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NOVEL 19-(CYCLOPROPYLAMINO)-ANDROST-4-EN-3,17-DIONE: A MECHANISM-BASED INHIBITOR OF AROMATASE

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The novel 19-(cyclopropylamino)-androst-4-en-3,17-dione (**5**, **CPA**), a mechanism-based inhibitor of aromatase has been synthesized from the 10β-aldehyde intermediate (**2**). The key reaction was the trifluoroacetic acid-catalysed condensation of **2** with cyclopropylamine in refluxing toluene to give the 19-cyclopropylimine (**3**). Enzyme inhibition studies show that **CPA** is a time-dependent, irreversible inhibitor of human placental microsomal aromatase (K_i = 92±2 nM). The inactivation of aromatase by **CPA** was NADPH-dependent and was protected by the presence of substrate testosterone (20 μM). In addition, the inactivation was not affected by the nucleophile, L-cysteine (0.5 mM), suggesting retention of the inhibitor in the enzyme's active site during the inactivation process.

KEY WORDS: Human placental aromatase, 19-(cyclopropylamino)-androst-4-en-3,17-dione, mechanism-based aromatase inactivator

INTRODUCTION

Human placental aromatase (estrogen synthetase) is a cytochrome P-450 enzyme which catalyses the conversion of androgens into estrogens. The potential therapeutic value of aromatase inhibitors in the treatment of estrogen-dependent diseases (e.g. breast cancer) has led to much interest in this area.[1] Some of the steroids which have been studied in this context are suicide or mechanism-based inhibitors whose effect is dependent upon activation by the enzyme, such as 10β-propargyl,[2] 10β-mercapto,[3] 19-mercapto,[4] 19,19-difluoro,[5] and 2,19-methyleneoxy[6] analogues of androstenedione (**AD**). One of these compounds, 10-(2-propynyl)-estr-4-en-3,17-dione is currently in clinical trials for breast cancer treatment.[7]

Cyclopropylamines have been reported as mechanism-based, irreversible inhibitors of rat liver cytochrome P-450s,[8–10] monoamine oxidase, a flavoprotein[11] and recently of human 17α-hydroxylase/$C_{17,20}$-lyase (17α-lyase) enzyme[12] and 14α-demethylase enzyme.[13] A proposed mechanism for these inhibitions involves one electron oxidation

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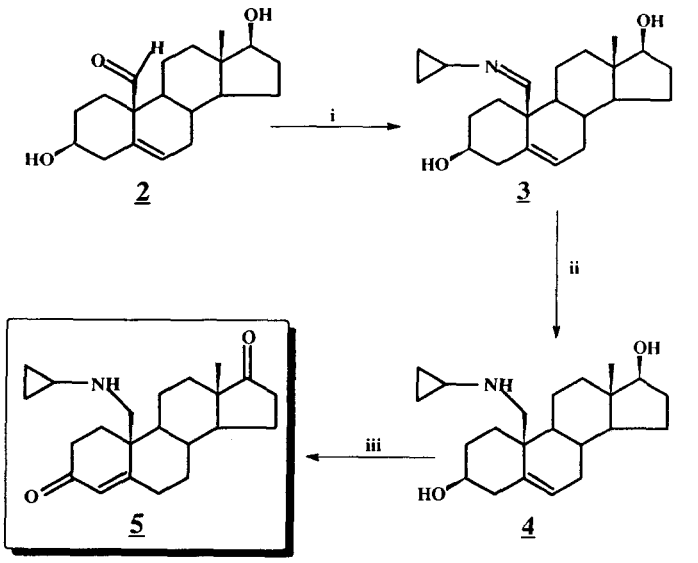


SCHEME 1 *Reagents and conditions*: i, cyclopropylamine, CF_3COOH, toluene, reflux; ii, $NaBH_4$, MeOH; iii, a modified Oppenauer oxidation.

of the amine to an aminium ion which could activate the adjacent cyclopropyl ring for covalent reaction with the enzyme.[8–10] Consequently, we embarked on the synthesis of the hitherto undescribed 19-(cyclopropylamino)-androst-4-en-3,17-dione (**5**, **CPA**, Scheme 1) on the basis that it may react covalently at the active site of aromatase, resulting in irreversible enzyme inhibition. This 19-amino compound is also especially interesting because attempts to prepare steroidal 19-primary amino-4-en-3-ones have been unsuccessful, although 19-amido,[14–16] 19-tertiary amino[14] and 19-aziridine[17] steroids have been described. We describe here the synthesis of a novel 19-cyclopropylamine steroid, and its evaluation as an inhibitor of microsomal aromatase.

