Chem. Pharm. Bull. 29(9)2646—2652(1981)

Studies on the Metabolism of Trilostane, an Inhibitor of Adrenal Steroidogenesis[1]

Yuji Mori,[a] Makoto Tsuboi,[a] Makoto Suzuki,*,[a] Akira Saito,[b] and Haruo Ohnishi[b]

Faculty of Pharmacy, Meijo University,[a] 15 Yagoto Urayama, Tempaku-ku, Nagoya 468, Japan and Research Laboratory of Pharmacology, Mochida Pharmaceutical Co., Ltd.,[b] Kamiya, Kita-ku, Tokyo 115, Japan

(Received February 18, 1981)

The metabolic fate of 2α-cyano-4α,5α-epoxy-17β-hydroxyandrostan-3-one (trilostane) in rats was studied. Five metabolites were isolated by successive column chromatography on XAD-2, LH-20, and silica gel, and they were characterized by gas chromatography–mass spectrometry, nuclear magnetic resonance spectroscopy, and infrared spectroscopy.

Unchanged trilostane and three metabolites, 2α-cyano-4α,5α-epoxyandrostane-3,17-dione (**M-1**), 2α-cyano-3α,16α-dihydroxy-4α,5α-epoxyandrostan-17-one (**M-2**), and 2α-cyano-4α,5α-epoxyandrostane-3α,16α,17β-triol (**M-3**) were identified by comparison with authentic samples. Structures were tentatively assigned to the other two metabolites (**M-4** and **M-5**).

In rats given trilostane orally, unconjugated metabolites were predominantly excreted in the urine, whereas conjugated ones were excreted in the bile.

On the basis of the data on biliary and urinary excretions and plasma level, the metabolic pathway of trilostane is proposed.

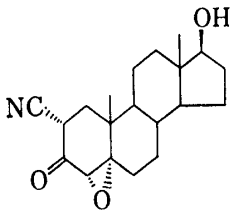
Keywords——2α-cyano-4α,5α-epoxy-17β-hydroxyandrostan-3-one; drug metabolism; metabolic pathway; rat; GC-MS; ion cluster technique

Trilostane (2α-cyano-4α,5α-epoxy-17β-hydroxyandrostan-3-one: TR), a synthetic steroid derived from testosterone, shows adrenal steroidogenesis-blocking activity.

Neumann *et al.*[2] reported the preparation of a number of 4,5-epoxysteroids as a part of their study on steroidal heterocycles, and showed that trilostane and some cyano-epoxy-steroids could inhibit adrenal cortical function. No report, however, has appeared on the behavior of trilostane and its metabolism in experimental animals.[3]

To investigate the metabolic pathway of trilostane in rats in detail, as well as the structure-activity relation of metabolites, the structures of the metabolites should be clarified.

We therefore examined the fate of trilostane in rat plasma, urine, and bile after oral administration by means of gas chromatography–mass spectrometry (GC–MS) and mass fragmentography (GC–MF), which was useful for the detection of metabolites with high specificity. Five metabolites M-1 to M-5 were isolated from rat urine and bile. The structures of these metabolites were elucidated on the basis of their infrared (IR), nuclear magnetic resonance (NMR), and mass spectra and those of three of the five new metabolites (**M-1**, **M-2**, and **M-3**) were finally established by chemical transformation from trilostane, as will be described in the following paper.[4]



trilostane

Chart 1

Our present study was undertaken with a dual purpose: i) isolation and identification of the urinary and biliary metabolites of trilostane in rats; ii) determination of plasma level and urinary and biliary excretions.

Experimental

Materials——All reagents were of special grade. β-Glucuronidase from *E. coli* (Type II) was purchased from Sigma Chemical Co., U.S.A. Trilostane-1-d_1 and authentic samples used in this study were synthesized

NII-Electronic Library Service

according to the method described in the following paper.[4]

Animal Studies——The animals used in these experiments were Wistar male rats weighing 200—250 g. Trilostane suspended in a 2.5% gum arabic solution was administered orally at a dose of 30 mg/kg after 12-h fasting for the quantitative determination of plasma level and urinary and biliary excretions. In the case of isolation of urinary metabolites, rats received the drug at a dose of 100 mg/kg. Urine and bile were collected for 3 and 9 days, respectively. In order to collect bile the rats' bile ducts were ligated.

