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43. Akira Kasahara, Takeshi Onodera, Haruo Tachizawa, Yasuo Oshima, and Masao Shimizu : Investigations on Steroids. VI.*1 Pharmacological Studies. (2). Endocrinological Properties and Acute Toxicity of 17β-Hydroxy-17α-methyl-5α-androstano[2,3-*c*]furazan (Androfurazanol).

(*Central Research Laboratory, Daiichi Seiyaku Co., Ltd.**2)

As reported previously,*1 17β-hydroxy-17α-methyl-5α-androstano [2,3-*c*] furazan (androfurazanol) proved to be a new anabolic steroid with a favorable myotrophic/androgenic ratio. In general, it has been known that anabolic agents have more or less undesirable side effects in clinical field. Interesting properties such as antagonism against cortisone[1~4] or dihydrotachysterol[5,6] have also been reported. Accordingly, it is very important to investigate various biological properties of androfurazanol. The present studies were undertaken to obtain information on other endocrinological properties and acute toxicity of androfurazanol.

Materials and Methods

Compounds and Animals——Androfurazanol[7] and stanozolol(17β-hydroxy-17α-methyl-5α-androstano [3,2-*c*]pyrazole)[8] were synthesized in this laboratory. Estrone, testosterone propionate, and DOCA (deoxycorticosterone acetate) were obtained from the Tokyo Kasei Kogyo Co., Ltd., and progesterone and cortisone acetate from Organon (Netherlands). Rats of Wistar-Imamichi strain and of Donryu strain were purchased from the Institute for Animal Reproduction (Ômiya) and the Central Laboratories for Experimental Animals (Tokyo), respectively. Mice of ddF strain and rabbits were obtained from Funabashi Nôjô (Funabashi).

Estrogenic Activity——The estrogenic activity was examined by the method described by Beyler, *et al.*[9] Female rats of Wistar-Imamichi strain were used in this experiment. After the animals were confirmed to exhibit a normal estrous cycle by the vaginal smear test, they were castrated at 71 to 75 days of age, and about 235 g. in body weight. The castrated animals were confirmed to be continuously in diestrus for one week and injected subcutaneously with the test compound in corn oil at 2.00 p.m. on the lst day and 10.00 a.m. on the 2nd day. Vaginal smears were taken with a saline-moistened swab as gently as possible at 4.00 p.m. on the 2nd day, at 10.00 a.m. and 4.00 p.m. both on the 3rd and 4th day. The smears were scored as positive when they contained only cornified cells or a mixture of cornified cells and nucleated cells but no leucocyte. Percentage of the rats which exhibited positive response was determined for each group. Estrone was taken as a reference standard.

Estrus-inhibiting Activity——The estrus-inhibiting activity was determined essentially according to the method described by Donini, *et al.*[5] Female rats of Wistar-Imamichi strain, 80 days of age and about 200 g. in body weight, were used in this experiment. This animals which exhibited a normal estrous cycle were selected for the assay by the vaginal smear test. Test compound was injected subcutaneously once daily, except Sunday, for 12 days. The vaginal smear test was performed daily,

*1 Part V : This Bulletin, **13**, 1460 (1965).

*2 Minamifunabori-cho, Edogawa-ku, Tokyo (笠原 明, 小野寺 威, 立沢晴男, 大島康夫, 清水正夫).

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except Sunday, at 9.00~10.00 a.m. during the assay period. Percentage of the rats which failed to exhibit the estrous stage was determined in each group. Testosterone propionate was taken as a reference standard.

Gonadotropin-inhibiting Activity——The parabiotic rats were used for the determination of gonadotropin-inhibiting activity of the test compound. Male and female rats of Wistar-Imamichi strain, approximately 30 days of age and weighing 60 to 80 g., were used. The male rat (right partner) was castrated and immediately paired with an intact female rat (left partner), under ether anesthesia, according to the technique described by Shipley.[10] The test compound in corn oil was injected subcutaneously to the castrated partner daily, except Sunday, for 10 days. On the day after the last injection, the animals were killed and the ovaries removed and weighed.

