Vol. 157, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1988 Pages 81-86

# [19-$^{14}$C]ANDROSTENEDIONE: A NEW SUBSTRATE FOR ASSAYING AROMATASE AND STUDYING ITS REACTION MECHANISM

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Received September 28, 1988

[19-$^{14}$C]Androstenedione has been prepared and utilized as a substrate for assaying microsomal human placental aromatase. Enzyme activity is determined by measuring the rate at which [$^{14}$C]formate is produced by aromatization of this $^{14}$C-labeled steroid. Isotope ratio experiments using [19-$^{14}$C]androstenedione and [1$\beta$-$^{3}$H]androstenedione demonstrate that an apparent kinetic hydrogen isotope effect exists for the aromatization of the tritiated steroid with $k_H/k_T \approx 1.09$. Metabolic switching occurs to a minor extent ($\approx$ 3%) during aromatization of [1$\beta$-$^{3}$H]androstenedione, but not during the aromatization of [19-$^{14}$C]androstenedione. © 1988 Academic Press, Inc.

Human placental aromatase is a cytochrome P-450 enzyme that converts androstenedione (**1**) into estrone (**4**) by the reaction pathway (**1** → **2** → **3** → **4**) shown in Scheme I (for a recent review of aromatase mechanistic studies see ref. 1). The most widely-used and convenient assays quantitate aromatase activity by measuring the amount of tritiated water produced during aromatization of either [1$\beta$-$^{3}$H]- or [1$\beta$,2$\beta$-$^{3}H_2$]androstenedione (2-4). [4-$^{14}$C]Androstenedione can also be used, but the assay is more laborious since product isolation is required. In this paper we report the preparation and use of [19-$^{14}$C]androstenedione as a new substrate for the aromatase assay. Since its aromatization liberates [$^{14}$C]formic acid that can be readily separated from the steroids by extraction, this compound retains the convenience associated with the tritiated androstenedione assays, while avoiding possible isotope effects resulting from the presence of tritium. In fact, using this $^{14}$C-labeled substrate in isotope ratio experiments with [1$\beta$-$^{3}$H]androstenedione we detected an apparent kinetic hydrogen isotope effect ($k_H/k_T \approx 1.09$) for the aromatization of [1$\beta$-$^{3}$H]androstenedione. We also found a new metabolite derived from [1$\beta$-$^{3}$H]androstenedione that is not formed from [19-$^{14}$C]androstenedione, which we believe to be the result of metabolic switching.

## MATERIALS AND METHODS

### General

The [1$\beta$-$^{3}$H]-, [19-$^{3}H_3$]-, [4-$^{14}$C]-, and [19-$^{14}$C]androstenedione (radiochemical purity $\geq$ 99%) were obtained from NEN Research Products, Boston, MA.

0006-291X/88 $1.50
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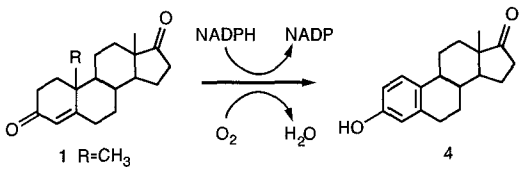


