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Minireview

Enzyme-activated Inhibitors of Steroidal Hydroxylases*

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Cytochrome *P*450 monooxygenases (CY*P*450) of the steroid biosynthetic pathways are highly substrate specific in comparison to the variable specificities of hepatic CY*P*450 enzymes. Both groups of enzymes catalyze the reductive cleavage of molecular oxygen with transfer of oxygen to the substrate to form hydroxylated derivatives. Those steroids formed in endocrine tissues represent highly specific endocrine/autocrine hormones with enhanced biological potency, while hepatic hydroxylation of steroids reduces their endocrine bioactivities and enhances urinary elimination. Changes of the hormonal milieu of endocrine and peripheral tissues are associated with the development of hyperplastic and/or malignant conditions. Hormone deprivation induces regression of endocrine dependent growth via apoptosis and may also alter growth of hormone insensitive cells by the induction of negative growth factors. Biosynthetic CY*P*450 enzymes of those steroids that mediate specific disease processes are potential therapeutic targets for selective intervention. This objective can be accomplished by the design of specific pseudo-substrate analogs that will be activated during enzyme-directed catalysis to produce a reactive functional group in the enzyme's active site that will either tightly or irreversibly bind and inactivate the host enzyme. The CY*P*450 enzymes that hydroxylate the C_{19} carbon of androgens (aromatase) and the C_{18} carbon of corticosterone (aldosterone synthase) were selected as target enzymes because they are terminal enzymes of biosynthetic pathways which hydroxylate specific angular methyl groups. Hypersecretion of their respective hormonal products, estrogens and aldosterone, are associated with specific disease conditions. Substrate analogs containing ethynyl, vinyl, or nitrile groups attached to the C_{19} or C_{18} methyl groups were enzyme-activated inhibitors. The ethynyl analogs, 19-acetylenic androstenedione (Plomestane) and 18-acetylenic deoxycorticosterone, had nanomolar inhibitory constants (K_i values) and were irreversible inactivators of their target enzymes in animal models.

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INTRODUCTION

Cytochrome *P*450 monooxygenases (CY*P*450) represent a diverse class of complex enzymes related by similarities in their catalytic functions and the presence of a heme prosthetic group. Oxidative reactions by these enzymes dominate hepatic metabolism due to their broad and overlapping selectivities for xenobiotic lipophilic substrates [1]. In contrast, CY*P*450 enzymes involved in physiological biosynthetic pathways exhibit selective substrate specificities, especially those associated with steroid hormones. The latter CY*P*450s regulate the biosynthesis of steroids with very diverse biological activities, e.g. estrogens, progestins and corticoids.

CY*P*450 hepatic drug metabolism and detoxification introduces hydroxyl functionalities that increase polarity and facilitate aqueous solubility and urinary excretion. These same enzymes can also activate common environmental polycyclic hydrocarbons, such as benzo[α]pyrene (BaP), via oxygen introduction at numerous carbon centers. These arene oxides can be catalytically opened by epoxide hydrase, an hepatic scavenger enzyme, to yield *trans*-dihydrodiols, which are processed by another *P*450 epoxidation cycle to generate a stereo-selective epoxide trans to the

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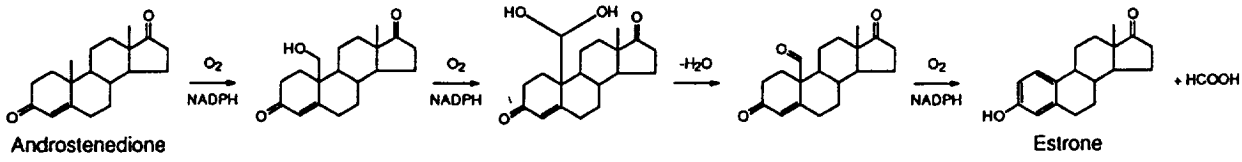
hydroxyl (e.g. BaP-diol epoxide I) which is more mutagenic than the parent or intermediate compounds [2]. The effectiveness of liver metabolism is also enhanced via differential induction of hepatic monooxygenases. Consequently, the metabolism of one substrate can also be altered by the residual effects of a preceding substrate or by more potent xenobiotic inducers which alter the substrate metabolic spectrum and product profile [3]. This concept of enhanced pharmacological inhibition of the metabolism of one substrate by other pseudo-substrates had been exploited with the use of monosubstituted acetylenic compounds as insecticide synergists [4, 5], but had not until recently been incorporated into rational drug design to provide selective pharmacological synergism and enhanced therapeutic benefits.

The steroidal biosynthetic pathway offered a unique situation for evaluation of this concept, since the diverse terminal products are the result of stereospecific hydroxylations of specific steroid substrates by unique CY*P*450 enzymes. We chose to utilize the steroidal substrate specificity of the biosynthetic CY*P*450 enzymes which hydroxylate the angular methyl groups at the steroidal ring junctures to design pseudo-substrates as selective enzyme inhibitors. The first CY*P*450 enzyme evaluated was aromatase ($CYP450_{Arom}$) which converts androgens to estrogens via two hydroxylations at carbon C_{19} (Scheme 1). The advantages of using aromatase as a therapeutic target enzyme are: (1) it is rate-limiting in the formation of its terminal product; (2) the $CYP450_{Arom}$-directed hydroxylations are confined to the C_{10} angular methyl (C_{19}) group, which is not a metabolic site of hepatic CY*P*450 enzymes [6, 7]; (3) the C_{10} methyl group is accessible for chemical modification; (4) the inhibition of estrogen biosynthesis is of therapeutic significance in hormone dependent diseases since estrogen receptor blockade by the antiestrogen, tamoxifen, is an effective breast cancer treatment [8] and extraglandular estrogen biosynthesis is inhibited by aminoglutethimide, a competitive aromatase inhibitor [9].

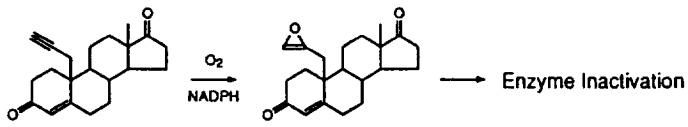
Following our initial successes in designing mechanism-based inhibitors of aromatase [10], we extended our concepts by designing inhibitors of aldosterone biosynthesis (Scheme 2). The C_{13} methyl (C_{18}) group undergoes two CY*P*450 catalyzed hydroxylations to form the mineralocorticoid, aldosterone, from corticosterone, a glucocorticoid, via the recently characterized aldosterone synthase ($CYP450_{Aldo}$) [11–13]. Our inhibitor studies [14, 15] established that two different CY*P*450 enzymes, 11β-hydroxylase and aldosterone synthase, were involved in the biosynthesis of corticosterone (CORT), 18-hydroxy-11-deoxycorticosterone (18-OH-DOC), 18-hydroxy corticosterone (18-OH-CORT) and aldosterone (ALDO) from 11-deoxycorticosterone (DOC). Aldosterone synthase is characterized by the same criteria which made aromatase a therapeutic target enzyme. Aldosterone is a terminal product of a rate-limiting CY*P*450 hydroxylation at C_{18} of corticosterone. Similarly, this angular methyl group at C_{13} is not subject to hepatic hydroxylations [6]. Finally, the mineralocorticoid receptor antagonist, spironolactone, is an effective antihypertensive agent and used clinically for treatment of primary aldosteronism and diuretic-induced secondary hyperaldosteronism.

We chose to design pseudo-substrates which would be potential mechanism-based enzyme inactivators for aromatase and aldosterone synthase. This class of inhibitors will presumably remain unreactive until the catalytic activity of the target enzyme generates an activated intermediate species which subsequently alkylates a proximal nucleophilic residue [16]. Such inhibitors are extremely specific, since they inactivate only those enzymes for which they are a pseudo-substrate. The return of enzyme activity is dependent upon *de novo* enzyme synthesis and not on the inhibitor's off-rate from the enzyme-inhibitor binding

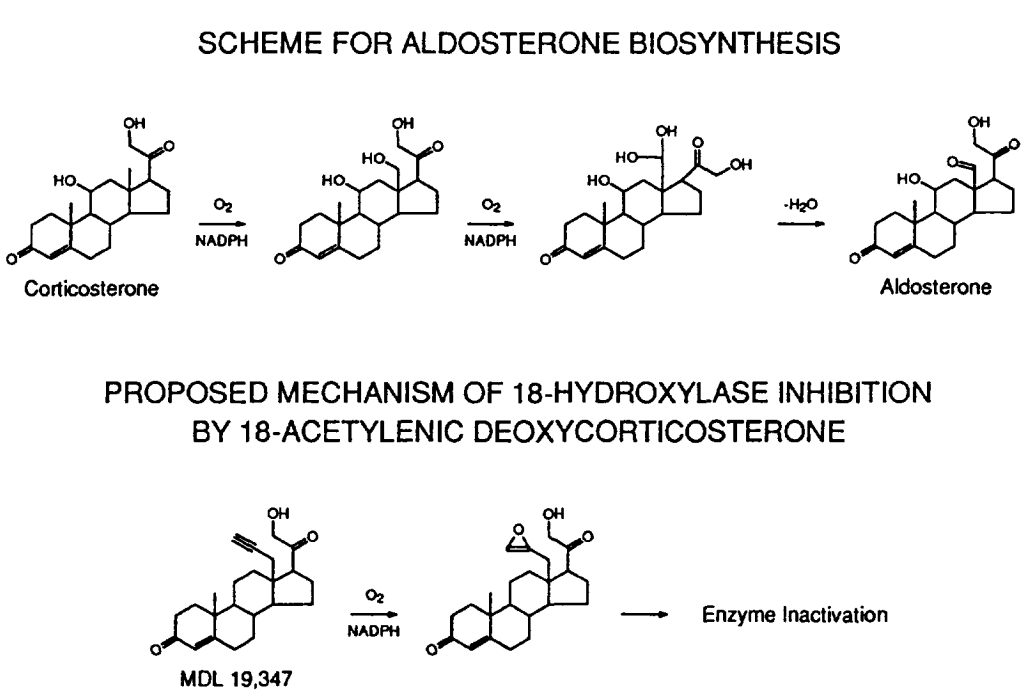
SCHEME FOR ESTROGEN BIOSYNTHESIS



PROPOSED MECHANISM OF AROMATASE INHIBITION BY PLOMESTANE



Scheme 1.

