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Biological Activities of a New Steroidal Inhibitor of Δ^4-5α-Reductase (41309)

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Abstract. 17β-*N,N*-Diethylcarbamoyl-4-methyl-4-aza-5α-androstan-3-one (4-MA), a known 5α-reductase inhibitor, was tested in a variety of bioassays designed to document its endocrinological spectrum. It proved to be a relatively nontoxic compound which showed little or no antifertility activity in either male or female rats, no significant estrogenic, progestational, androgenic, or gonadotropin-inhibiting potency, and it did not affect serum prolactin levels. 4-MA did, however, cause feminization of male fetuses carried by treated pregnant female rats. It also caused a cessation in growth of the ventral prostate and seminal vesicles of young adult male rats. Both the feminization of male fetuses and the inhibition of male sex accessory growth are believed to be a result of the inhibition of 5α-reductase by 4-MA.

In some androgen responsive tissues the action of testosterone (T) is mediated primarily through its 5α-reduced metabolite, dihydrotestosterone (DHT) (1, 2). The prostate gland (1–3), skin (4–6), hamster flank organ (7, 8), urogenital sinus, and urogenital tubercle (9) have all been shown to convert T to DHT, and it is believed that the latter androgen is utilized by these tissues. The bioconversion of T to DHT is catalyzed by the enzyme Δ^4-5α-reductase, and it was suggested that if 5α-reductase were inhibited, the formation of DHT should be reduced and its specific effects attenuated or prevented (10–12).

Several steroids including progesterone (11), 4-androstan-3-one-17β-carboxylic acid and its methyl ester (10), megestrol acetate (13), and medrogestone (14) were subsequently reported to be active *in vivo* against 5α-reductase of human skin (11), hamster flank organ (10), and human prostate (13, 14). Those findings encouraged the view that effective therapy for such androgen-related conditions as acne, hirsutism, baldness, and prostatic hyperplasia might be afforded by 5α-reductase inhibitors (12, 15, 16).

In our laboratories, test systems were initiated to search for inhibitors of 5α-reductase and these studies led to the discovery of 17β-*N,N*-diethylcarbamoyl-4-methyl-4-aza-5α-androstan-3-one (4-MA). This compound is a potent 5α-reductase inhibitor (17) which can preserve T-dependent phenomena while greatly attenuating those due to DHT. The present report summarizes the results of a variety of assays which showed it to be a relatively nontoxic compound which is essentially free of hormonal activities.

Materials and Methods. For the experiments described below, solutions were prepared by dissolving test compounds in absolute ethanol, then adding 9 parts sesame oil. The compounds were usually given by subcutaneous (sc) injection but for some studies a different route of administration was chosen.

Fertility studies in mature male rats. Mature male rats (Holtzman) of proven fertility were used. 4-MA was given sc at levels of 0, 10, or 50 mg/kg/day for 67 days. Weights were taken weekly and the dose adjusted accordingly. Twice a week throughout the treatment period each male was caged overnight with a proestrous female. A vaginal smear was taken the following morning to determine whether sperm were present and, if so, the female was autopsied 17–20 days later to determine whether she was pregnant. The males were sacrificed on the 68th day and the weights of various organs recorded.

Mating and fertility study in female rats. Adult virgin female rats (Holtzman) were used to determine whether treatment with 4-MA in amounts sufficient to inhibit pros-

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tatic 5α-reductase in male rats would affect mating and the ability to conceive. They were treated sc daily for 5 days with 1 or 10 mg of 4-MA before being caged with fertile males and treatment was continued through the 9th postmating day. If mating had not occurred by the 15th day after a particular pair had been caged together, her treatment was discontinued. Mated females were sacrificed on Day 10 of pregnancy and the number of implantations determined.

Fetal male feminization assay. This experiment was conducted to determine whether 4-MA affected development of the external genitalia of male fetuses. Mated female rats (Holtzman) were treated sc over Days 13–21 of pregnancy. The nonsteroidal antiandrogen, flutamide, was used as a reference compound and given at levels of 0.333, 1, or 3 mg/day. Dosage levels of 4-MA were 0.333, 1, 3, or 9 mg/day. The rats were sacrificed on Day 22 and their fetuses removed. The distance from the anal to the urethral opening was measured with calipers to the nearest 0.1 mm. The body cavity was then opened to determine whether the fetus possessed testes or ovaries.

