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Investigations on Steroids. X.[1] Pharmacological Studies. (4). Conversion of 17β-Hydroxy-5α-androstano[2,3-c]furazan into 17-Oxo Compound by Rat Liver *in Vitro*

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17β–Hydroxy–5α–androstano[2,3–c]furazan (I) was incubated with rat–liver mince in the presence of NAD and nicotinamide in oxygen atmosphere. It has been demonstrated that at least 23% of the steroid (I) is converted into 17–oxo–5α–androstano[2,3–c]furazan. This oxidation rate at the 17–position is the same as that observed for testosterone, indicating the substituted furazan ring has no significantly different effect on the metabolic oxidation at this position. The relation of this fact to myotrophic activity of I by oral route has been discussed.

In Part IX it was demonstrated that 17β–hydroxy–5α–androstano[2,3–c]furazan (I) exhibits myotrophic activity when given by injection but not by oral route, whereas its 17α–methyl derivative is highly effective by either route. The decreased activity, associated with oral administration, of the compounds without the 17α–methyl group has been exemplified by testosterone[3] and 17β–hydroxy–5α–androstano[2,3–*d*]isoxazole.[4] In the case of testosterone, the low activity is explained by the rapid metabolic transformation in the liver to the corresponding 17–ketone which is accepted as the key intermediate for other metabolites.[5a,b] On the other hand, 17β–acetoxy–1–methyl–5α–androst–1–en–3–one, a myotrophic steroid possessing no 17α–methyl group, is orally effective and its 17–hydroxy derivative suffers the metabolic oxidation at the 17–position in minor degree than testosterone, indicating the effect due to the modification of the ring A.[6,7]

As a part of our study on the metabolism of androstano[2,3–c]furazans, the effect of the substituted furazan ring on the metabolic transformation of the compound (I) to the ketone was examined by incubating with rat liver mince under the same conditions as used for testosterone.[8]

Materials and Methods

Test Steroids and Reagents——The steroids used as substrates were 17β–hydroxy–5α–androstano[2,3–c]furazan (I) and 17β–hydroxy–17α–methyl–5α–androstano[2,3–c]furazan (furazabol) (II). 17–Oxo–5α–androstano[2,3–c]furazan (III) and its 2,4–dinitrophenylhydrazone (IV) were taken as reference standards for identification of incubation products. These compounds (I to III[9]) and IV[10]) were synthesized in this

1) Part IX: *Chem. Pharm. Bull.* (Tokyo), **16**, 1456 (1968).
2) Location: *Minamifunabori–cho, Edogawa–ku, Tokyo.*
3) K. Miescher and E. Tschopp, *Schweiz. Med. Wochschr.*, **68**, 1258 (1938).
4) A.J. Manson, F.W. Stonner, H.C. Neumann, R.G. Christiansen, R.L. Clarke, J.H. Ackerman, D.F. Page, J.W. Dean, D.K. Phillips, G.O. Potts, A. Arnold, A.L. Beyler, and R.O. Clinton, *J. Med. Chem.*, **6**, 1 (1963).
5) *a*) L.T. Samuels and C.D. West, *Vitamins and Hormones*, **10**, 251 (1952); *b*) B.H. Levedahl and L.T. Samuels, *J. Biol. Chem.*, **186**, 857 (1950).
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8) P. Ofner, *Biochem. J.*, **61**, 287 (1955).
9) G. Ohta, T. Takegoshi, K. Ueno, and M. Shimizu, *Chem. Pharm. Bull.* (Tokyo), **13**, 1445 (1965).
10) *Anal.* Calcd. for $C_{25}H_{30}O_5N_6$: C, 60.71; H, 6.11; N, 17.00. Found: C, 60.29; H, 6.06; N, 17.09. $\lambda_{max}^{CHCl_3}$ mμ (ε): 367 (23000). mp 269—270° (decomp.).

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laboratory and their structural formulae are shown in Chart 1. Nicotinamide, NAD (nicotinamide adenine dinucleotide), *m*-dinitrobenzene, and 2,4-dinitrophenylhydrazine were obtained from the Daiichi Pure Chemicals Co., Ltd. (Tokyo). Girard-T reagent (trimethylaminoacetohydrazide hydrochloride) was purchased from the Tokyo Kasei Kogyo Co., Ltd., and alumina from the Nippon Rikagaku Yakuhin K.K. (Tokyo).

