IDENTIFICATION BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY OF INTERMEDIATES IN THE AROMATIZATION OF MODIFIED C_{19} STEROIDS BY HUMAN PLACENTAL MICROSOMES[1]

W. Emmett Braselton, Jr.,[2] James C. Orr[3] and Lewis L. Engel[4]

From the John Collins Warren Laboratories of the Collis P. Huntington Memorial Hospital of Harvard University at the Massachusetts General Hospital and the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts, 02115.

Received: 7/29/74

ABSTRACT

Analogs of 4-androstene-3,17-dione and testosterone were tested as substrates for the aromatizing enzyme complex of human placenta. Compounds modified in rings B, C, and D were found to be aromatized via a pathway similar to that postulated for 4-androstene-3,17-dione and testosterone, in which oxidation to the 19-hydroxy and 19-oxo (or corresponding gem-diol) intermediates occurs. No evidence of additional intermediates was obtained.

Many compounds[5] have been tested for their acceptability as substrates for the aromatizing enzyme system of human placental microsomes (for reviews see: 6,7,8). In those studies the major interest was in the capability of the aromatizing system to form phenolic products. No attempts appear to have been made to identify intermediates or to determine at what stage in the sequence the reaction stopped if no estrogens were detected. Some of those studies also lacked rigorous identification of products. Identification of intermediate metabolites could be of value toward elucidation of the mechanism of aromatization of the modified precursors. The development of a procedure for identification of the intermediates of steroid aromatization, using gas chromatography-mass spectrometry (9) made possible a study of the course of aromatization of a number of structurally modified substrates by human placental microsomes. It was also possible, with the above

procedure, to show the aromatization of 4,6-androstadiene-3,17-dione, a compound previously thought not to be a substrate (10).

MATERIALS AND METHODS

Steroids. 4,6-Androstadiene-3,17-dione and 17β-hydroxy-1,4-6-androstatrien-3-one were obtained from Steraloids, Inc.; 17β-hydroxy-4,9(11)-androstadien-3-one from the Medical Research Council Reference Collection; 17β,19-dihydroxy-17α-methyl-4-androsten-3-one was a gift from Dr. M. Wolff; and 19-hydroxy-4,6-androstadiene-3,17-dione a gift from Dr. G. Kruger.

4-Androsten-3-one was prepared as follows: 5-Androsten-3β-ol was prepared by Wolff-Kishner reduction of 3β-hydroxy-5-androsten-17-one (40 g) with hydrazine (40 ml) and a solution of sodium (10 g) in diethylene glycol under reflux for 70 hours. The product was partially evaporated, poured into water, washed, recrystallized from methanol, and then from hexane to give felted needles, mp 137° identical with the published value (11). It was shown to be homogeneous and of the correct molecular weight by GC-MS (M^+274).

Oxidation of the 3β-alcohol with chromium trioxide in aqueous acetone (12) and recyrstallization from methanol gave, unexpectedly, 3,3-dimethoxy-5-androstene, mp 113-116°, λ^{EtOH}_{max} 240 nm, ε0.805, NMR ($CDCl_3$) δ0.732 ppm (18-H_3), 1.020 (19-H_3), 3.12, 3.22 (3,3-bis-OCH_3), 5.37 (broad doublet), J = 6Hz due to 6-H coupled to 7β-H (13). Analysis: calculated for $C_{21}H_{34}O_2$: C, 79.19; H, 10.76; 0, 10.05; found C, 78.70, 78.09; H, 10.03, 10.74, 0, 11.68, 11.03. GC-MS gave two peaks of approximately equal height, both of M^+ 286, presumably corresponding to flash-heater loss of methanol to give 3-methoxy-2,4-androstadiene and 3-methoxy-3,5-androstadiene.

Treatment of the ketal in benzene with aqueous hydrochloric acid gave 4-androstene-3-one, recrystallized three times from methanol to mp 104°, identical to the literature value (14). This enone was shown to be homogeneous by TLC and GC-MS, M^+ 272. The NMR spectrum ($CDCl_3$) 0.770 (18-H_3), 1.201 (19-H_3) and 5.79 (4-H, singlet) is in good accord with the assigned structure.

4-Androstene-3α,17β-diol was prepared by reduction of testosterone (2 g) with sodium borohydride in ethanol. Chromatography on alumina (80 g, activity III) and elution with ether gave 4-androstene-3β, 17β-diol crystallized from methanol to mp 151-154°, and characterized by NMR (15). Elution with 5% to 25% methanol gave 4-androstene-3α,17β-diol, recyrstallized from methanol to mp 230-235°.

Aromatizing enzymes. Washed human placental microsomes were prepared according to the procedure of Ryan (16) and stored at -70° in sealed ampoules.

Incubations. Each flask contained 200 nmoles of substrate, 12 μmoles NADP, 56 μmoles glucose-6-phosphate, 2 unit of glucose-6-phosphate dehydrogenase, and 50 mM sodium phosphate buffer, pH 7.0, in a total volume of 3 ml. The flasks were incubated for 30 min. in air at 37° to generate NADPH, then the aromatization reaction was started by addition of 2 ml of microsomes (equivalent to 10 g wet weight of tissue) and continued for 1 hr. Incubations with 19-hydroxy-17α-methyltestosterone (5), 4-androsten-3-one, and epitestosterone were with 400 nmoles of substrate. The 19-hydroxy-4,6-androstadiene-3,17-dione and 19-hydroxyandrostenedione were incubated for 15, 30, and 60 min. The reactions were stopped by addition of 5 ml of dichloromethane and vigorous shaking.

Purification of steroids. Steroids were isolated by extraction of the incubation medium with dichloromethane, evaporation, and partition of the residue between 90% methanol and hexane. The residue from the methanol phase was partitioned between ethyl acetate and water, and the residue from the ethyl acetate phase chromatographed on hydroxyalkoxypropyl Sephadex, as described in detail elsewhere (9).

Thin layer chromatography. The fractions recovered from hydroxyalkoxypropyl Sephadex chromatography that contained steroids were fractionated further by TLC on plates of fluorescent Silica gel G (Quantum Industries). Solvent systems used were: A, diethyl ether/benzene (5:4); B, chloroform/methanol (99:1); C, chloroform/ethyl acetate/methanol (1:1:1); and D, diethylether/benzene/cyclohexane (1:1:1). Compounds were located by examination under ultraviolet light, and by spraying with sulfuric acid/ethanol (1:1) and heating (110°). Phenolic steroids were detected specifically by spraying the plates with Phenol reagent (Fisher) followed by exposure to ammonia vapor (17). Following incubation of 19-hydroxy-4,6-androstadiene-3,17-dione, TLC was carried out in two dimensions. System A was used in the first direction, the plates were turned 90° and the zones concentrated by running 0.5 cm past the starting spot twice with system C. They were then developed in system B.