Effect of high treatment levels of 4-MA on organ weights. In addition to determining the effect of high levels of 4-MA on the weight of various organs, this test was run to determine at what dosage level toxicity became evident.

Young adult intact male rats (Holtzman) weighing about 220 g were treated sc daily for 14 days with 50, 150, or 450 mg of 4-MA. They were weighed twice a week and the dose adjusted according to weight change. At sacrifice on the 15th day, various organ weights were recorded.

Effect on sex accessory gland growth in young intact male rats. Intact male rats (Marland Farms) weighing about 200 g were treated sc for 14 days using 4-MA at dosage levels of 0 or 50 mg/kg/day. Body weights were taken every other day and the dose adjusted for changes in weight. Beginning on the day after treatment began, five rats each from the control and treated groups were sacrificed daily during the treatment period. At sacrifice, body, ventral prostate, and stripped seminal vesicle weights were recorded.

Recovery of male rat sex accessory gland weights after discontinuation of treatment with 4-MA. Intact male rats (Charles River) weighing about 200 g were treated sc for 7 days with 4-MA using dosage levels of 0 or 50 mg/kg/day. The animals were weighed at 2- to 3-day intervals and the dose adjusted accordingly. On the day following the final injection and at weekly intervals thereafter over a period of 28 days, eight rats from each group were sacrificed. At sacrifice, weights were taken of the body, ventral prostate, and stripped seminal vesicles.

Effect on serum prolactin levels in young male rats. Intact male rats (Charles River) weighing 250–300 g were used. They were treated by ip injection for 1, 2, or 3 days using dosage levels of 0, 50, or 100 mg/kg/day of 4-MA. Blood was obtained by decapitation 15, 30, and 60 min after dosing on the first day and at 30 min post-treatment on Days 2 and 3. Serum was stored at −80° until prolactin levels were determined by radioimmunoassay. The prolactin reference standard RP-1 was used and all test results are expressed in terms of RP-1.

General bioassays. In addition to the tests described above, several bioassays were performed using standard procedures. These were: uterotropic assay (18, p. 96); vaginal cornification assay (18, p. 68); progestational assay (19); and pituitary gonadotropin inhibition assay (20). In each of these assays, 4-MA was given subcutaneously.

Results. *Effect of 4-MA on organ weights and fertility in male rats.* Average body weight gains of rats receiving 10 or 50 mg/kg/day of 4-MA for 67 days were slightly but not significantly reduced. Similarly, there were no significant differences between groups in average organ weights with the exceptions of the ventral prostate and seminal vesicles which were smaller ($P < 0.01$) in both treated groups and epididymides which were heavier ($P < 0.05$) in rats receiving 10 mg/kg/day of 4-MA. These results are shown in Table I.

Rats of all groups mated and remained fertile for the entire period of the test. Their fertility data are found in Table II.

Effect of 4-MA on fertility in female rats. The highest dose of 4-MA, 10 mg/day, appeared to cause a reduction in the willing-

TABLE I. BODY WEIGHT CHANGE AND ORGAN WEIGHTS OF MATURE MALE RATS FOLLOWING 67 DAYS TREATMENT WITH SUBCUTANEOUSLY ADMINISTERED 4-MA

Group	Dose/kg/day	N	Body wt change (g)	Testes (mg)	Ventral prostate (mg)	Seminal vesicles (mg)	Epididymides (mg)
Control	1 ml vehicle	6	139 ± 12[a]	3272 ± 123	617 ± 25	798 ± 44	1070 ± 16
4-MA	10 mg	6	114 ± 12	3488 ± 115	396 ± 37***	530 ± 33***	1155 ± 23*
4-MA	50 mg	6	111 ± 8	3529 ± 83	271 ± 14***	270 ± 16***	1055 ± 31
			Kidneys (mg)	Adrenals (mg)	Thymus (mg)	Thyroid (mg)	Pituitary (mg)
Control	1 ml vehicle	6	2923 ± 52	47 ± 1	209 ± 13	21 ± 1	12 ± 0.5
4-MA	10 mg	6	2805 ± 91	48 ± 2	221 ± 12	18 ± 1	12 ± 0.3
4-MA	50 mg	6	2847 ± 102	50 ± 2	256 ± 24	21 ± 2	12 ± 0.5