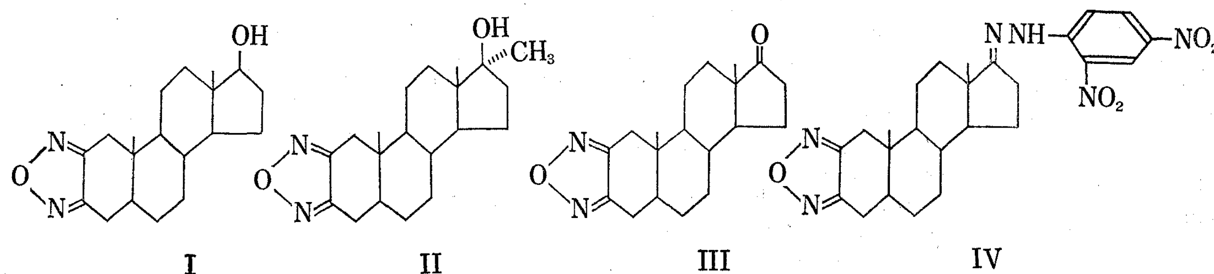

Chart 1. Structural Formulae of Steroids used

Incubation Conditions——Male Donryu rats obtained from the Central Laboratories for Experimental Animals (Tokyo), 72 days of age and about 200 g in body weight, were killed by decapitation followed by exsanguination. The liver of the animals was removed and immediately immersed in ice-cold saline solution. Left lobe of the liver was minced by pushing the tissue out of a sieve (about 20 mesh) made of stainless steel. Incubation conditions were essentially the same as the method described by Ofner.[8] The medium consisted of 0.04 M phosphate buffer (KH_2PO_4–Na_2HPO_4; pH 7.8) and 0.04 M nicotinamide in which NAD was dissolved at a concentration of 6 μmoles per 25 ml of the solution. One ml each of an ethanolic solution of I (1 μmole/ml) in five flasks was evaporated to dryness at a temperature below 50°. The residue was then dissolved in 0.2 ml of EtOH and 0.50 g of the freshly prepared mince was washed into each of the flasks with 25 ml of the medium. The flasks were filled with oxygen and incubated in a bath at 37.5°±0.2° for 1 hr. After the incubation, the enzymic action was stopped by boiling the contents under a reflux for 5 min.

Extraction of Incubation Products——The extraction procedures for the incubation products are shown in Chart 2. The content of five flasks was extracted with ether (100 ml×6) and centrifuged, and then the

