	—	—	αOH	keto	—	4	25	9	11	51	—	17	78	5	—
138	αOH	—	—	—	αOH	keto	—	3	9	8	14	64	—	6	85	9	—
139	keto	—	—	—	αOH	keto	—	6	12	7	12	60	—	8	83	8	—
140	βOH	—	keto	—	αOH	keto	Br	5	47	7	6	35	—	21	73	6	200
141	βOH	—	keto	βBr	αOH	keto	—	3	42	9	5	41	—	7	86	7	—
142	βOCOCH$_3$	—	keto	βBr	αOH	keto	—	2	43	9	5	41	—	4	89	8	—
143	keto	αBr	keto	—	αOH	keto	OCOCH$_3$	36	16	9	5	26	114	82	15	4	19
144	βOH	—	—	αC≡N	—	keto	—	1	28	8	12	51	—	31	62	7	34
145	βOCOCH$_3$	—	—	αC≡N	—	keto	—	2	30	9	10	49	—	9	85	6	—
146	keto	—	—	αC≡N	—	keto	—	1	29	9	11	50	—	3	88	8	—
147	βOCOCH$_3$	—	—	αNCH$_3$HCl*	—	keto	—	0	16	7	7	66	—	0	91	8	—

* NCH$_3$HCl, aminomethylhydrochloride.

TABLE VIII

EFFECT OF 19-NOR-ANDROSTANE DERIVATIVES ON HUMAN PLACENTAL CONVERSION OF DEHYDROEPIANDROSTERONE AND PREGNENOLONE

Control No	Substituents on position 3	4	5	17	Products from dehydroepiandrosterone (%) D (0.7 ± 0.8)	A (27.2 ± 8.0)	T (9.9 ± 1.0)	E^1 (9.4 ± 1.4)	E^2 (50.5 ± 6.2)	I_{50} (μM)	Products from pregnenolone (%) P (1.0 ± 1.0)	Pro (90.7 ± 1.0)	5aPro (8.2 ± 1.1)	I_{50} (μM)
148	keto	Δ	—	βOH	7	34	48	2	8	—	86	17	3	81
149	keto	Δ	—	βOH, aethinyl	55	11	5	3	25	116	71	25	4	77
150	keto	Δ	—	$\beta OCOCH_3$	9	25	40	2	17	—	31	47	22	160
151	keto	—	$\beta C\equiv N$	βOH, aethinyl	1	25	7	12	54	—	2	89	10	—
152	—	—	$\alpha C\equiv N$	βOH, $\alpha CH_2\text{-}C(CH_3)=CH_2$	2	25	6	13	54	—	2	90	8	—
153	keto	Δ	—	20 spiro	3	27	10	9	51	—	1	91	8	—

TABLE IX

EFFECT OF ESTRATRIENE DERIVATIVES ON HUMAN PLACENTAL CONVERSION OF DEHYDROEPIANDROSTERONE AND PREGNENOLONE

Control No.	Substituents on position 2	3	4	16	17	Products from dehydroepiandrosterone (%) D (0.7 ± 0.8)	A (27.2 ± 8.1)	T (9.9 ± 1.0)	E^1 (9.4 ± 4.1)	E^2 (50.5 ± 6.2)	I_{50} (μM)	Products from pregnenolone (%) P (1.0 ± 1.0)	Pro (90.7 ± 1.0)	5aPro (8.2 ± 1.1)	I_{50} (μM)
154	—	OH	—	—	keto	1	21	8	46	23	—	78	19	3	315
155	—	CH_3O	—	—	keto	0	28	12	33	28	—	1	88	10	—
156	—	CH_3O	—	—	$\beta N(CH_3)_2\alpha CH_3$	0	32	12	33	23	—	3	90	8	—
157	—	CH_3O	—	—	$\beta OH\alpha C\equiv N$	0	27	10	39	25	—	3	88	9	—
158	—	OH	—	—	βOH	1	17	37	15	20	—	86	12	2	188
159	—	CH_3O	—	—	$\beta OCOCH_3$	2	17	23	19	40	—	78	17	4	196
160	—	CH_3O	—	—	βOH	0	15	28	15	42	—	4	90	6	—
161	Br	OH	Br	—	βOH	9	36	14	10	31	—	81	15	4	56
162	—	OH	Br	—	βOH	6	9	23	20	40	—	69	27	4	71
163	—	CH_3O	—	—	$\beta OCOCH_3\alpha C\equiv N$	1	34	6	8	42	—	0	89	11	—
164	—	CH_3O	—	Δ	$-C\equiv N$	0	40	10	7	43	—	2	89	19	—
165	—	CH_3OCO	—	Δ	$-C\equiv N$	7	38	8	7	41	—	62	32	6	110

stituted estrogen (165) is more potent than either estrone (154) or estradiol-17β (158).

