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SYNTHESIS OF DEUTERIUM- AND TRITIUM-LABELED 4-HYDROXYANDROSTENE-3,17-DIONE, AN AROMATASE INHIBITOR, AND ITS METABOLISM *IN VITRO* AND *IN VIVO* IN THE RAT

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Abstract—The metabolism of the aromatase inhibitor 4-hydroxyandrostenedione (4-OHA) was studied *in vitro* and *in vivo* in the rat. To accomplish this, deuterium- and tritium-labeled 4-OHA were prepared from 4-hydroxyandrosta-4,6-diene-3,17-dione. The latter was synthesized from 4-androstene-3,17-dione. Using deuterated 4-OHA in *in vitro* incubations of rat ovarian microsomes, 4-hydroxytesterone (4-OHT) was identified by gas chromatography/mass spectroscopy as the major metabolite. 4-OHT constituted approximately 20% of the total radioactivity from [6,7-^{3}H]-4-OHA in the ovarian microsomal incubations. Conversion of [6,7-^{3}H]-4-OHA to 4-hydroxyestrone was approximately 0.1%. The major metabolite of [6,7-^{3}H]-4-OHA *in vivo* identified in the free, neutral fraction of rat blood was 3β-hydroxyandrostane-4,17-dione. This metabolite accounted for approximately 5% of the total radioactivity in the blood, whereas 4-OHT accounted for only 0.1%. 4-OHT inhibited *in vitro* ovarian aromatization by 59%, but 3β-hydroxyandrostane-4,17-dione had little effect. It was concluded that the *in vivo* effects of 4-OHA previously reported are largely due to its own activity although additional effects of its metabolic products cannot be excluded.

We have previously demonstrated that 4-hydroxyandrostene-3,17-dione (4-OHA) is an effective *in vitro* inhibitor of aromatase (estrogen synthetase) [1]. *In vivo* administration of the compound was found to significantly reduce ovarian secretion of estrogen in rats [1] and to inhibit the peripheral conversion of androgens to estrogens in monkeys [2]. The possibility of controlling estrogen-dependent processes is being investigated [3], and we have found that 4-OHA inhibits ovulation and causes marked regression of 7,12-dimethylbenz[*a*]anthrocene (DMBA)-induced, hormone-dependent tumors in the rat [1]. Potentially, 4-OHA could be useful in the treatment of patients with estrogen-dependent breast cancer [4].

To understand more fully the mechanism of action of this interesting compound, we investigated its metabolism *in vitro* and *in vivo* in the rat. To carry out these studies we synthesized deuterium- and tritium-labeled 4-OHA.

MATERIALS AND METHODS

Synthesis

4-Hydroxyandrostene-3,17-dione (4-OHA). Synthesis of this compound has been described previously [1].

4-Hydroxytestosterone 17-acetate. This compound was prepared according to the procedure of Camerino *et al.* [5].

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4-Hydroxytestosterone (4-OHT). 4-Hydroxytestosterone 17-acetate was hydrolyzed at room temperature (4 hr) with an aqueous methanolic solution (80 ml of 70% methanol) of K_2CO_3 (2.25 g). The product was neutralized with 1 N HCl and poured onto ice-water, and the precipitate was separated by filtration. The solid was purified by preparative plate chromatography and crystallized from ethyl acetate, giving a white solid: u.v. (MeOH) λ_{max} = 282 nm; 60 MHz PMR (δ, $CDCl_3$) 0.96 (s, 3, 18-CH_3), 1.20 (s, 3, 19-CH_3), 2.60 (broad, D_2O exchangeable, 17-OH), 3.67 (t, 1, 17-CH), 6.17 (broad, D_2O exchangeable, 4-OH); gas chromatographic/mass spectroscopic (GC/MS) data: gas chromatography gave one peak with an appropriate mass spectroscopic pattern (see Table 1).

4-Hydroxyandrosta-4,6-diene-3,17-dione. A freshly prepared solution of potassium *t*-butoxide (34.5 mmoles in 45 ml *t*-butanol) was added to a solution of 3 g (10.5 mmoles) of androst-4-ene-3,17-dione in 120 ml *t*-butanol with stirring. The course of the reaction was followed by thin-layer chromatography (TLC) on silica gel in benzene–ethyl acetate (1:1) and was halted when little or no starting material could be detected (approximately 20 hr). At this point the u.v. spectrum of an aliquot showed maxima at 254 and 318 nm, characteristic of a 4,6-diene. The reaction mixture was neutralized with glacial acetic acid and the *t*-butanol was removed under vacuum at 40°. The residue was dissolved in acetone–methanol (1:1), and ether was added to precipitate the potassium acetate. The organic material was chromatographed on silica gel column (benzene–ethyl acetate, 1:1) and recrystallized from methanol to give 4-hydroxyandrosta-4,6-diene-

3,17-dione with m.p. 206–207°; λ_{max} 320 nm, ε = 20,000 (methanol); m/e = 300 (M^+). Anal. Calc. for $C_{19}H_{24}O_3$: C, 75.96; H, 8.05. Found: C, 75.80; H, 8.50.