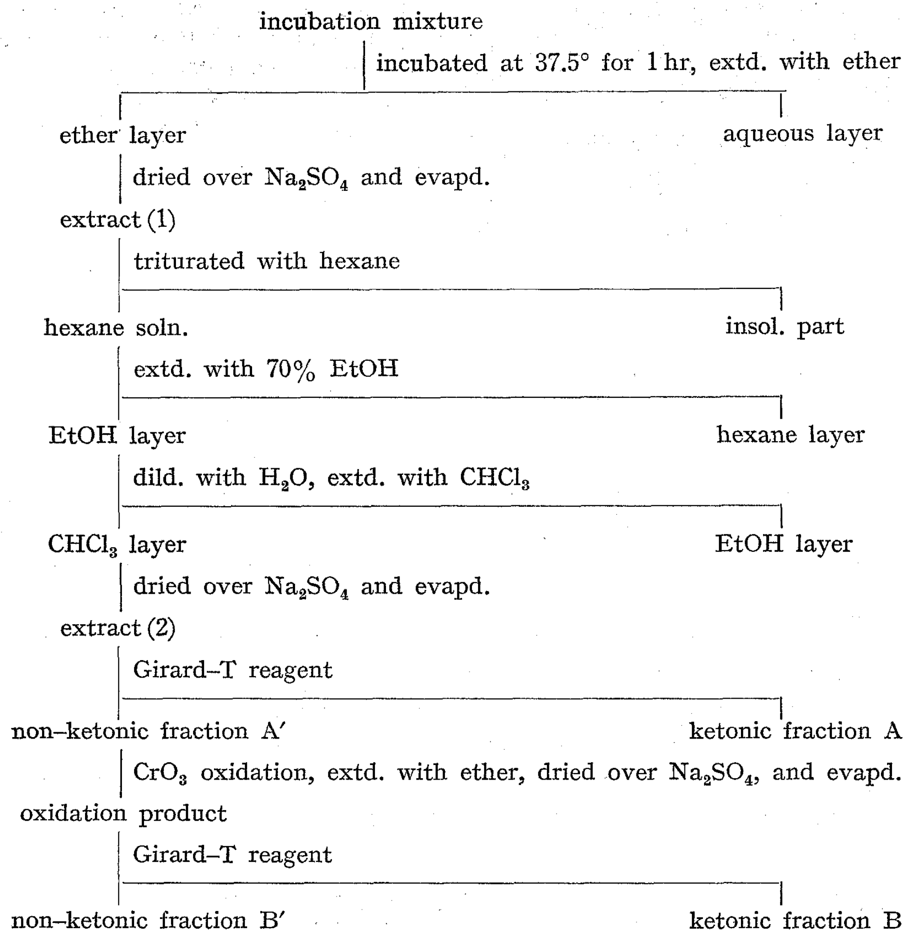

Chart 2. Extraction and Fractionation of Incubation Products

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ether layer was separated. The combined ether extract was evaporated to dryness, and the residue (extract 1) was triturated with hexane (80 ml). The hexane solution was separated and extracted with 70% EtOH (125 ml×2). After dilution of the EtOH layer to 30% EtOH with H_2O, the resultant milky-white emulsion was extracted with $CHCl_3$ (100 ml×2). Following the separation of the $CHCl_3$ layer by centrifugation, the extract was evaporated to dryness under a reduced pressure below 50°.

Separation of Ketonic Fraction from Non-ketonic Fraction——Girard-T reagent was used for the separation of ketonic fraction from non-ketonic faction. The extract to be separated was dissolved in a mixture of MeOH (2.5 ml) and AcOH (0.2 ml) containing Girard-T reagent (300 mg). The reaction mixture was left to stand overnight at room temperature and then 15 ml of ice water added. After neutralization of the solution with 10% NaOH, non-ketonic substance was removed by repeated extraction of the mixture with ether (20 ml×3). Non-ketonic fraction was obtained by evaporation of the ether layer after washing with 10 ml of H_2O. The aqueous layer was acidified with 3 ml of conc. HCl and was left to stand for 2 hr at room temperature. The regenerated ketonic substance was then recovered by ether extraction (20 ml×3). After washing of the ether layer with 2.5% NaOH (10 ml) and H_2O(10 ml×2), ketonic fraction was obtained by evaporation of the solvent.

Gas Chromatography——A Barber-Colman Model 10 chromatographic unit provided with an argon ionization detector was used. U-shaped column, 1.8 m×6 mm (int. diam.), was packed with 80—100 mesh Gas Chrom P coated with 2% nitrile silicone (GE XE-60, cyanoethylmethyl type). Temperature of the column, detector, and flash heater were 240°, 210°, and 240°, respectively. Argon flow rate was 75 ml/min. The sample to be examined was dissolved in acetone and injected with a microsyringe. Since retention time is relative, identity was confirmed by the increased peak height in the chromatogram of a material mixed with an authentic sample.

Column Chromatography——A chromatographic tube of 7 mm inside diameter was packed with 2 g of alumina (Merck) which was suspended in a mixture of benzene and hexane (1:1).

Thin-layer Chromatography——Glass plates (5×20 cm) were coated with "Silica Rider" (Daiichi Pure Chemicals Co., Ltd.) to 0.25 mm in thickness. Zimmermann's reaction was carried out by spraying 2% *m*-dinitrobenzene and 2.5 N NaOH in EtOH followed by heating of the plate.

2,4-Dinitrophenylhydrazone of Ketonic Fraction——Essentially according to the method described by Reich, *et al.*,[11] 0.2 ml of an absolute EtOH solution of the ketonic fraction A or B was mixed with 0.4 ml of dinitrophenylhydrazine solution,[12] and the mixture was kept at room temperature overnight. After addition of 0.4 ml of Benedict's reagent and 0.4 ml of H_2O, the mixture was heated for 10 min in a bath at 95° to destroy an excess of 2,4-dinitrophenylhydrazine, and, after cooling, extracted with $CHCl_3$ (5 ml×2). The combined $CHCl_3$ solution was dried over anhyd. Na_2SO_4 and evaporated to dryness.