1 R=$CH_3$

2 R=$CH_2OH$

3 R=CHO

4

+ HCOOH

Scheme I

[19-$^{14}$C]Androstenedione

5α,10α-Epoxyestrane-3,17-*bis*-cycloethyleneketal (0.78 mmol)(ref. 5), in ether (10 ml), was added to [$^{14}$C]methyl magnesium bromide (3.9 mmol, 59 mCi/mmol) in ether (5 ml) and refluxed (24 hr). $NH_4Cl$ (2 M, 0.5 ml) was added and the solvents were evaporated. Water (50 ml) and $CH_2Cl_2$ (50 ml) were added and the $CH_2Cl_2$ layer was washed successively with 1N HCl, 5% $NaHCO_3$, and water, then dried ($Na_2SO_4$). Following solvent evaporation, the crude solid was dissolved in acetone (40 ml) and 10% $H_2SO_4$ (6 ml) and stirred for 1 hr. Water (25 ml) was added and the mixture was concentrated *in vacuo*. The mixture was extracted with ethyl acetate (30 ml) and the extract was washed with 5% $NaHCO_3$, and water, dried ($Na_2SO_4$) and evaporated. The residue was dissolved in methanolic NaOH (0.1 N, 45 ml) and stirred for 2 hr; water (30 ml) was added and the mixture concentrated (to ≈ 15 ml) and extracted with ethyl acetate (40 ml). The extract was washed with water, dried ($Na_2SO_4$), and evaporated. The crude product was partially purified by preparative thin layer chromatography on silica gel (1:1 hexane/ethyl acetate). Final purification was achieved on a DuPont Zorbax-ODS semi-preparative HPLC column using methanol/water/tetrahydrofuran (40:57:3) as the mobile phase. The yield was 2.5% (1.1 mCi, sp. act. 57 mCi/mmol). The radiochemical purity was ≥ 99% as determined by reverse isotope dilution and HPLC analysis.

[19-$^{13}$C]Androstenedione

A similar procedure was used to prepare this steroid except that [$^{13}$C]dimethyl magnesium (99% $^{13}$C) was used for introduction of the carbon label. From 5α,10α-epoxyestrane-3,17-*bis*-cycloethyleneketal (1.56 mmol) the purified product was obtained in 20% yield (88.5 mg). The product was characterized by its $^1$H and $^{13}$C NMR spectra. It had: $^1$H NMR(300 MHz, $CDCl_3$) δ 0.93 (s, 3, $CH_3$, $C_{18}$), 1.22 (d, $^1J_{HC}$ = 126.6 Hz, 3, $CH_3$, $C_{19}$), 5.75 (s, 1, HC=C); $^{13}$C NMR(75 MHz, $CDCl_3$) δ 17.36 (q, $^1J_{CH}$ = 126.6 Hz, $C_{19}$); $^{13}C\{^1H\}$ NMR δ 17.39 (s, $C_{19}$).

Aromatase Assay

The preparation and assay of microsomal aromatase were as reported previously (6). Briefly, steroids (3 nmol) were dissolved in ethanol (0.06 ml) and incubated in a shaker bath at 37° C in air with microsomes and 100 μM NADPH in pH 7.5 assay buffer (2.94 ml of 10mM potassium phosphate containing 100 mM KCl, 1 mM EDTA, 4% propylene glycol). At various times aliquots (0.5 ml) were removed, added to chloroform (5.0 ml), vortexed (45 sec), centrifuged (1470 x *g* for 5 min), and a portion of the aqueous layer (100 μl) taken for scintillation counting to determine the extent release of radiolabel, from which turnover was calculated.

Lyophilization Procedure for [$^{14}$C]- and [$^{13}$C]Formic Acid Recovery

[19-$^{14}$C]Androstenedione (3 nmol, 2.84 x $10^5$ cpm in 0.06 ml ethanol) was incubated in assay buffer (2.94 ml) with microsomes (266 μg protein) and 100 μM NADPH at 37° C for 1 hr. An aliquot (0.5 ml) was removed and processed for turnover determination as described above. The remainder of the incubation was adjusted to pH 10 with 0.4M NaOH, shell frozen in liquid nitrogen and lyophilized. The lyophilisate was trapped in a U-tube immersed in liquid nitrogen. After warming, the mass of lyophilisate was

determined gravimetrically and an aliquot was assayed for radioactivity by scintillation counting. The residue from the lyophilisate was dissolved in 1.0 M *p*-toluenesulfonic acid (1.0 ml), shell frozen in liquid nitrogen and lyophilized. The lyophilisate was trapped and assayed for radioactivity.

The same procedure was used to isolate [$^{13}$C]formic acid produced by the aromatization of [19-$^{13}$C]androstenedione (3 nmol) except that aliquots were not taken for scintillation counting and product analysis was by $^{13}$C NMR spectroscopy.

