Isolation, Synthesis, and Biological Activity of Five Metabolites of Danazol

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Metabolites of danazol (17α-pregna-2,4-dien-20-yno[2,3-*d*]isoxazol-17-ol), an orally effective pituitary gonadotropin inhibitory agent devoid of estrogenic and progestational activities, were isolated from urine of a female subject who had taken danazol orally at a dose of 800 mg/day for 7 days. The metabolites isolated were 17-hydroxy-17α-pregn-4-en-20-yn-3-one (**11**), 17-hydroxy-2α-(hydroxymethyl)-17α-pregn-4-en-20-yn-3-one (**5**), 17-hydroxy-2-(hydroxymethyl)-17α-pregna-1,4-dien-20-yn-3-one (**7**), 6β,17-dihydroxy-2α-(hydroxymethyl)-17α-pregn-4-en-20-yn-3-one (**8**), and 6β,17-dihydroxy-2-(hydroxymethyl)-17α-pregna-1,4-dien-20-yn-3-one (**10**). None of these metabolites exhibited pituitary inhibiting activity comparable to danazol.

The synthesis of danazol, 17α-pregna-2,4-dien-20-yno-[2,3-*d*]isoxazol-17-ol (**1**) (Scheme I), was disclosed by our laboratory in 1963 in a paper on androstano[2,3-*d*]isoxazoles and related compounds.[1] Danazol inhibits pituitary gonadotropin secretion in rodents, rhesus monkeys, and humans and it possesses neither estrogenic nor progestational activities and it has only a weak impeded androgenic activity.[2] It is effective in the treatment of various clinical syndromes such as endometriosis, benign fibrocystic mastitis, and precocious puberty.

The clinical efficacy of danazol has now been well documented.[3] Only a few examples of the metabolism of heterocyclic-fused steroids have been reported. The metabolism of stanozolol, 17-methyl-2′*H*-5α-androst-2-eno[3,2-*c*]pyrazol-17β-ol, was reported to give only the pyrazole ring opened derivative 17β-hydroxy-2α,17β-dimethyl-5α-androstan-3-one azine.[4] In contrast, the metabolism of furazabol, 17-methyl-5α-androstano[3,2-*c*]-furazan-17β-ol, was reported to give several metabolites, all of which retained the intact furazan ring.[5]

Recently, the metabolism of radioactive danazol was reported by our Drug Metabolism group.[6] Simultaneously with their study the present authors studied the metabolism of unlabeled danazol. Our objective was the identification of metabolites and the synthesis of large quantities of these metabolites so that their biological activity could be compared to those of danazol. The metabolites isolated and identified in this report were only five of a much greater number seen on thin-layer chromatographic (TLC) plates. A secondary objective of our study was the synthesis of likely metabolites for comparison of their R_f on TLC plates with those of unidentified metabolites of danazol. The only such compound disclosed in this report is 2-(aminomethylene)-17-hydroxy-17α-pregn-4-en-20-yn-3-one (**2**). Although it was not isolated in this study it is a logical intermediate in our postulated pathway to the metabolites characterized in this study.

Metabolism studies were initiated in monkeys (*Macaca mulatta*). These studies indicated that the drug was rapidly metabolized to give products in which the isoxazole ring had been cleaved. An extract of the monkey's urine showed several metabolites on TLC. Ethisterone, 17-hydroxy-17α-pregn-4-en-20-yn-3-one (**11**), was isolated and characterized.

The metabolism of danazol was next studied in humans. In addition to ethisterone, four other metabolites were isolated and characterized. By far, the major metabolite proved to be 17-hydroxy-2α-(hydroxymethyl)-17α-pregn-4-en-20-yn-3-one (**5**). The next most prominent metabolite was 17-hydroxy-2-(hydroxymethyl)-17α-pregna-1,4-dien-20-yn-3-one (**7**). The other two metabolites isolated have tentatively been identified as 6β,17-dihydroxy-2α-(hydroxymethyl)-17α-pregn-4-en-20-yn-3-one (**8**) and 6β,17-dihydroxy-2-(hydroxymethyl)-17α-pregna-1,4-dien-20-yn-3-one (**10**).