[a] Values are means ± SEM.
* Significantly different from control at $P < 0.05$.
*** Significantly different from control at $P < 0.001$.

ness of females to mate since only four of the eight at that level did mate. However, when they were examined 10 days later, all mated rats were carrying normal numbers of grossly normal fetuses. Thus, the compound had no influence on the ability of these rats to conceive and implant. At the lower dose, 1 mg/day, seven of eight treated animals mated and they too were carrying a normal number of fetuses. All eight control rats mated and were pregnant when sacrificed. The results are presented in Table III.

Fetal male feminization assay. The average anogenital distance in males born to control, vehicle-treated females was 4.5 mm. That of males born to females treated with 333 μg, 1 mg, or 3 mg flutamide per day averaged 4.6, 3.7, and 3.3 mm, respectively, and in males born to females given 333 μg, 1 mg, 3 mg, or 9 mg of 4-MA per day the anogenital distances were 4.9, 4.7, 3.8, and 3.4 mm, respectively.

The 1 and 3 mg/day levels of flutamide and the 3 and 9 mg/day doses of 4-MA caused anogenital distance in males to be significantly less than in controls. Approximately the same results were obtained with 1 mg flutamide as with 3 mg 4-MA.

No significant changes in anogenital distance were noted in female fetuses from dams treated with either flutamide or 4-MA. The results are presented in Table IV.

Effect of high treatment levels of 4-MA on organ weights. Results of this test are presented in Table V. Treatment levels much higher than those required to cause significant reduction in prostatic DHT concentration (17) were used. The most obvious effects were the marked decreases ($P < 0.001$) in weight of the ventral prostate and seminal vesicles of animals given 50 or 150 mg/kg/day. Testis weights were not reduced by those treatment levels, suggesting gonadotropins were not inhibited although epididymal weights were decreased ($P < 0.01$) by the 150 mg/kg/day level. The lack of effect on adrenal and thymus weights suggests the rats were not stressed and that ACTH levels were unaffected. Weight gain (60 g) was significantly less ($P < 0.01$) at 150 mg/kg/day than in the control (80 g) or the 50 mg/kg/day group (84 g).

Five of eight rats treated with 450 mg/kg/day died before completion of the test. When autopsied there was no grossly evident cause of death but increased adrenal

TABLE II. FERTILITY OF MALE RATS THROUGH A 67-DAY PERIOD DURING WHICH A DAILY SUBCUTANEOUS INJECTION OF 4-MA WAS ADMINISTERED

Group	Dose/kg/day	N	Females pregnant/females mated									
			Week 1	2	3	4	5	6	7	8	9	10
Control	1 ml vehicle	6	10/10	10/10	9/10	11/11	10/11	11/11	11/11	9/9	11/11	9/9
4-MA	10 mg	6	10/10	10/11	9/9	13/13	8/8	9/10	12/12	12/12	8/8	8/8
4-MA	50 mg	6	11/11	10/10	9/11	7/11	6/7	9/11	9/11	6/9	4/7	7/7

TABLE III. EFFECT OF DAILY SUBCUTANEOUS ADMINISTRATION OF 4-MA ON FERTILITY OF FEMALE RATS

Group	Dose/day	Pregnant/mated	Mean No. fetuses
Control	0.25 ml vehicle	8/8	14.5
4-MA	1 mg	7/8	13.7
4-MA	10 mg	4/8	14.5

Note. The females were treated from 5 days prior to exposure to males through the 9th day after mating. Females were not treated longer than 20 days. Rats were sacrificed 10 days after mating.

and reduced thymus weights of the three survivors indicated generalized stress.

Effect on sex accessory gland growth in young intact male rats. The results of this experiment are illustrated in Fig. 1. Over the course of the treatment period, 4-MA did not cause an actual reduction in weight of either the ventral prostate or seminal vesicles. However, it did almost completely block further growth of these organs. Body weight gains were not influenced by 4-MA.

Sex accessory gland weights of control and treated rats were not significantly different for the first 2 days of treatment. However, from Day 3 through Day 14 seminal vesicle weights were always significantly lower in treated animals. Ventral prostate weights of treated animals were significantly lower on Day 4 of the treatment period and also on Days 7 through 14.