Isotope Ratio Conversion Studies

A solution of androstenedione (2.89 nmol), [1$\beta$-$^{3}$H]androstenedione (0.11 nmol), and [19-$^{14}$C]androstenedione (3 nmol) in ethanol (0.12 ml) was added to assay buffer (5.88 ml) containing 100 $\mu$M NADPH and microsomes (265 $\mu$g protein) and incubated at 37° C. At various times, aliquots (0.5 ml) were removed for turnover determination as described previously. Scintillation counting was done on both the $^{3}$H and $^{14}$C channels and corrected for spillover. A correction for the amount of 1$\alpha$-$^{3}$H (15% or 22% as specified by supplier) present in the [1$\beta$-$^{3}$H]androstenedione samples was made also. The apparent intermolecular hydrogen kinetic isotope effect was determined from the $^{3}$H/$^{14}$C product ratio using the formula (Equation 1) reported by Melander and Saunders (7).

$$k_H/k_T = \log(1 - F_1)/\log[1 - (F_1R_p/R_o) \quad \text{(Equation 1)}$$

Metabolic Switching Studies

[1$\beta$-$^{3}$H]Androstenedione (6 nmol, 1.25 x $10^{6}$ cpm) in ethanol (0.06 ml) was added to assay buffer (5.94 ml) containing 100 $\mu$m NADPH and microsomes (400 $\mu$g protein) and incubated at 37° C under 100% $O_2$ for 1.5 hr. An aliquot (0.5 ml) was removed and assayed for radioactivity. The remaining incubate was applied to a $C_{18}$ Baker extraction column and the steroids eluted with methanol (2.0 ml). The methanol was evaporated and the residue was redissolved in methanol (0.06 ml). A portion (5 $\mu$l) was used to quantitate steroid recovery by scintillation counting and another portion (5 $\mu$l) was used for HPLC analysis (Radiomatic FLO-ONE HP detector) on 2 tandem connected Alltech #60085 5$\mu$ silica cartridge columns (250 x 4.6 mm) eluted at 2 ml/min with 4:6 hexane/ethyl acetate.

## RESULTS

The incubation of [19-$^{14}$C]androstenedione (500 pmol/0.5 ml) with microsomal aromatase (66 $\mu$g protein/0.5 ml) for 1.0 hr at 37° C resulted in release of 96% of the $^{14}$C label into the water layer (Figure 1). HPLC analysis of the recovered steroids from the incubation showed the remaining 4% of the label to be in a peak that had the same retention time as [19-$^{14}$C]androstenedione. No other radioactive peaks were detected. During the standard assay time (5 min) the reaction velocity was linear with respect to time and protein concentration (22 - 66 $\mu$g/0.5 ml).

The water layer was analyzed for volatile radioactivity. Following alkalinization, the aqueous layer was lyophilized. The lyophilisate contained $\approx$ 0.6% of the total counts present in the initial water layer. Acidification and lyophilization of the residue resulted in localization of 72% of the initial radioactivity in the lyophilisate. The non-volatile residue contained 7% of the radioactivity, in good agreement with the 4% of label found in unreacted substrate (*v.s.*). A portion of the initial radioactivity, 21%, was not recovered.

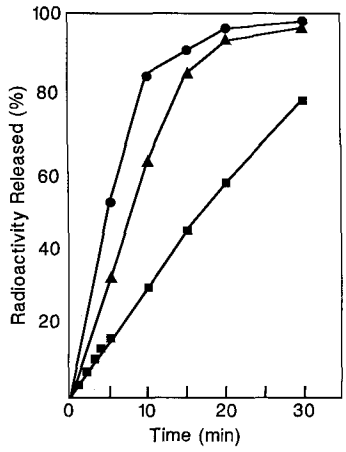


Fig. 1. Release of [$^{14}$C]Formic acid from [19-$^{14}$C]Androstenedione. Microsomal protein = 22 (■), 44 (▲), and 66 (●) μg/0.5 ml (n = 3; precision = 2%).