MATERIALS AND METHODS

General

Most of the chemicals were purchased from Aldrich Chemical Company or Janssen Chimica. 5-Bromo-6β,19-epoxy-5α-androstane-3β,17β-diacetate (**1**) was obtained as a gift from Dr. C.H. Robinson of Johns Hopkins University School of Medicine, Baltimore, MD, USA.

Melting points were determined on a Kofler micro hot-stage and are uncorrected. IR spectra were recorded on a Perkin-Elmer 398 spectrophotometer as KBr discs. UV spectrum was obtained in ethanol on a Perkin-Elmer Lambda 5 instrument.

^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ or CD_3OD and referenced to $SiMe_4$ with a Varian XL-400 MHz spectrometer; *J* values are recorded in Hz. Mass spectra were obtained with a Finnigan MAT 90 spectrometer and an A.I.E. MS 30/Kratos DS 55 system. Elemental analysis were performed by Ms. M. Reinert of the Institut für Organische Chemie, Universität des Saarlandes, Saarbrücken, Germany. Analytical TLC was performed on Macherey-Nagel Düren glass plates (silica gel 60) with fluorescent indicator (254 nm). Flash column chromatography (FCC) was performed by the method of Still[18] using silica gel (60 A.C.C) purchased from Solvants Documantation Syntheses (SDS), Rastatt, Germany. Pet. ether refers to petroleum ether (b.p. 40–60°C).

Chemical Synthesis

19-Oxoandrost-5-en-3β,17β-diol (**2**). This compound, prepared from **1** as we reported previously,[17] provided spectral and analytical data as described.[17]

19-(Cyclopropylimino)-androst-5-en-3β,17β-diol (**3**). A solution of the 19-oxo-diol **2** (360 mg, 1.1764 mmol) in dry toluene (20 ml), excess cyclopropylamine (0.5 ml) and CF_3CO_2H (3 drops from a pasteur pipette) was refluxed under a Dean-Stark trap for 2 h before being cooled and concentrated *in vacuo* to give a light yellow solid (390 mg). This was purified by FCC (silica gel, 12:1, CH_2Cl_2-EtOH) to give *titled compound* **3** (white solid, 364 mg, 91%), m.p. (fragments at 105–109°C) 185–189°C. IR (KBr)/cm^{-1} 3300br. (OH) and 1640 (C=N), ^1H NMR δ 0.68 (3H, s, 18-H_3), 0.76–0.88 (4H, m, cyclopropyl-Hs), 2.85 (1H, m, cyclopropyl-H), 3.60 (2H, m, 3α and 17α-Hs), 5.63 (1H, br, s, 6-H) and 7.67 (1H, s, 19-H); ^{13}C NMR δ (100 MHz, $CDCl_3$) 7.8 (C-21), 7.9 (C-22), 10.7 (C-18), 21.4 (C-11) 30.3 (C-15), 30.5 (C-16), 31.3 (C-2), 32.4 (C-8), 33.3 (C-7), 36.6 (2C, C-10, C-12), 41.6 (C-1), 42.7 (C-4), 43.2 (C-13), 46.0 (C-9), 50.3 (C-14), 51.1 (C-20), 71.5 (C-3), 81.8 (C-17), 123.9 (C-6), 137.1 (C-5) and 164.7 (C-19); CIMS m/z 344 (MH^+, 37.4%), 343 (71.4), 325 (100), 297 (20), 258 (37.7) and 190 (37.8) (Found: M^+, 343.2510. $C_{22}H_{33}O_2N$ requires M, 343.2520; Found: C, 77.29; H, 9.82; N, 3.95. $C_{22}H_{33}O_2N$ requires C, 76.91; H, 9.69; N, 4.08%).