For identification of steroids by GC-MS, the zones were scraped from unsprayed plates and the zones containing steroids eluted with methanol/ethyl acetate (1:1).

Gas chromatography-mass spectrometry. Trimethylsilyl ethers and methoxime-trimethylsilyl ethers were prepared for solid injection as described previously (18,9). GC-MS analyses were performed on an LKB 9000 chromatograph -mass spectrometer under operating conditions previously described (70 eV) (9). Quantitation was achieved from the total ion current tracing by measuring the peak height of the steroid relative to that of an internal standard (5β-pregnane-3α,20α-diol diacetate) and extrapolating from a standard curve.

Incubation with 17β-estradiol dehydrogenase. The α-configuration of the phenolic diol isolated after incubation of epitestosterone was established by the fact that it was recovered unchanged after

Table 1. Metabolism by human placental microsomes of substrate analogs modified in ring D.

Substrate Metabolites	TLC[a]		GC-MS[b]		
	R_f	Phenol reagent	Derivative	Rrt[c]	M^+
17β,19-Dihydroxy-17α-methyl-4-androsten-3-one	0.12(0.10)[d]	-	19-TMSi-MO	1.05	419
17β-Hydroxy-17α-methyl-3-oxo-4-androsten-19-al	0.32	±[e]	$(MO)_2$	1.05	374
17α-Methyl-1,3,5(10)-estratriene-3,17β-diol	0.50 (0.49)	+	3-TMSi	0.80	358
Epitestosterone	0.21(0.19)	-	TMSi	0.55(0.55)	360
19-Hydroxyepitestosterone	0.05	-	$(TMSi)_2$	0.95	448
19-Oxoepitestosterone	0.14	±[e]	$(MO)_2$-TMSi	1.00	432
17α-Estradiol	0.41(0.40)	+	$(TMSi)_2$	0.62(0.60)	416
4-Androsten-3-one	0.52(0.53)[f]	-	MO	0.47(0.47)	301
19-Hydroxy-4-androsten-3-one	0.19[f]	-			
1,3,5(10)-Estratrien-3-ol	0.55[f]	+	TMSi		328

[a]The solvent system for TLC was ether/benzene (5:4) except where otherwise noted.

[b]1% OV-1 on Gas-ChromQ; column size 1.8 m x 4 mm (id); column temperature 200°; helium flow 27 ml per min.

[c]Retention time relative to 5α-cholestane.

[d]Numbers in parentheses refer to values for authentic reference compounds.

[e]19-Aldehydes give a faint positive reaction with phenol reagent (9).

[f]The solvent system for TLC was ether/benzene/cyclohexane (1:1:1).

incubation with human placental 17β-estradiol dehydrogenase (kindly supplied by Dr. D.J.W. Burns). This enzyme has absolute stereospecificity for 17β-hydroxyl groups (19). The conditions of the incubation were as follows: Each flask contained 0.2 mM NAD+, 57 mU of 17β-estradiol dehydrogenase, 20μmoles of either estradiol-17β, estradiol-17α, or the phenolic compound isolated from the incubation, in a total of 3.0 ml of 0.1 M sodium bicarbonate buffer, pH 8.75. The incubations were for 10 min at room temperature. The aqueous solution was extracted twice with dichloromethane; estrogens were separated by TLC in solvent system A, and detected by Phenol reagent as described above.

RESULTS

The metabolites identified following incubation of ring D modified steroids are shown in Table 1. The compounds were characterized by their TLC mobilities, ultraviolet absorption on fluorescent TLC plates, a color reaction specific for phenolic steroids (17), GLC retention times, and mass spectra.

After incubating 19-hydroxy-17α-methyltestosterone with placental microsomes, the 19-aldehyde and 17α-methylestradiol were identified. The mass spectrum of the isolated 17α-methyl-19-oxotestosterone as the bis-methoxime is shown in Fig. 1. Under the conditions employed, the tertiary 17β-hydroxyl group does not form the trimethylsilyl ether. The spectrum is similar to that of the bis-methoxime of 19-oxotestosterone shown, but with differences of 14 a.m.u. in the ions containing ring D. The prominent fragment at M-31 is due to loss of a methoxyl group, characteristic of methoximes. The groups of fragnents at M-58, M-59, M-62 and M-63, and at M-89 and M-90 are characteristic of all 19-oxo-steroid methoximes studied to date (9). The ions at M-58 and M-89 are probably due to loss of the C-19 group ($-C=NOCH_3$), and C-19 plus an additional methoxyl group.

The 17α-methylestradiol recovered from TLC was identified by GC-MS as its 3-trimethylsilyl ether, with molecular ion at m/e 358 and other ions of interest at M-15, M-18, and M-(15+18). Fragments