In an attempt to account for the unrecovered radioactivity, [$^{14}$C]formic acid was incubated with NADPH and unlabeled androstenedione, in the presence or absence of microsomes, and subjected to the same lyophilization experiments. The efficiency of trapping [$^{14}$C]formic acid was only 81%. Thus, the unrecovered volatile radioactivity generated in the aromatization of [19-$^{14}$C]androstenedione may be accounted for by the inefficiency of trapping [$^{14}$C]formic acid.

We also prepared [19-$^{13}$C]androstenedione and used proton decoupled $^{13}$C NMR(125 MHz) to obtain spectroscopic evidence for the loss of $C_{19}$ as [$^{13}$C]formic acid. Authentic samples of $^{13}$C-labeled formic acid, sodium carbonate, and sodium bicarbonate had $^{13}$C resonances at 171.3, 168.6, and 161.2 ppm, respectively; and $T_1$ values of 21.0, 66.3, and 5.6 sec, respectively. A $^{13}$C resonance at 171.4 ppm which we attribute to [$^{13}$C]formic acid was detected in the sample recovered from the aromatization experiment. No resonances were observed at 168.6 and 161.2 ppm.

Isotope ratio experiments were then carried out with [19-$^{14}$C]- and [1β-$^{3}$H]androstenedione to determine the apparent hydrogen kinetic isotope effect for removal of the 1β-$^{3}$H in the aromatase reaction. The apparent intermolecular isotope effect ($k_H/k_T$) was calculated according to Equation 1. The data from one of five experiments is shown in Table I. For the five experiments, the average values ± SEM (n = 5) for the isotope effect at 4, 8, 12, 16, and 20 min were 1.13 ± 0.01, 1.07 ± 0.02, 1.08 ± 0.01, 1.07 ± 0.01, and 1.07 ± 0.02 respectively. The average value of $k_H/k_T$ is 1.09 ± 0.02.

Since the presence of tritium at $C_{19}$ of androstenedione causes metabolic switching (8), we wished to determine if the presence of tritium at the β-position on $C_1$ would similarly result in metabolic switching. The results are presented in Table II. Incubation of [1β-$^{3}$H]androstenedione under conditions of complete substrate turnover resulted in

Table I. Representative $^{3}H/^{14}C$ Product Ratios and Calculations of $k_H/k_T$ for [1β-$^{3}$H]Androstenedione Aromatization

| Time (min) | $^{3}H_2O$[a] (cpm) | $H^{14}COOH$[a] (cpm) | $F_1$[b] | $R_o$[c] | $R_p$[d] | $k_H/k_T$ |
|---|---|---|---|---|---|---|
| 4 | 3,410 | 1,247 | 0.109 | 3.027 | 2.734 | 1.11 |
| 8 | 6,245 | 2,257 | 0.198 | 3.027 | 2.766 | 1.10 |
| 12 | 9,130 | 3,260 | 0.285 | 3.027 | 2.801 | 1.10 |
| 16 | 11,825 | 4,145 | 0.363 | 3.027 | 2.853 | 1.08 |
| 20 | 14,310 | 5,047 | 0.442 | 3.027 | 2.835 | 1.09 |

[a] Cpm are per 0.5 ml and have been corrected for spillover during scintillation counting and for the rel % of 1α-$^{3}$H in [1β-$^{3}$H]1.
[b] $F_1$ = cpm $H^{14}COOH$/total cpm [19-$^{14}$C]1 at time t.
[c] $R_o$ = cpm [1β-$^{3}$H]1 (34,587)/cpm [19-$^{14}$C]1 (11,425) @ t = 0.
[d] $R_p$ = cpm $^{3}H_2O$/cpm $H^{14}COOH$ at time t.

68.2 % of the initial radioactivity being liberated into the water layer and 24.3 % of the initial radioactivity being recovered in the steroid products. HPLC analysis showed that 2.5 % of the initial radioactivity was present in a metabolite of unknown structure. Although identification of this unknown metabolite was not attempted because it occurred in such small amounts, we believe it to be a product of metabolic switching for the following reasons.