Recovery of male rat sex accessory gland weights after discontinuation of treatment with 4-MA. On Days 1, 8, and 15 following the end of treatment, weights of both the ventral prostate and seminal vesicles of treated animals were significantly less than those of control rats (Fig. 2). By Day 22, however, weights of the respective sex accessory glands were not significantly different in the two groups. Body weights were almost identical in treated and control animals throughout the recovery period.

Effect on serum prolactin levels in young male rats. Results of this experiment are shown in Table VI. There was a slight increase in serum prolactin on Day 3 in both treated groups but it was not dose related and was considered to be of doubtful physiological significance. That was also true for the decrease noted at 60 min post-treatment on Day 1 in the 100 mg/kg group. The conclusion drawn from this experiment was that 4-MA did not affect serum prolactin levels in male rats.

General bioassays. In the uterotropic assay a total amount of 0.5 μg of estrone given over a 3-day period caused a significant increase in uterine weight of immature

TABLE IV. FEMINIZATION OF MALE RAT FETUSES AS A RESULT OF SUBCUTANEOUS TREATMENT OF THEIR DAMS WITH FLUTAMIDE OR 4-MA OVER DAYS 13–21 OF PREGNANCY[a]

Group	Dose/day	No. dams	Anogenital distance (mm) ± SEM	
			Males	Females
Control	0.5 ml vehicle	2	4.5 ± 0.1 (8)[b]	2.6 ± 0.04 (14)
Flutamide	333 μg	4	4.6 ± 0.1 (20)	2.7 ± 0.03 (24)
Flutamide	1 mg	4	3.7 ± 0.1*** (19)	2.9 ± 0.1 (22)
Flutamide	3 mg	4	3.3 ± 0.1*** (20)	2.9 ± 0.04 (24)
4-MA	333 μg	4	4.9 ± 0.1 (19)	3.0 ± 0.04 (25)
4-MA	1 mg	4	4.7 ± 0.1 (19)	2.9 ± 0.03 (33)
4-MA	3 mg	4	3.8 ± 0.1** (15)	3.0 ± 0.1 (13)
4-MA	9 mg	4	3.4 ± 0.1*** (29)	2.7 ± 0.1 (13)

[a] The fetuses were removed from females in their 22nd day of pregnancy.
[b] Numbers in parentheses refer to number of fetuses.
** Significantly different from control at $P < 0.01$.
*** Significantly different from control at $P < 0.001$.

TABLE V. EFFECT OF 14 DAYS OF TREATMENT WITH HIGH SUBCUTANEOUS LEVELS OF 4-MA ON BODY AND ORGAN WEIGHTS OF YOUNG ADULT MALE RATS

Group	Dose/kg/day	N	Body wt change (g)	Testes (mg)	Ventral prostate (mg)	Seminal vesicles (mg)	Epididymides (mg)
Control	4 ml vehicle	7	90 ± 5[a]	3026 ± 353	262 ± 20	224 ± 16	785 ± 63
4-MA	50 mg	8	84 ± 3	3233 ± 66	137 ± 10***	122 ± 9***	764 ± 33
4-MA	150 mg	8	60 ± 7**	3259 ± 53	92 ± 8***	82 ± 7***	638 ± 22**
4-MA	450 mg	3	−3 ± 3	2573 ± 298	52 ± 11	54 ± 6	397 ± 98
				Thymus (mg)	Adrenals (mg)	Thyroid (mg)	Pituitary (mg)
Control	4 ml vehicle	7		385 ± 28	49 ± 3	20 ± 1	11 ± 0.4
4-MA	50 mg	8		450 ± 35	52 ± 3	19 ± 1	11 ± 0.5
4-MA	150 mg	8		373 ± 26	52 ± 2	20 ± 1	10 ± 0.4
4-MA	450 mg	3		133 ± 81	67 ± 10	16 ± 2	10 ± 0.5

[a] Values are means ± SEM.
** Significantly different from control at $P < 0.01$.
*** Significantly different from control at $P < 0.001$.

rats. Total doses of from 0.5 to 4.5 mg of 4-MA failed to affect uterine weight, indicating the compound had little or no estrogenic activity. In a more specific test of estrogenicity, the vaginal cornification assay, total amounts of 33, 100, or 300 mg per rat of 4-MA failed to cause cornification of the vaginal epithelium whereas this response was elicited in six of eight treated rats given a total dose of 4.5 μg of estrone.