Determination of Trilostane Metabolites in Urine and Bile——(1) Isolation of Metabolites: Urine and bile collected were diluted with 10—20 volumes of water and digested with β-glucuronidase at 37°C for 12 h in 0.2 M phosphate buffer (pH 6.8). The digests were extracted with ethyl acetate. These samples were worked up as shown in Chart 2. Silica gel chromatography of the organic layer obtained from bile was carried out with solvents of increasing polarity ($CHCl_3$/$CHCl_3$–acetone/$CHCl_3$–MeOH). From the fractions of 10 to 40% acetone in $CHCl_3$, **M-1** (540 mg) and **M-2** (22 mg) were obtained. In the case of urine, trilostane (204 mg) and **M-2** (25 mg) were eluted from the silica gel column (20 to 50% acetone in $CHCl_3$) followed by **M-3** (40 mg) and a mixture of **M-4** and **M-5** (11 mg) (5 to 10% MeOH in $CHCl_3$).

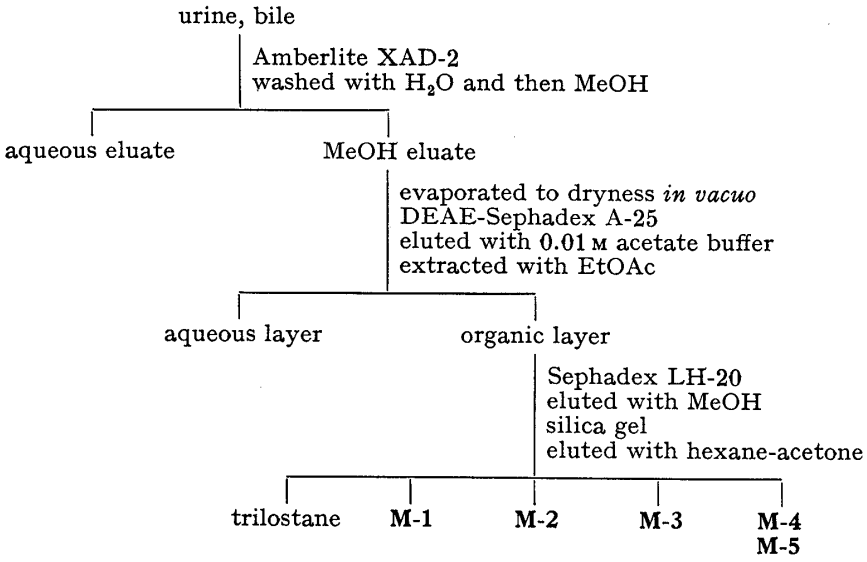


Chart 2. Isolation of the Urinary and Biliary Metabolites of Trilostane in Rats

The substances obtained were identified by thin-layer chromatography (TLC), nuclear magnetic resonance (NMR) spectroscopy, infrared (IR) spectroscopy, and mass spectrometry (MS).

(2) Thin-Layer Chromatography (TLC): TLC was carried out on plates precoated with Kieselgel HF_{254} (Merck) 0.25 mm thick. The following solvent systems were used: A, acetone–hexane (1: 1); B, $CHCl_3$–MeOH (9: 1); and C, EtOAc–MeOH–iso-PrOH (97: 2: 1). Metabolites on TLC plates were detected by spraying with 50% H_2SO_4 and by heating at 110°C for 15 min.