6,(7)-*Deutero-4-hydroxyandrost-4-ene-3,17-dione.* A mixture of 100 mg of 4-hydroxyandrosta-4,6-diene-3,17-dione, 50 mg of 10% Pd/C and 10 ml of dioxane was shaken with D_2 at atmospheric pressure for 20 min. The shift in the u.v. spectrum (320→ 280 nm) indicated completion of the reaction. After removal of catalyst and dioxane, the product was chromatographed on silica gel (benzene–ethyl acetate). A white solid was obtained with m.p. 205.5–207.5°; m.p. 4-hydroxyandrost-4-ene-3,17-dione = 205.5–207.5°. Its mass spectrum indicated 67% D_2 and 33% D_1. Ultraviolet and i.r. spectra and TLC R_f values [in benzene–ethyl acetate (1:1) and ethyl ether–hexane (3.1)] were identical to those for 4-OHA, as was the PMR spectrum except for a decrease in the C-6α proton signal at δ 3.0.

6,(7)-*Tritio-4-hydroxyandrost-4-ene-3,17-dione.* Under high vacuum, 2.4 ml of tritium (measured at S.T.P.) was added to 30 mg of 4-hydroxyandrosta-4,6-diene-3,17-dione and 15 mg of 10% Pd/C in 3 ml of dioxane cooled in liquid nitrogen. After warming to room temperature, the mixture was shaken (still under vacuum) for 2 hr, filtered, and evaporated. An ultraviolet spectrum of the residue indicated that only a small portion of the starting material had been converted to the product. The product was dissolved in 3 ml of dioxane and treated with 15 mg of 10% Pd/C and hydrogen for 25 min at STP. The mixture was filtered and washed with benzene and evaporated. An ultraviolet spectrum of the residue in methanol then indicated that the reaction was complete. The product was purified on a silica gel column (as described for deuterated 4-OHA).

Reverse isotope dilution with carrier 4-OHA followed by crystallization indicated that the radioactive product was 97% pure when constant specific activity was achieved. Acetylation (acetic anhydride/pyridine) and recrystallization of the product from ethanol did not alter the specific activity.

In vitro *metabolism*

Preparation of the aromatase enzyme system. Microsomes were prepared as described previously [6] from ovaries of rats that had been pretreated with 100 I.U. of pregnant mares' serum gonadotropin on alternate days for 12 days. The ovaries were homogenized in 0.25 M sucrose and the homogenate was centrifuged at 10,000 *g*. The 10,000 *g* supernatant fraction then was spun at 105,000 *g* to yield the microsomal pellet. This was suspended in 0.1 M phosphate buffer (pH 7.4).

Experiment 1 (*see Table* 1)

Substrate preparation. 4-OHA and 6,7-deuterated 4-OHA were admixed so that a sample showed equal M and M + 2 fragmentation on GC/MS analysis. Due to the presence of some monodeuterated species, M + 1 fragments were also prominent. The mixture gave a characteristic "triple peak" fragmentation pattern with high masses at 302, 303 and 304. [6,7-^{3}H]-4-OHA of negligible mass was added to the substrate to give it a specific activity of 16,200 dpm/μg.

Incubation and metabolite purification. Eight identical incubations were carried out with 90 μg (0.66 μCi) of the above 6,7-deuterated, 6,7-tritiated 4-OHA substrate, microsomes from 450 mg wet weight of ovarian tissue, 10 mg of NADP, 20 mg of glucose-6-phosphate, and 30 units of glucose-6-phosphate dehydrogenase in a total volume of 15 ml of sodium phosphate buffer (pH 7.4). The incubations were shaken at 37° under oxygen for 1.5 hr. The contents were then combined and extracted with ethyl acetate which removed about 90% of the incubated radioactivity.