CrO_3 Oxidation of Non-ketonic Fraction A'——To a solution of one-half the amount of non-ketonic fraction A' in acetone (1 ml), 0.2 ml of a solution of CrO_3 (26.7 g) in H_2O (50 ml) and conc. H_2SO_4 (23 ml) was added and, after standing for 10 min at room temperature, the mixture was poured into 10 ml of ice water. The solution was neutralized with 1 N NaOH solution and the oxidation product extracted with ether (10 ml×2).

Results

Separation and Qualitative Examinations of Incubation Products

Five μmoles (1.58 mg) of I was incubated with rat-liver mince as described above, and ether extraction of the mixture yielded 284 mg of extract (1), a yellow-brown crystalline substance. Extract (2) obtained from extract (1), using a purification procedure shown in Chart 2, weighed 49.3 mg, which was yellow-brown, semi-solid oil. The extract (2) was then separated into ketonic fraction A and non-ketonic fraction A' using Girard-T reagent. The ketonic fraction A, a pale-yellow and partly crystalline solid, weighed 6.6 mg after drying over $CaCl_2$ *in vacuo* overnight. A portion of the ketonic fraction A was submitted to gas chromatographic analysis and the results obtained are shown in Fig. 1. The fraction gave two distinct peaks, a small peak (A) and a larger one (B), which gave a retention time of 12.7 min and 14.3 min, respectively. The retention time of the peak (B) was in agreement with that of III, but the peak (A) could not be identified. These two peaks were distinguished from that of I added as a substrate, which showed a retention time of 11.6 min.

11) H. Reich, S.J. Sanfilippo, and K.F. Crane, *J. Biol. Chem.*, **198**, 713 (1952).
12) The dinitrophenylhydrazine solution was prepared by dissolving 125 mg of 2,4-dinitrophenylhydrazine in a mixture of 15 ml of absolute EtOH and 0.4 ml of conc. HCl with gentle warming.

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Thin-layer chromatographic analysis of the ketonic fraction A with three different solvent systems showed the presence of III. In each solvent system only one spot with a blue-violet color was detected by Zimmermann's reaction as found in III. *Rf* values of the spot were 0.67, 0.71, and 0.07 in the solvent systems of benzene—ethyl acetate (2:1), benzene—methanol (9:1), and benzene—chloroform (2:1), respectively, which were in agreement with those of III.

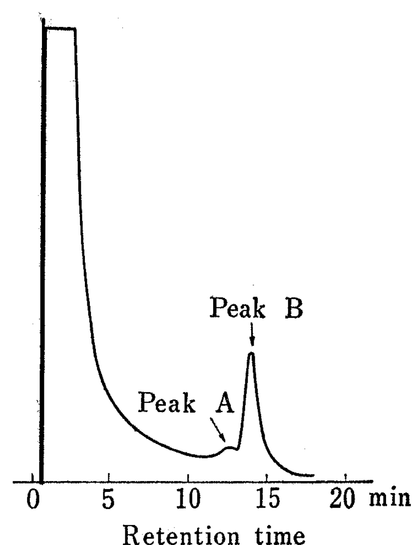

Fig. 1. Gas Chromatogram of Ketonic Fraction A

One-half the amount of total ketonic fraction A was converted into 2,4-dinitrophenylhydrazones. The resultant products were dissolved in 4 ml of benzene—hexane (1:1) and chromatographed on alumina, to separate the crude hydrazone mixture into its constituent, by successive elution with 20 ml of benzene, 12 ml of benzene—chloroform (9:1), and 10 ml of benzene—chloroform (3:1). Each fraction of 2 ml was evaporated to dryness and dissolved in 5 ml of chloroform. The chloroform solution was submitted to thin-layer chromatographic and photometric analyses. In the thin-layer chromatography, as shown in Fig. 2, fraction Nos. 4 to 7 showed the same *Rf* value as that of IV in the four different solvent systems, while *Rf* values of fraction No. 1 and 2 were different from that of IV. These results indicate that the ketonic fraction A contains at least two ketonic substances.