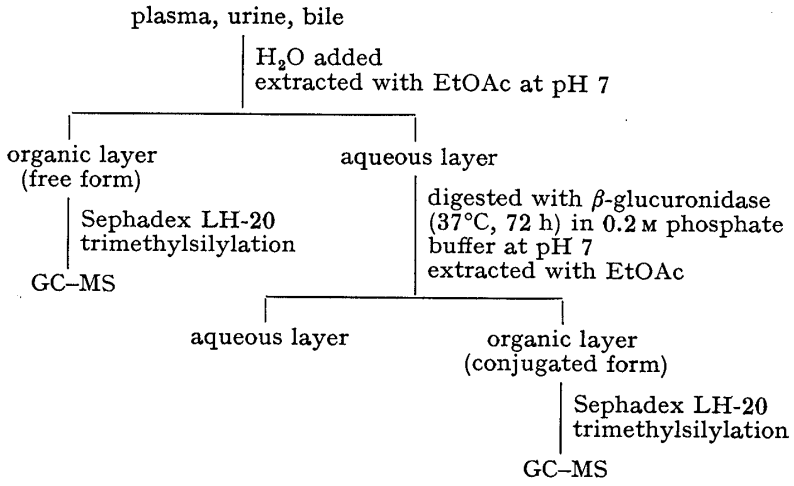


Chart 3. Procedure for the Determination of Trilostane and Its Metabolites in Plasma, Urine, and Bile

NII-Electronic Library Service

Plasma Level of Trilostane and Its Metabolites——Three rats were given 30 mg/kg of trilostane and blood samples were withdrawn at 1/6, 1/2, 1, 3, 6, 24, and 48 h after administration. Each plasma sample was fractionated into free and conjugated forms of metabolites according to the procedure described in Chart 3. Calibration curves were prepared by the use of similarly treated blood plasmas to which known amounts of trilostane and its metabolites had been added as standards. Using these calibration curves, the amounts of trilostane and its metabolites were calculated as trilostane and expressed as μg equivalent of trilostane/ml.

Urinary and Biliary Excretion of Trilostane and Its Metabolites——Three rats were given 30 mg/kg of trilostane and their urine was collected for 2 d. Cannulation of the bile duct was carried out in three other rats and the bile was collected for 2 d. The urine and the bile (each 1 ml) were fractionated according to the method shown in Chart 3, and quantitative determination was carried out in the same way as with blood plasma.

Gas Chromatography-Mass Spectrometry (GC-MS)——Plasma, urine, and bile samples were trimethylsilylated with 30 μl of a 25% solution of N,O-bis(trimethylsilyl)acetamide in acetonitrile at 60°C for 30 min and 3 μl aliquots of the solution were injected for GC-MS analysis.

GC-MS and mass chromatography were carried out on a JMS D-300 mass spectrometer equipped with a glass column (2 m × 2 mm i.d.) containing 3% OV-17 on Gas Chrom Q (80—100 mesh). The column temperature was programmed at 3°C/min from 200°C to 250°C. Helium was used as a carrier gas at a flow-rate of 30 ml/min.

Mass fragmentography (MF) was performed on an LKB-9000 B machine coupled to a glass column (0.5 m × 3 mm i.d.) packed with 3% OV-17 on Gas Chrom Q at 240°C. The ionization voltage was 70 eV and the acceleration voltage was 3.5 kV. The ions selected for quantification of the metabolites were *m/e* 473 for trilostane, *m/e* 399 for **M-1**, *m/e* 474 for **M-2**, and *m/e* 473 for **M-3**.

Results and Discussion

Identification of Biliary and Urinary Metabolites

In order to trace the administered compound, the ion cluster technique of GC–MS was used.[5] Monitoring the doublet ions of $M^{+\cdot}$ and $[M+1]^{+\cdot}$ (ion cluster), which are important indexes for the original compound, permits easy detection of drug-derived peaks.

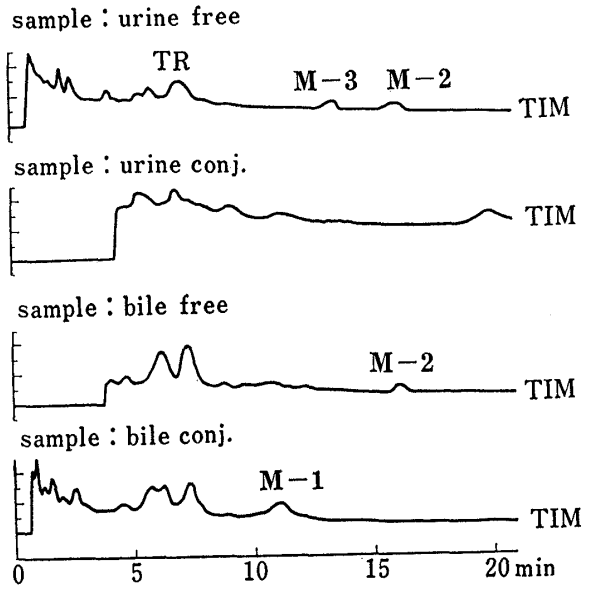


Fig. 1. Total Ion Monitoring of Urinary and Biliary Metabolites of Trilostane (TR)