The extract was then chromatographed on TLC in ether–hexane (3:1). The area with an R_f between 4-OHA and androstenedione, and which would include 4-OHT, was eluted and rechromatographed on a small Sephadex LH-20 column. This gave one major fraction containing 21% of the radioactivity originally applied to the plate.

The fraction was subjected to 100 MHz PMR, u.v. spectrophotometry and gas chromatography/mass spectroscopy. Following overnight acetylation of the fraction with pyridine/acetic anhydride and purification on TLC in benzene–ethyl acetate (7:3), similar analyses were also carried out to further identify any of the possible metabolites of the incubated 4-OHA.

Experiment 2 (*see Table* 2)

Incubation and metabolite purification. Prior to experiment 2, [6,7-^{3}H]-4-OHA was purified by column chromatography (methanol–water–heptane–toluene 4:1:4:1). Reverse isotope dilution of the chromatographed material with carrier 4-OHA showed it to be 95% pure. Four sets of incubation flasks were set up in triplicate. Each incubation flask contained one drop of propylene glycol, 3.15 μCi (0.15 μg) of [6,7-^{3}H]-4-OHA plus 1.4 μg 4-OHA and the following steroids. Set 1 contained no androstenedione, Set 2 contained 0.5 μg androstenedione, Set 3 contained 1 mg 4-hydroxyestrone (4-OHE_1), and Set 4 contained 0.5 μg androstenedione and 1 mg 4-OHE_1. Volatile solvents were evaporated under nitrogen. Sodium phosphate buffer (pH 7.4), 1.4 ml containing 0.2% EDTA, was added to each flask followed by microsomes prepared from 50 mg wet weight of ovarian tissue suspended in 1 ml of the same buffer. The reaction was initiated by the addition of 1 mg NADP, 2 mg glucose-6-phosphate and 2.5 units of glucose-6-phosphate dehydrogenase. The flasks were incubated at 37° in an oxygen atmosphere for 30 min. The reactions were terminated by adding 10 ml of ethyl acetate and freezing. The incubates were stored frozen until analyzed.

The incubation mixtures were extracted three times with 10 ml of ethyl acetate. The ethyl acetate extracts (30 ml) from each were combined and washed with 10 ml of water, 10 ml of saturated NaCl, and filtered through anhydrous sodium sulfate. The ethyl acetate extract was then evaporated. The residues were dissolved in 5% ethanol in benzene and assayed for radioactivity.

To 1% aliquots of each extract were added 0.3 mg each of 4-OHA and 4-OHT plus 0.6 mg of 4-OHE_1. The mixtures were chromatographed on 0.5 mm silica gel plates in ether–hexane (4:1) at 4°. The carrier

steroids were located by brief exposure to u.v. light and then eluted with acetone. The 4-OHA and 4-OHE_1 having similar R_f values were eluted as a mixture, whereas the 4-OHT was eluted from a zone that was well separated from them. The acetone was removed in a stream of nitrogen and the eluted steroids were immediately converted to their methyl ethers by extractive methylation. For this, steroids were dissolved in 5 ml dichloromethane containing 0.25 ml iodomethane. Tetrahexyl ammonium hydroxide (0.1 M in 0.1 ml methanol) was added, followed by 5 ml of freshly prepared 0.5 N sodium hydroxide through which nitrogen had been bubbled for 15 min. The mixture was shaken vigorously in a nitrogen atmosphere for 5 min, and the methyl ethers were isolated from the dichloromethane phase.

The 4-OHT 4-methyl ether was purified by TLC first in benzene–ethyl acetate (3:2), which separated 4-OHA 4-methyl ether from 4-OHE_1 3,4-dimethyl ether. Further purification was achieved by TLC in dichloromethane–methanol (50:1).

The recoveries of carrier steroids were determined by u.v. spectrophotometry of the isolated methyl ethers in ethanol using standard curves constructed from the spectra of the respective authentic methyl ethers. The radioactivity was determined by liquid scintillation spectrophotometry and was corrected for recovery by that calculated for the carrier steroids.

In vivo *metabolism* (*see Table* 3)

A female rat was injected i.v. with 600 μCi [6,7-^{3}H]-4-OHA (30 μg 4-OHA). After 1 hr the rat was decapitated and the trunk blood was collected. The blood was extracted as described previously [7] to give the "free, neutral" fraction that contained about 30% of total blood radioactivity with ethyl acetate. 4-OHA and 4-OHT, 5 mg of each, were added separately to 20% aliquots of the free, neutral blood extract. Each aliquot was subjected to chromatography on TLC followed by crystallization. Constant specific activity (tritium to weight) of 4-OHT was obtained on the third and fourth recrystallizations and on the second and third recrystallizations for 4-OHA.