A possible pathway to these metabolites is shown by dotted lines in Scheme I. Chemical and microbiological conversions are shown with a solid line.

A reductive cleavage of the N–O bond in the isoxazole ring would afford the enamino ketone **2** which upon hydrolysis would afford the hydroxymethylene steroid **3**. Reduction of **3** would then give the major metabolite **5**. Hydroxylation of **5** at C-1 followed by dehydration may be the route to **7** or 6β-hydroxylation of **5** may afford **8**. Metabolite **10** may arise either by 6β-hydroxylation of **7** or perhaps by C-1 hydroxylation of **8** followed by dehydration of the 1-hydroxyl group. Ethisterone may be produced by the elimination of formaldehyde from **5** by a reverse aldol condensation. The possible intermediates **2** and **3** were not detected in the present study.

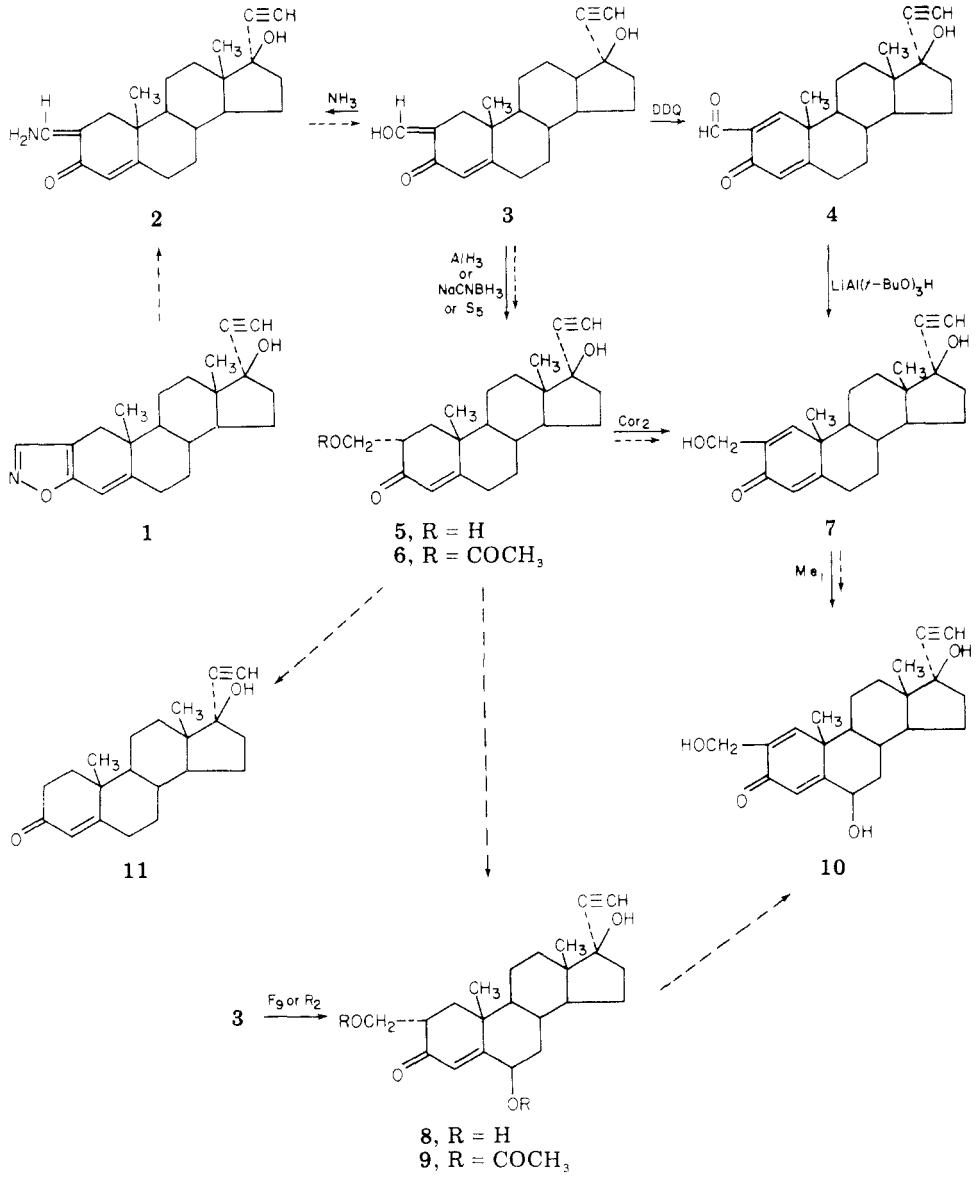
The synthesis of **5** had previously been accomplished in this laboratory in 10% yield by the action of *Rhizopus stolonifer* on **3**.[7] In the present work the conversion of **3** to **5** was increased to 71% using the organism *Streptomyces roseochromogenus*. The synthesis of **5** from **3** by chemical methods was much less promising. Treatment of **3** with aluminum hydride (AlH_3) according to the procedure of Corey[8] afforded **5** in only 8% yield. Reduction of **3** with sodium cyanoborohydride ($NaCNBH_3$) afforded **5** in 48% yield.