Progestational Activity——Assay for progestational activity was performed essentially according to the method of Clauberg.[11] Immature female rabbits, 0.73 to 1.05 kg. in body weight, were used in groups of 3 to 4 rabbits per dose level. Five subcutaneous pretreatments of animals with 3 μg./day of estrone were followed by subcutaneous injection of the test compound once daily for 5 days. The vehicle was corn oil. The animals were sacrificed and the uterus removed immediately on the day after the last injection. Middle part (about 0.5 cm. in length) of left uterine horn was cut and fixed for 24 hr. in Bouin's solution. The tissue was embedded in paraffin, sectioned with a microtome, and stained with Hematoxylin-Eosin in a customary manner. The degree of the progestational proliferation of uterine endometrium was scored by the McPhail scale.[12] Progesterone was taken as a reference standard.

Antagonism to Cortisone Acetate——Male rats of Donryu strain, 28 days of age and about 56 g. in body weight, were used in this experiment. Cortisone acetate and a test compound were administered once daily, except Sunday, for 10 days by subcutaneous injection. The test compound and cortisone acetate were injected simultaneously at distant sites to avoid chemical interaction of these compounds. Cortisone acetate was suspended in 2% acacia and test compounds were dissolved or suspended in cottonseed oil. The animals of each group were weighed daily, and killed by exanguination on the day after the last injection. The adrenals were removed immediately, weighed, and fixed in neutral 10% Formalin for a histological examination. The paraffin sections prepared from one of the adrenals removed from each animal were stained with Hematoxylin-Eosin. The other one was cut with a freezing microtome and the sections stained with Sudan Ⅲ and Hematoxylin.

Effect on Electrolyte Excretion——This experiment was carried out essentially according to the method of Marcus, *et al.*[13] Female rats of Wistar-Imamichi strain were bilaterally adrenalectomized under ether anesthesia at 90 days of age. On the day following the operation, 2 hr. after removal of food and water, each animal received an intraperitoneal injection of a 0.9% NaCl solution (5 ml./100 g.) and a subcutaneous injection of a cottonseed oil solution of the test compound, and was transferred into metabolism cages. Before the injection the urinary bladder was emptied by treatment with ether and by suprapubic pressure. Five-hour urine specimens were collected and analyzed for sodium and potassium in the urine with Hitachi flame photometer (Model FPF-2). DOCA was taken as a reference standard.

Acute Toxicity——The acute toxicity of androfurazanol was determined in mice after a subcutaneous, intraperitoneal, and oral administration. Mice of ddYF strain, 40 days of age and about 25 g. in body weight, were used in these experiments. LD_{50} (24-hr.) was calculated according to the method of Litchfield and Wilcoxon.[14] The test compound was suspended in 5% acacia.

Results and Discussion

Estrogenic Activity

Table I is the result of the experiment wherein the cornification responses of the vaginal epithelium of castrated female rats were observed. The experimental data show that the subcutaneous injection of estrone exhibited apparently the positive response in total doses of 0.005 to 0.1 mg./kg. although the response was not found at 0.0015 mg./kg. On the other hand, androfurazanol was not estrogenic within a dose range of 0.1 to 10 mg./kg. These facts indicate that androfurazanol is practi-

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Table I. Estrogenic Activity of Estrone and Androfurazanol on Vaginal Epithelium of Ovariectomized Rats

Compound	Total dose (mg./kg., s.c.)	No. of animals used	Positive response (%)
Estrone	0.0015	9	0
	0.005	9	11
	0.01	8	100
	0.03	9	100
	0.1	9	100
Androfurazanol	0.1	8	0
	1	8	0
	10	8	0

cally devoid of estrogenic effect. It was reported that no estrogenic effect was found also in another steroidal heterocycles such as stanozolol[9] and androisoxazole.[5]

Estrus-inhibiting Activity

Table II shows the data obtained from the vaginal smear test in rats treated with various compounds. From these data, the median estrus-inhibiting dose (ID_{50}), the dose at which 50% of the animals failed to exhibit the estrous stage, was estimated.