19-(Cyclopropylamino)-androst-5-en-3β,17β-diol (**4**). To a stirring solution of the imine **3** (300 mg, 0.8746 mmol) in dry methanol (50 ml) at 0°C, $NaBH_4$ (421 mg, 11.08 mmol) was added over a 30 min period. The solution after being stirred for 20 h, was treated with more $NaBH_4$ (210 mg) and the reaction continued for a further 12 h. The reaction mixture was concentrated to a volume of 5 ml, diluted with 0.1 N NaOH (20 ml) and the product extracted with 10% methanol in EtOAc (15 ml×3). The combined extracts were washed with brine, dried (Na_2SO_4) and concentrated to give a white solid which was subjected to column chromatography (silica gel, 40–60 microns, 18 cm×2.5 cm, CH_2Cl_2 saturated with ammonia; collecting *ca*. 20 ml fractions) to give recovered starting material (60 mg) and the *titled compound* **4** (217 mg, 72%), m.p. 195–196°C (from EtOAc); IR (KBr)/cm^{-1} 3360br. (OH, NH); ^1H NMR δ 0.22–0.43 (4H, m, cyclopropyl-Hs), 0.75 (3H, s, 18-H_3), 2.54, 2.57 (1H, B-part of an AB-system, *J* 11.8, one of 19-H), 3.01, 3.04 (1H, A-part of an AB-system. *J* 11.8, one of 19-H), 3.56 (1H, m, 17α-H), 3.60 (1H, m, 3α-H) and 5.62 (1H, br, s, 6-H); ^{13}C NMR δ 7.5 (2C,

C-21, C-22), 12.8 (C-18), 23.0 (C-11), 24.9 (C-15), 31.4 (C-2), 32.5 (C-16), 32.6 (C-8), 33.2 (C-7), 35.5 (C-12), 38.0 (C-10), 38.9 (C-1), 41.8 (C-4), 43.7 (C-13), 44.8 (C-9), 52.3 (C-14), 53.3 (C-19), 54.3 (C-20), 73.1 (C-3), 83.1 (C-17), 126.7 (C-6) and 139.3 (C-5); (Found: M^+, 345.2662. $C_{22}H_{35}O_2N$ requires M, 345.2677; Found: C, 75.66; H, 9.75; N, 4.00. $C_{22}H_{35}O_2N.1/4C_2H_5OCOCH_3$ requires C, 75.15; H, 10.15; N, 3.81%).

*19-(Cyclopropylamino)-androst-4-en-3,17-dione (***5***,* **CPA***).* A solution of the amine **4** (110 mg, 0.3188 mmol) in dry toluene (10 ml) and 4-methylpiperidone (1.0 ml) was refluxed under a Dean-Stark trap until *ca*. 2 ml of distillate had collected. Dry aluminium isopropoxide (214 mg, 1.05 mmol) was added to the stirred reaction mixture which after being heated under reflux for 3 h was cooled, diluted with EtOAc and washed successively with 5% aq. $NaHCO_3$ and brine and dried (Na_2SO_4). Evaporation of the solvent gave a yellow oil which was purified by column chromatography (silica gel, 40–60 microns, 15 cm×2.5 cm, CH_2Cl_2 saturated with ammonia; collecting *ca*. 20 ml fractions) to give *compound* **5** (45 mg, 40%), m.p. 58–60°C; IR (KBr)/cm^{-1} 3420 (NH), 1740 (C=O) and 1670 (conj. C=O); UV EtOH λ_{max} 238 nm (ε 11,000); ^{1}H NMR δ 0.20–0.45 (4H, m, cyclopropyl-Hs), 0.85 (1H, m, cyclopropyl-H), 0.94 (3H, s, 18-H_3), 2.85, 2.86 (1H, B-part of an AB-system, *J* 11.5, one of 19-H), 2.91 (1H, m) 3.18, 3.21 (1H, A-part of an AB-system, *J* 11.5, one of 19-H) and 5.90 (1H, s, 4-H); ^{13}C NMR δ 6.6 (C-21), 6.9 (C-22), 14.1 (C-18), 20.9 (C-11), 21.9 (C-15), 31.3 (C-12), 31.5 (C-7), 31.8 (C-6), 33.4 (C-2), 33.7 (C-8), 35.6 (C-16), 35.9 (C-1), 42.8 (C-10), 47.8 (C-13), 51.3 (C-14), 53.3 (C-9), 55.0 (2C, C-19, C-20), 126.9 (C-4), 167.0 (C-5), 200.4 (C-3) and 220.0 (C-17) (Found: M^+, 341.2352. $C_{22}H_{31}O_2N$ requires M, 341.2364; Found: C, 76.91; H, 8.75; N, 3.89. $C_{22}H_{31}O_2N$ requires C, 77.37; H, 9.16; N, 4.10%).