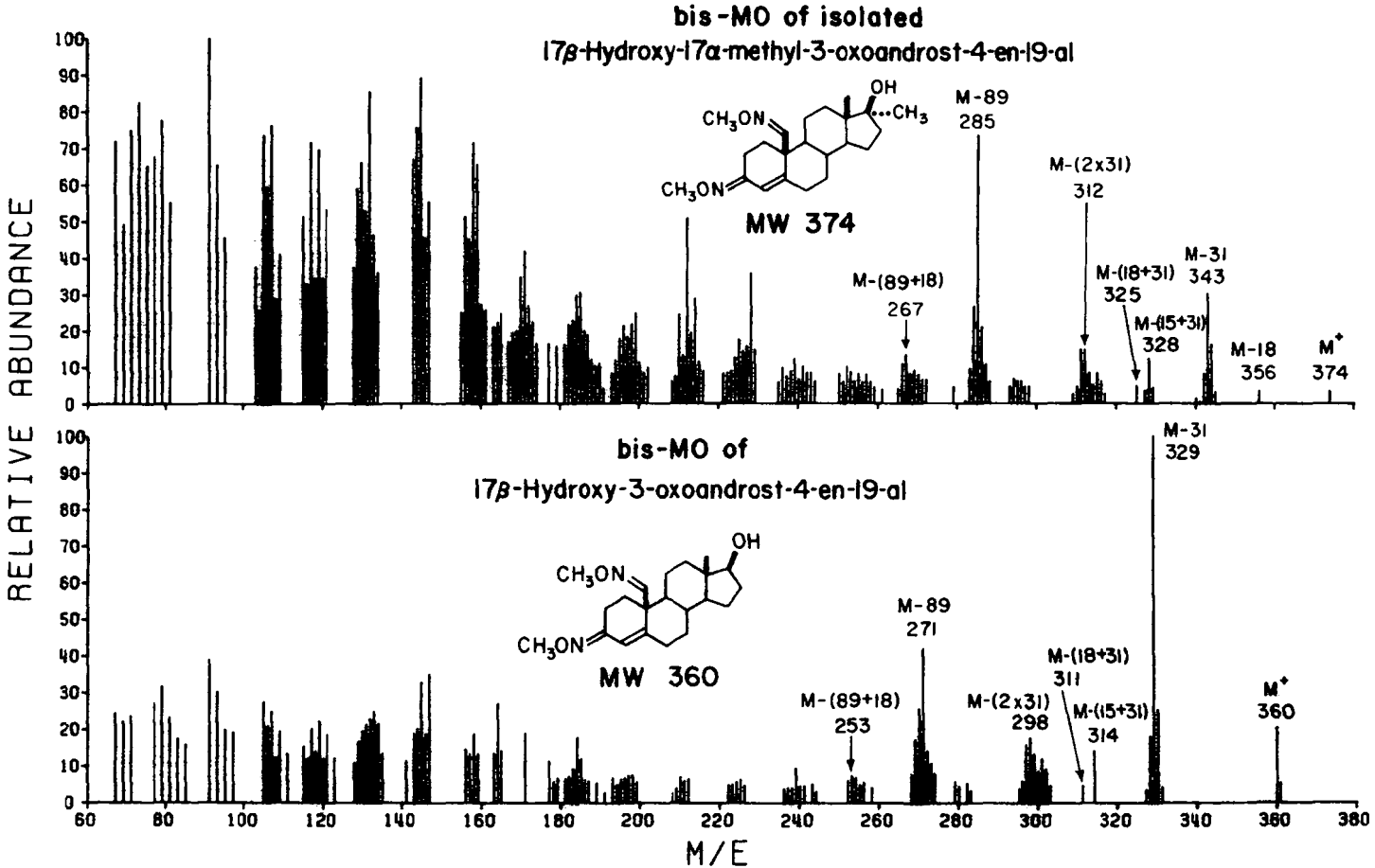

Fig. 1. Mass spectra of bis-methoxime of 17α-methyl-19-oxotestosterone isolated from incubation of 19-hydroxy-17α-methyltestosterone(top), and bismethoxime of 19-oxotesterone reference steroid (bottom).

characteristic of estrogens and derived from rings A, B, and C (20, 21, 22) were also present (Table 2) and the spectrum was identical to that of an authentic reference sample.

Epitestosterone was aromatized to estradiol-17α by the human placental microsomes; the 19-hydroxy and 19-oxo intermediates were identified (Table 1). The mass spectrum of the isolated 19-hydroxy-epitestosterone $(TMSi)_2$ was similar to that of 19-hydroxytestosterone $(TMSi)_2$. Fragments of interest were M^+ 448, M-30, M-90, M-103, due to loss of the primary alcohol trimethylsilyl ether (C-19)(9), M-(90+30), M-(90+103), m/e 129 which arises from fragmentation of ring D (23) and m/e 103 (C-19 group).

The mass spectrum of the recovered 19-oxoepitestosterone $(MO)_2$-TMSi (Fig. 2) was similar to that of 19-oxotestosterone $(MO)_2$-TMSi (9). The spectrum was characterized by the groups of fragments at M-58, M-59, M-62, M-63 and M-89, M-90 as discussed above in relation to other compounds containing the 19-aldehyde methoximes.

The mass spectrum of the trimethylsilyl ether of the isolated estradiol-17α was that of a typical estrogen (Table 2) and identical to that of a sample of authentic estradiol-17α $(TMSi)_2$. Since the 17α- and 17β-hydroxy epimers cannot easily be distinguished by their mass spectra, the isolated compound was identified as the 17α-epimer by incubating it with the soluble human placental 17β-estradiol dehydrogenase. The isolated phenolic compound and authentic estradiol-17α remained unchanged, while estradiol-17β was completely oxidized to estrone.

4-Androsten-3-one was aromatized (Table 1) but the intermed-

Table 2. Prominent mass spectral ions of phenolic steroids isolated after aromatization of substrate analogs by human placental microsomes.

Compounds	Ions (m/e)												
	M^+	M-15	M-18	M-33	M-90	n[a]	k[a]	b[a]	h[a]	f[a]	c[a]	m/e 232[b]	m/e 129
Estradiol $(TMSi)_2$	416	401	-[d]	-	326	285	388	258	257	244	218	+[c]	+
Estrone TMSi	342	327	-	-	-	285	314	-	257	244	218	+	-
17α-Methylestradiol 3-TMSi	358	343	340	325	-	285	-	258	257	244	218	+	-
17α-Estradiol$(TMSi)_2$	416	401	-	-	326	285	-	258	257	244	218	+	+
1,3,5(10)-Estratrien-3-ol TMSi	328	313	-	-	-	285	300	-	257	244	218	-	-
$\Delta^{9(11)}$-Estrone TMSi	340	325	-	-	-	283	312	-	-	244	218	-	-
Δ^6-Estrone TMSi	340	325	-	-	-	283	312	256	255	242	216	+[e]	-
Δ^6-Estradiol$(TMSi)_2$	414	399	-	-	324	283	-	256	255	242	216	+[e]	+

[a]Fragment ions of estradiol $(TMSi)_2$ and estrone TMSi described in (21)

[b]Fragment described in (19)