Table II. Estrus-inhibiting Activity of Testosterone Propionate, Androfurazanol, and Stanozolol in Rats

Compound	Dose (mg./kg./day, s.c.)	No. of animals used	Estrus inhibition (%)
Testosterone propionate	0.03	5	0
	0.1	5	60
	0.3	5	100
Androfurazanol	0.1	5	0
	0.3	5	20
	1	5	80
	3	5	100
Stanozolol	0.3	5	0
	1	4	25
	3	5	100

Testosterone propionate brought about a 60% response in estrous inhibition in a dose of 0.1 mg./kg./day, although the inhibition was not observed in the animals receiving 0.03 mg./kg./day. In a dose of 0.3 mg./kg./day, the inhibition was found in all animals. Thus, ID_{50} was roughly estimated to be between 0.03 and 0.1 mg./kg./day for testosterone propionate, and similarly, between 0.3 and 1 mg./kg./day for androfurazanol and between 1 and 3 mg./kg./day for stanozolol. On the basis of these evaluations, androfurazanol and stanozolol were approximately 0.1 times and 0.03 times, respectively, as active as testosterone propionate in estrous inhibition. Donini, *et al.*[5] reported, in a similar experiment with androisoxazole, that the inhibition was not found in a dose of 1 mg./kg./day, and 50% inhibition was found in doses of 10 and 20 mg./kg./day. These facts indicate that the difference of a heterocyclic ring fused at 2,3-position of androstane molecule has a considerable effect on the degree of the effect.

Gonadotropin-inhibiting Activity

The gonadotropin-inhibiting activities of anabolic steroids have been examined, in general, by the use of parabiotic rats[15~18] or by determination of gonadotropin in the rat treated with a test compound. In the present experiments, parabiotic rats were used. Results obtained from the experiments are presented in Table Ⅲ. The hyper-

Table Ⅲ. Gonadotropin-inhibiting Activity of Testosterone Propionate, Androfurazanol, and Stanozolol in Parabiotic Rats

Compound	Dose (μg./day, s.c.)	No. of pairs	Mean ovarian weight ± S.E.[a] (mg.)
Control (corn oil 0.1 ml./day)		6	135.4± 8.14
Testosterone propionate	1	6	138.6± 7.70
	10	6	19.8± 3.00[b]
	100	6	21.6± 3.12[b]
Androfurazanol	1	6	132.6± 6.48
	10	6	82.0±19.66[b]
	100	6	17.4± 1.20[b]
	1000	6	13.4± 1.54[b]
Stanozolol	1	6	122.6± 8.56
	10	6	144.6± 8.12
	100	6	55.8±16.68[b]
	1000	6	15.8± 2.12[b]
(Unpaired female rats)		(10)	(21.4± 1.58)[b]

a) S.E.: Standard error.
b) Significantly different ($P < 0.05$) from control (t test).

secretion of pituitary gonadotropin caused by castration was indicated by a significant increase in ovarian weight of female partners in the control group compared to that of unpaired female rats. When testosterone propionate was administered subcutaneously into castrated partners of parabiotic rats in a daily dose of 10 or 100 μg., the increase in ovarian weight of female partners was significantly suppressed although the suppression was not found in a dose of 1 μg. It was found that a daily dose of 10 μg. or more of testosterone propionate produced an apparent gonadotropin inhibition. Also in the case of androfurazanol, the inhibition was observed in a daily dose of 10 μg. or more. The comparison of the effect observed in androfurazanol with that in testosterone propionate indicated, however, that the former was significantly less active than the latter in gonadotropin inhibition. In addition, the effect of stanozolol was not found in a daily dose of 10 μg. but found in 100 and 1000 μg. As shown already, estrus-inhibiting effect of androfurazanol was observed in 20% of rats at a dose of 0.3 mg./kg./day, and in 80% at 1 mg./kg./day. As mentioned above, the minimum dose of androfurazanol required to produce gonadotropin-inhibiting effect was 10 μg./rat/day, which corresponds approximately to 0.1 mg./kg./day since the castrated male partners were regarded as about 100 g. in the mean body weight during the assay period. Although this dose is slightly lower than the dose (0.3 to 1 mg./kg./day) in which the estrous inhibition was found, it appeared reasonable to suppose that the gonadotropin-inhibiting effect of androfurazanol plays an important role in the appearance of estrous inhibition.