Another aliquot, equal to half of the total blood extract, was chromatographed on TLC in the system benzene–ethyl acetate–methanol (75:20:5) and then scanned for radioactivity in a Vanguard radiochromatogram scanner. The major single peak was eluted and the characteristics of the eluate were compared to those for several steroidal compounds. The eluate had an R_f similar to authentic 3β-hydroxyandrostane-4,17-dione (3-OH-dione). Thus, the latter compound (24.4 mg) was added to the eluate and the mixture was recrystallized four times from ethyl acetate. The crystals from the fourth recrystallization were acetylated with pyridine and acetic anhydride (see Table 4). The resulting acetate was recrystallized twice from methanol.

The percentage of radioactivity in the total blood extract identified as labeled 4-OHA, 4-OHT and as 3β-OH-dione in the total blood extract was calculated (Table 3).

Metabolites assayed for aromatase inhibition

Authentic samples of 4-OHT and 3-OH-dione were incubated with ovarian microsomes from 12-day PMSG primed rats as described previously [1] and as outlined in Table 5. The percent aromatization of [1,2^{3}H]androstenedione (New England Nuclear Corp., Boston, MA) was compared with and without addition of the compounds.

RESULTS

Identification and quantitation of 4-*hydroxytestosterone in* in vitro *incubations*

6,(7)-Deutero-4-hydroxyandrost-4-ene-3,17-dione was prepared and purified with approximately 40% yield. Calculations based on PMR and MS data indicated that deuteration occurred at least at 53% in the α-position. The u.v., i.r. and chromatographic data were all consistent with the structure of 4-OHA.

The 6,7-deutero-4-OHA was used as substrate to determine the metabolism of 4-OHA by rat ovarian microsomes in the first experiment. The 100 MHz PMR spectrum of the material in the major fraction from TLC of the incubation extract (21% of the radioactivity incubated) showed three peaks in the C_{18} and C_{19} region. A multiplet in the δ 3.75 region suggested a C-17 α-H under a 17β-OH. The $u.v._{max}$ 280 nm suggested that there had been no change in the A ring. A GC/MS analysis of the metabolite showed several GC peaks, only one of which showed the M, M + 1, M + 2 "triple peak pattern" of the original substrate. These were at *m/e* 304, 305 and 306, indicating the addition of 2 protons to the substrate. Since there are only three sites of unsaturation where 2 protons could be added to 4-OHA (i.e. positions 3, 4–5 and 17) and since the u.v. spectrum indicated that the "A" ring was intact, a logical consideration for the structure of the metabolite was 4-OHT. The GC/MS of 4-OHT showed a similar retention time to the metabolite. A comparison of the fragmentation pattern of the two compounds (Table 1) indicates much similarity although there were some differences due probably to the difference in the relative concentration of compound versus "background" at the point at which the GC peak was sampled and to some overlap of the d-containing peaks with others. On TLC in benzene–ethyl acetate (7:3), the metabolite and 4-OHT had identical R_f values. Following acetylation, the R_f of the metabolite on TLC was identical to that of 4-OHT diacetate. The GC/MS of the metabolite derivative showed three different peaks on GC with the "triple peak" fragmentation pattern. The minor GC peak with the shortest retention time (4 min, 3% OV-1, 5 ft column, 220°) remains unidentified.* A second minor GC peak with a retention time of 10 min corresponded in retention time, fragmentation pattern, and relative intensity to a sample of 4-OHT 17-acetate (Peak "B", Table 1). The third and major GC peak had a retention time of 18 min and compared well to a sample of authentic 4-OHT diacetate

* Fragmentation: 305, 19%; 292, 63%; 278, 100%; 263, 45%; 232, 41%; 217, 54%; 214, 50%; 202, 40%; and 199, 83%.

Table 1. Mass spectral data for the metabolite and the acetates of the metabolite in comparison to 4-hydroxytestosterone and its acetate derivatives*

	Mass peak (nm)									
	346	304	289	286	271	268	262	258	243	225
Metabolite†	—	100	22	20	20	23	33	17	24	35
4-Hydroxytestosterone	—	100	16	20	14	8	19	12	13	33
Metabolite acetate† Peak "B"	71	100	43	100	44	57	—	35	43	61
4-Hydroxytestosterone-17-acetate	67	100	29	100	36	39	—	23	28	52
Metabolite acetate+ Peak "C"	100	66	27	96	30	34	—	36	21	43
4-Hydroxytestosterone diacetate	100	56	4	76	20	23	—	8	6	15

* Expressed as percent parent peak.