A 1:1 mixture of trilostane and trilostane-1-d_1 was administered to rats and the bile and the urine collected were treated according to the method described in Chart 3. Their total ion monitoring (TIM) traces are shown in Fig. 1, where clear doublet ions diagnostic for the detection of metabolites appeared at retention time (t_R) of 7.1, 11.0, 12.2, and 15.9 min.

In bile, the peak with a t_R value of 11.0, which was named **M-1**, was detected in the fraction of conjugated metabolites and **M-2** at t_R 15.9 was detected in that of free metabolites. In urine, the peak at t_R 7.1 corresponded to unchanged trilostane. Two other peaks were named in order of their t_R values, *i.e.*, **M-3** (t_R 12.2) and **M-2** (t_R 15.9).

Metabolites **M-1**, **M-2**, and **M-3** were isolated according to the procedure summarized in Chart 2. Vary small amounts of two other metabolites named **M-4** and **M-5**, which were not detected by the ion cluster method, were isolated as a mixture from the fraction of **M-3**.

The mass spectrum of **M-1**, mp above 300°C, showed a molecular ion peak at *m/e* 327, indicating a shift to lower level by 2 u, which suggested that **M-1** was an oxidation product of trilostane. In the IR spectrum of **M-1**, the bands at 2180 and 1730 cm^{-1} were assigned to

NII-Electronic Library Service

nitrile and 5-membered ring ketone, respectively. In the NMR spectrum, the signal of C-4-H on the epoxide ring appeared at δ 3.53 in addition to the signals assigned to the protons of the C-18 and C-19 methyl groups at δ 0.89 and 0.94. On the basis of this evidence, it was concluded that **M-1** is 2α-cyano-4α,5α-epoxyandrostane-3,17-dione, and it was confirmed to be identical with an authentic sample.[4]

Metabolite **M-2**, mp 223—226°C, showed bands at 3400, 2230, and 1745 cm^{-1} in the IR spectrum, indicating the presence of hydroxy, nitrile, and 5-membered ring ketone moieties, respectively. The mass spectrum of **M-2** showed a peak at *m/e* 345 ($M^{+\cdot}$) which shifted to *m/e* 489 upon trimethylsilylation (Fig. 2). The increase of 144 u suggested the presence of two trimethylsilyl groups. The NMR spectrum of **M-2** indicated the presence of two methyl groups at δ 0.96 and 1.08 and one methine proton (C-4-H) on the epoxide ring at δ 2.70 as a doublet (J=4.5 Hz). Two signals at δ 4.13 (1H, t, J=4.5 Hz) and δ 4.30 (1H, m) suggested that **M-2** possessed two secondary hydroxy groups, one of which was expected to be at C-3 from the coupling constant (J=4.5 Hz) between the C-3 and C-4 protons. The infrared carbonyl band at 1745 cm^{-1} of **M-2**, displaced from that of **M-1** at 1735 cm^{-1}, indicates that one secondary hydroxy group is in a position affecting the C-17 substituent, perhaps at C-16. The absence of absorption between 1425 and 1400 cm^{-1} indicated that there was no unsubstituted methylene group adjacent to the ketone function. Consideration of these results led to the plane structure corresponding to 2α-cyano-3α,16α-dihydroxy-4α,5α-epoxyandrostane-17-one for **M-2**. The stereochemistry of **M-2** was established by comparison with an authentic sample, preparation of which will be reported in the following paper.[4]