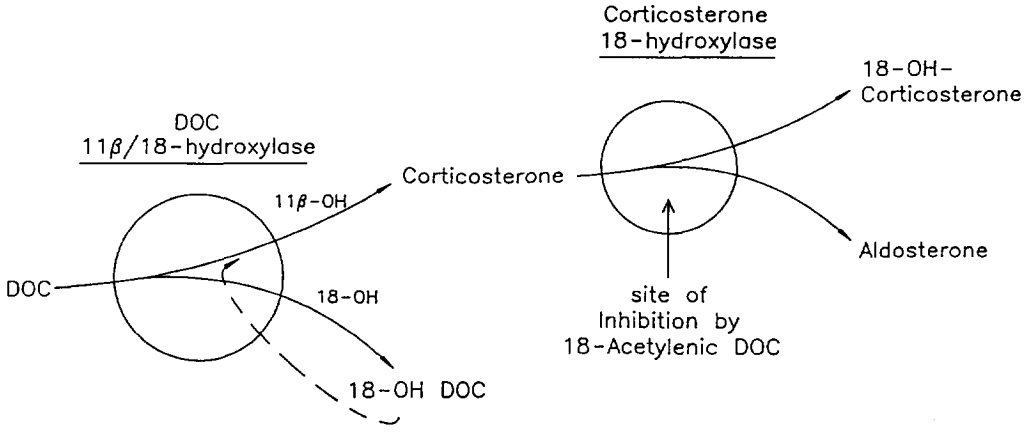


Scheme 2.

complex $[E + I \leftrightarrow E - I]$ as is the case with competitive inhibitors. Therefore, mechanism-based enzyme inhibitors achieve prolonged pharmacological effects, since the enzyme may remain inactivated even after drug clearance [17].

19-Acetylenic-androstenedione was one of the initial pseudo-substrates designed as mechanism-based inhibitors of aromatase [10]. This acetylenic compound was synthesized because synthetic 17α-ethynyl steroids (norethisterone or ethynylestradiol) selectively induced self-catalyzed inactivation of hepatic CY*P*450 enzyme activity [18], reduced CY*P*450 concentrations and heme levels [19], and also reduced phenobarbital-induced hepatic CY*P*450 isozymes [20]. Various terminal acetylenic substrates mediate NADPH-dependent enzyme-catalyzed inactivation of hepatic CY*P*450 enzymes [21, 22]. Thus, we proposed that the addition of an acetylenic functional group to the C_{10} methyl of androstenedione or C_{13} methyl group of corticosterone or DOC would provide pseudo-substrates which would act as selective enzyme-activated inhibitors of aromatase and aldosterone synthase, respectively. These inhibitors would not be expected to readily inactivate hepatic CY*P*450 enzymes due to the regioselectivity and stereospecificity associated with these angular methyl groups [6].

This report describes the biochemical and endocrine pharmacological experiments conducted to establish chemical specificity and enzymatic selectivity of rationally designed enzyme-activated inhibitors of aromatase. These concepts are extended with novel data on the enzyme irreversibility of steroidal aromatase inhibitors which have been clinically investigated. New clinical Phase 1 data are included for Plomestane. Concepts



Scheme 3. Site of aldosterone synthase inhibition by 18-acetylenic deoxycorticosterone.

established via chemical modification at C_{19} were utilized in the design of C_{18} analogs, which represent the first enzyme-activated inhibitors of aldosterone biosynthesis. Additional biochemical and endocrine pharmacological experiments are described which extend our previous meeting reports on these novel inhibitors.

MATERIALS AND METHODS

Chemicals and reagents

All inorganic chemicals and organic solvents were of reagent grade and those used in aromatase assays have been previously described [23, 24]. Tritium-labeled steroids: [1-^{3}H]androstenedione, 28 Ci/mmol; [1,2-^{3}H]testosterone, 40 Ci/mmol; [1,2,6,7-^{3}H]-corticosterone, 105 Ci/mmol; [17α-methyl-^{3}H]17β-hydroxy-estra-4,9,11-trien-3-one (methyltrienelone, R1881), 82 Ci/mmol; and [7α-methoxycarbonyl-^{3}H]15β,16β-methylene-3-oxo-17α-pregn-4-ene-21, 17-carbolactone (ZK 91587), 82 Ci/mmol, were obtained from NEN Research Products (Boston, MA) and [1α,2α-^{3}H]DOC, 36–46 Ci/mmol, was purchased from Amersham Corp. (Arlington Heights, IL). These labeled steroids were chromatographed (HPLC) to ensure radiochemical purity (99 + %). Purity of nonradioactive steroids obtained from commercial sources was determined prior to use. Dextran coated-charcoal (DCC) was prepared 24 h prior to use as a 1:10 suspension (w/w) of Dextran T-70 (Pharmacia Fine Chemicals, Inc., Piscataway, NY) and washed activated charcoal, Norit-A (MCB, Norwood, OH), in assay buffer to adsorb unbound tritiated steroids in aromatase and receptor binding assays. Aqueous scintillation cocktail, Atomlight® (NEN Research Products), was used to enhance radioactivity detection for aromatase and receptor binding assays. Scintillation fluid, Flo-Scint II® (Packard Instrument Co., Meriden, CT), was used for radioactivity detection in HPLC-based aldosterone assays.

Steroidal inhibitors

The synthesis of 19-acetylenic androstenedione [Plomestane, MDL 18,962, 10-(2-propynyl)estr-4-ene-3,17-dione], 17β-hydroxy Plomestane [17β-hydroxy-10-(2-propynyl)estr-4-ene-3-one], 6β-hydroxy Plomestane [6β-hydroxy-10-(2-propynyl)estr-4-ene-3,17-dione] and 19-allenyl androstenedione [10-(1,2-propadienyl)estr-4-ene-3,17-dione] have been previously described [10, 25]. 18-Acetylenic DOC [18-Ac-DOC, 21-hydroxy-13-(2-propynyl)-18-nor-preg-4-ene-3,20-dione] was prepared from bis-norcholenaldehyde (3-oxopregn-4-ene-20β-carboxaldehyde, Upjohn Co., Kalamazoo, MI) in a manner analogous to the preparation of 11β-hydroxy-18-acetylenic progesterone [11β-hydroxy-13-(2-propynyl)-18-nor-preg-4-ene-3,20-dione] and 18-acetylenic progesterone [13-(2-propynyl)-18-nor-preg-4-ene-3,20-dione] [26]. The 21-hydroxyl substitution was introduced via peracid epoxidation of a suitably protected trimethylsilyl enol ether [27]. Preparation of 18-allenyl progesterone [13-(1,2-propadienyl)-18-nor-preg-4-ene-3,20-dione] and 18-vinylidene analogs of progesterone (18-vinyl progesterone) and deoxycorticosterone (18-vinyl DOC) have been reported [28]. 18-Cyanoprogesterone [18-cyanopregn-4-ene-3,20-dione] was prepared in an analogous fashion to 11β-hydroxy-18-cyanoprogesterone [11β-hydroxy-18-cyanopregn-4-ene-3,20-dione] [26, 29]. Other androstenedione analogs that were time-dependent aromatase inhibitors [30–32] were evaluated for comparative purposes. These compounds were Formestane (4-hydroxyandrostenedione), prepared as reported [33], and Atamestane (1-methylandrosta-1,4-dien-3,17-dione) obtained from Schering AG (Berlin, Germany). Other steroids or compounds not obtained from commercial sources were synthesized and characterized by standard methods in the Research Chemistry Department, Marion Merrell Dow Research Institute (MMDRI), Cincinnati, OH. Stock solutions (10 mM) of nonlabeled steroids were prepared in dimethyl sulfoxide (DMSO) and stored at 4°C. For assays, serial dilutions (1:10, v/v) were made with assay buffer to obtain desired concentrations. Compounds not soluble at 1 mM in 10% DMSO/buffer were rediluted from the stock solution into buffer containing 40% ethanol and then serially diluted with assay buffer. Steroid diluents were also prepared as solvent/buffer solutions at their highest assay concentration to ensure that these compounds remained in solution under assay conditions.

Animals

Sprague–Dawley (SD) rats were obtained from USDA-certified animal laboratories and maintained in a controlled environment with a 14 h photoperiod (6:00–20:00 h). These animals were fed laboratory rodent chow (Purina Mills Inc., St Louis, MO) or, when indicated, a sodium deficient diet, Hartcroft–Eisenstein type (ICN Nutritional Biochemicals, Cleveland, OH), and deionized water *ad libitum*. Bilateral adrenalectomy or gonadectomy was performed using inhalation anesthesia (Metofane, Pitman-Moore Inc., Mundelein, IL). Adrenalectomized rats were maintained on saline containing 1% sucrose *ad libitum*. At necropsy, rats were sacrificed by cervical dislocation or decapitation while under CO_2-induced anesthesia. Male SD rats (175–225 g) that had been maintained on a Na-deficient diet for at least 2 weeks were used as a source of adrenal glomerulosa tissue. Similarly treated rats received single subcutaneous (s.c.) injections of 1–30 mg/5 ml/kg of 18-Ac-DOC or 5 ml/kg of aqueous vehicle (10% ethanol in 0.05% carboxymethylcellulose). These animals were sacrificed at 2, 4 or 6 h following treatment. These animals were bled via cardiac puncture while under CO_2-induced anesthesia. Sera from these bleedings were stored at

−80°C until assayed for aldosterone levels by RIA methodology. All animal protocols were approved by the MMDRI Animal Care and Use Committee.