[c]Ion present

[d]Ion absent or not prominent

[e]Ion present at m/e 230

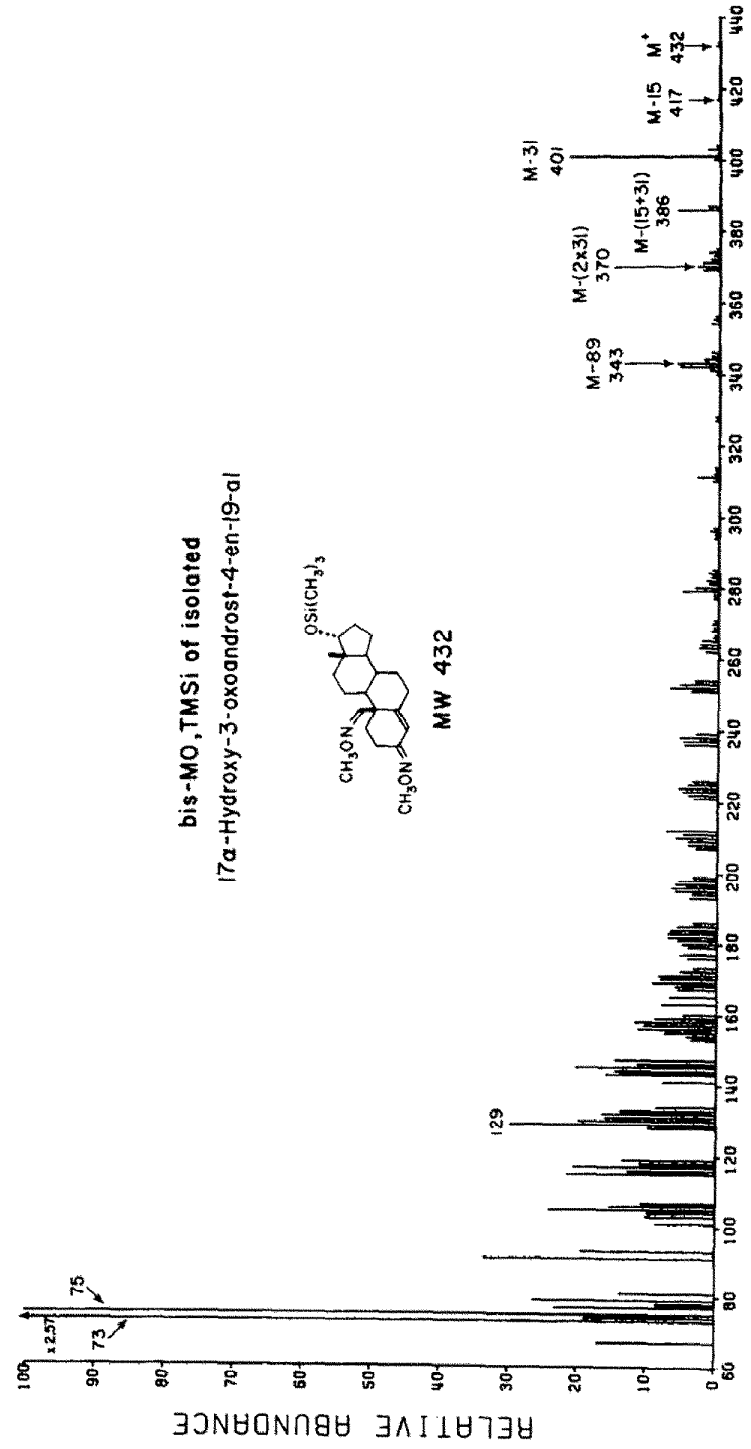

Fig. 2. Mass spectrum of the bis-methoxime-trimethylsilyl ether of 19-oxoepitestosterone isolated from incubation of epitestosterone with placental microsomes.

iates were insufficient in amount to be identified positively. The mass spectral fragmentation of 1,3,5(10)-estratrien-3-ol TMSi was similar to that of the other estrogens except for the ions containing ring D (Table 2).

The substrate modified in ring C, $\Delta^{9(11)}$-testosterone, was aromatized to $\Delta^{9(11)}$-estrone (Table 3). The 19-hydroxylated intermediate was also identified, but the 19-oxo intermediate was not detected. The mass spectrum of the methoxime-bis-trimethylsilyl ether of 19-hydroxy-$\Delta^{9(11)}$-testosterone (Fig. 3) was characterized by the molecular ion M^+ 475, fragments due to loss of the C-19 group (M-103, M-(103+31)), loss of fragments containing trimethylsilanol (M-90, M-(90+30) and ring D fragmentation typical of 17-trimethylsilyloxy compounds (m/e 129).

The mass spectrum of the isolated $\Delta^{9(11)}$-estrone as the trimethylsilyl ether was identical to that of the authentic reference compound. The spectra of the $\Delta^{9(11)}$-estrogens differ somewhat from the typical pattern seen in the ring C saturated compounds due to the ability of the former to form fully conjugated systems. A Thorough discussion of the fragmentation of the $\Delta^{9(11)}$-estrogens is given by Djerassi, et al. (20).

The ring B modified substrates, 4,6-androstadiene-3,17-dione and 19-hydroxy-4,6-androstadiene-3,17-dione, were aromatized (Table 4) and the 19-hydroxy- and 19-oxo-intermediates identified by GC-MS. The mass spectrum of the bis-methoxime trimethylsilyl ether of 19-hydroxy-4,6-androstadiene-3,17-dione obtained from incubation of 4,6-androstadiene-3,17-dione with placental microsomes is compared

Table 3. Metabolism by human placental microsomes of substrate analogs modified in ring C.

Substrate Metabolites	TLC[a]		GC-MS		
	R_f	Phenol reagent	Derivative	Rrt[b]	M^+
$\Delta^{9(11)}$-Testosterone	0.33(0.33)[c]	-	MO-TMSi	0.66(0.67)	387
19-Hydroxy-$\Delta^{9(11)}$-testosterone	0.05	-	MO-$(TMSi)_2$	1.04	475
$\Delta^{9(11)}$-Estradiol	0.41(0.42)	+			
$\Delta^{9(11)}$-Estrone	0.59(0.59)	+	TMSi	0.55(0.55)	340

[a]The solvent system for TLC was ether/benzene (5:4).

[b]Retention time relative to 5α-cholestane; chromatographic conditions as in Table 1, footnote b.

[c]Numbers in parentheses refer to values for reference compounds.