Preparation of Aromatase

The enzyme was obtained from the microsomal fractions of freshly delivered human term placenta according to the method of Thompson and Siiteri.[19] The microsomes were resuspended in a minimum volume of phosphate buffer (0.05 M) and stored at −70°C. No loss of activity was observed within four months. Protein concentration was measured according to the method of Lowry *et al.*[20]

Aromatase Inhibition Studies

Enzyme activity was monitored using the tritiated water method of Thompson and Siiteri.[19] The incubations were performed as previously described.[21] The tritiated water formed during the conversion of [1β,2β-^{3}H]-testosterone to estradiol was determined after separation of the steroids by dextran-coated charcoal (DCC). Following centrifugation, the radioactivity of a 200 μl supernatant aliquot was determined. For the determination of IC_{50} values, compounds were tested at six appropriate concentrations. The molar concentration causing 50% inhibition of aromatase activity was determined by plotting the percentage inhibition *vs*. the concentration of inhibitor on a semilog plot.

The K_i value was determined using the same procedure as described above but with modifications as follows: concentrations of [^{3}H]-testosterone 0.05–0.4 μM,

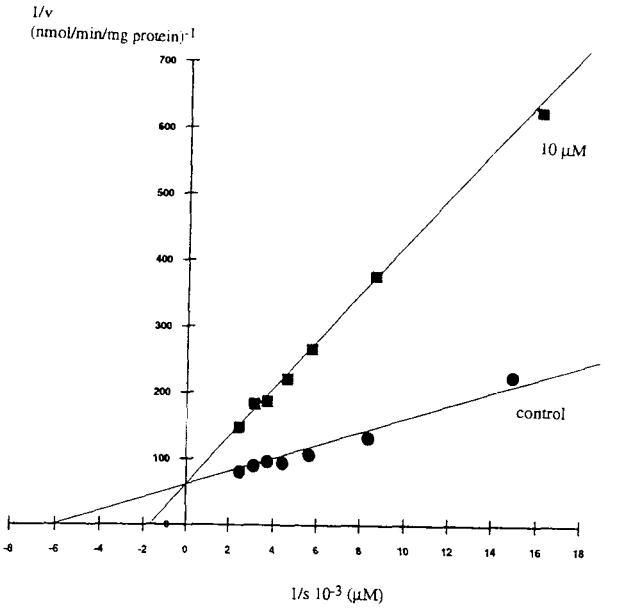


FIGURE 1 Lineweaver-Burk analysis (1/v *vs* 1/s) of 19-cyclopropylamine **CPA** at 10 μM. K_m for testosterone = 167±5 nM. Each point is a mean of three determinations.

microsomal protein, 20–40 mg/incubation, 1 mM NADP and NADPH generating system (glucose-6-phosphate, 10 mM and glucose-6-phosphate dehydrogenase, 1 EU). The incubations (0.25 ml) with or without inhibitor were carried out for 15 min at 30°C under initial velocity conditions. The K_i value was calculated from the Lineweaver-Burk plot (Figure 1) and the K_m value for testosterone (substrate) was also determined.