Enzyme assays

Aromatase assays. Lyophilized placental microsomes were prepared from 5–10 frozen term placentas from non-smoking women and stored at −80°C [23]. Aromatase activities for these preps were 17–35 pmol estrogen formed/min/mg protein at V_{max} with K_m values for androstenedione of 22–40 nM. Pseudo-substrate analogs of androstenedione were evaluated as aromatase inhibitors using both competitive and time-dependent kinetic analyses [23, 24, 34]. Selected compounds were evaluated for aromatase active-site directed inhibition by determining the time-dependent inactivation of aromatase activity by the inhibitor when co-incubated with 1, 3, or 10 times the K_m of the natural substrate, testosterone [23, 34]. The requirement for NADPH cofactor for time-dependent inhibition was determined by the effect of its presence or absence during the preincubation phase of time-dependent assays [23, 34]. Disassociation of enzyme-inhibitor complexes, $[E - I]$, were evaluated in experiments where inhibition kinetics were determined before and after the addition of additional enzyme to the reaction tubes [23]. These latter parameters were also evaluated by centrifugal precipitation of the microsomal pellet ($[E - I]$-complexes), which were washed with excess buffer and reconstituted in assay buffer prior to determining residual aromatase activity [24, 34].

Aldosterone synthase assays. Adrenals from male SD rats fed a Na-deficient diet for 2–4 weeks were removed and decapsulated to obtain the glomerulosal zone tissue. These glomerulosa/capsular tissues were homogenized in ground-glass homogenizers with 0.5 ml of Tris assay buffer (8.5 mM $MgCl_2$, 2.7 mM $CaCl_2$, 3.13 mM KCl, 7.59 mM NaCl, 0.1% $(C_2H_5)_3N$ and 50 mM Tris–HCl adjusted to pH 7.4), diluted with Tris buffer to provide 5–12 mg of glomerulosal tissue per ml of buffer and centrifuged at 800 *g* for 10 min. Aliquots (300 µl) of this adrenal cytosolic supernatant were added as the enzyme source to 35 ml glass centrifuge tubes containing 100 µl of an NADPH-generating system (2.92 mg/ml glucose-6-phosphate, 1.68 mg/ml NADP, and 4 IU glucose-6-phosphate dehydrogenase) and 50 µl of steroidal inhibitor. Following preincubation in a Dubnoff shaking incubator at 1 atm of 95% O_2/5% CO_2 at 25°C for varying time intervals of 0–60 min, the assays were started with addition of 50 µl of [^{3}H]DOC (~235,000 dpm, 500 pmol) or [^{3}H]CORT (~865,000 dpm, 500 pmol) in 0.1% DMSO/Tris buffer for 10 min assays. Time-course assays were conducted over a 1–2 h incubation interval in duplicate assay tubes. Assay components were increased in volume to permit sequential sampling of assay reactants (0.5 ml) at each time point. These reactants were quenched by the addition of 5 ml of ethyl acetate and 6.7 µg of nonradioactive steroids (e.g. DOC, 18-OH-DOC, CORT, 18-OH-CORT, and ALDO) were added to enhance recovery of radioactive steroidal products. The samples were extracted twice with 5 ml of ethyl acetate and evaporated under a N_2 stream at 30–40°C. Residues were dissolved in 105 µl of MeOH:H_2O (40:60, v/v) containing 0.1% $(C_2H_5)_3N$, and transferred to microvials for auto injection (Wisp 712, Waters, Millipore Corp., Milford, MA) into a HPLC system (Beckman Instruments Inc., San Ramon, CA) for analysis. Separation of products was achieved on a C18 reverse phase column (5 µ ODS-Hypersil, 4.6 × 250 mm; Shannon, Altech Associates, Inc., Deerfield, IL) with 1 ml/min flow rate using a MeOH:H_2O gradient of solvent A (10:90 v/v) and solvent B (90:10 v/v) containing 0.1% $(C_2H_5)_3N$. The gradient solvent elution cycle conditions in respect to duration (min) and the change in percent solvent B are: 0.0–0.01 min, 40–41% B; 0.01–6 min, 41–42% B; 6–8 min, 42–48% B; 8–12 min, 48–50% B; 12–14 min, 50–55% B; 14–16 min, 55–60% B; 16–18 min, 60–70% B; 18–32.5 min, 70% B; 32.5–32.7 min, 70–40% B; 32.7–40 min, 40% B. Nonlabeled substrate remaining and products formed were monitored by the UV absorbance at 246 nm with an in-line Kratos Spectroflow 757 spectrometer (Kratos Analytical Instruments, Ramsey, NJ) and radioactive components were measured by an in-line Flow-one β-scintillation spectrometer (Radiomatic Instruments and Chemical Co., Inc., Meriden, CT). Retention times for chromatographic standards were: ALDO, 16.9 min; 18-OH-CORT, 18.5 min; 19-OH-DOC, 20.2 min; 11-Dehydro-CORT, 22.8 min; 19-Oxo-DOC, 23.7 min; 18-OH-DOC, 25.1 min; CORT, 26.8 min; 19-Nor-DOC, 29.2 min; DOC, 31.6 min.

All assays were run in duplicate including solvent/buffer controls, except for evaluations of corticoid biosynthesis in glomerulosal tissues from drug-treated rats. In these assays, adrenal capsules with attached glomerulosal cells from each rat were placed in 10 ml ground-glass homogenizers containing 300 µl of cold assay buffer. These capsules were homogenized, centrifuged at 800 *g* for 10 min, and the remaining assay ingredients were added to the homogenization tubes as described above.

The mass of steroidal product formed was the multiple of the mass of substrate added times the percent of the ratio of radioactivity (cpm) of each steroidal product peak, corrected for cpm of solvent blank, to the total cpm (solvent blank corrected) determined for each respective chromatogram. Kinetic parameters for steroidal products of aldosterone synthase were estimated using adrenal glomerulosal tissue obtained from rats on a Na-deficient diet [34]. Apparent K_m values were 320 nM for 18-OH-corticosterone and 240 nM for aldosterone using DOC as the substrate with respective V_{max} values of 308 and 74 pmol/mg of glomerulosal tissue/h.

Steroid receptor binding assays

Mineralocorticoid receptor assays. Male SD rats (175–225 g) were adrenalectomized 18–36 h prior to sacrifice by decapitation. Kidneys were isolated *in situ* by ligation of the aorta anterior and posterior to the renal arteries. Each kidney was perfused by a renal arterial catheter with ~2 ml of cold (~4°C) isotonic 10 mM Tris buffered-saline (pH 7.4) via a push–pull perfusion procedure. The blanched kidneys were removed, weighed, sliced, and homogenized in cold TEMG assay buffer (10 mM Tris, 1.5 mM EDTA, 10 mM Na molybdate and 20% glycerol, pH 7.4) at 1:3 ratio (w/v) with a Polytron Pt-10 (Brinkman Instruments, Westbury, NY) for three intervals of 5 s. Homogenates were centrifuged at 800 *g* for 10 min, and the resulting supernatants were then centrifuged at 105,000 *g* for 60 min at 4°C. Kidney cytosolic supernatants were used as the source of mineralocorticoid receptor (MR) binding proteins. Cytosolic protein concentrations were determined by dye binding procedure [35] (Bio-Rad Inc., Hercules, CA). Steroidal compounds evaluated for their MR binding affinities were diluted to 5× the desired assay concentrations (10 pM–10 μM) with TEMG assay buffer. Radiolabeled ligand, [^{3}H]ZK 91587, was solubilized in TEMG buffer to provide ~50,000 dpm/100 μl (~280 fmol/assay). The binding assays were initiated by the addition of 100 μl of [^{3}H]ZK 91587, 300 μl of kidney cytosol and 100 μl of test steroids to 12 × 75 mm borosilicate glass tubes. After vortexing, duplicate samples were incubated in a Dubnoff shaking water bath at 22°C for 1 h, then 500 μl of 1% DCC were added to each assay tube. Samples were vortexed and placed in an ice bath for 10 min prior to centrifugation at 2600 *g* for 10 min. Supernatants containing receptor bound [^{3}H]labeled ligand were decanted into 7 ml glass scintillation vials and 5 ml of scintillation fluid were added to each vial. Radioactivity was determined in a β-scintillation spectrometer (1900 CA, Packard Instrument Co.). Nonspecific binding was determined in the presence of 1 μM of nonlabeled ZK 91587 and subtracted from total binding to estimate specific ligand binding. Relative binding affinity (RBA) for each steroid was the percent ratio of the molar concentration for half-maximal binding (IC_{50}) of the nonlabeled ligand to the IC_{50} value of the test steroid. The mean IC_{50} value for ZK 91587 from four assays in duplicate was 0.97 ± 0.16 nM.

Androgen receptor assays. Male SD rats (150–200 g) were gonadectomized 18–36 h prior to necropsy. Ventral prostate tissues were removed, trimmed, weighed, rinsed with cold saline, homogenized in cold TETG buffer (50 mM Tris–HCl, 1 mM EDTA, 12 mM thioglycerol and 30% glycerol, pH 7.5) at 1:4, ratio (w/v), and cytosol prepared as described for MR assays. Prostate cytosolic supernatants were used as the source of androgen receptor (AR). Cytosol was diluted at least 1:4 (v/v) with cold TETG buffer (~1 mg protein/ml) and 300 μl aliquots were added to 12 × 75 mm glass assay tubes containing ~55,000 dpm (~300 fmol) of [^{3}H]R1881 in 100 μl of TEG assay buffer (10 mM Tris–HCl, 1 mM EDTA and 20% glycerol, pH 7.5). Steroidal compounds evaluated for their AR binding affinities were diluted to 5× the desired assay concentrations (10 pM–1 μM) with TEG buffer and added to the assay as 100 μl aliquots. After vortexing, duplicate samples were incubated at 4°C for 18 h, then 500 μl of cold 0.5% DCC were added to each assay tube. The tubes were vortexed and placed in an ice bath for 5 min prior to centrifugation at 2600 *g* for 5 min at 4°C. Supernatants were prepared for measurement of specific AR bound [^{3}H]R1881 and RBA values as described for MR assays, except 1 μM R1881 was used to measure nonspecific binding. The mean IC_{50} value for R1881 from seven assays in duplicate was 1.35 ± 0.11 nM.