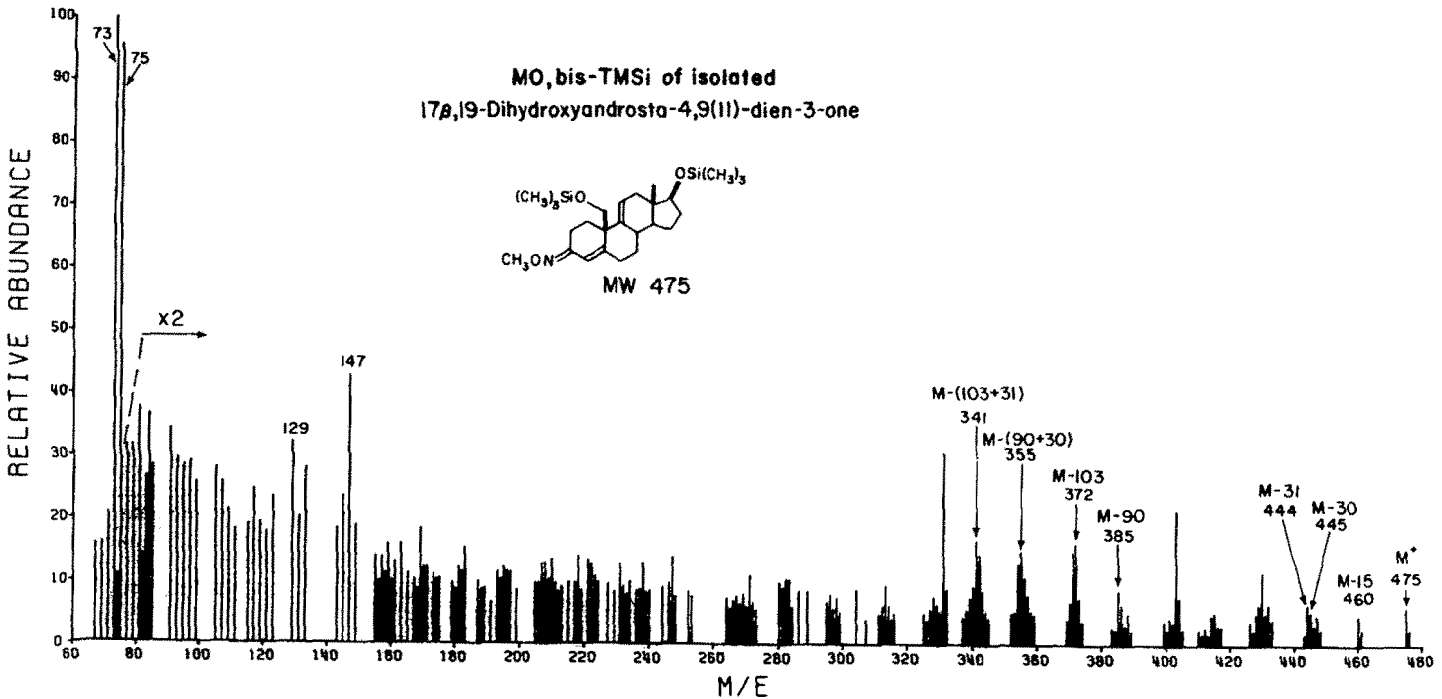

Fig. 3. Mass spectrum of the methoxime bis-trimethylsilyl ether of 19-hydroxy-$\Delta^{9(11)}$-testosterone isolated from incubation of $\Delta^{9(11)}$-testosterone with placental microsomes.

Table 4. Metabolism by human placental microsomes of substrate analogs modified in ring B.

Substrate Metabolites	TLC R_f	TLC Phenol reagent	GC-MS Derivative	GC-MS Rrt[a]	GC-MS M^+
4,6-Androstadiene-3,17-dione	0.41(0.42)[b,c]	-	$(MO)_2$	0.72(0.72)	342
Δ^6-Testosterone	0.29	-	MO-TMSi	0.78	387
19-Hydroxy-Δ^6-testosterone	0.05	-	MO-$(TMSi)_2$	1.02	475
19-Hydroxy-4,6-androstadiene-3,17-dione	0.09	-	$(MO)_2$-TMSi	0.99, 1.05 (syn & anti)	430
Δ^6-Estradiol	0.41(0.42)	+	$(TMSi)_2$	0.71(0.71)	414
Δ^6-Estradiol	0.41(0.42)	+	free	0.64(0.63)	270
Δ^6-Estrone	0.56	+	TMSi	0.56(0.56)	340
19-Hydroxy-4,6-androstadiene-3,17-dione	not determined	not determined	$(MO)_2$-TMSi	0.98, 1.04 (syn & anti)	430
19-Hydroxy-Δ^6-testosterone	not determined	not determined	$(TMSi)_2$	1.02	446
19-Oxo-Δ^6-testosterone	not determined	not determined	$(MO)_2$-TMSi	1.06	430
Δ^6-Estradiol	not determined	not determined	$(TMSi)_2$	0.72(0.72)	414
Δ^6-Estrone	not determined	not determined	TMSi	0.57(0.56)	340

[a]Retention time relative to 5α-cholestane; chromatographic conditions as in Table 1, footnote b.

[b]The solvent system for TLC was ether/benzene (5:4).

[c]Numbers in parentheses refer to values for reference compounds.

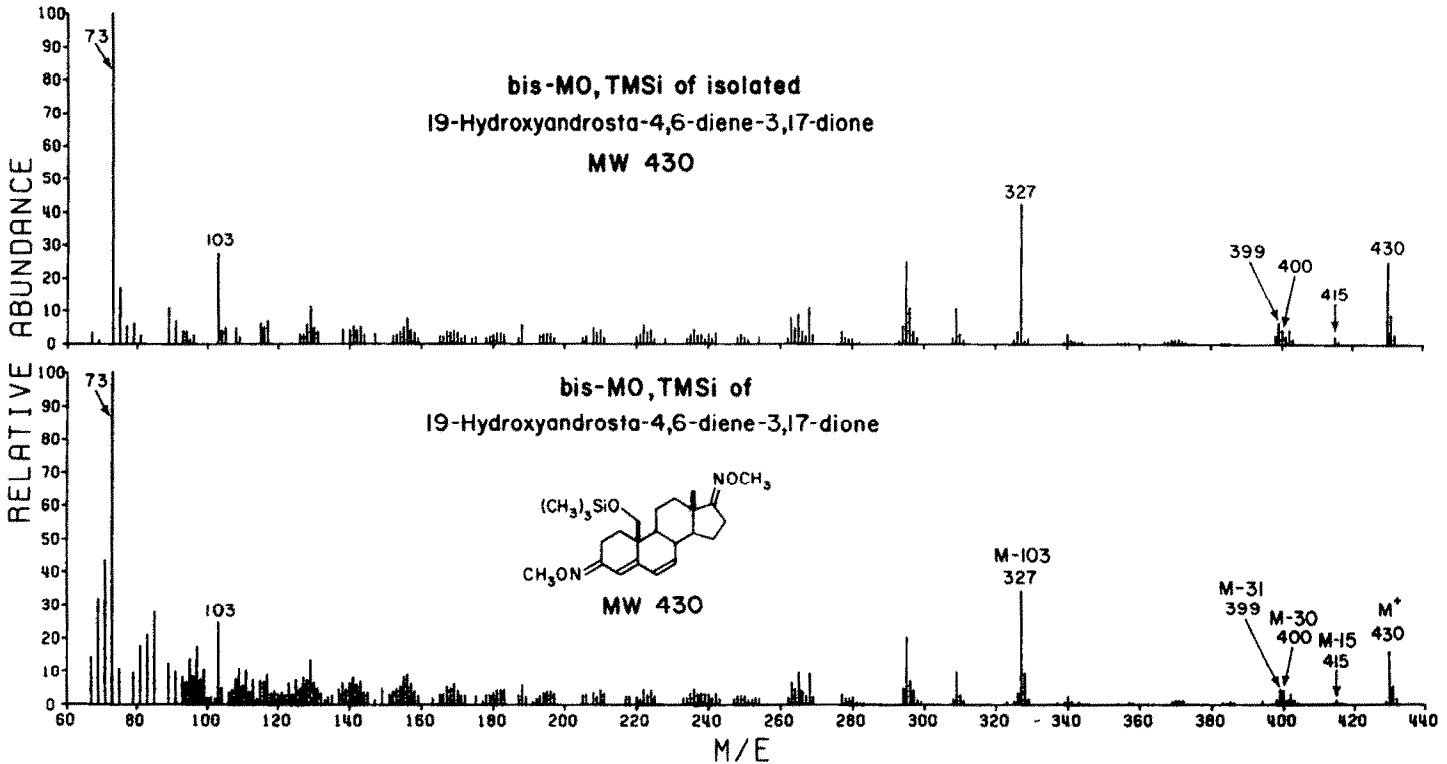

Fig. 4. Mass spectra of the bis-methoxime trimethylsilyl ether of 19-hydroxy-4,6-androstadiene-3,17-dione(top)isolated from incubation of 4,6-androstadiene-3,17-dione and of reference compound(bottom).