The assay for irreversible inhibition of aromatase was performed as previously described,[21] by incubating microsomal aromatase with NADPH and inhibitor (10 μM) for 30 min. After separation of the inhibitor (shaking with DCC followed by centrifugation), aromatase activity in the supernatant was determined at 6, 12 and 18 min intervals. The substrate used in this assay was also [^{3}H]-testosterone (0.5 μM).

The time-dependent inactivation assay was performed as follows: The inhibitor (10 μM) incubated with microsomal aromatase, NADPH, in 0.05 M phosphate buffer, pH 7.4 in a total volume of 0.5 ml at 30°C in air. Aliquots (50 μl) were removed at various time intervals (5–30 min). Following DCC treatment, each aliquot was assayed for aromatase activity, using [^{3}H]-testosterone (1.0 μM) as substrate.

RESULTS AND DISCUSSION

Although a few other steroidal cyclopropylamines have been reported,[12,13] we expected difficulties in the present synthesis because of the sterically hindered

19-position. Attempts to transform 19-oxoandrost-5-en-3β,17β-diol (**2**) into 19-(cyclopropylamino)-androst-5-en-3β,17β-diol (**4**) by reductive amination of **2** with cyclopropylamine failed. We eventually synthesized the desired 19-cyclopropylamino steroid (Scheme 1) by condensation of the key intermediate **2** with cyclopropylamine in refluxing toluene, followed by reduction of the resulting imine with sodium borohydride ($NaBH_4$). The key intermediate in our synthesis, **2**, was prepared as reported previously.[17]

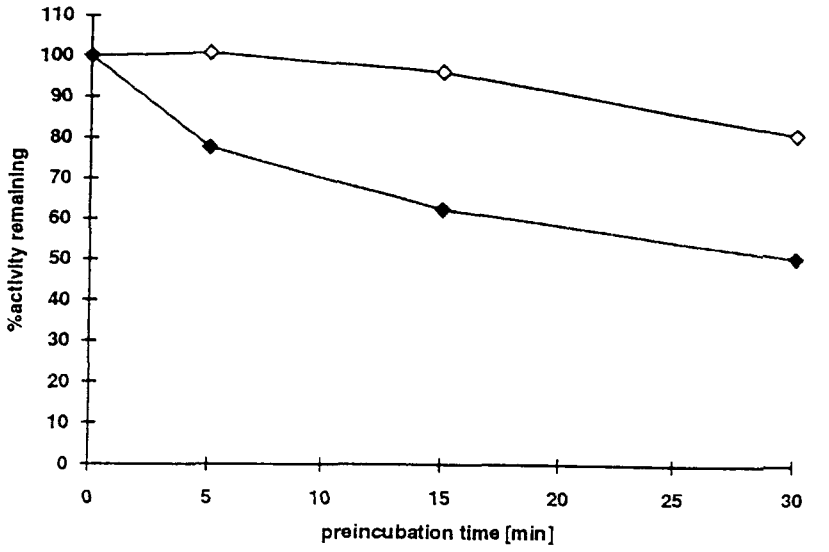
Reaction of the 10β-aldehyde (**2**) with excess cyclopropylamine and trifluoroacetic acid (CF_3CO_2H) as catalyst (acetic acid was ineffective) in dry toluene at relux for 2 h with removal of water (Dean-Stark trap) gave the desired 19-imine (**3**) in 91% yield. It is necessary to point out that no trace of the imine was formed (as observed by TLC) in the absence of CF_3CO_2H even after prolonged (*ca.* 12 h) refluxing in toluene. Compound **3** was reduced with $NaBH_4$ in methanol at room temperature to give the 19-cyclopropylamine (**4**) in 72% yield. Oxidation of **4** to the desired 19-(cyclopropylamino)-androst-4-en-3,17-dione (**5**, **CPA**) was accomplished by a modified Oppenauer oxidation.[22] To our knowledge, **5** is the first 19-secondary amino-4-en-3-dione steroid described in the literature. All compounds gave satisfactory analytical and spectroscopic data ^{13}C NMR chemical shifts were assigned by comparison with reported values for closely related compounds.[23,24]

We first assessed the inhibitory potency of **CPA** using human placental microsomal aromatase as described in the Experimental section[21] and found that it is a modest inhibitor of aromatase (IC_{50} value = 10 μM, using 2.5 μM testosterone as substrate). 4-Hydroxyandrostenedione (**4-OHA**, formestane), a potent mechanism-based inhibitor of aromatase was also tested for comparison. Under our assay conditions, **4-OHA** was more potent than **CPA**, with an IC_{50} value of 0.6 μM.