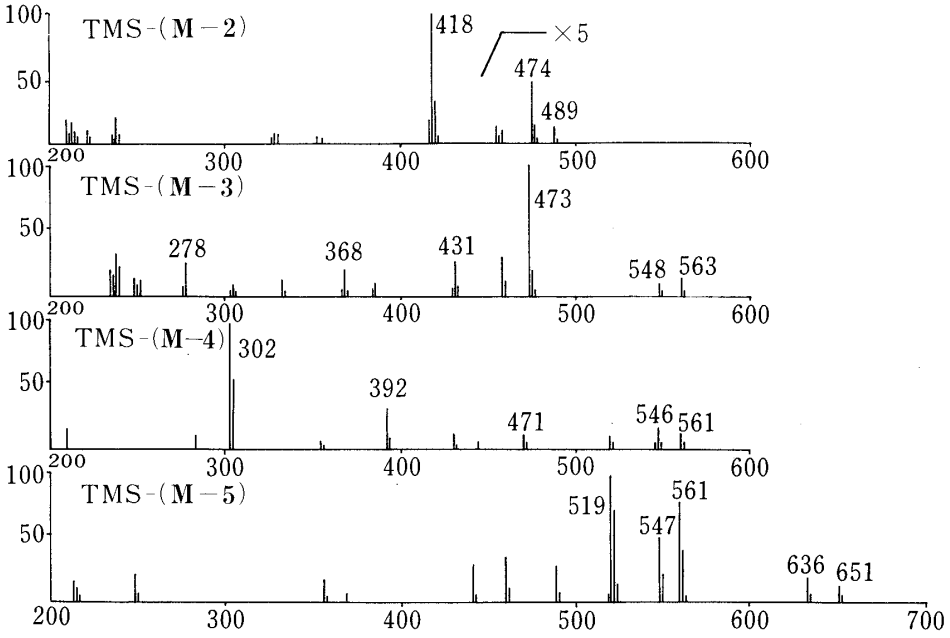


Fig. 2. Mass Spectra of Trimethylsilylated **M-2**, **M-3**, **M-4**, and **M-5**

The GC–MS spectrum of **M-3** showed the molecular ion peak at *m/e* 563, indicating that **M-3** had three trimethylsilyl (TMS) groups (Fig. 2). Therefore, the molecular weight of **M-3** is 347, an increase of 2 u over **M-2**.

Sodium borohydride reduction of **M-2** in THF gave two products. Comparison of the GC–MS spectra of the two products showed the same molecular ion peak (*m/e* 563) but the values of t_R (10.8 and 12.2) and the relative intensities of the fragment ion peaks were apparently different. The peak with larger t_R value and its mass spectrum were found to be identical with those of **M-3**. These data imply that the C-17 ketone of **M-2** has been reduced

NII-Electronic Library Service

to a hydroxy group. The structure of **M-3** can thus be assigned as 2α-cyano-4α,5α-epoxy-androstane-3α,16α,17β-triol. The configurations of the hydroxyl groups were established by comparison of the metabolite with an authentic sample.[4]

The mass spectrum of trimethylsilylated **M-4** showed the molecular ion peak at *m/e* 561 (Fig. 2). Allowing for three TMS groups, **M-4** has a molecular weight of 345 and can be assigned as a monohydroxylated derivative of trilostane. On the basis of the metabolic pathway of trilostane, the hydroxyl group was tentatively placed on the C-16 position.

The mass spectrum of **M-5** showed the molecular ion peak of the TMS derivative at *m/e* 651, which suggested that **M-5** had one additional trimethylsilyloxy group compared to **M-3** (Fig. 2) and thus was a hydroxylated product of **M-3**.

Since only small amounts of **M-4** and **M-5** had been isolated, further evidence of the structures of **M-4** and **M-5** could not be obtained.

Metabolism in Rats

The urinary and biliary excretions of trilostane and its metabolites after oral administration of the drug are given in Tables I and II, respectively. The total amount of the metabolites in urine was about 3% of the dose during 24 h. The amount of trilostane recovered was 2.7%. In the first 24 h, 0.3% of the dose was excreted as **M-2**. Other metabolites **M-1**, **M-3**, **M-4**, and **M-5** were present in such a small amount (less than 0.1%) that their amounts could not be determined. In addition, conjugated metabolites in urine were not detected in these experiments.

On the other hand, in the rats with ligated bile duct, 30.2% of the dose was excreted in the bile. The main biliary metabolite was conjugated **M-1**. The bile collected during 24—48 h after administration contained 3.7% of **M-1** in a conjugated form, while there was no more than a trace of other metabolites.