In a progestational assay an average endometrial proliferation score of +2 was

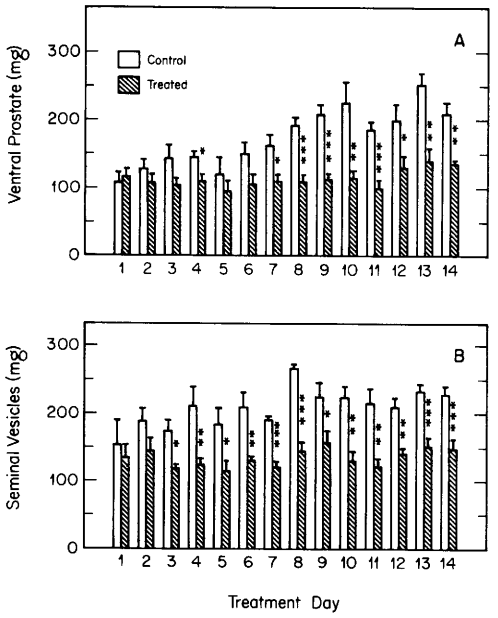

FIG. 1. Effect of the sc administration of 50 mg/kg/day of 4-MA on weights of the (A) ventral prostate and (B) seminal vesicles of young adult male rats over a 14-day treatment period. At each datum point $N = 5$. Values are means ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

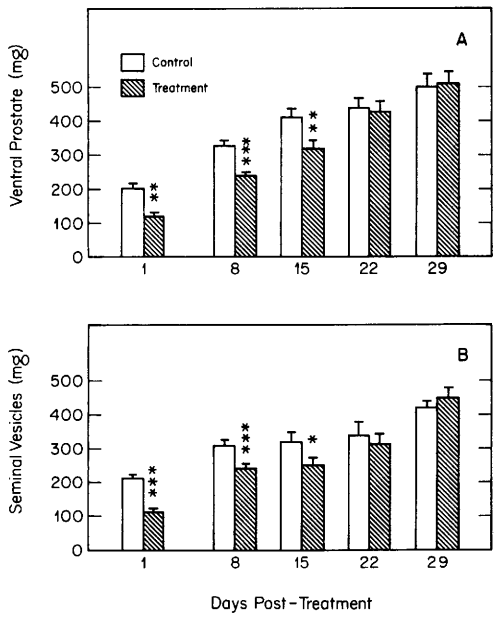

FIG. 2. Recovery of (A) ventral prostate and (B) seminal vesicle weights of young adult male rats following 7 days of subcutaneous treatment with 4-MA at a level of 50 mg/kg/day. Eight rats are represented at each datum point. Values are means ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

TABLE VI. RAT SERUM PROLACTIN (AS ng/ml RP-1) FOLLOWING THE INTRAPERITONEAL ADMINISTRATION OF 4-MA[a]

		Serum prolactin				
		Day 1			Day 2	Day 3
Group	Dose/kg/day	15 min	30 min	60 min	30 min	30 min
Control	0	14.2 ± 5.0	5.2 ± 0.9	13.0 ± 5.8	12.0 ± 4.0	7.7 ± 1.4
4-MA	50 mg	10.2 ± 2.0	7.5 ± 1.9	7.8 ± 2.0	12.2 ± 3.0	16.8 ± 4.0
4-MA	100 mg	5.0 ± 1.3	5.3 ± 1.3	2.7 ± 1.1	4.3 ± 1.0	12.7 ± 4.1

[a] Young adult male rats were treated for 1, 2, or 3 days and blood was taken at 15, 30, or 60 min post-treatment on the day indicated. Each datum point in the table represents the average prolactin level of six animals. Values are expressed as means ± SEM.

obtained in rabbits injected with 100 μg of progesterone daily. Daily treatment levels ranging from 200 μg to 180 mg of 4-MA produced no endometrial proliferation and the compound was judged to have no significant progestational activity.