Treatment of **5** with the organism *Anthrobacter simplex* gave **7** in 70% yield. The latter compound was also prepared in 64% yield by the reduction of the unsaturated aldehyde **4** with lithium tri-*tert*-butoxyaluminum hydride [$LiAl(t\text{-}BuO)_3H$]. The aldehyde **4** was prepared in 82% yield from **3** by dehydrogenation with 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) according to the method of Edwards.[9]

Compound **8** was prepared in 20% yield by treatment of **3** with *Rhizopus stolonifer* or *Fusarium reticulatum*. The assigned structure is based on the following evidence. The elemental analysis and mass spectrum are compatible with the empirical formula $C_{22}H_{30}O_4$; its UV absorption spectrum, λ_{max} (95% EtOH) 238 nm, is close to 235 nm which is characteristic of 6β-hydroxy-Δ^4-3-keto steroids.[10] The most convincing evidence for **8** was provided by calculating the chemical shift for the C-18 and C-19 angular methyl groups in the diacetate **9** from **6** and adding the Zürcher value for a 6β-acetoxy group as reported by Cohen.[11]

	6	Zürcher value	Calcd for 9	Obsd for 9
C-18 CH_3	0.90	0.06	0.96	0.95
C-19 CH_3	1.24	0.11	1.35	1.34

Scheme I



Compound **10** was produced from **7** in 45% yield by treatment with *Melanospera parasitica*. The assigned structure is based on the following evidence. The elemental analysis and its mass spectrum are compatible with an empirical formula of $C_{22}H_{28}O_4$; its UV absorption spectrum, λ_{max} (95% EtOH) at 250 nm, is not far from several 6β-hydroxy-$\Delta^{1,4}$-3-keto steroids, e.g., 6β,17β-dihydroxyandrosta-1,4-dien-3-one which has λ_{max} (95% EtOH) at 246 nm.[10] The NMR shows two vinyl protons at δ 7.07 and 6.03 which are similar to the positions of the vinyl protons in **7**. The aminomethylene derivative **2** was prepared from **3** by treatment with NH_3 according to the procedure of Gerali.[12]

Biological Results. The oral pituitary gonadotropin inhibitory activity of the metabolites **5**, **7**, **8**, **10**, and **11** and danazol was compared in mature male rats. Danazol at 100 mg/kg administered orally in ethanol–oil for 14 days produced highly significant decreases in the weights of the ventral prostrate and seminal vesicles as well as a significant decrease in testicular weight. Compounds **5**, **7**, **8**, **10**, and **11** when administered orally at the same dose in the same vehicle had no significant effect on the sex accessory organs or on testicular weight.