Mineralocorticoid bioassays

Mineralocorticoid activity of steroidal compounds were evaluated using a modified Kagawa bioassay [36]. Male SD rats (125–150 g) were used within 3–7 days of adrenalectomy. Rats were placed at 08:00 h in individual metabolic cages equipped with stainless steel collection funnels. Food was removed the previous evening. Rats were provided deionized water *ad libitum* during the treatment day. At 10:00 h, rats were forced to urinate using subpubic pressure and a whiff of ether to provide the pretreatment urine samples. Animals were then s.c. injected with test steroid or 10% ethanolic saline vehicle (0.2 ml/rat) and with two 1.5 ml injections of 0.154 M NaCl. Urine samples were collected during the 3 h interval following drug treatment. Urine volumes were recorded and urinary creatinine values were determined by the Jaffee reaction using a Coulter DACOS spectrometer (Coulter Electronics Inc., Hialeah, FL). Urinary Na^+ and K^+ were measured by flame photometry (Coleman Model 51 Ca, Bacharach, Inc., Pittsburgh, PA). Mineralocorticoid bioactivity of test steroids that induce Na retention will alter urinary electrolyte excretion by decreasing the Na/K ratio. Aldosterone, 0.1 μg/rat, was used as the standard agent for demonstrating Na retention in adrenalectomized rats [37].

Plomestane clinical tolerance trial

A double blind, placebo-controlled, crossover Phase I single dose oral tolerance study of Plomestane was conducted using normal male volunteers. After completion of pretreatment clinical tests, safety evaluations and sample collections, Plomestane or placebo was orally administered to subjects on Study Day 1. Safety monitoring and sample collections were continued for the next 72 h. On Study Day 8, the alternate treatment was administered and all tests and evaluations were repeated during the subsequent 72 h. Clinical safety

data at each dose level was evaluated before proceeding to the next dose level. All 72 subjects remained in the study facility (Harris Laboratories, Inc., Phoenix, AZ) for the duration of their participation in the study. Both phases of the cross-over study were completed by 63 subjects. Nine subjects who were in the initial placebo treatment group were dismissed due to elevated hepatic enzyme values, SGPT > 50 U/l. The doses of Plomestane were 0.1–20 mg/kg representing 14 different dose levels with four volunteers per dose group, except for 0.4 mg/kg (12 subjects) and 1.6 mg/kg (3 subjects). Drug and placebo dosage tablets of 25, 50 and 100 mg were provided by the Pharmacy Department at MMDRI. Serum levels of Plomestane and 17β-hydroxy Plomestane were determined after solid phase extraction on C_{18}-columns, reconstitution of dried eluent in 50% acetonitrile/water, separation by Waters HPLC system with a RCM-100 radial compression module containing an 8C18 Novapak, 4 μ column, and detection by UV absorption at 247 nm. Absorption data was digitized and serum concentrations estimated by peak height ratios of standard concentration curves to those of the internal standard, Norethindrone (19-nor-17α-ethynylandrosten-17β-ol-3-one). The assay sensitivity was 1 ng/ml for Plomestane and 2 ng/ml for 17β-hydroxy Plomestane. Only volunteers receiving 0.6–20 mg/kg had detectable serum concentrations of Plomestane (35 subjects) or 17β-hydroxy Plomestane (30 subjects) [38].

Radioimmunoassays

Aldosterone concentrations in rat sera were measured by adapting a solid-phase coated tube [^{125}I]-radioimmunoassay (RIA) kit (Diagnostic Products Corp., Los Angeles, CA). The assay requirement of 200 μl of human serum was replaced with 25 μl of rat serum and 175 μl of "zero calibrator" diluent. Estimates of aldosterone concentrations were linear when 10–50 μl of rat serum or plasma were diluted to 200 μl with "zero calibrator". The crossreactivity of 18-Ac-DOC with the anti-aldosterone antibody was less than 0.01%.

Estradiol and estrone serum levels of five normal male volunteers in the Plomestane Phase I oral tolerance study were determined following column chromatographic separation using previously described RIA methods [39]. The estrogen antisera used in these assays by Dr D. C. Collins (University of Kentucky, Lexington, KY) did not cross react with Plomestane or its metabolites. Initially, serum estradiol and estrone levels were analyzed in two different University Research Clinical Chemistry Laboratories using different commercially available estrogen antisera and standard RIA procedures. Estrogen RIA values of sera from subjects receiving 6.4–20 mg/kg of Plomestane were compromised due to immunological crossreactivities with presumptive Plomestane metabolite(s).

Statistical and kinetics analyses

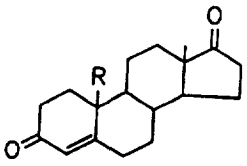
Statistical analyses used standard procedures with data expressed as mean $\pm$ SEM [40]. Michaelis–Menten enzyme kinetic constants, apparent K_m, K_i and V_{max} values were determined by least-squares linear and nonlinear regression analyses [41–43]. In time-dependent enzyme inhibition experiments, the kinetic constants, apparent $K_{i(inact.)}$, k_{cat} and τ_{50}, the $t_{1/2}$ interval for enzyme inactivation at infinite inhibitor concentration, were estimated by the method of Kitz and Wilson [44]. The receptor binding affinities, IC_{50} values, were graphically estimated from semilog plots of percent ligand binding vs log molar concentration of ligand. For pharmacokinetic analyses of the Plomestane single oral dose tolerance study, data from 25 subjects receiving 1.2–20 mg/kg dose with quantifiable serum levels of Plomestane or its metabolite from 9–24 h post treatment were used to estimate the terminal exponential half-lives by least squares regression analysis of the log of the serum concentration vs time. Areas under the serum concentration–time curves (AUC), 0–24 h, for both Plomestane (21 subjects) and metabolite (16 subjects) were calculated using the trapezoidal rule [45]. Based on 0–24 h data from four subjects receiving 15 mg/kg of Plomestane, these calculations captured 98 and 91% of the AUC for Plomestane and 17β-hydroxy Plomestane, respectively, for the estimated AUC for 0 h to infinity.

RESULTS

Aromatase inhibition

Pseudo-substrate analogs designed as enzyme activated inhibitors must exhibit a high affinity for the enzyme active site and possess latent functionalities which can be activated via the normal catalytic action of the target enzyme. The hydroxylation of the C_{10} methyl by aromatase dictated the use of C_{10} positional analogs of androstenedione. The C_{10} carbon was modified by the synthesis of a series 1,2-propadienyl, 1- and 2-alkynyl, 2-propenyl and cyano derivatives (Table 1). These pseudo-substrate analogs were evaluated in time-dependent aromatase assays. These data were used to calculate enzyme inhibition kinetic parameters [44]. Compounds could be ranked for efficiency of enzyme-catalyzed inactivation by calculating the ratio of their catalytic inactivation rate, $k_{(cat)}$, to their molar concentration at half-maximal inactivation, apparent $K_{i(inact.)}$. The magnitude of this ratio reflects the inhibitor's effectiveness as an enzyme inactivator. The most potent of this series of analogs was the 2-propynyl analog (Plomestane) which had a $K_{i(inact.)}$ value of 3.2 ± 0.5 nM, $k_{(cat)}$ value of 1.2×10^{-3} s^{-1} and τ_{50} value of 9.4 ± 0.7 min representing mean values from five experiments as illustrated in Fig. 1(A). These data demonstrate both concentration and time-dependent inhibition of human placental aromatase,

Table 1. Enzyme kinetic parameters for androstenedione analogs in human placental aromatase assays



	$K_{i(inact)}$ (nM)	t_{50} (min)	k_{cat} (s^{-1})	$k_{cat}/K_{i(inact)}$
CH_2-C ≡ CH*	3.2 ± 0.5	9.4 ± 0.7	1.2×10^{-3}	384,060
CH_2-C ≡ C-CH_3	14.6	23.4	4.9×10^{-4}	33,560
C ≡ C-CH_3	NI	—	—	—
CH(OH)-C ≡ CH_2	49 ± 8	32.3 ± 4.5	3.6×10^{-4}	7380
CO-C ≡ CH_2	350	NTD	—	—
CH_2-CH = CH_2	12.5	NTD	—	—
CH = C = CH_2	13.6	25.9	4.5×10^{-4}	32,800
CH_2-C ≡ N	22.5	NTD	—	—
C ≡ N	NI	—	—	—
17β-Hydroxy plomestane†	28.1	7.8	1.5×10^{-3}	52,710
6β-Hydroxy plomestane†	13.4	36.7	3.1×10^{-4}	23,130
Formestane‡	30 ± 9	3.2 ± 0.9	3.7×10^{-3}	121,440
Atamestane§	66.0	64.0	1.8×10^{-4}	2740

*Plomestane.
†Data from reference [25].
‡Data are mean of reported values [32, 46–48].
§Data from reference [32].
NI, no inhibition at 100 nM. NTD, not time dependent, apparent K_i value reported.

indicating that Plomestane has a high competitive affinity for the active site as well as an enzyme-activated mechanism of inhibition. The observed saturation kinetics, i.e. τ_{50} value >0, indicates that a kinetic step subsequent to formation of the reversible Michaelis–Menten complex, $[E + I \leftrightarrow E - I]$, was rate-limiting. The binding of Plomestane to aromatase was active site directed, since enzyme inactivation could be blocked or protected by increasing substrate concentration during the preincubation phase [Fig. 1(B)]. The $t_{1/2}$ of inactivation of human placental aromatase activity by 5 nM Plomestane was 23 min in the absence of testosterone substrate in the 0–40 min preincubation phase of time-dependent assays. The addition of 135 nM testosterone to the preincubation phase slowed the enzyme inactivation rate to $t_{1/2} = 74$ min. Thus the inhibitor and the substrate were competing for binding to the same active site. Time-dependent enzyme inactivation required NADPH cofactor [Fig. 1(B)], indicating that Plomestane must be transformed by aromatase to a reactive intermediate before enzyme inactivation could occur in the active site (Scheme 1). The absence of a lag time in the onset of inhibition that occurs in the presence of 10 nM dithiothreitol in the incubation medium supports the direct action of the inhibitor in the enzyme active site without involving a diffusible alkylating species. The reversibility of aromatase inhibitors was determined following incubation of inhibitors for 15 min with placental microsomes and isolation of the microsomes by centrifugation. The residual microsomal pellets were washed and resuspended in buffer prior to determining residual aromatase activity [24]. Plomestane did not exhibit any disassociation from the enzyme (Fig. 2). The competitive inhibitor, 2,10-ethylene androstenedione ($K_i = 35$ nM) [34], completely disassociated from the enzyme-complex, while the time-dependent inhibitors, Atamestane ($K_{i(inact.)} = 66$ nM) [32] and Formestane ($K_{i(inact.)} = 10$–55 nM) [32, 46–48] exhibited partial disassociation from the enzyme-complex, with Formestane having a greater affinity for the aromatase active site.