† Only the M peak is given although M + 1 and M + 2 peaks are present in the spectrum (i.e. mono- and di-deuterated metabolite).

(Peak "C", Table 1). A sample of 4-OHT, acetylated in the same manner as the metabolite, showed both the 10- and 18-min (GC) peaks even though no monoacetate could be detected by TLC. The 10-min peak may be due to GC column degradation of the diacetate molecule.

The recovery of the 6,(7)tritiated-4-hydroxyandrost-4-ene-3,17-dione was 5×10^{11} dpm indicating a 4% yield of product, assuming incorporation of two ^3H atoms/molecule. The specific activity was found to be 6.25 Ci/mmole. This material was used as substrate for the second *in vitro* experiment. The results are shown in Table 2. Incubations carried out with or without androstenedione (the natural substrate for aromatase) did not appear to alter the pattern of metabolism of 4-OHA. 4-OHT was found to represent approximately 20% of the total radioactivity in Experiment 2. Very little aromatization to 4-OH-estrone occurred even in the presence of added 4-OHE_1. However, the addition of this estrogen to the incubation resulted in a reduction in the conversion of 4-OHA to 4-OHT.

Identification of 3β-hydroxy-5α-androstane-4,17-dione in vivo

Following injection of the rat with [6,7-^3H]-4-OHA, the blood extract yielded a single major and several minor peaks after TLC. The eluted area corresponding to the major peak was subsequently chromatographed against several authentic steroids and found to have an R_f similar to 3β-hydroxy-5α-androstane-4,17-dione (3-OH-dione) (Table 3). Constant specific activity was achieved after two crystallizations with added authentic carrier (3-OH-dione) and no further change in specific activity occurred after crystallization of the acetate derivative (Table 4).

Evaluation of metabolites as aromatase inhibitors

The two metabolites of 4-OHA, 3-OH-dione and 4-OHT, were incubated with rat ovarian microsomes. As indicated in Table 5, 3-OH-dione had little inhibitory effect (10%) on aromatization, while 4-OHT had about 65% of the inhibitory activity of 4-OHA.

Table 2. Incubation of [6,7-^3H]-4-hydroxyandrostenedione with rat ovarian microsomes*

Substrates incubated	% [6,7-^3H]-4-OHA recovered as products		
	4-OHA	4-OHT	4-OHE_1
(1) None	47.0	19.7	—
	52.1	19.2	0.26
(2) A	53.9	21.7	0.10
	53.9	22.6	0.09
(3) 4-OHE_1	58.1	7.5	0.13
	81.3	5.9	0.58
(4) A + 4-OHE_1	67.3	7.6	0.07
	63.2	7.9	0.11

* Rat ovarian microsomes (equivalent to 50 mg tissue) were incubated with [6,7-^3H]-4-OHA (3.15 μCi). Androstenedione (A), 0.5 μg, was added to incubations 2 and 4, and 4-hydroxyestrone (4-OHE_1), 1 mg, was added to incubations 3 and 4. Each incubation was carried out in 2.5 ml phosphate buffer (pH 7.4) for 30 min at 37° under oxygen. The reaction was initiated with cofactors 1 mg NADP, 2 mg glucose-6-phosphate, and 2.5 units of glucose-6-phosphate dehydrogenase. Incubation 1, conversion to 4-OHE_1, was not determined.

Table 3. Metabolites of [6,7-^{3}H]-4-OHA isolated from rat blood*

Products	% of total extract
4-OHA	1.4
4-OHT	0.5
3-OH-Dione	18.4

* The rat was injected i.v. with 600 μCi [6,7-^{3}H]-4-OHA (30 μg). Blood was collected after 1 hr. Abbreviations: 4-OHA, 4-hydroxyandrostenedione; 4-OHT, 4-hydroxytestosterone, and 3-OH-dione, 3β-hydroxyandrostane-4,17-dione.

Table 4. Purification of the 3β-hydroxyandrostane-4,17-dione metabolite of 4-OHA from rat blood*

Recrystallization (dpm/mg)	Recrystallization acetate (dpm/mg)
2nd, 1288	1st, 1316
3rd, 1230	2nd, 1265
4th, 1341	

* 3β-Hydroxyandrostane-4,17-dione was crystallized from ethyl acetate. Following overnight acetylation with pyridine and acetic anhydride, the acetate was recrystallized. Results (dpm/mg) are corrected for molecular weight differences back to the parent compound.