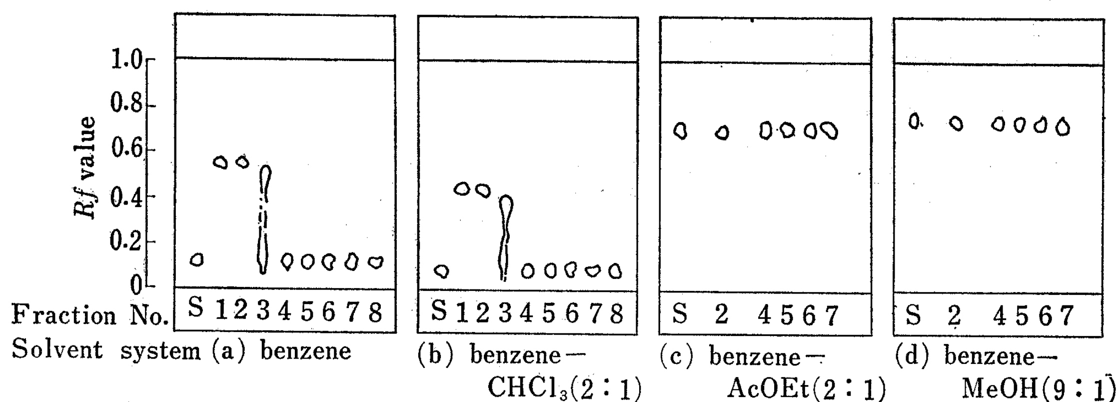

Fig. 2. Thin-layer Chromatograms of 2,4-Dinitrophenylhydrazones obtained from Ketonic Fraction A

S: compound (IV) All spots are yellow.

Fraction No. 3 is probably a mixture of 2,4-dinitrophenylhydrazones of these ketonic substances. These facts are consistent with the presence of two peaks in the gas chromatogram of the ketonic fraction A. Furthermore, as presented in Table I, absorption curves of fraction No. 4 to 7 showed maximum at 366 or 367 mμ which was in agreement with that of IV. On the other hand, fraction No. 1 to 3, and No. 8 did not show the absorption maximum at or near 367 mμ.

Thus, from the gas chromatographic, thin-layer chromatographic, and photometric examinations, it was ascertained that one of the incubation products is III, although other products are still unknown.

In addition, a similar incubation experiment was performed with furazabol but no ketonic metabolite was found by gas chromatographic analysis.

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Table I. Photometric Properties of 2,4-Dinitrophenylhydrazones obtained from Ketonic Fraction A

Fraction No.[a]	Color[b]	$\lambda_{max}^{CHCl_3}$ (mμ)	$E_{367\,m\mu}^{CHCl_3}$	Amount as compound (IV) (μg)
1	light yellow	<340	0.186	
2	deep yellow	364	0.582	
3	yellow	359	0.314	
4	yellow	367	0.325	35
5	deep yellow	366	1.284	139
6	yellow	367	0.775	84
7	light yellow	366	0.286	30
8	almost colorless	<340	0.234	
9—16	almost colorless			
Total				288

a) Number of each fraction chromatographed on alumina.
b) color of each fraction in $CHCl_3$

Quantitative Determination of a Ketonic Substance

Since a ketonic substance obtained from the incub
quantitative determination of the substance was attem
experiment are presented in Table I. The amount of the substance as its 2,4-dinitrophenylhydrazone was determined from the extinctions of the fraction No. 4 to 7 described above, using a calibration curve prepared with IV, since only these fractions were consistent with IV in thin-layer chromatographic and photometric observations. Since 288 μg of the hydrazone was estimated from 1/2 the amount of the ketonic fraction A, total amount of the hydrazone was calculated to be 576 μg (1.16 μmoles) which corresponds to 23% of that of I (5 μmoles) added as a substrate.

Examinations of Non-ketonic Fraction A′

Gas chromatography and thin-layer chromatography of non-ketonic fraction A′ were attempted but they failed to reveal the presence of unchanged I. The fraction was then oxidized with chromium trioxide and the reaction mixture was separated into ketonic fraction B and non-ketonic fraction B′ by treatment with Girard-T reagent. In gas chromatography of the ketonic fraction B, a small peak was found, which corresponds to that of III in retention time (Fig. 3). Also, by thin-layer chromatography, the formation of III was proved, using three solvent systems, benzene—ethyl acetate (2:1), benzene—chloroform (2:1), and benzene—methanol (9:1).