Plomestane or placebo tablets were orally administered to 63 healthy male volunteers enrolled in a single dose, double-blind placebo-controlled crossover Phase 1 tolerance study. The maximum tolerated dose was not established in this study since, there were no changes in ECG, vital signs, laboratory clinical parameters, or adverse events that differentiated Plomestane (0.1–20 mg/kg) from placebo treatment. RIA analysis of serum estradiol and estrone levels from two different research laboratories demonstrated significant ($P < 0.05$) decreases in estrogen levels at 12–24 h, with the maximum difference observed at 24 h after receiving a single oral dose of Plomestane (0.1–20 mg/kg) in comparison to their placebo treatment. The apparent minimum and maximum effective doses were 0.2–0.3 and 6.4 mg/kg of Plomestane. Analysis of urine and serum from volunteers receiving concentrations of Plomestane greater than 6.4 mg/kg were apparently compromised by immuno-crossreactivity from a presumptive drug metabolite(s). In addition, serum samples from 5 volunteers were also analyzed in a third research laboratory [39], where the apparent immunocrossreactivites were not observed in sera from volunteers receiving 6.4–20 mg/kg of Plomestane (Fig. 3).

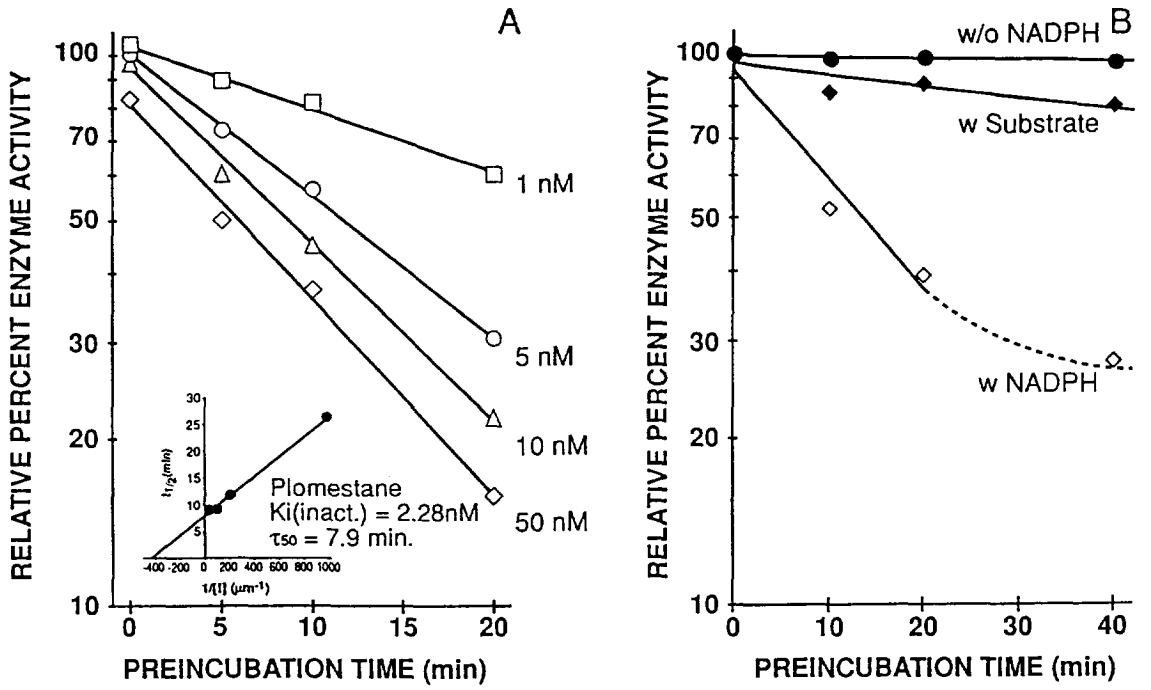


Fig. 1. Inactivation of aromatase activity induced by Plomestane. (A) Concentration and time-dependent inhibition of human placental aromatase activity were induced by 1–50 nM Plomestane. The insert represents a Kitz–Wilson plot [44] of linear regression analysis of enzyme inactivation rates ($t_{1/2}$) vs reciprocal of Plomestane concentrations. The Y-intercept is τ_{50} value (7.9 min) and X-intercept is $1/K_{i(inact.)}$ (2.28 nM). Percent residual enzyme activity is relative to respective buffer controls to corrected for inherent time course loss of aromatase activity (10–15%). (B) Human placental microsomes were preincubated for varying time intervals with 5 nM Plomestane in the presence of NADPH (◇), $t_{1/2} = 22.9$ min; in the absence of NADPH (●), $t_{1/2} = 928$ min; or in the presence of NADPH plus 135 nM testosterone as a competitive substrate (◆), $t_{1/2} = 73.7$ min. The relative residual enzyme activity values were adjusted so that the "0" min of preincubation values equaled 100% activity to allow inter assay comparisons. The $t_{1/2}$ values are from linear regression analyses of non-adjusted data.

Based on these limited observations, the suppression of estrogen levels observed in initial analyses may not reflect the true magnitude of estrogen suppression. Median terminal half-lives for Plomestane and 17β-hydroxy Plomestane were 4.69 and 6.82 h, respectively. Maximum concentrations of both compounds were observed at 3 h post dosing. No half-life dose dependencies were observed in these studies. At doses equal to or greater than 10 mg/kg, pharmacokinetic analyses suggested that both saturation and dissolution rate-limited absorption can occur. At higher concentrations of Plomestane, proportionally less 17β-hydroxy metabolite was observed in the serum concentration–time curves relative to parent drug. This suggests that the metabolism of the Plomestane to its 17β-hydroxy metabolite can also be saturable.

Aldosterone inhibition

After establishing that C_{19}-acetylenic analogs of androstenedione were enzyme-activated inhibitors of aromatase, we designed and synthesized similar C_{18}-analogs of progesterone and DOC as potential inhibitors of C_{18} hydroxylation of corticosterone [14, 15, 26, 28]. These compounds were evaluated in adrenal glomerulosa cytosolic preparations from rats maintained on a sodium-deficient diet for at least 2 weeks. Time-course experiments established that after 11 days, this dietary regimen induced a 60-fold increase in serum aldosterone levels (9.2 ± 1.6 vs 0.29 ± 0.03 ng/ml; $P < 0.01$) when compared to serum from rats fed normal rat chow. Glomerulosa/capsular cytosol from these rats exhibited significant ($P < 0.01$) concomitant increases in biosynthesis of [^{3}H]18-OH-CORT (1.29 ± 0.14 vs 0.15 ± 0.03 pmol/mg/h) and [^{3}H]ALDO (0.61 ± 0.09 vs 0.09 ± 0.005 pmol/mg/h) when incubated with 20 nM [^{3}H]CORT for a 30 min assay at 30°C.

For time-dependent assays, 1 μM [^{3}H]DOC was used as the substrate because it allowed evaluation of 11β/18-hydroxylase activity as well as 18-hydroxylase activities of corticosterone methyl oxidase. In these assays, linear first-order rate kinetics were observed during the initial 5–10 min for [^{3}H]-product formation for both primary (18-OH-DOC and CORT) and secondary (18-OH-CORT and ALDO) products of [^{3}H]DOC. The 18-acetylenic analog of DOC was a better inhibitor of aldosterone biosynthesis than 18-acetylenic analogs of 11β-hydroxy progesterone or

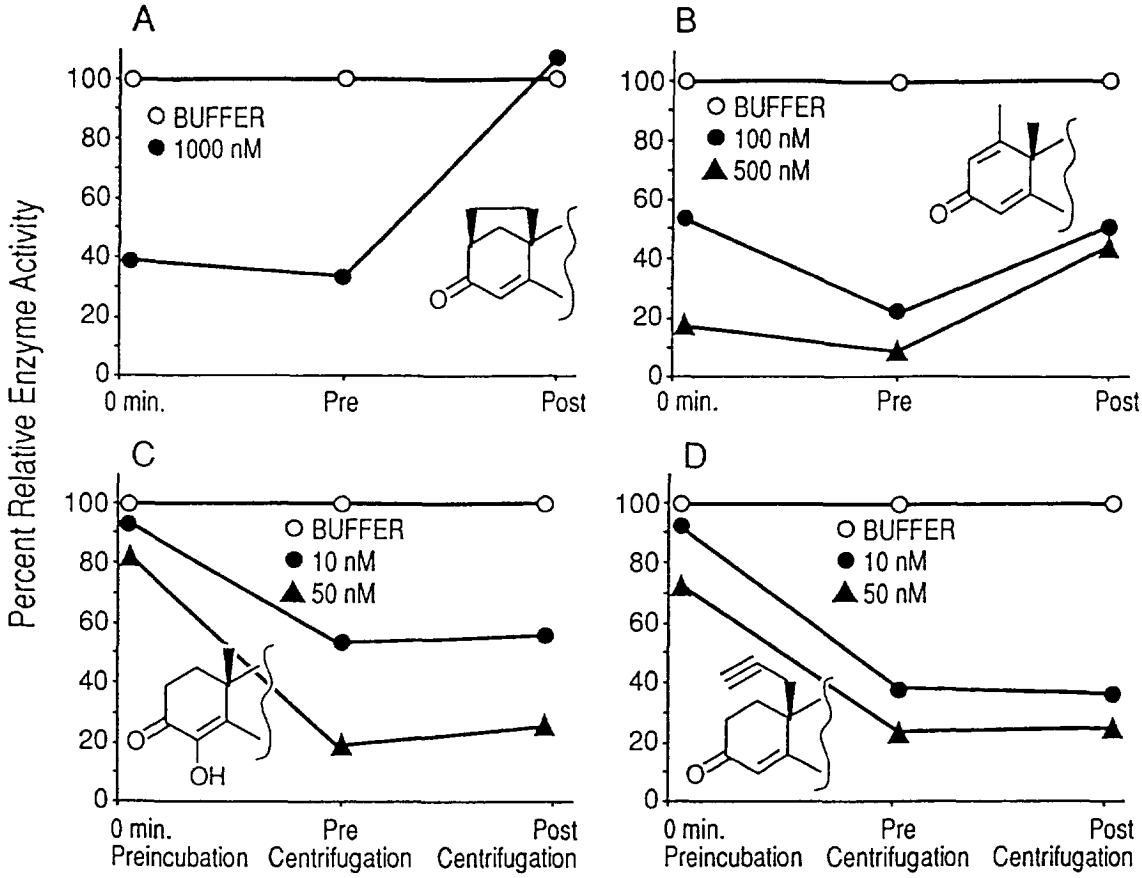


Fig. 2. The reversibility of aromatase binding of androstenedione analogs. Recovery of aromatase activity was determined following incubation of inhibitors for 15 min with human placental microsomes and isolation of microsomal pellet by centrifugation. After washing the pellet, the microsomes were reconstituted in buffer and assayed for aromatase activity. Percent residual enzyme activity is relative to respective buffer controls to corrected for procedural losses. Inhibitors evaluated were (A) 2,10-ethylene androstenedione, (B) Atamestane, (C) Formestane and (D) Plomestane.