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Progestational Activity

The results are shown in Fig. 1, wherein progestational responses in various compounds scored by the McPhail scale were plotted against the log doses of the compounds. The effective doses which produced the McPhail scale 3 were estimated graphically on the assumption that the responses are in linear relation to the log doses in each compound. Thus, the effective doses were estimated to be 0.47 and 13.0 mg. per animal, respectively, for progesterone and androfurazanol. On the basis of these data, androfurazanol is only 0.036 times as active as progesterone. In addition, stanozolol was found to be less active than androfurazanol. Desaulles, *et al.*[17] presented dose-response relations in various anabolic steroids. Judging from their data, androfurazanol seems to be in the same order as 4-chlorotestosterone acetate in progestational activity.

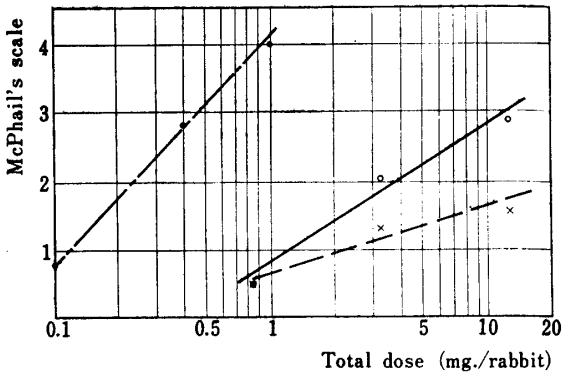

Fig. 1. Progestational Response of Progesterone, Androfurazanol and Stanozolol

●--- Progesterone; ○— Androfurazanol; ×— — Stanozolol

Antagonism to Cortisone Acetate

It has been demonstrated that various steroids prevent the growth inhibition and adrenal atrophy observed in cortisone-treated rats.[1~4] This fact led us to examine whether androfurazanol is able to prevent such actions of cortisone. The results obtained from these experiments are presented in Table IV. As shown in the Table,

Table IV. Effect of Various Steroids on Growth and Adrenal Weight in Male Rats

Treatment[a]	No. of animals used	Mean increment of body weight ±S.E.[b] (g.)	Mean adrenal weight ±S.E.	
			Absolute (mg.)	Relative (mg./100 g.b.w.)
Control (untreated)	9	61.1±3.21	24.0±1.05	20.0±0.29
CA[c](2)	9	7.1±2.33[f]	2.5±0.24[f]	3.7±0.36[f]
CA[c](2)+AF[d](1)	9	18.1±2.28[f,g]	10.0±0.60[f,g]	13.3±0.85[f,g]
CA[c](2)+AF[d](2)	9	16.9±2.37[f,g]	10.9±0.71[f,g]	15.1±1.21[f,g]
CA[c](2)+AF[d](4)	9	16.3±2.86[f,g]	10.7±0.56[f,g]	15.1±1.50[f,g]
CA[c](2)+ST[e](1)	9	18.6±2.80[f,g]	6.1±0.79[f,g]	7.3±0.77[f,g]
CA[c](2)+ST[e](2)	9	15.4±2.42[f,g]	5.9±0.63[f,g]	7.6±0.71[f,g]
CA[c](2)+ST[e](4)	9	20.7±2.23[f,g]	6.4±0.53[f,g]	8.4±0.63[f,g]

a) Figures in parentheses are doses (mg./day) used.
b) S.E.: Standard error
c) CA: Cortisone acetate
d) AF: Androfurazanol
e) ST: Stanozolol
f) Significantly different ($P < 0.05$) from control (t test).
g) Significantly different ($P < 0.05$) from CA (2).