DISCUSSION

The present report confirms that homogenates of human placenta convert dehydroepiandrosterone to androstenedione, testosterone, estradiol-17β and estrone, and pregnenolone to progesterone and 5α-progesterone. The thin-layer systems used are capable of separating the androgens from the estrogens and progesterone from pregnenolone. The method permits determination of enzymatic activity in small amounts of tissue and allows measurement of multiple samples rapidly and efficiently. The method used labelled substrate to which additional cold substrate has been added to saturate the enzyme, and uses the conditions whereby the formation of products is proportional both to time and to amount of enzyme. Unlike many studies where simply label is used, conditions in these experiments use rate-limiting conditions of enzyme concentration. Consequently, the present quantitative estimations are considerably more valid than when substrate is the rate-limiting component.

This study illustrates several pharmacologic principles suggested earlier for the design of active-site-directed inhibitors[6]. Potent inhibition of the conversion of dehydroepiandrosterone and pregnenolone is exhibited by several natural substrates including dehydroepiandrosterone, androstenedione, and testosterone. These inhibitory capacities can be unaffected, reduced or increased by certain nucleophilic substituents at or near the site on the steroid nucleus catalyzed by the enzyme. In some cases, these substitutions can make active inhibitors of non-inhibitory steroids. For example, Δ^{16}-17-cyano substitution (2, 13) reduces the inhibitory capacity of the corresponding parent compounds (1, 12) with each substrate 2–3-fold. Although 17α-cyano substitution (16) decreases the degree of inhibition of the conversion of dehydroepiandrosterone produced by Compound 12 5-fold, the degree of inhibition of conversion of pregnenolone is increased. 2-Hydroxymethylene (63) and 2α-bromo (56–65) substitutions increase the inhibitory potency of the parent compounds (55, 60) with pregnenolone as substrate 7–50 times. Moreover, Compound 63, unlike the parent compound, is a very potent inhibitor with dehydroepiandrosterone as substrate. In like manner, 16α-cyano (101, 144) but not 16β-cyano (105), 16α-cyano-20-oxime (114), 6-methyl (122), and 6,16β-dimethyl (123) derivatives are considerably more potent inhibitors than their respective parent compounds (95, 133). Moreover, 16α-cyano substitution (86, 87) makes inhibitors of a non-inhibitory parent compound (71). Recently, utilizing such principles, a labelled 6β-bromotestosterone-acetate has been synthesized and stoichiometric inhibition of crystalline Δ^{5-4},3-ketosteroid isomerase from *Pseudomonas testosteroni* has been demonstrated[7]. Covalent binding of inhibitor and enzyme has been demonstrated by gel electrophoresis. Two other labelled steroids with nucleophilic substituents, 2α- and 6β-bromoprogesterone have been shown to be active-site-directed inhibitors of purified 20β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans*[8]. After inactivation and hydrolysis, the enzyme's active site component bound to the inhibitor was shown to be cysteine.

We have made preliminary reports of our observation of inhibition of testicular steroidogenesis in the rat selectively at the level of gonadal Δ^5,3β-hydroxysteroid

dehydrogenase system, 17α-hydroxylase and C_{17-21} lyase by certain nitrile-substituted C_{19} derivatives of dehydroepiandrosterone[4,9,10]. A 17β-ureido derivative of testosterone and a 16β-bromo derivative of 5α-pregnane-3β,17α-diol-11,20-dione also selectively inhibit rat gonadal 17α-hydroxylase, C_{17-20} lyase, and Δ^5,3β-hydroxysteroid dehydrogenase only with pregnenolone as substrate[9]. Steroidal excretion patterns of rats treated with these inhibitors are completely consistent with the proposed mode of action of these inhibitors[10].