Table 2. Enzyme kinetic parameters for corticoid analogs in aldosterone synthase assays

Compound	IC_{50} (μM)	$K_{i(inact)}$ (μM)	τ_{50} (min)	k_{cat} (s^{-1})	$k_{cat}/K_{i(inact)}$
Progesterone analogs					
18-Cyano progesterone	20	NTD*			
11β-Hydroxy-18-cyano prog	14	10.1	26.8	4.3×10^{-4}	43
18-Allenyl progesterone	0.2‡	TD†			
18-Vinyl progesterone	0.02‡	TD			
11β-OH-18-acetylenic prog	20	TD			
18-Acetylenic progesterone	0.035	0.04	9.6	1.2×10^{-3}	30,000
18-Acetylenic-progesterone (17α-acetyl isomer)	NI§				
Deoxycorticosterone analogs					
18-Acetylenic DOC	0.014	0.038	4.8	2.4×10^{-3}	63,160
18-Vinyl DOC	MR agonist				
Other inhibitors					
Ketoconazole	0.6‡	NTD			
Spironolactone	7.0	NTD			

Data derived from time-dependent assays using adrenal glomerulosa/capsular homogenates from SD rats on an Na-deficient diet for at least 2 weeks. Steroidal compounds were preincubated for varing intervals from 0–40 min prior to adding 500 pmol of [^{3}H]DOC for 10 min assay. Labeled aldosterone formed was quantitated by HPLC methods.

*NTD, not time-dependent.

†TD, time-dependent.

‡Compound was not specific for aldosterone synthase, but also inhibited 11β/18-hydroxylase.

§NI, no inhibitory activity at 0.05 μM.

progesterone (Table 2). The presence of an 11β-hydroxy group on 18-acetylenic progesterone reduced its inhibitory IC_{50} value from 35 nM to 20 μM. Changes in the steric orientation of the C_{17} acetyl group from beta to alpha of 18-Ac-progesterone reduced enzyme inhibitory activity. 18-Vinyl progesterone was 1000 times more effective than the 18-cyano analogs in time-dependent assays. At 10–50 nM, 18-vinyl progesterone exhibited time-dependent inhibition of the 18-hydroxylation of corticosterone, as reflected by concentration and time-dependent increases in the percent of [^{3}H]DOC converted to [^{3}H]18-OH-CORT and [^{3}H]ALDO, without blocking the formation of [^{3}H]CORT and [^{3}H]18-OH-DOC. At higher concentrations, 0.1 and 1.0 μM, 18-vinyl progesterone produced a time-dependent inhibition of 11β/18-hydroxylation of [^{3}H]DOC. Ketoconazole, a competitive non-steroidal inhibitor, was 12 times more potent than the competitive inhibition exhibited by Spironolactone, a steroidal mineralocorticoid receptor antagonist. Both of these compounds displayed competitive inhibition of 11β/18-hydroxylation of [^{3}H]DOC and 18-hydroxylation of [^{3}H]corticosterone (Table 2).

As with the aromatase inhibitors, acetylenic substrate analogs were the most potent and selective inhibitors of hydroxylation of the C_{18}-angular methyl group. As shown in Fig. 4, 18-Ac-DOC was twice as effective as 18-Ac-PROG in time-dependent inhibition of 18-hydroxylase activity as reflected in $k_{(cat)}/K_{i(inact.)}$ values of 63,160 and 30,000, respectively. Neither compound had a significant effect on the 11β/18-hydroxylation of DOC at concentrations that produced 90% time-dependent inhibition of aldosterone biosynthesis. The time-course product curves in Fig. 5(A) display the product profile of [^{3}H]DOC metabolism in glomerulosa cytosolic preparations of adrenal tissue from rats fed a normal sodium replete diet. The depletion of sodium from the diet induced an increase in the rate of DOC utilization and a 10-fold increase in corticosterone 18-hydroxylase activity [Fig. 5(B)]. The incubation of 0.1 μM 18-Ac-DOC for 20 min prior to the addition of [^{3}H]DOC inhibits the 18-hydroxylase activity without altering the 11β/18-hydroxylation of DOC [Fig. 5(C)]. This selective inhibition of 18-hydroxylation of corticosterone by 18-Ac-DOC normalizes the DOC metabolic profiles of rats with hyperaldosteronism [compare Fig. 5(A) with Fig. 5(C)].

The *in vivo* specificity of 18-Ac-DOC was evaluated in a 10 min assay following the addition of 500 pmol [^{3}H]DOC to adrenal glomerulosa preparations obtained 2 h after rats had been s.c. injected with 1–30 mg/kg of 18-Ac-DOC. The dose of 18-Ac-DOC that produced half-maximal inhibition, ED_{50}, of aldosterone biosynthesis was 3 mg/kg s.c. at 2 h post treatment. The data in Fig. 6 shows the selective effects of a single 15 mg/kg subcutaneous injection of 18-Ac-DOC. Only the corticosterone 18-hydroxylase activity was inhibited. The steroidal precursors to the second enzyme, CORT and 18-OH-DOC, are increased, while the products of the second enzymatic step, corticosterone-18-hydroxylase, are significantly inhibited ($P < 0.01$). Sera from these rats showed a sustained decrease in aldosterone levels which reflects the inhibition of aldosterone biosynthesis measured in the *ex vivo* adrenal enzyme assays (Fig. 7).

Steroid receptor binding assays

The potential hormonal activities of these aromatase and aldosterone synthesis inhibitors were evaluated by determining their ability to bind to androgen and mineralocorticoid receptors isolated from prostate and kidney tissue (Table 3). Of the androstenedione analogs, only Plomestane's 17β-hydroxy metabolite, 19-Acetylenic testosterone, exhibited any androgen receptor binding activity (RBA = 3.91%). None of the C_{21} steroids or their analogs effectively bound to the androgen receptor protein. Spironolactone exhibited weak affinity (RBA = 1.59%) for the androgen receptor.

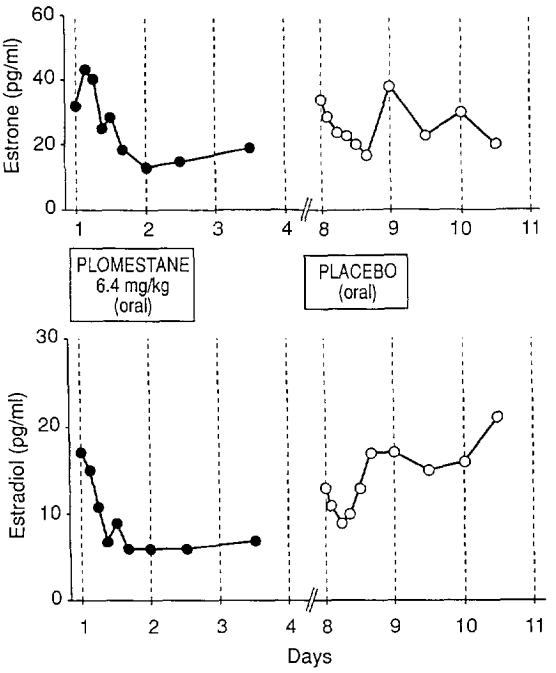


Fig. 3. Serum estrogen levels from a Phase I single oral dose tolerance study of Plomestane. A healthy male volunteer in a double-blind, placebo-controlled crossover study received an oral dose of 550 mg of Plomestane on Day 1 and 550 mg placebo on Day 8. Serum concentrations of Plomestane decreased from 237.0 ng/ml at 3 h post dose (C_{max}) to 17.2 and 2.7 ng/ml at 12 and 24 h post treatment, respectively. Serum concentrations of 17β-hydroxy Plomestane decreased from 50.8 ng/ml at 3 h post dose (C_{max}) to 8.0 and 4.0 ng/ml at 12 and 24 h post treatment, respectively. The apparent terminal half-life for Plomestane and 17β-hydroxy Plomestanes were 4.81 and 12.02 h, respectively. The area under the serum concentration–time curves (0–24 h) for Plomestane was 1096.5 ng/ml/h and 310.0 ng/ml/h for 17β-hydroxy Plomestane.

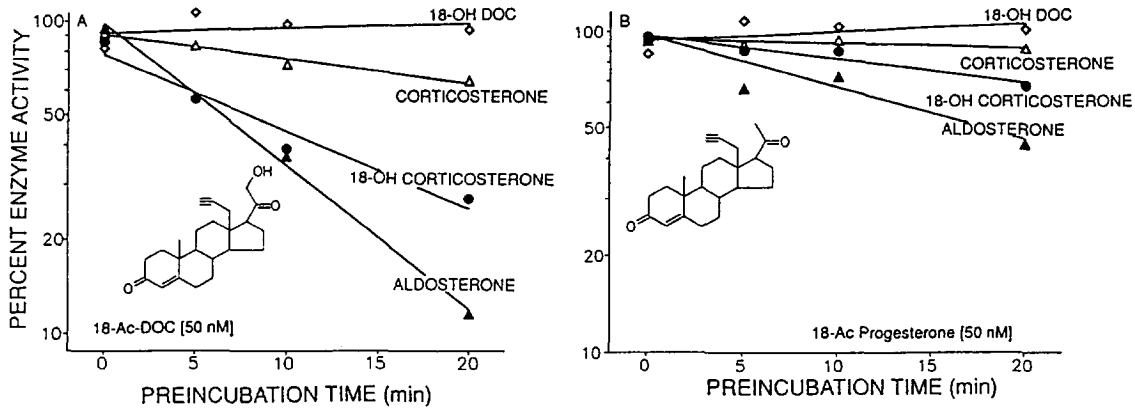


Fig. 4. Time-dependent inhibition of 11β/18-hydroxylation of DOC and 18-hydroxylation of corticosterone induced by 18-acetylenic DOC and 18-acetylenic progesterone. Adrenal glomerulosa preparations from rats on a Na-deficient diet were incubated with 50 nM of inhibitors for 0–20 min prior to addition of 500 pmol [^{3}H]DOC for a 10 min assay. Products quantified by HPLC chromatography and online scintillation spectrometry. Percent residual enzyme activity is relative to respective buffer controls.