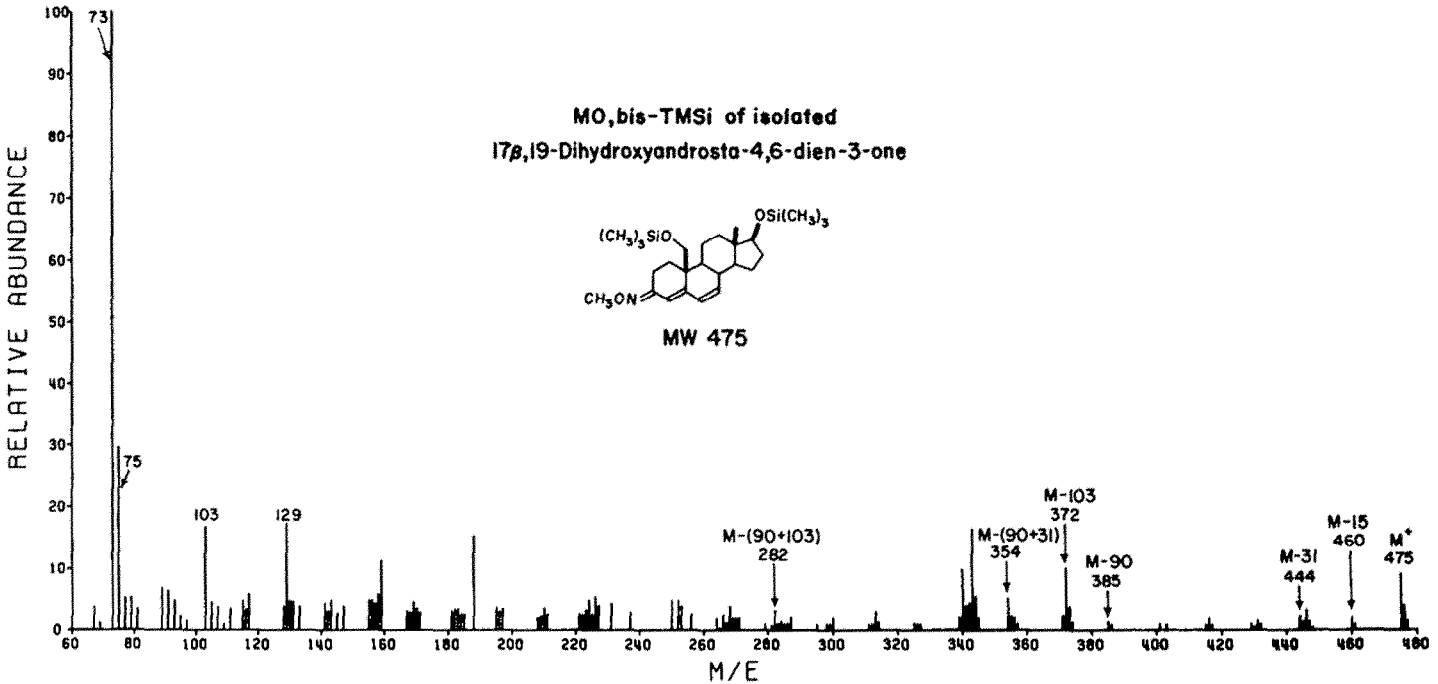

Fig. 5. Mass spectrum of the methoxime bis-trimethylsilyl ether of 19-hydroxy-Δ^6-testosterone isolated from incubation of 4,6-androstadiene-3,17-dione with placental microsomes.

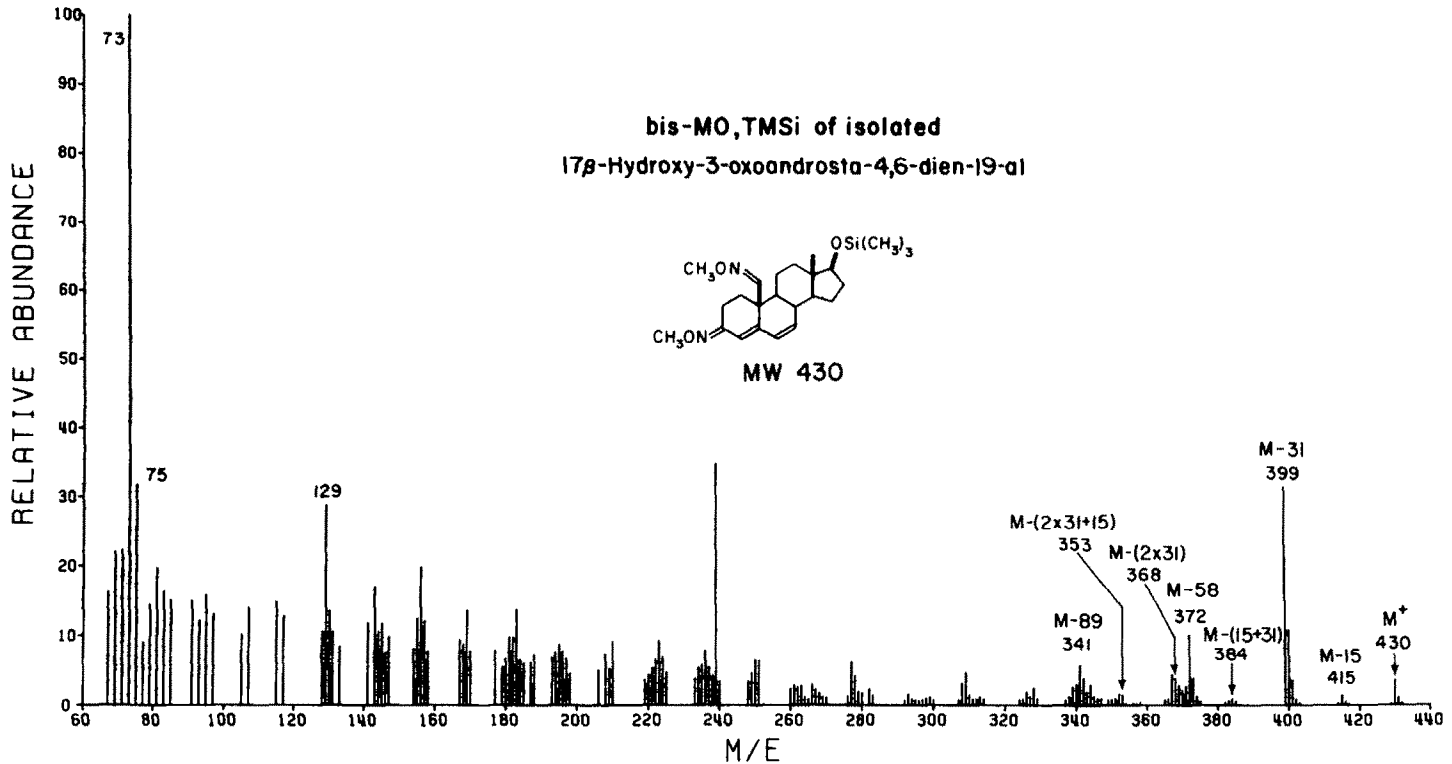

Fig. 6. Mass spectrum of the bis-methoxime trimethylsilyl ether of 19-oxo-Δ^6-testosterone isolated from incubation of 19-hydroxy-4,6-androstadiene-3,17-dione with placental microsomes.