The pituitary gonadotropin inhibitory activity of these compounds was evaluated subcutaneously in mature female rats. Danazol at a dose of 20 mg/kg for 2 weeks significantly decreased ovarian weights and the number of vaginal estrous days. Compounds **5**, **8**, and **10** at a dose of 50 mg/kg and compounds **7** and **11** at a dose of 25 mg/kg had no effect on either ovarian weight or the number of vaginal estrous days.

The above data show that the danazol metabolites **5**, **7**, **8**, **10**, and **11** have no significant pituitary inhibiting effect at doses wherein danazol is effective.

Experimental Section

All melting points were determined in capillary tubes and are uncorrected. UV spectra were measured in EtOH on a Cary Model 15 spectrophotometer except for material isolated from metabolism studies which was run in methanol in a Beckman DB recording spectrophotometer. Mass spectra were measured on a Joelco JMS-1-OCS mass spectrograph. IR spectra were determined on a Model-21 Perkin-Elmer infrared spectrophotometer. NMR spectral measurements were made on Varian A-60 or HA-100 spectrometers. Silica gel used for column chromatography was Davison, Grade 923, 100–200 mesh.

A typical fermentation run consisted of inoculating 10 l. of sterile nutrient solution with a 10% suspension of a 48–72-h vegetative growth of the organism. This inoculum was prepared

Table I

	R_f values			
	System 1	System 8	λ_{max}^{MeOH}, nm	M^+
Danazol	0.52	0.36	285	337
Ethisterone	0.44	0.28	241	312
Monkey metabolite	0.44	0.29	241	312

by transferring the organism from agar slants into flasks containing 500 ml of the same medium on a shaker rotating at 220 rpm at 27 °C. The culture was allowed to grow for 24 h with an air supply of 4 l./min and agitation at 450 rpm after which time the substrates for conversion were added. Fermentation was continued for 1–6 days.

Preliminary purification for microbiological transformation products involved extraction with a large volume of CH_2Cl_2 which was washed in succession with 0.2 N NaOH, 0.2 N HCl, and H_2O. The solvent was removed on a rotary evaporator and the residue dissolved in MeOH–H_2O (9:1) which was extracted several times with hexane. The methanolic solution was then evaporated and the crude product was purified as described under the appropriate experiment.

2-(Aminomethylene)-17-hydroxy-17α-pregn-4-en-20-yn-3-one (2). To a solution of 7.76 g of **3**[1] in 360 ml of pyridine was added a solution of 3.6 g of NH_4Cl and 4.8 g of NaOAc·$3H_2O$ in 20 ml of H_2O. Ammonia was bubbled through the solution for 1 h and after standing an additional hour the solvent was removed by distillation on a rotary evaporator in a water bath kept at <35 °C. Water was added to the residue and the product filtered: yield 7.88 g, mp 245–247 °C; 7.58 g recrystallized from 100 ml of DMF–H_2O (3:1) gave 6.00 g, mp 250–252 °C; UV λ_{max} (95% EtOH) 245 nm (ε 14 000), 353 (9900); IR λ_{max} 2.99, 3.15, 3.08 (≡CH), 4.48 (–C≡C–), 6.11 (CO), 6.20, and 6.30 μm. Anal. ($C_{22}H_{29}NO_2$) C, H, N.

17-Hydroxy-3-oxo-17α-pregna-1,4-dien-20-yne-2-carboxaldehyde (4). A solution of 50 g (0.22 mol) of DDQ in 400 ml of dioxane was added to a solution of 68 g (0.20 mol) of **3**. Almost immediately DDHQ precipitated and after 2 min the reaction mixture was filtered and diluted with 3 l. of CH_2Cl_2. The solution was washed three times with 2 l. of 0.5 N NaOH and then with 2 l. of H_2O; the dried ($MgSO_4$), filtered solvent was removed and the residue, crystallized from CH_3CN, gave three crops: 55.6 g (82% yield); mp 126–135 °C. This material was of sufficient purity for further transformations. The analytical sample was prepared by recrystallization from benzene: mp 146–149 °C; $[\alpha]^{25}D$ –116.6° (1% in $CHCl_3$); UV λ_{max} 222 nm (ε 11 000), 247 (13 200); M^+ 338; NMR δ 0.94 (3, C-18 CH_3), 1.33 (3, C-19 CH_3), 252 (1, ≡CH), 2.74 (1, OH), 6.71 (1, =CH), 7.82 (1, =CH), 10.26 (1, CHO). Anal. ($C_{22}H_{36}O_3$) C, H.