Table 3. Steroid receptor binding affinities

	Androgen*		Mineralocorticoid*	
Ligands	IC_{50}† (nM)	RBA† (%)	IC_{50}† (nM)	RBA† (%)
Labeled ligand‡	1.35	100	0.97	100
Endogenous androgens				
Dihydrotestosterone	2.59	52.1	NT	—
Testosterone	6.8	19.9	NT	—
Androstenedione	610	0.22	1.1 μM	0.09
Androgen analogs				
Plomestane	2 μM	0.07	13.5 μM	0.007
17β-Hydroxy plomestane	34.5	3.91	4 μM	0.024
6β-Hydroxy plomestane	>50 μM	<0.003	>50 μM	<0.002
Formestane	690	0.20	NT	—
Endogenous corticoids				
Deoxycorticosterone	300	0.45	0.84	115.5
19-Hydroxy DOC	>50 μM	<0.003	25.0	3.88
18-Hydroxy DOC	>10 μM	<0.01	450	0.22
Corticosterone	1.75 μM	0.08	3.25	29.9
18-Hydroxy corticosterone	>10 μM	<0.01	1.2 μM	0.08
Aldosterone	>10 μM	<0.01	1.74	55.8
11-Dehydrocorticosterone	NT	—	62.5	1.55
5α-Dihydrocorticosterone	NT	—	1.0 μM	0.01
Progesterone	210	0.64	7.10	13.7
Corticoid analogs				
18-Cyanoprogesterone	3.0 μM	0.05	1.0 μM	0.01
11β-OH-18-cyanoprogesterone	3.0 μM	0.05	420	0.23
18-Vinyl progesterone	288	0.47	15.0	6.47
18-Allenyl progesterone	NT	—	20.0	4.85
11β-OH-18-acetylenic prog	650	0.21	325	0.30
18-Acetylenic progesterone	275	0.49	50	1.94
18-Acetylenic DOC	1.1 μM	0.12	11.7	8.29
18-Vinyl DOC	NT	—	1.05	92.4
Other steroidal compounds				
Dexamethasone	NT	—	11.12	8.72
Spironolactone	85	1.59	6.0	16.2

*Prostate and kidney tissue cytosols, respectively, were prepared from castrated and adrenalectomized male SD rats at 24 h post-surgery.

†Relative binding affinities (RBA) are expressed as the percentage of the ratio of the half-maximal binding concentration (IC_{50}) of the labeled ligand to the IC_{50} value of the competitive ligand. RBA values represent the mean of duplicates from 1–8 assays per compound. NT, not tested.

‡Labeled ligands were [^{3}H]methyltrienolone (R1881) and [^{3}H]ZK 91587 for androgen and mineralocorticoid receptors, respectively.

Mineralocorticoid binding proteins present in rat kidney cytosols exhibited type-I high affinity binding (<1 nM) which was consistent for the physiologic aldosterone receptor [49]. The high affinity binding of DOC to the type-I mineralocorticoid receptors has been reported by others [49, 50]. Hydroxylation of the angular methyl carbons at C_{19} or C_{18} of DOC decreases its receptor affinities by 30- and 500-fold, respectively. A similar decrease in binding affinity (370-fold) was observed with 18-hydroxylation of corticosterone. However, hydroxylation at C_{11} of the steroid (DOC vs corticosterone) reduces binding by only 4-fold, while hydroxylation of progesterone at C_{21} yielding DOC increases affinity by 8.4-fold. Addition of vinylidene or alkynyl substitution at C_{18} of progesterone or DOC did not alter receptor binding to the degree that was observed for C_{18}-cyano analogs or hydroxylation at C_{18}. The 18-vinyl analogs of DOC and progesterone reduced affinity by only 1.4- and 2-fold, respectively. Interestingly the analogous acetylenic analogs exhibited a 14- and 7.7-fold reduction. These slight structural differences may account for the significant difference observed in the mineralocorticoid bioactivities between 18-vinyl DOC and 18-Ac-DOC. Spironolactone displayed about twice the binding affinity observed for dexamethasone.

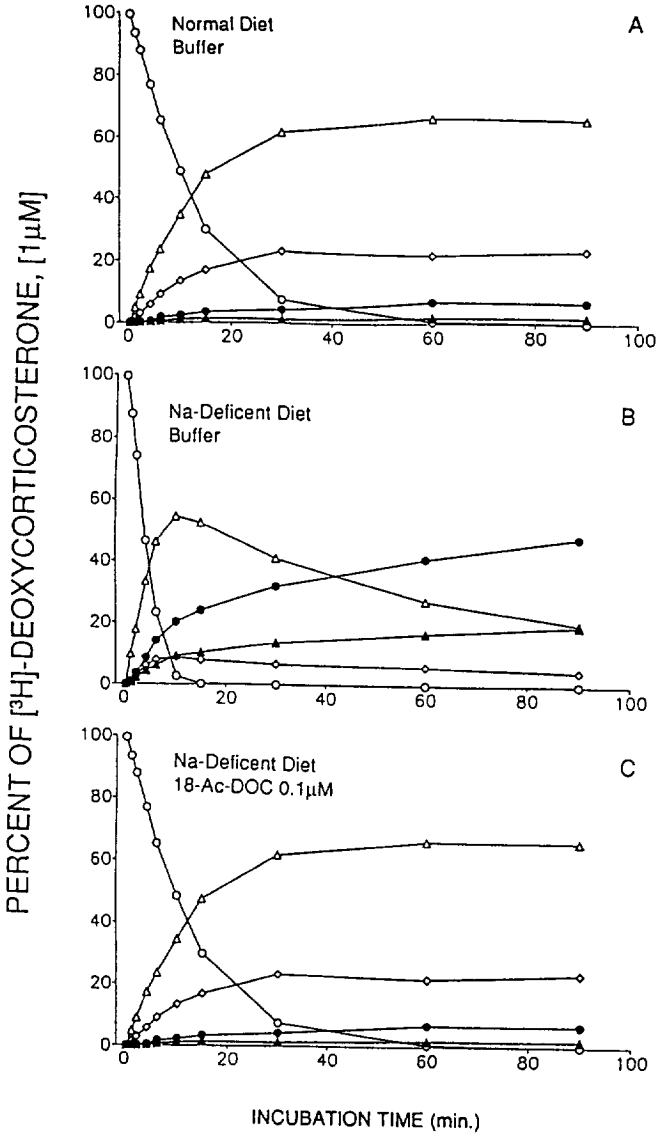


Fig. 5. Time-course for corticoids formed from [^{3}H]deoxycorticosterone during incubation of rat adrenal glomerulosa preparations. (A) Adrenal tissue from rats that were on a normal diet. (B) Adrenal tissue from rats that were on a Na-deficient diet. Glomerulosa preps were preincubated with buffer for 20 min prior to adding 500 pmol [^{3}H]DOC. (C) Adrenal tissue from rats that were on a Na-deficient diet. Glomerulosa preps were preincubated with 0.1 μM of 18-Ac-DOC for 20 min prior to adding 500 pmol [^{3}H]DOC. The [^{3}H]corticoids measured were DOC, ○; 18-OH DOC, ◇; corticosterone, △; 18-OH corticosterone, ●; and aldosterone, ▲.

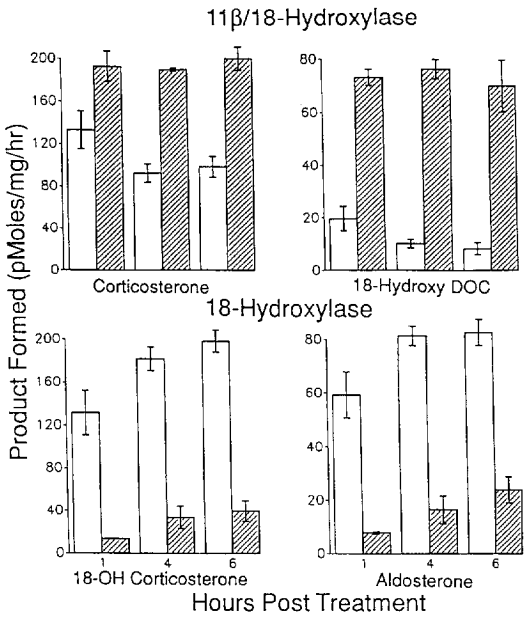


Fig. 6. Steroidogenesis of adrenal glomerulosa from rats on a Na-deficient diet treated with 18-acetylenic DOC. Male rats (4 per group) received either a single subcutaneous injection of 15 mg/kg of 18-Ac-DOC, ▨, or 5 ml/kg of vehicle, □. Individual glomerulosa preps from each of 4 rats per treatment interval were incubated with 500 pmol [^{3}H]DOC for 30 min assay. The tritiated products of 11β/18-hydroxylase and 18-hydroxylase of aldosterone synthase were measured.