with that of the reference compound in Fig. 4. The corresponding 19-hydroxy-Δ^6-testosterone was also formed during incubation, and the mass spectrum of its methoxime bis-trimethylsilyl ether is shown in Fig. 5. The mass spectrum of the bis-methoxime trimethylsilyl ether of the isolated 19-oxo-Δ^6-testosterone is given in Fig. 6. The fragmentation is similar to that of the other 19-oxo compounds discussed and comprises the following: an intense ion at M-31, and characteristic groups of fragments at M-58, M-59, M-62, M-63 and M-89, M-90. The ion at m/e 129 is derived from ring D.

The mass spectra of the trimethylsilyl ethers of the 6-dehydroestrogens obtained from incubation of the ring B modified substrates are outlined in Table 2, and were identical to the spectra of reference steroids. The spectra are similar to those of estrone and estradiol (Table 2) with a shift of 2 a.m.u. lower due to the additional double bond. A highly characteristic ion of the 6-dehydroestrogens was found at m/e 229 and is due to the formation of a favored benztropylium ion (20, 21).

The rate of formation of estrogens from B and C ring modified substrates is compared with the formation of estrone and estradiol from androstenedione in Table 5. After 60 min, $\Delta^{9(11)}$ -testosterone gave only 11%,4,6-androstadiene-3,17-dione, 26% as much phenolic steroid as did the corresponding unsubstituted compounds. The yield of estrogen from 19-hydroxy-4,6-androstadiene-3,17-dione was about half that obtained from 19-hydroxyandrostenedione.

The A and B ring modified substrate analog 17β-hydroxy-1,4,6-androstatrien-3-one was aromatized in low yield. The phenolic products were detected by TLC (phenol reagent positive) but

Table 5. Extent of aromatization of natural substrates and substrate analogs by human placental microsomes.

Substrate	Incubation time (min)	Estrone or analog (nmoles)	Estradiol or analog (nmoles)	Total (nmoles)	Extent relative to: Androstenedione	Extent relative to: 19-Hydroxyandrostenedione
Androstenedione	30	15	70	85	1.0	
"	60	23	117	140	1.0	
Testosterone	30	18	66	84	1.0	
$\Delta^{9(11)}$ Testosterone	60	15	trace	15	0.1	
4,6-Androstadiene-3,17-dione	60	5.5	31	37	0.3	
19-Hydroxy-androstenedione	15	5.5	98	104	--	1.0
"	30	10	165	175	2.0	1.0
"	60	12	147	159	1.1	1.0
19-Hydroxy-4,6-androstadiene-3,17-dione	15	7.8	53	61	--	0.6
"	30	5.1	74	79	0.9	0.4
"	60	6.5	90	96	0.7	0.6

insufficient product was obtained for GC-MS identification. The 19-hydroxy or 19-oxo intermediates were not seen.

Several compounds incubated with the placental microsomes were not aromatized; these were compounds that were modified in ring A. 4-Androstene-3α,17β-diol remained unchanged after incubation for 1 hour; 17β-hydroxy-5α-androstan-3-one was reduced to 5α-androstane-3β,17β-diol. No evidence of 19-hydroxylation could be found for either of the above substrate analogs.

DISCUSSION

The results provide evidence that aromatization by placental microsomes of a number of steroids modified in different parts of the molecule involves the same sequence of oxidations as has been elucidated for the major substrates, androstenedione and testosterone. Thus the pathway C_{19} steroid → 19-hydroxysteroid → 19-oxosteroid (or corresponding gem-diol) → C_{18} aromatic steroid is still followed. This is not to say that other mechanisms do not operate on certain types of compounds, such as the 19-nor steroids which obviously do not follow this pathway. Meigs and Ryan (24) have shown that the aromatization of the 19-nor compounds is sensitive, aromatization of the $\Delta^{1,4}$ compound partially sensitive, and aromatization of the normal substrates insensitive to carbon monoxide. The mechanisms of the carbon monoxide sensitive systems have not been elucidated to date.

At the outset of these experiments it had been hoped that changes in structure from the normal substrate might cause a change in the rate limiting step in the aromatization sequence.

Thus intermediates between the 19-oxo and the phenolic product might accumulate and give further information on the mechanism of elimination of C-19 and the 1β hydrogen. This was not the case with the compounds chosen. 19-Hydroxylation usually became slower, and intermediates were seen in lesser, rather than greater amounts than in the cases of the natural substrates. An esception was the incubation with epitestosterone, in which case proportionally larger amounts of 19-oxo-epitestosterone resulted, as judged by total ion current chromatogram peak heights; quantitative results were not obtained.

Other workers have found that Δ^4-3-ketones and Δ^5-3β-hydroxy compounds (25) are substrates for 19-hydroxylase. It is not known if 3β,19-dihydroxy-5-androsten-17-one or the corresponding 17β-hydroxy compound, both potential intermediates, can serve as substrates for the second 19-hydroxylation step or must first be attacked by the Δ^5-3β-hydroxysteroid dehydrogenase-isomerase system that is also present in placental microsomes.

Reduction of the Δ^4-enone system to the saturated ketone or shift of the double bond to the Δ^1-position both prevent aromatization. Neither 5α-androst-1-ene-3,17-dione, the ocrresponding 17β-hydroxy compound (26), nor 17β-hydroxy-5α-androstan-3-one (5) is aromatized by the placental microsome system. In the present study, neither 17β-hydroxy-5α-androstan-3-one nor 4-androstene-3α,17β-diol was aromatized. In both cases the block appeared to be at or prior to the 19-hydroxylation step since no intermediates or phenolic products were found.