17-Hydroxy-2α-(hydroxymethyl)-17α-pregn-4-en-20-yn-3-one (5). A. Microbiological. Transformation by the organism *Streptomyces roseochromogenus* (S_5) (ATCC 13 400): six runs of 8 g of **3** for 3–6 days; residue from benzene–Et_2O, 19.5 g, mp 154–156 °C; second crop from EtOAc–cyclohexane, 14.5 g, mp 153–155 °C; total yield 34.0 g (71%); TLC indicated <2% impurities; recrystallized from EtOAc, mp 158–159 °C (lit.[7] mp 164–165 °C). Anal. ($C_{22}H_{30}O_3$) C, H.

B. AlH_3 Reduction of 3. To a mechanically stirred slurry of 0.3 mol of NaH (12.65 g of 57% mineral oil dispersion) in 100 ml of THF in a nitrogen atmosphere was added 34 g (0.1 mol) of **3** in 150 ml of THF. After stirring for 1 h in an ice bath, 150 ml (0.1 mol) of AlH_3 in THF (prepared from $LiAlH_4$ and 100% H_2SO_4 by the method of Brown[13]) was added. After stirring an additional hour, saturated NH_4Cl was added and the reaction mixture filtered through filter cel. The filter cake was washed with CH_2Cl_2 and the filtrate diluted with Et_2O and extracted with dilute NaOH to remove unchanged **3**. The solution was dried ($MgSO_4$), filtered, evaporated, and chromatographed on 2 kg of silica gel. Elution with Et_2O–CH_2Cl_2 (1:1) gave fractions containing **5** which after pooling and recrystallization from EtOAc gave 2.74 g (8%) of **5**, mp 156–158 °C. This material was identical in IR ($CHCl_3$ solution), mass spectrum, UV, and NMR with material from procedure A.

C. $NaCNBH_3$ Reduction of 3. To a stirred solution of 3.4 g (0.01 mol) of **3** in 50 ml of EtOH, 50 ml of THF, 10 ml of H_2O, and 5 ml of HOAc was added 0.63 g (0.01 mol) of $NaCNBH_3$. Dilute HCl was added from time to time so that a pH of ~4 was maintained. After 5 h the solvent was removed (rotary evaporator) and the residue was partitioned between EtOAc and dilute NaOH. Acidification of the NaOH extract gave 0.83 g of **3**. The EtOAc was dried ($MgSO_4$) and solvent removed. A 1.05-g aliquot of the 3.5 g of residual foam was placed on six 20 × 40 cm preparative plates (coated with Brinkmann PF_{254} silica gel in 1–1.5 mm thickness). The plates were developed with 3% MeOH in $CHCl_3$. The appropriate band was removed and the product was eluted with CH_2Cl_2–Et_2O (1:1). Evaporation gave a yellow oil which on recrystallization from EtOAc–cyclohexane gave 0.49 g (48%), mp 156–158 °C. This material was identical in TLC and IR ($CHCl_3$) with material from procedure A. Treatment of **5** with Ac_2O–Py gave **6**, mp 86–89 °C (lit.[7] mp 86–89 °C).