Table I. Urinary Excretion of Trilostane (TR) and Its Metabolites in Rats

Period (h)	Free			Conjugate		
	TR	M-1	M-2	TR	M-1	M-2
0—24	2.7±0.6	N.D.	0.3±0.1	N.D.	N.D.	N.D.
24—48	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

values are means (% of dose)±S.D. for threee rats.

Table II. Biliary Excretion of Trilostane (TR) and Its Metabolites in Rats

Period (h)	Free			Conjugate		
	TR	M-1	M-2	TR	M-1	M-2
0—24	N.D.	N.D.	0.9±0.2	N.D.	25.7±0.9	N.D.
24—48	N.D.	N.D.	N.D.	N.D.	3.6±0.3	N.D.

Values are means (% of dose)±S.D. for three rats.

These results show significant differences between the urinary and biliary excretions: the biliary metabolites are largely present in conjugated form, in contrast to the free form for the urinary metabolites, and biliary excretion is more important in trilostane metabolism than urinary excretion.

The moderate percentage (32.3%) of the intake excreted in the urine and the bile during two days after administration suggests the possibility of fecal excretion of trilostane. Moreover, trilostane might also be metabolized in part to a water-soluble polyhydroxylated compound which could not be isolated by the procedure described in Chart 2.

NII-Electronic Library Service

Figure 3 shows the plasma levels of trilostane and **M-1** in rats. The time of maximum plasma concentration of trilostane was 2.0 h after administration, suggesting rapid formation of **M-1** from trilostane. Since **M-1** is the major metabolite in bile, it is reasonable to consider that **M-1** might be the first intermediate in the metabolism of trilostane. The formation of **M-1** could be explained by oxidation of the C-17 hydroxy group, which is common in the metabolism of androstanes.[6]

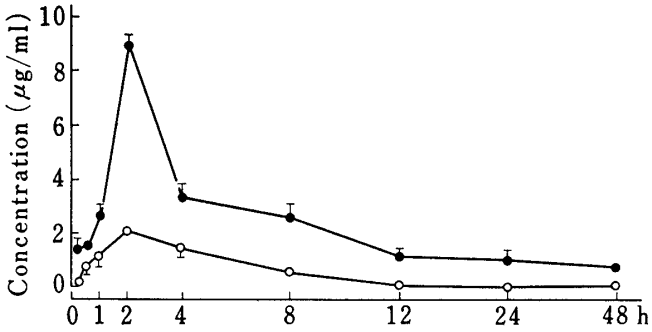


Fig. 3. Plasma Levels of Trilostane and **M-1**
—○— trilostane, —●— **M-1**.

On the basis of these results, we proposed the metabolic pathways of trilostane shown in Chart 4. At this point, we should take into account the possibility of two pathways to **M-3**. Oxidation of the D-ring of **M-1** followed by reduction of the A-ring might lead to **M-2**, or *vice versa*. Metabolite **M-3** should be formed by the reduction of **M-2** and **M-4**. It is natural to assume that the pathway to **M-3** *via* **M-2** might be a major route, because **M-2** is present in a larger amount than **M-4** in urine and bile.