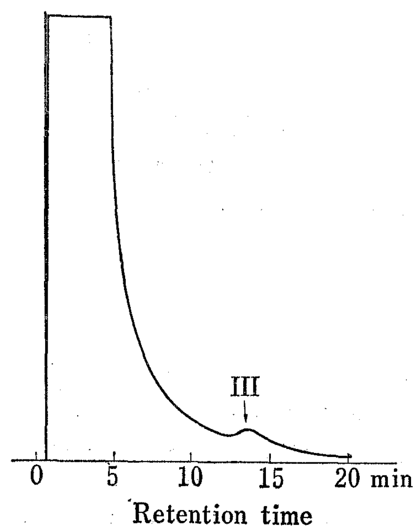

Fig. 3. Gas Chromatogram of Ketonic Fraction B

In addition, ketonic fraction B was converted into its 2,4-dinitrophenylhydrazones to confirm the formation of III. The reaction products were dissolved in benzene—hexane (1:1), chromatographed on alumina and eluted with benzene. Each fraction (1.5 ml) was examined by thin-layer chromatography. The appearance of several spots, probably those of different 2,4-dinitrophenylhydrazones, indicates the formation of various ketonic substances by the oxidation of non-ketonic fraction A′. Among these spots, the one which was the same as that of IV in *Rf* value was found in fraction No. 5, 6, and 7. The results indicated the presence of unchanged I in non-ketonic fraction A′, but the scarcity of the material prevented the accurate quantitative estimation.

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Discussion

Samuels, *et al*[13] investigated the ability of the liver of various species of
bolize testosterone, and observed that, in mammals, NAD-dependent reaction leads to the formation of 17-ketosteroids. Further, Levedahl, *et al.*[14] have demonstrated, in comparative studies on *in vitro* metabolism of androgens by liver tissue, that the substances giving the Zimmermann reaction were formed in the case of testosterone but not in the case of methyltestosterone. Later, conversion of testosterone into androst-4-ene-3,17-dione in rat-liver mince was confirmed by Ofner.[8] Since evidences were presented that the 17-ketone is the key intermediate in the metabolic transformation,[5a,b] these facts offered a rational explanation for the observation by Miescher, *et al.*[3] that methyltestosterone exceeds testosterone in androgenic effect by oral administration.

In the present work, 17β-hydroxy-5α-androstano[2, 3-*c*]furazan (I) was incubated with rat-liver mince in the presence of NAD and nicotinamide to investigate whether the compound similarily forms the corresponding 17-ketosteroid. The incubation product was examined by gas chromatography, alumina-column chromatography, thin-layer chromatography, and by photometric analysis. The incubation procedure was the same as that used for testosterone by Ofner[8] who has demonstrated that at least 20% of testosterone is converted into androst-4-ene-3,17-dione. In our experiments with testosterone for comparison, the rate of 25% was obtained. The experiments with I showed that at least 23% of I was converted into 17-oxo-5α-androstano[2,3-*c*]furazan (III), whereas such an oxidation product was not observed for furazabol. The unchanged I in the incubation products was detectable but not measurable. The same order in the rate of conversion of the 17β-alcohol to the 17-ketone observed for I and testosterone indicates that the substituted furazan ring has no significantly different effect on the metabolic oxidation at the 17-position. The Δ^1-1-methyl grouping in 17β-hydroxy-1-methyl-5α-androst-1-en-3-one has been shown to prevent the oxidation of the 17β-hydroxyl group[6,7] and a recent investigation with an enzyme from Pseudomonas Testosteroni has revealed that various structural modification at ring A affects the rate of such an oxidation.[15]

As reported previously,[1] the myotrophic activity of I is markedly less than that of furazabol by oral route whereas both compounds show comparable activity when given subcutaneously. In addition III is almost inactive either by oral or by subcutaneous administration. Although other metabolites of I including the conjugates of I have not been examined, the same oxidation rate with respect to I and testosterone in the liver suggests that the formation of III from I plays an important part to effect the difference between the oral activity of I and that of furazabol.

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