17-Hydroxy-2-(hydroxymethyl)-17α-pregna-1,4-dien-20-yn-3-one (7). Chemical Preparation. To a solution of 20.0 g (0.0575 mol) of **4** in 300 ml of THF was added 25.4 g (0.1 mol) of $LiAl(t\text{-}BuO)_3H$. The reaction was stirred overnight and after treatment with dilute HCl, the THF was removed on a rotary evaporator. The residue was dissolved in EtOAc, washed with dilute HCl, dilute NaOH, and H_2O, dried ($MgSO_4$), and filtered, and solvent was removed on a rotary evaporator and the residue from CH_3CN. Three crops totaling 13.3 g (67% yield), identical TLC, were recrystallized from CH_3CN: mp 213–214 °C; $[\alpha]^{25}D$ –54.5° (1% in $CHCl_3$); UV λ_{max} 248 nm (ε 16 500); NMR δ 2.52 (1, C≡CH), 3.11 (1, OH), 3.38 (1, OH), 4.40 (2, OCH_2), 6.10 (1, C=CH), 7.08 (1, C=CH). Anal. ($C_{22}H_{34}O_3$) C, H.

Microbiological Preparation. Fermentation by the organism *Arthrobacter simplex* (ATCC 6946) (Cor_2): five runs of 4 g of **5** for 24 h; residue crystallized from $CHCl_3$–Et_2O, 13.9 g (70% yield), mp 214–215 °C; identical in TLC, IR, UV, and NMR with material from chemical preparation.

6β,17-Dihydroxy-2α-(hydroxymethyl)-17α-pregn-4-en-20-yn-3-one (8). Preparation A. Organism *Rhizopus stolonifer* (ATCC 12939) (R_2): six runs of 4.0 g of **3** for 6 days; residue on 1.4 kg of silica gel prewet with EtOAc–hexane (1:3). The column was developed with increasing EtOAc in EtOAc–hexane fractions. Material of similar R_f on TLC combined and recrystallized from EtOAc–Et_2O: yield 5.40 g; mp 186–189 °C. This material combined with material from preparation B.

Preparation B. Organism *Fusarium reticulatum* (CBS 18 435) (F_9): five runs of 4.0 g of **3**. Crude product was crystallized from

Table II

	R_f values							λ_{max}^{MeOH},	
TLC systems	1	2	3	4	5	6	7	nm	M^+
Metabolite 1	0.46	0.34	0.56	0.47				241	312
Ethisterone (11)	0.46	0.34	0.56	0.47				241	312
Metabolite 2	0.40	0.18	0.45					241	342
Compound 5	0.39	0.18	0.44					241	342
Metabolite 3	0.22	0.13	0.42			0.23		248	340
Compound 7	0.22	0.13	0.42			0.23		248	340
Metabolite 4	0.25		0.32		0.48			238	358
Compound 8	0.25		0.32		0.48			238	358
Metabolite 5			0.27				0.55	250	356
Compound 10			0.28				0.54	250	356
Danazol (1)	0.53	0.40	0.57					285	337

EtOAc-Et_2O, yield 4.30 g, and combined with 5.40 g from preparation A and recrystallized from EtOAc: yield 7.9 g; mp 188–190 °C; $[\alpha]^{25}D$ -34.8° (1% in 95% EtOH); M^+ 358; UV λ_{max} 238 nm (ϵ 12 800); NMR δ 0.88 (3, C-18 CH_3), 1.33 (3 C-19 CH_3), 3.29 (1, ≡CH), 3.60 (2, OCH_2), 4.40 (1, $HOCH_2$), 5.07 (1, 6β-OH), 5.27 (1, 17β-OH), 5.67 (1, C_4=CH). Anal. ($C_{22}H_{30}O_4$) C, H.

Treatment of **8** with Ac_2O-Py afforded the diacetate **9** which was used without purification for NMR (see discourse).

6β,17-Dihydroxy-2-(hydroxymethyl)-17α-pregna-1,4-dien-20-yn-3-one (10). Organism *Melanospora parasitica* (ATCC 11 103) (Me_1): five runs of 2.0 g of **7** for 48 h; crude product from EtOAc; yield 4.5 g; mp 226–227 °C; $[\alpha]^{25}D$ -81° (1% in 95% EtOH); M^+ 356; UV λ_{max} 250 nm (ϵ 15 800); NMR δ 0.87 (3, C-18 CH_3), 1.41 (3, C-19 CH_3), 3.23 (1, C≡C-H), 4.20 (2, OCH_2), 4.40 (1, CHO), 4.85 (1, CH_2OH), 5.21 (1, CHOH), 5.30 (1, C-17 OH), 6.03 (1, =CH), 7.07 (1, =CH). Anal. ($C_{22}H_{28}O_4$) C, H.