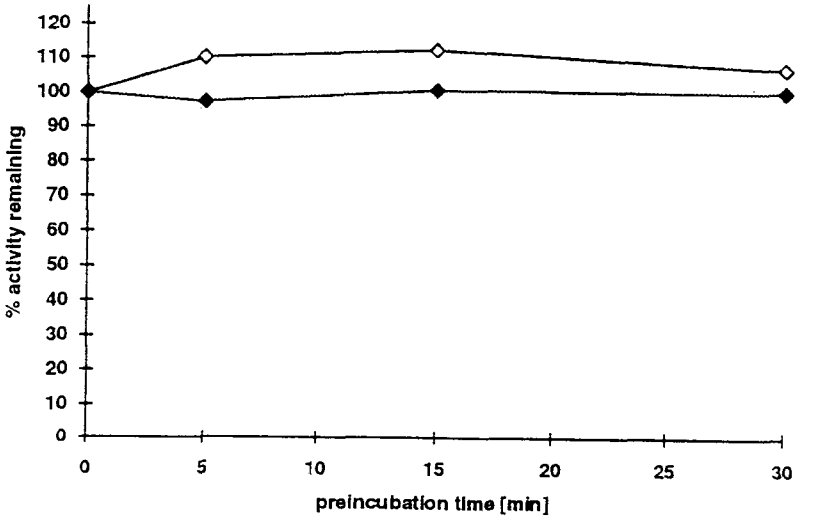
Following the IC_{50} determination, **CPA** was evaluated further to determine the apparent K_i value (from Lineweaver-Burk plot, Figure 1). **CPA** exhibited kinetics indicative of an excellent competitive inhibitor of placental aromatase with a K_i value of 92±2 nM [K_m for testosterone (substrate) is 167±5 nM].

Assays designed to examine possible time-dependent inactivation of the enzyme a 30°C show that **CPA** brought about a time-dependent loss of enzymatic activity over 30 min in the presence of NADPH (Figure 2(a)). No inactivation was observed when **CPA** was incubated with the enzyme without added NADPH (Figure 2(b)). Thus, the loss in enzyme activity is totally dependent on the presence of NADPH, implying that the inactivation process involves oxidative enzymatic activation of **CPA**. Addition of substrate testosterone (20 μM) to the preincubation medium reduced the inhibition due to **CPA** by *ca.* 50%. Thus, the inhibition by **CPA** was active site directed. In addition, the inhibitory potency of **CPA** was not affected by the presence of the nucleophile, L-cysteine (0.5 mM, data not shown). These findings suggest that **CPA**, after binding at the steroid site forms a tight bond with the enzyme; i.e., circumstantial evidence that a reactive alkylating agent is not released from the active site.

To further define the mechanism of inhibition of **CPA**, we performed an assay for irreversible inhibition as previously described.[21] Our inhibitor caused a 42% irreversible inhibition of the enzyme (Figure 3). The reason(s) for this partial inactivation of aromatase by **CPA** is unknown at this time. However, a similar observation



(a)



(b)

FIGURE 2 Time dependent inactivation experiment: Interaction of **CPA** with aromatase (a) in the presense of NADPH, (b) in the absense of NADPH; ◊ no inhibitor (control); ♦ 10 mmol dm^{-3} of **CPA**. Each point represents the average of duplicate analyses and the deviations were within a range of ±4% of the mean.