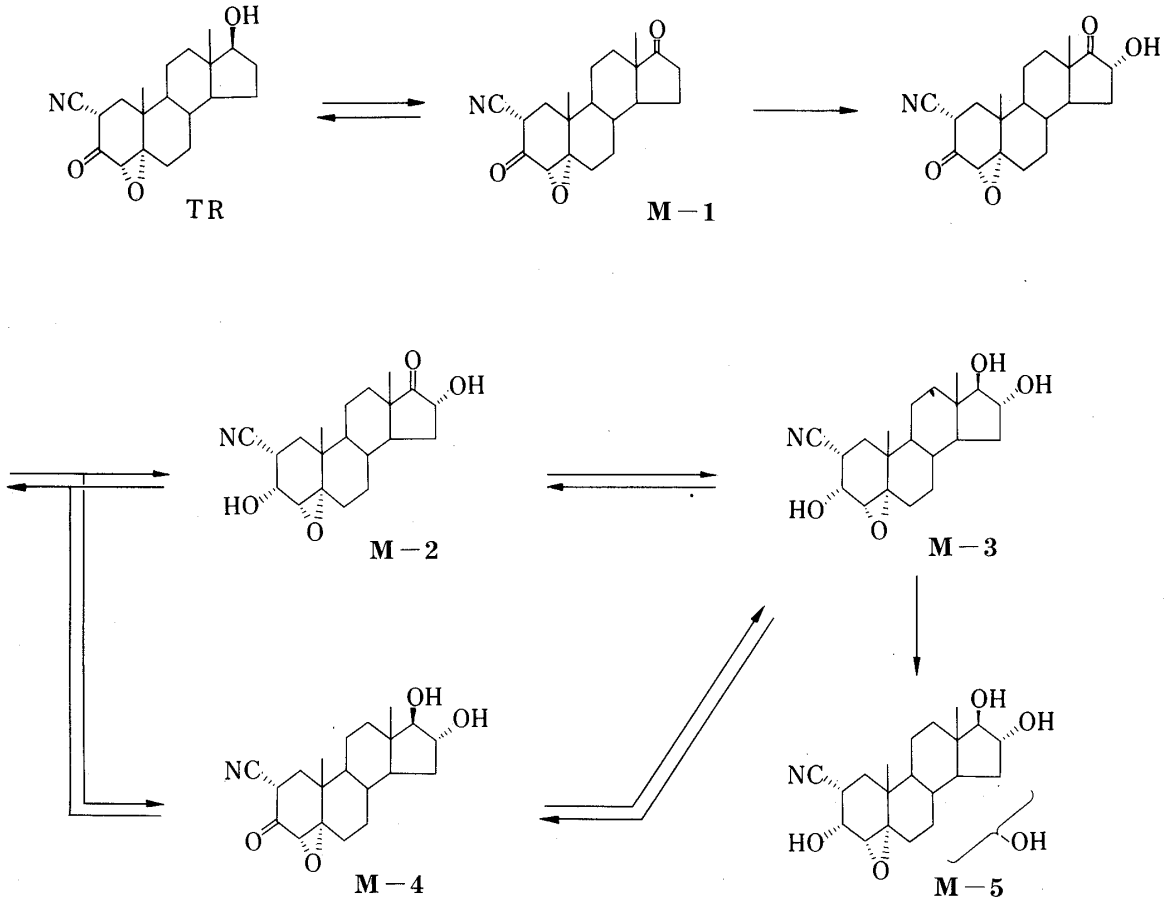


Chart 4. Proposed Metabolic Pathways of Trilostane

Although the identified metabolites account for about 32% of the dose, it is of particular interest that all of these metabolites contain an epoxide ring, which is readily subject to ring-opening reactions both *in vivo* and *in vitro*. We could not find any epoxy ring-opened metabolite of trilostane in urine or bile, but further work using [^{14}C]-labeled trilostane is in progress.

NII-Electronic Library Service

References and Notes

1) A preliminary communication of this work appeared in *J. Pharm. Dyn.*, 3(7), s-10 (1980).
2) H.C. Neumann, G.O. Potts, W.T. Ryan, and F.W. Stonner, *J. Med. Chem.*, **13**, 948 (1970).
3) After this paper had been prepared, a study on trilostane was reported: J.F. Baker, D. Benziger, B.W. Chalecki, S. Clemans, A. Fritz, P.E. O'Melia, L. Shargel, and J. Edelson, *Arch. int. Pharmcodyn. Thér.*, **243**, 4 (1980).
4) Y. Mori, M. Tsuboi, and M. Suzuki, *Chem. Pharm. Bull.*, **29**, 2478 (1981).
5) A. Tatematsu, H. Miyazaki, M. Suzuki, and Y. Maruyama, "Practical Mass Spectrometry for Medical and Pharmaceutical Sciences," Kodansha Ltd., Tokyo, 1979, pp. 135—184.
6) K. Fortherby, *Biochem. J.*, **67**, 259 (1957); J.E. Bridgeman, P.C. Cherry, A.S. Clegg, J.M. Evans, E.R.H. Johnes, A. Kasal, V. Kumar, G.D. Meakins, Y. Morisawa, E.E. Richards, and P.D. Woodgate, *J. Chem. Soc. (C)*, **1970**, 250.

NII-Electronic Library Service