When a portion of the steroids recovered from each incubation was re-incubated, there was no significant change in the relative percentage of the combined estrone and estradiol peaks and the unknown metabolite (Table II). If the unknown metabolite were some intermediate in the aromatase reaction whose conversion to estrogen products was slowed by the small kinetic isotope effect we observed for the aromatization of [1β-

Table II. Analysis of Tritium Distribution in the Products Produced by the Incubation of [1β-$^{3}$H]Androstenedione and Microsomal Aromatase

| Steroid Incubated | Radioactivity Distribution (% Total cpm Recovered ± SEM, n = 4) | | | |
|---|---|---|---|---|
| | Water Layer | Steroid Fraction[a] | | |
| | | $E_1 + E_2$[b] | Unk[c] | Other[d] |
| [1β-$^{3}$H]1[e] | 68.2 ± 2.3 | 20.5 ± 1.9 (84.3)[f] | 2.5 ± 0.8 (10.4)[f] | 1.3 ± 0.2 (5.3)[f] |
| Recovered Steroids[g] | 7.8 ± 2.6 | 54.6 ± 2.9 (83.1)[f] | 8.0 ± 1.1 (12.2)[f] | 3.1 ± 0.5 (4.7)[f] |

[a] Steroid fraction was analyzed by HPLC as described in the methods.
[b] $E_1$ = estrone; $E_2$ = estradiol. HPLC retention times = 5.1 and 6.5 min, respectively.
[c] Unk = unknown metabolite. HPLC retention time = 15.8 min.
[d] Other = Total radioactivity found in inconsistently appearing trace metabolites (≤ 0.5% ea).
[e] Counts incubated = 1.45 - 2.04 x $10^6$ cpm.
[f] Numbers in parentheses are relative % of cpm in products detected by HPLC analysis.
[g] Counts incubated = 2.33 - 2.83 x $10^5$ cpm.

$^{3}$H]androstenedione, then the second incubation should have made it possible for at least a portion of this metabolite to complete the aromatization reaction. This did not occur since the relative percentage of estrogens and the unknown metabolite did not change. In addition, the unknown metabolite was not detected in incubations of [4-$^{14}$C]androstenedione.

## DISCUSSION

[19-$^{3}H_3$]Androstenedione was introduced as a substrate for assaying human aromatase because it made possible independent measurement of the initial $C_{19}$ hydroxylation steps ($^{3}H_2O$ release) and final aromatization step ($^{3}$HCOOH release) of the aromatase reaction (9,10). However, the large hydrogen isotope effect associated with $C_{19}$-hydroxylation of [19-$^{3}H_3$]androstenedione causes metabolic switching (1$\beta$- and 2$\beta$-hydroxylation) to occur with this substrate(8). To avoid these metabolic switching products while retaining an aromatase assay based on cleavage of the $C_{10}$-$C_{19}$ bond, we prepared [19-$^{14}$C]androstenedione and studied its aromatization. Because primary carbon isotope effects are very small (*ca.* 1.06)( ref.11), we expected no metabolic switching in the aromatization of [19-$^{14}$C]androstenedione and no evidence for it was found.

Using [19-$^{14}$C]androstenedione we determined a small apparent intermolecular hydrogen isotope effect in the aromatization of [1$\beta$-$^{3}$H]androstenedione by human placental aromatase. This $^{14}$C-labeled steroid will be useful for establishing the oxidation state of $C_{19}$ when it is lost during the aromatization or demethylation (leading to 19-norandrogen formation) of androstenedione by related enzymes from other tissues and species. It will also be a valuable starting material for the synthesis of 19-$^{14}$C-labeled suicide substrates of aromatase (for a review of aromatase suicide substrates see ref. 1).

## ACKNOWLEDGEMENTS

We thank Dr. Paul Sherwin for helpful advice regarding the isotope ratio experiments and Dr. Daniel Canney for assistance in obtaining the 125 MHz $^{13}$C NMR spectra. This research was supported by NIH Grant CA 23582 (D.F.C.). Assistance was provided by the Washington Univ. High Resolution NMR Service Facility, supported in part by NIH 1 S10 RR00204 and a gift from the Monsanto Company.

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