The aromatization of 17α-methyltestosterone (6,27), 4,16-androstadien-3-one (28) and epitestosterone (6,29,30) have been reported. Horn and Finkelstein (31) found little aromatization of epitestosterone with a freeze-dried preparation of the 28,000 x g pellet from placental microsomes that had been stored at -20°. 4,6-Androstdiene-3,17-dione, shown here to be aromatized at about one-fourth the rate of androstenedione, was previously reported as not aromatized (10). When the 19-hydroxylated analog of this compound was used as a substrate, the double bond at C-6 became less of an impediment to aromatization, so that 19-hydroxy-4,6-androstadiene-3,17-dione was aromatized at about one-half the rate of 19-hydroxyandrostenedione (Table 5). The aromatization of 19-hydroxy-4,6-androstadiene-3,17-dione and also of $\Delta^{9(11)}$ -testosterone are reported here for the first time.

FOOTNOTES AND REFERENCES

1. This is Publication No. 1447 of the Cancer Commission of Harvard University. This work was supported by USPHS grant CA 01393 and American Cancer Society grant BC 17-N. The gas chromatograph-mass spectrometer was purchased with a grant from the American Cancer Society, Massachusetts Division.

2. Present address: Department of Medicine, Medical College of Georgia, Augusta, Georgia 30902.

3. Present address: Laboratory of Human Reproduction and Reproductive Biology, Harvard Medical School, 45 Shattuck Street Boston, Mass. 02115.

4. American Cancer Society Professor of Biological Chemistry, Harvard Medical School. To whom inquiries should be addressed at the Laboratory of Human Reproduction and Reproductive Biology, Harvard Medical School, 45 Shattuck Street, Boston, Mass. 02115.

5. The following non-standard names and abbreviations are used: 19-hydroxy-Δ^6-testosterone, 17β,19-dihydroxy-4,6-androstadien-3-one; 19-oxo-Δ^6-testosterone, 17β-hydroxy-3-oxo-4,6-androstadien-19-al; $\Delta^{9(11)}$-testosterone, 17β-hydroxy-4,9(11)-androstadien-3-one; 19-hydroxy-$\Delta^{9(11)}$-testosterone, 17β,19-dihydroxy-4,9(11)-androstadien-3-one; 19-hydroxy-17α-methyltestosterone, 17β,19-dihydroxy-17α-methyl-4-androsten-3-one; 17α-methyl-19-oxotestosterone, 17β-hydroxy-17α-methyl-3-oxo-4-androsten-19-al; epitestosterone, 17α-hydroxy-4-androsten-3-one; 19-hydroxy-epitestosterone, 17α,19-dihydroxy-4-androsten-3-one; 19-oxo-epitestosterone, 17α-hydroxy-4-oxo-4-androsten-19-al; 17α-methylestradiol, 17α-methyl-1,3,5(10)estratriene-3,17β-diol; $\Delta^{9(11)}$-estrone, 3-hydroxy-1,3,5(10),9(11)-estratetraen-17-one; $\Delta^{9(11)}$-estradiol, 1,3,5(10),9(11)-estratetraen-3,17β-diol; Δ^6-estrone, 3-hydroxy-1,3,5(10),6-estratetraen-17-one; Δ^6-estradiol, 1,3,5(10),6-estratetraene-3,17β-diol; TMSi, trimethylsilyl ether; MO, O-methyloxime, GC-MS, gas chromatography-mass spectrometry, TLC, thin layer chromatography.

6. Ryan, K.J., PROCEEDINGS OF THE FIFTH INTERNATIONAL CONGRESS OF BIOCHEMISTRY, Vol. VII, Pergamon Press, Oxford, 1963, p.381.

7. Dorfman, R.I., and Ungar, F., METABOLISM OF STEROID HORMONES, Academic Press New York, 1965, p. 132.

8. Engel, L.L., in HANDBOOK OF PHYSIOLOGY, Editor, R.O. Greep, Sect. 7, Endocrinology Vol. II, P. 467, American Physiological Society, Bethesda, Md., 1973.

9. Braselton, W.E., Jr., Orr, J.C., and Engel, L.L., ANAL. BIOCHEM. 53, 64 (1973).

10. Gual, C., Morato, T., Hayano, M., Gut, M., and Dorfman, R.I., ENDOCRINOLOGY 71, 920 (1962).

11. Wagner, A., Wolff, N., and Wallis, E., J. ORG. CHEM. 17, 529 (1952).

12. Bowden, K., Heilbron, I.M., Jones, E.R.H., and Weedon, B.C.L., J. CHEM. SOC. 39 (1946).

13. Orr, J.C., and Broughton, J.M., J. ORG. CHEM. 35, 1126 (1970).

14. Shapiro, R.H., and Djerassi, C., J. A M CHEM. SOC. 86, 2825 (1964).

15. Ward, M.G., Orr, J.C., and Engel, L.L., J. ORG. CHEM. 30, 1421 (1965).

16. Ryan, K.J., J. BIOL. CHEM. 234, 268 (1959).

17. Lisboa, B.P., and Diczfalusy, E., ACTA ENDOCRINOL. 40, 60 (1962).

18. Engel, L.L., and Orr, J.C., in BIOCHEMICAL APPLICATIONS OF MASS SPECTROMETRY, Editor, G.R. Waller, Wiley-Interscience, New York, 1972, p. 537.

19. Langer, L.J., Alexander, J.A., and Engel, L.L., J. BIOL. CHEM. 234, 2609 (1959).

20. Djerassi, C., Wilson, J.M., Budzikiewicz, H., and Chamberlin, J.W., J. AM. CHEM. SOC. 84, 4544 (1962).

21. Budzikiewicz, H., Djerassi, C., and Williams, D.H., STRUCTURE ELUCIDATION OF NATURAL PRODUCTS BY MASS SPECTROMETRY, Vol. 2, Holden-Day, San Francisco, 1964, p. 50.

22. Okerholm, R.A., Clark, S.J., and Wotiz, H.H., ANAL. BIOCHEM. 44, 1 (1971).

23. Vihko, R., ACTA ENDOCRINOL. SUPPL. 109, 1 (1966).

24. Meigs, R.A., and Ryan, K.J., J. BIOL. CHEM. 246, 83 (1971).

25. O'Kelly, D.A., and Grant, J.K., EUR. J. STEROIDS 2, 209 (1967).

26. Ofner, P., Ryan, K.J., Smith, O.W., Fried, J., and Munson, P.L., CANCER CHEMOTHERAPY REPORTS 16, 285 (1962).

27. Breuer, H., Knuppen, R., Ortlepp, R., Pangels, G., and Puck, A., BIOCHIM. BIOPHYS. ACTA 40, 560 (1960).

28. Knuppen, R., and Breuer, H., ACTA ENDOCRINOL. 42, 129 (1963).

29. Higuchi, T., and Villee, C.A., ENDOCRINOLOGY 86, 912 (1970).

30. Saroff, J., Keenan, R.E., Sandberg, A.A., and Slaunwhite, W.R., Jr., Steroids 10, 15 (1967).

31. Horn, H. and Finkelstein, M., Endocrinology 88, 271 (1971).