An unexpected principle emerges from the observation that substitution of the 3β-hydroxyl group of 3β-hydroxy-5α-androstane-17-one with a nucleophilic group, such as, 3β-chloro (40) or 3β-chloroformate (41) increases inhibitory capacity of the parent compound (39) from 1–3-fold. The steric specificity of this substitution is illustrated by the fact that the 3α-chloroformate (51) substitution is completely inactive. Curiously, 3β-chloro substitution (25) eliminates inhibitory capacity of dehydroepiandrosterone (1).

Stoichiometric inhibitors have also been used to demonstrate species variations in isozymic specificity of dihydrofolic reductases which may have not been demonstrated by other means[6]. Unlike the inhibition of the conversion of dehydroepiandrosterone and pregnenolone in the gonads, adrenals, and placenta of rats, cows, and guinea pigs, the enzymic activity of the dehydrogenase with either substrate in the human placenta is not inhibited by cyanoketone (2α-cyano-4,4,17α-trimethyl-17β-hydroxy-5-androstene-3-one) or 17β-hydroxy-4,4,17α-trimethyl-5-androstene-(2,3d)-isoxazole. Thus, in the human, as well as the rabbit[11] and chick (Idelman, S., personal communication), Δ^5,3β-hydroxysteroid dehydrogenase with either substrate, is not affected by these inhibitors and the species variation of this enzyme to this inhibition is apparent. The variation in binding may be due to the presence of the 4,4-dimethyl group, since conversion of pregnenolone is inhibited by a 2α-cyano analog (23) which has a cyclopropyl group in place of the 4,4-methyl group in ring A. Inhibition of this enzyme is also observed with a similar 4,4-cyclopropyl-2-hydroxymethylene analog (22). It may be speculated that the smaller molecular distances between the two methyl groups of the cyclopropyl group allows for easier access to the placental enzyme's active site. However, when the 17β-hydroxy,17α-methyl group of cyanoketone is substituted by a 17-20 spiro (furan) substitution (24), the most potent inhibitor of each enzyme is obtained. Thus, in this case, the spiro configuration apparently overcomes the 4,4-dimethyl effect. The interpretation of the present lack of inhibition of the dehydrogenases in placental homogenates by cyano-ketone is further complicated by the previous reports[13,14] that cyanoketone inhibits conversion of dehydroepiandrosterone to estrogens by human placental microsomes. Although microsomes derived from 20 g of placental were inhibited with 3 μM but not by 0.85 μM of cyanoketone[13] suggests nonstoichiometric conditions of this microsomal inhibition, the exact nature of the differences between the lack of inhibition of the homogenates by cyanoketone in the present study and the presence of inhibition of cyanoketone in microsomes[13,14] remains to be resolved.

Although enzymes are characterized by substrate specificity, previous kinetic studies have not demonstrated differences in the dehydrogenase converting C_{19} or C_{21} substrates. The active site of the enzyme converting pregnenolone must have isozymic variations in inhibitor binding sites from that converting dehydroepiandrosterone as indicated by selective inhibition with the presence of the C_{21} side-chain

and 17α-hydroxyl group of 108, the 16,17d-isoxazole of 129, 130, 16α-cyano-substitution of 144, 5α-saturation of 56, 65, and a phenolic ring A of the estrogen series. This indicates separate identity of the human placental isozymes converting dehydroepiandrosterone and pregnenolone. Consequently, the name of the enzymes should be changed to imply this substrate specificity, namely, C_{19}-3β-hydroxysteroid oxidoreductase and C_{21}-3β-hydroxysteroid oxidoreductase.

Compounds 24 (2α-cyano-4,4-dimethyl-2o-spirox-5-en-3-one), 123 (6,16β-dimethyl-5-pregnene-2o-one-16α-nitrile) and 63 (2-hydroxymethylene-17β-hydroxy-17α-methyl-5α-androstane-3-one) are highly potent inhibitors with either substrate. Preliminary studies by gas–liquid chromatography–mass spectrometry indicate that female rats treated with 123 excrete Δ^5,3β-hydroxysteroids of adrenal and ovarian origin[10]. The marked virilizing properties of Compound 63 limits the theoretical usefulness of this derivative of dihydrotestosterone acetate.

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