Table 5. Effect of 4-OHA metabolites on aromatization*

	% Inhibition
None	0
3-OH-Dione	10
4-OHT	59
4-OHA	91

* Rat ovarian microsomes were incubated with [1,2^{3}H]androstenedione (200,000 dpm), 1.7 μM androstenedione and a 10.2 μM concentration of test compounds. Incubation conditions were the same as for Table 2.

DISCUSSION

Our previous studies indicate that 4-OHA, an inhibitor of aromatization, is highly effective in causing regression of hormone-dependent mammary tumors in rats [1]. These results suggest that 4-OHA might be useful in the treatment of human breast cancer [4]. Besides inhibition of aromatase, and thus, of estrogen production, other actions of the compound may be involved in mammary tumor regression. In this study we have investigated whether two apparent metabolites of 4-OHA have activity as aromatase inhibitors which might account for some of the effects of 4-OHA *in vivo*.

Using deuterated and tritiated 4-OHA, we identified and quantitated the formation of 4-OHT by ovarian microsomes *in vitro*. Conversion to [^{3}H]-4-OHE$_1$ was low, even when 4-OHE$_1$ was added to trap any radioactive 4-OHE$_1$ that was produced. However, the presence of 4-OHE$_1$ in the incubation reduced the conversion of 4-OHA to 4-OHT. Although verification is needed, this finding suggests that 4-OHE$_1$ may act as an inhibitor for 17β-hydroxysteroid dehydrogenase. An alternative possibility is that there is competitive conversion of 4-OHE$_1$ to 4-OHE$_2$. The first possibility might have the effect of retarding the conversion by the dehydrogenase of estrone to the more potent estrogen, estradiol. On the other hand, Martucci and Fishman [8] observed that 4-OHE$_1$ was estrogenic. However, the amount reaching the circulation from the ovary would probably be low. Since marked tumor regression occurs with 4-OHA, it seems unlikely that any 4-OHE$_1$ produced would be sufficient to result in tumor stimulation.

4-OHT was also identified in rat blood following injection of [6,7-^{3}H]-4-OHA. Although this compound has about 65% the aromatase inhibitory activity of 4-OHA and could therefore contribute to its effectiveness *in vivo*, it was not the major metabolite in the free fraction of rat or monkey blood [7] and represented only 0.5% of the total extract.

Tomorkeny *et al.* [9] identified 3β-hydroxy-5α-androstane-4,17-dione as the major metabolite of 4-OHA from aerobic cultures of *Mycobacterium phlei*. We therefore compared 3-OH-dione with the major metabolite present in the neutral extract of rat blood. Proof of identity through several recrystallizations and quantitation revealed that 3-OH-dione accounted for the largest proportion, approximately 20% of the free extract and 5% of the total blood radioactivity. The production of this metabolite is likely to be extraovarian, the conversion probably occurring in the liver, 3-OH-Dione was determined to have little aromatase inhibitory activity. It is not known whether this compound has other biological activities.

The above findings suggest that the *in vivo* effects of 4-OHA are largely due to the compound itself, although additional (but lesser) effects of its metabolic products cannot be excluded.

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REFERENCES

1. A. M. H. Brodie, W. C. Schwarzel, A. A. Shaikh and H. J. Brodie, *Endocrinology* **100**, 1684 (1977).
2. A. M. H. Brodie and C. Longcope, *Endocrinology* **106**, 19 (1980).
3. A. M. H. Brodie, D. A. Marsh, J. T. Wu and H. J. Brodie, *J. Steroid Biochem.* **11**, 107 (1979).
4. A. M. H. Brodie, *Hormones and Cancer* (Eds. S. Iacobelli, R. J. B. King, H. R. Lindner and M. E. Lippman), Vol. 14, p. 507. Raven Press, New York (1980).
5. B. Camerino, B. Patteli and A. Vercellone, *J. Am. chem. Soc.* **78**, 3540 (1956).
6. A. M. H. Brodie, W. C. Schwarzel and H. J. Brodie, *J. Steroid Biochem.* **7**, 787 (1976).
7. A. M. H. Brodie, L. P. Romanoff and K. I. H. Williams, *J. Steroid Biochem.* **14**, 693 (1981).
8. C. P. Martucci and J. Fishman, *Endocrinology* **105**, 1288 (1979).
9. E. Tomorkeny, G. Toth, G. Horvarh and K. G. Buki, *Acta chim. hung.* **87**, 409 (1975).