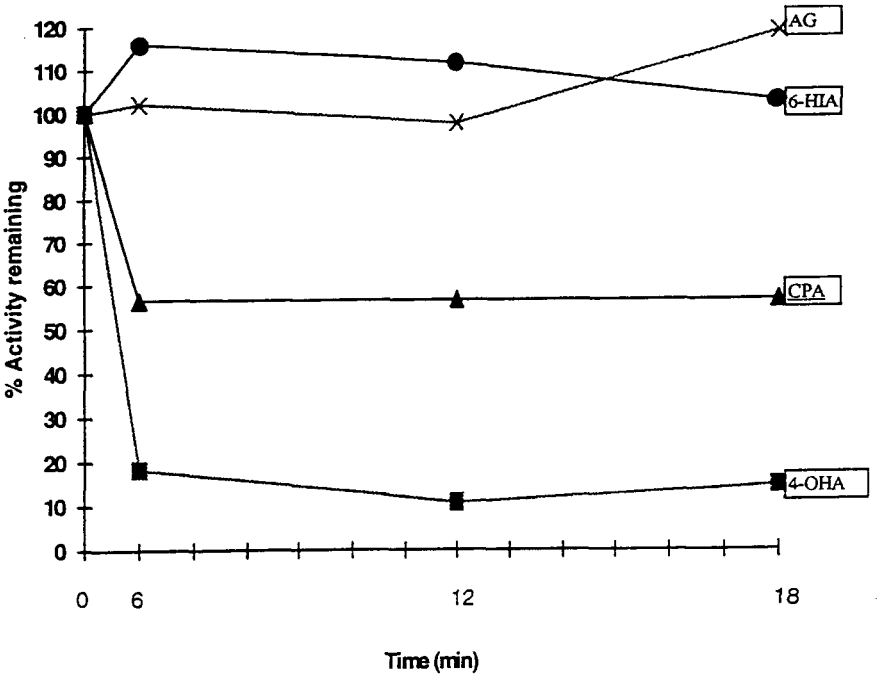


FIGURE 3 Irreversible inhibition studies with compound **CPA**, **4-OHA**, **6-HIA** and **AG**: Time course assay of aromatase activity after separate treatment of the enzyme (preincubation time of 30 min) with the inhibitors **CPA**, **4-OHA**, **6-HIA** and **AG** [10 μM each, substrate: [^{3}H]-testosterone (1 μM)] followed by removal of the inhibitors from the microsomes by dextran coated charcoal (DCC) treatment and centrifugation. Each point represents an average of two replicates with three experiments and the deviations were within a range of ±5% of the mean.

has been reported by Macdonald *et al.*[9] in their studies of the inactivation of rat hepatic cytochrome P-450 by cyclopropylamine inhibitors. The irreversible inhibitory potencies of three known aromatase inhibitors, **4-OHA**, (E)-6-hydroximinoandrost-4-en-3,17-dione (**6-HIA**) and aminoglutethimide (**AG**) were also determined for comparison (Figure 3). As expected, **4-OHA** caused *ca.* 90% irreversible inhibition of the enzyme while the inhibitions caused by **6-HIA** and **AG** respectively, were completely reversible.

Our present findings show that **CPA** is a modest mechanism-based irreversible inhibitor of human placental microsomal aromatase. We believe that covalent modification of the enzyme probably takes place; however, this point needs to be demonstrated with radiolabeled inhibitor. On the basis of our results and of data obtained with other cyclopropylamine inhibitors of cytochrome P-450 enzymes,[8–10,12] we propose that **CPA** is activated by enzymatic (aromatase P-450) one-electron oxidation of the amino nitrogen, followed by opening of the cyclopropyl ring and

formation of an aminium cation radical which might proceed to form a covalent bond with the enzyme while still bound in the active site. However, additional experiments are needed to characterize fully the mechanism of aromatase inhibition by **CPA**.

In summary, we have synthesized the novel 19-cyclopropylamino steroid (**5**, **CPA**) and have studied its interaction with human placental microsomal aromatase. The results of our experiments show that **CPA** is a modest mechanism-based irreversible inhibitor of aromatase. A possible mechanism for this inactivation process of aromatase is enzymatic generation of reactive aminium ion through one-electron oxidation of **CPA**.

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