cortisone acetate markedly inhibited the growth of rats when injected alone subcutaneously in a daily dose of 2 mg. On the other hand, when 1 mg./day of androfurazanol was injected subcutaneously in combination with cortisone acetate, the growth inhibition was prevented to a certain degree. However, the effect of androfurazanol at 2 or 4 mg. was in the same order as that observed at 1 mg. Furthermore, the comparison of adrenal weights (both in absolute and in relative weights) showed that the simultaneous injection of androfurazanol in a daily dose of 1 mg. apparently inhibited the adrenal atrophy in the cortisone-injected rats. Also in this case, a higher degree

of prevention was not found in spite of an increase of the dose to 2 or 4 mg. Stanozolol was also found to have such effects against the actions of cortisone acetate as mentioned above, but it should be noted that the ability of androfurazanol to prevent adrenal atrophy was significantly larger than that of stanozolol.

In addition, histological examinations of the adrenals were made. As shown in Fig. 2, in control group, glomerular zone and outer part of the fascicular zone indicated heavy concentration of sudanophilic lipid and quantity of the lipid decreased gradually toward the reticular zone. The sudanophobic zone, which is known to be characteristic in rats, separated the glomerulosa from the fasciculata. The subcutaneous injection of 2 mg./day of cortisone acetate produced a marked depletion of the

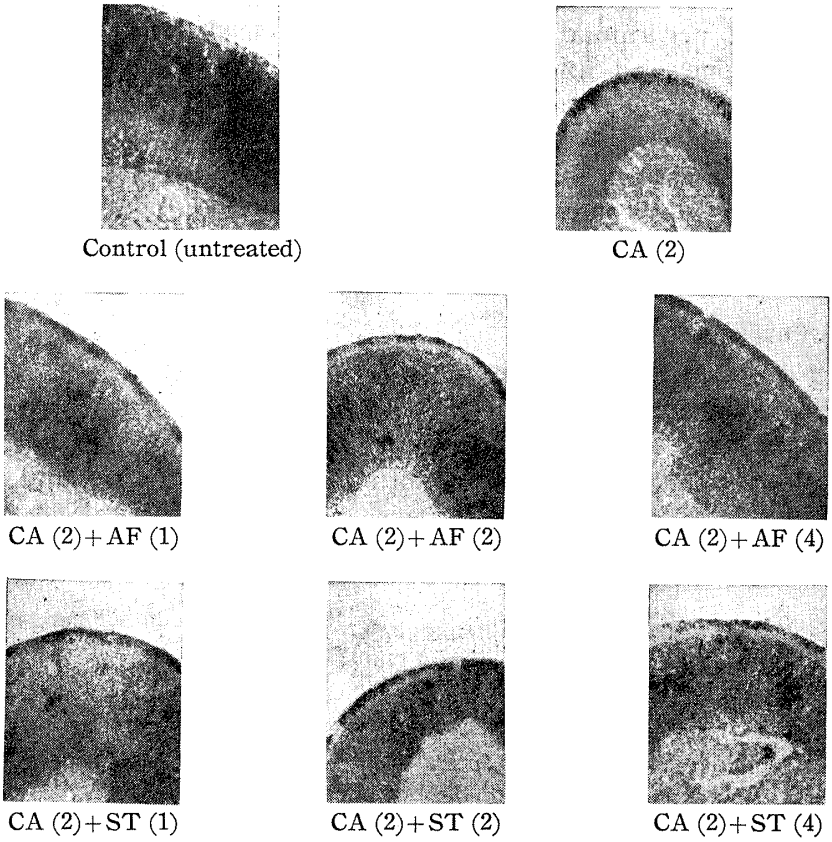

Fig. 2. Distribution of Sudanophilic Lipid in Frozen Sections of Rat Adrenals

Figures in parentheses indicate doses (mg./day) used.
CA, cortisone acetate; AF, androfurazanol; ST, stanozolol

lipid from an outer part of the fascicular zone although an intense sudanophilia was observed in the glomerular zone. However, when androfurazanol was injected simultaneously with cortisone acetate, in a daily dose of 1, 2, or 4 mg., the lipid depletion was largely prevented. A similar effect was found in treatment with stanozolol in the same dose but no difference could be found between androfurazanol and stanozolol in the degree of this effect.