Ovarian weights of parabiosed female rats were significantly reduced by treatment of their castrate male partners with 6 μg of testosterone acetate daily. Treatment of similar males with daily doses of 0.5 to 9.0 mg of 4-MA failed to reduce ovarian weights of their female partners, indicating 4-MA lacked significant gonadotropin inhibiting potency.

Discussion. A dose-related significant reduction in sex accessory gland weights occurred in mature male rats treated with 4-MA for 67 days. That was expected since the compound inhibits DHT formation and also directly antagonizes DHT action to some extent (17). The rats exhibited no apparent change in libido as they mated regularly throughout the experiment and most of those matings resulted in pregnancies. The complete cycle of spermatogenesis in the rat requires approximately 50 days (21) and if allowance is made for an additional 12 days in passage through the epididymis (22), it is evident that the sperm responsible for fertilizing ova in the last weeks of the test were formed after treatment had already begun. Furthermore, histological examination of testes from treated rats revealed normal spermatogenesis in all instances.

A similar absence of an antifertility effect was also noted in female rats treated with 4-MA over a period from 5 days prior to their being exposed to males until 9 days postmating. Apparently normal pregnancies were established in each animal which mated. That result indicated 4-MA did not interfere with the functioning of the hypothalamic–pituitary–gonadal axis, fertilization, ovum transport, or fetuses.

There was a relatively marked effect on development of the external genitalia in male rat fetuses whose dams had been treated with 4-MA. Similar reduction in anogenital distance, an index of sexual differentiation (23), was obtained by administering either 3 mg/day of 4-MA or 1 mg/day of flutamide to the pregnant females. Since the peripheral anti-androgenicity of 4-MA is very much less than that of flutamide (17), the effects seen in fetal males lend credence to the thesis of Imperato-McGinley *et al.* (24) that DHT is the androgen chiefly responsible for proper development of the external male genitalia. Our results were consistent with that hypothesis if it is assumed that 4-MA inhibited the formation of DHT from T secreted by the fetal testis.

In male rats given 50 or 150 mg/kg/day for 14 days, no significant changes in testis weight occurred. This was yet another indication that 4-MA is not a gonadotropin inhibitor. In addition, at those treatment levels the compound did not significantly affect thymus or adrenal weights, a result suggesting it was not a stressor agent (25) and had no glucocorticoid activity. However, at 450 mg/kg/day 4-MA was toxic and the majority of rats treated with that level of compound died before the end of the experiment.

The chronic administration of 50 mg/kg/

day of 4-MA to young adult male rats caused no alteration in body weight gains by these rapidly growing animals. However, it did result in a rapid, and almost complete, cessation of growth of the ventral prostate and seminal vesicles (Fig. 1). This static effect of 4-MA on sex accessory gland growth was fully reversible since by the 22nd day of a recovery period following a 7-day period of treatment with 4-MA, weights of these organs were similar in control and treated animals (Fig. 2).

High treatment levels of 4-MA elicited no change in serum prolactin. That was of interest since both stress and estrogens stimulate prolactin release (26). Thus, the failure of 4-MA to cause a rise in serum prolactin is evidence indicating it lacks toxicity and estrogenic potency at the doses used.

Results of several tests suggested 4-MA was not a gonadotropin inhibitor and had no estrogenic, androgenic, or progestational potency. First, it did not affect ovarian weights of parabiosed female rats, a result indicating it had not inhibited gonadotropin secretion. In parabiosed animals, estrogens, androgens, and progestins reduce ovarian weights (27). Further, 4-MA did not increase sex accessory gland weights in the castrate, parabiosed male partner, an effect which is usually, but not always, seen if the test substance is androgenic (27). In the uterotropic assay 4-MA caused no increase in uterine weight, a response caused primarily by estrogens but also androgens and, in sufficiently high doses, progestins. Cornification of the vaginal epithelium of castrate rats is a classic test for estrogenic activity and here too 4-MA was inactive. Finally, the compound exhibited no progestational potency in a standard assay.

On the basis of the assay results obtained, it was concluded that 4-MA is a relatively nontoxic 5α-reductase inhibitor which antagonizes certain androgen-dependent actions but is without significant estrogenic, progestational, gonadotropin-inhibiting, androgenic, or antifertility activities.

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