Metabolite Isolation. The following TLC systems were utilized in metabolite studies using silica gel PF_{254} plates: (1) EtOAc; (2) EtOAc-cyclohexane (1:1); (3) CH_2Cl_2-CH_3OH (9:1); (4) EtOAc-cyclohexane-isopropylamine (25:24:1); (5) EtOAc-MeOH (9:1); (6) Et_2O; (7) EtOAc-MeOH (7:3); (8) EtOAc-*n*-hexane (1:1).

Aliquots of crude extracts of urine were applied in narrow bands on TLC plates and developed in one of the above solvent systems. Components were visualized under 253-nm UV light or by spraying sections of the plate with 50% EtOH-H_2SO_4 and heating for color development. Bands were removed and the silica gel was placed in a glass micro column and eluted with MeOH. After evaporation the material was put on another plate which was developed in a second solvent system. Isolated metabolites were compared with reference compounds in several solvent systems.

Monkey Study. A 24-h urine sample was collected from three female Rhesus monkeys medicated orally with danazol in Tween 80 at 50 mg/kg b.i.d. for a total of 664 mg. A 100-ml sample was extracted with *n*-hexane (2 × 200 ml) which was discarded. The sample was then extracted with CH_2Cl_2 (2 × 200 ml) which was dried (Na_2SO_4) and evaporated and the residue purified by TLC with system 1 and then system 8. Several metabolites were observed but only one, ethisterone (**11**), was obtained in sufficient purity to allow identification (Table I).

Human Study. Urine from a female subject administered 800 mg of danazol orally was collected for 24 h. A 1.8-l. aliquot was extracted with CH_2Cl_2 (2 × 4 l.). After being washed with 1 l. of 0.1 N NaOH, 1 l. of 0.1 N HCl, and 1 l. of water, the CH_2Cl_2 solution was dried (Na_2SO_4) and the solvent removed by distillation. The residue was chromatographed using system 3 to give five components which were rechromatographed and the products isolated and identified: metabolite 1, purified in system 2, was identical with ethisterone (**11**); metabolite 2, purified in system 1, was identical with **5**; metabolite 3, purified in system 6, was identical with **7**; metabolite 4, purified in system 5, was identical with **8**; metabolite 5, purified in system 7, was identical with **10**. See Table II.

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Ring D Oxygenated Spirolactones. Characterization of a Human Metabolic Product of Spironolactone

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15α-Hydroxycanrenone (**1b**) was prepared from canrenone (**1a**) by microbiological oxidation with a *penicillium* species. The product was identical with one obtained from the metabolism of spironolactone (**3**) in human. Oxidation of **1b** with Jones reagent furnished the corresponding 15-oxocanrenone (**1d**) which underwent base-catalyzed β elimination to generate an α,β-unsaturated cyclopentenone system. 15α-Hydroxycanrenone (**1b**) failed to show antimineralocorticoid activity at the screening dose of 2.4 mg while the oxo derivative **1d** exhibited approximately 15% the activity of **3**. Since the activity of canrenone is 38% that of spironolactone, introduction of the carbonyl group at the 15 position of canrenone resulted in a reduction in activity. This effect is opposite to that observed with 6-dehydroprogesterone.

In earlier studies on the antimineralocorticoid activity of various progesterone derivatives, the effect which an oxygen function at the 15 position had on this activity was examined.[1] In extending these studies, we sought to introduce an oxygen function into C-15 of 3-(17β-hydroxy-3-oxoandrosta-4,6-dien-17α-yl)propionic acid γ-lactone (canrenone, **1a**) in order to determine whether it would alter the mineralocorticoid-blocking activity of this