Effect on Electrolyte Excretion

In Tabie V are summarized the results observed in rats injected with DOCA or androfurazanol. In these experiments, the groups treated with 0.01 and 0.1 mg. of DOCA showed a marked diminution in urine output. In addition, the sodium excretion in these groups was significantly less than that in the control. Potassium excretion

Table V. Effect of Deoxycorticosterone Acetate (DOCA) and Androfurazanol on Urine Volume, and Sodium and Potassium Excretion in Adrenalectomized Female Rats

Compound	Dose (mg./rat, s.c.)	No. of animals used	Urine volume ±S.E.[a] (ml.)	Sodium ±S.E. (meq.×100)	Potassium ±S.E. (meq.×100)
Control (cottonseed oil 0.2 ml./rat)		4	2.10±0.49	60.6±5.6	31.9±2.5
DOCA	0.01	4	0.78±0.11[b]	20.2±1.2[b]	31.5±2.7
	0.1	4	0.78±0.24	16.1±3.0[b]	36.6±1.7
Androfurazanol	0.1	4	1.48±0.51	53.6±9.9	25.8±0.5
	2	4	1.43±0.31	49.7±9.0	28.9±3.6

a) S.E.: Standard error *b*) Significantly different ($P < 0.05$) from control (t test).

was not significantly affected by treatment with DOCA at the dose levels used. On the other hand, subcutaneous injection of androfurazanol showed a tendency to decrease the urine volume, and sodium and potassium excretions in doses of 0.1 and 2 mg., but the changes were not statistically significant. These facts indicate that androfurazanol has little effect on the metabolic balance of water, sodium, and potassium.

Acute Toxicity

LD_{50} values of androfurazanol in mice are tabulated in Table VI. Intraperitoneal LD_{50} of the compound was 0.494 g./kg. and oral LD_{50}, 2.330 g./kg. With lethal doses, rolling, tonic convulsion, salivation, loss of righting reflex, and ataxia were observed.

Table VI. Acute Toxicity of Androfurazanol in Mice

Route	No. of animals used	LD_{50} (g./kg.)[a]
Intraperitoneal	28	0.494 (0.353～0.692)
Oral	24	2.330 (1.820～2.980)
Subcutaneous	10	>4

a) Figures in parentheses are the 95% fiducial limit.

The tonic convulsion, however, differed from that by strychnine, showing no enhancement of reflex to sound. On the other hand, all the animals receiving androfurazanol by subcutaneous injection survived over 2 weeks even in a dose of 4 g./kg. This is probably due to poor absorption of androfurazanol through subcutaneous route since a considerable amount of its deposit was found at the site of injection in the autopsied mice.

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Summary

Various biological effects of androfurazanol, 17β-hydroxy-17α-methyl-5α-androstano[2,3-*c*]furazan, were tested on rats and rabbits as compared with those of stanozolol, 17β-hydroxy-17α-methyl-5α-androstano[3,2-*c*]pyrazole. Androfurazanol was practically devoid of estrogenic effect and was approximately less active than testo-

sterone propionate in estrous inhibition and gonadotropin inhibition. The gonadotropin-inhibiting effect of the compound is supposed to play an important role in the appearance of estrous inhibition. Furthermore the compound was found to be 0.036 times as active as progesterone in progestational activity. The inhibitions of growth and adrenal weights due to cortisone acetate were antagonized by androfurazanol. The result was comfirmed also with histological examinations of the adrenals. Subcutaneous injection of androfurazanol showed a tendency to decrease the urine volume, and sodium and potassium excretions in doses of 0.1 and 2 mg. but the changes were not statistically significant. Intraperitoneal LD_{50} was 0.494 g./kg. and oral LD_{50} 2.330 g./kg. but all the animals given this compound by subcutaneous injection survived over 2 weeks even in a dose of 4 g./kg.

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