Mineralocorticoid bioactivities

The modified Kagawa bioassay [36] was utilized to evaluate the mineralocorticoid bioactivity of the progesterone and DOC analogs. The Na/K ratios of urine collected during the 1–3 h interval following s.c. injection of drug or vehicle were used as an index of sodium retention. Na/K ratios of 5 or higher were observed for vehicle treatment or compounds devoid of sodium retaining activity. Maximal Na/K ratios responses of 1.3–1.6 were measured following treatment with aldosterone at 0.1 μg/rat (~0.5 μg/kg) or DOC at 10 μg/rat (Fig. 8). The half-maximal response, ED_{50} for aldosterone and DOC were 0.04 and 2.5 μg/rat, respectively. Neither 18-vinyl nor 18-acetylenic progesterone exhibited any sodium retaining activity at 100 μg/rat. However, 18-vinyl DOC at 10 μg/rat was equipotent to 10 μg/rat of DOC (Na/K ratio = 2.2 ± 0.3 vs 2.8 ± 0.3) while 18-acetylenic DOC did not exhibit any mineralocorticoid bioactivity at 100 μg/rat. These *in vivo* observations are consistent with the 10-fold greater binding affinity displayed by 18-vinyl DOC for the type I mineralocorticoid receptor than that observed for 18-acetylenic DOC.

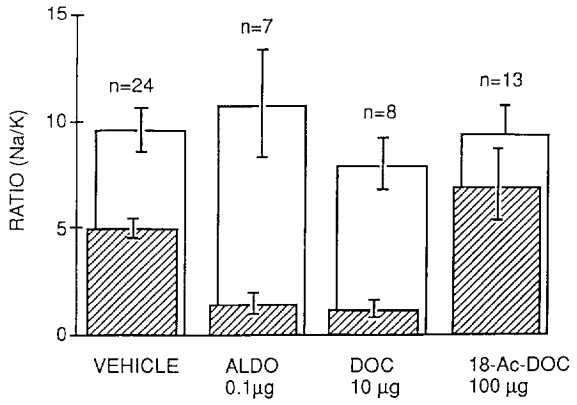


Fig. 8. The mineralocorticoid bioactivities of steroidal compounds. Na/K ratio of urine collected from adrenalectomized rats during a 2 h pretreatment interval, □, and a 3 h post treatment interval, ▨. Data shown for rats s.c. injected with 0.1 μg of aldosterone, 10 μg of DOC or 100 μg of 18-acetylenic DOC. The number of rats per treatment group is shown above each bargraph.

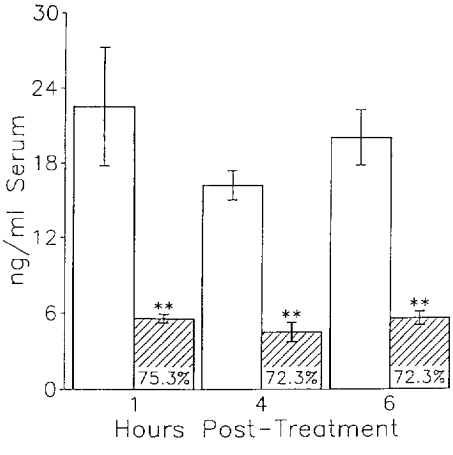


Fig. 7. Aldosterone serum levels of rats on a Na-deficient diet treated with 18-acetylenic DOC. Male rats (4 per group) received either a single s.c. injection of 15 mg/kg of 18-Ac-DOC, ▨, or 5 ml/kg of vehicle, □. The percent reduction of aldosterone levels relative to their respective vehicle controls are shown within each treatment bargraph. This reduction in aldosterone levels was statistically significant at $P \leq 0.01$.

DISCUSSION

The CY*P*450 enzymes that hydroxylate the angular methyl groups of the steroid nucleus lead to the formation of hormones that are highly specific but extremely diverse in their biological actions. In the formation of estrogen from androgens, aromatase readily inserts oxygen into the C—H bonds of the C_{19} methyl during the first two oxidative steps to produce the 19-carboxaldehyde derivative. These reactions are typical CY*P*450 hydroxylations utilizing two equivalents of molecular oxygen and NADPH (Scheme 1). The third catalytic step is proposed to involve the nucleophilic attack by the CY*P*450 heme's ferric peroxide intermediate on the aldehyde group of the 19-oxo-steroid [51, 52]. With oxidative cleavage of the C_{10}–C_{19} bond, the aldehyde is lost as formic acid with the concomitant introduction of a double bond into the unsaturated sterol A-ring which tautomerizes to phenolic estrogen. The emergence of new active-site models for aromatase [53] has supported our original

approach to design C_{19} analogs of androstenedione as enzyme-activated inhibitors [10]. Several series of compounds were synthesized [10, 24, 34], however, 19-acetylenic androstenedione (Plomestane) was the most active of these substrate analogs. Pseudo-first-order time-dependent loss of aromatase activity was observed with a catalytic inactivation rate proportional to inhibitor concentration, i.e. $K_{i(inact.)} = 3.2$ nM and $k_{(cat)} = 0.0012\ s^{-1}$ [Fig. 1(A)]. Plomestane was active-site directed and required NADPH cofactor for its time-dependent inactivation, implying that enzyme activation of the inhibitor was required [Fig. 1(B)]. Irreversible binding of Plomestane to placental microsomes was supported by dialysis studies where aromatase activity of microsomal preparations incubated with Plomestane failed to return to pretreatment levels following 24 h of dialysis [23]. Ongoing studies have demonstrated covalent binding of [^{14}C]Plomestane to purified human placental aromatase following enzyme incubation. Labeled inhibitor remained associated with the CY*P*450$_{Arom}$ protein during SDS gel electrophoresis and autoradiography. In addition, a [^{14}C]-labeled peptide fragment was isolated by HPLC chromatography of tryptic digests of [^{14}C]Plomestane/aromatase complex [54]. Irreversible inactivation of aromatase by Plomestane was observed in trophoblast cell cultures [55] and *in vivo* when intratumoral aromatase activity was measured following treatment of nude mice with human tumor xenografts [56]. In these later studies, intratumoral aromatase activity remained suppressed even after [^{14}C]Plomestane levels had decreased to non-detectable level in tumor tissue, suggesting that the return of aromatase activity awaited *de novo* enzyme synthesis. Peripheral aromatase activity in female baboons was significantly inhibited following treatment with intravenous or oral Plomestane [57]. Having demonstrated the lack of inherent hormonal activities and no adverse toxicology [58], Phase 1 clinical studies were initiated using an intravenous preparation of Plomestane [59]. Serum estrogen levels were significantly decreased in this clinical study, thus a single oral dose study was initiated. As shown in Fig. 3, a single oral dose of Plomestane induced a sustained suppression of estrogen biosynthesis that persisted even after Plomestane and its major bioactive metabolite [25] were no longer detectable in the serum from a male volunteer. These studies confirm that rationally designed mechanism-based enzyme inhibitors offer significant therapeutic potential by the specificity of their action. Prolonged pharmacological effects are achieved even after drug clearance, since irreversible inhibition will require new enzyme synthesis to restore enzymatic activity.

Based on the effectiveness of 19-acetylenic androstenedione in inhibiting only the hydroxylation at C_{19} and the lack of inhibitory activity against hepatic CY*P*450 enzymes [60], we designed similar analogs of progesterone, 11β-hydroxy progesterone and deoxycorticosterone as potential inhibitors of 18-hydroxylase activity required for aldosterone biosynthesis [26, 28, 29]. The zona glomerulosa cells of the adrenal cortex are the only cells in the body expressing mitochondrial CY*P*450 of aldosterone synthase. This enzyme controls the final steps of aldosterone biosynthesis from corticosterone (Scheme 2). Steroidogenesis in the zona fasciculata, the adjacent cellular zone to the glomerulosa, is regulated almost exclusively by pituitary adenocorticotropin (ACTH). In contrast, steroidogenesis in the zona glomerulosa cells is stimulated via membrane receptors for various peptide hormones, biogenic amines and regulatory lipids. These agonists stimulate only the early steps of steroid biosynthesis that precede pregnenolone formation. Thus their effects on aldosterone synthesis are completely dependent upon the level of aldosterone synthase activity. This enzyme is very sensitive to alterations in electrolyte composition of extracellular fluid, especially potassium ion concentration which contributes to its physiologic control of electrolyte homeostasis. Consequently aldosterone, as a mineralocorticoid, can selectively regulate plasma electrolyte balance by enhancing Na^+ ion retention and promoting K^+ ion excretion by the distal renal tubules. However, the responsiveness of aldosterone biosynthesis to alterations in sodium or potassium intake occurs slowly over a time course of hours to days [60, 61]. Due in part to the differential regulation of the early and final steps of aldosterone biosynthesis, numerous conditions contribute to hypersecretion during various pathophysiological states. Hypersecretion of aldosterone can be induced by either conditions of primary hyperaldosteronism (i.e. adrenal adenomas) or in states of secondary hyperaldosteronism as in response to diuretic therapy. Increased aldosterone biosynthesis is also associated with conditions of hypertension, edema, congestive heart failure and even cardiac arrhythmias in conditions of severe hypokalemia.

The relatively slow responsiveness of aldosterone biosynthesis to dietary regulation via the rate limiting step of 18-hydroxylation of corticosterone makes this enzyme an excellent therapeutic target for mechanism-based enzyme inhibition. Such inactivating compounds could potentially normalize conditions of hyperaldosteronism where competitive inhibitors would be less effective due to their reversible kinetics. Based on the biological specificity imparted by the sequential hydroxylation at C_{18} angular methyl of corticosterone, we designed several potential mechanism-based inhibitors of aldosterone synthase [14, 26, 28, 29]. As we had observed with aromatase, the acetylenic anologs were the most potent inactivators. The utilization of glomerulosal preparations from rats fed sodium-deficient diets significantly enhanced aldosterone synthase activity. These preparations when evaluated in radioenzymatic assays with quantification of labeled products in both time-dependent and time-course

assays allowed for a clear characterization of these inhibitors (Figs 3 and 4). These procedures detected the inhibitory effects of 18-vinyl progesterone on both 11β/18-hydroxylase and aldosterone synthase. Similar observations have not been reported by others who used non-stimulated rat adrenal tissues in conventional enzyme assays with quantification of only aldosterone by RIA procedures [62–64]. The comparisons of the 18-vinyl and 18-acetylenic analogs demonstrate the specificity associated with the latter compounds. In addition to the inhibitory effect of 18-vinyl progesterone on corticosterone synthesis, this compound had a 3-fold greater affinity for the mineralocorticoid receptor than 18-acetylenic progesterone. These differences were greater for the DOC analogs. 18-Vinyl DOC was almost equipotent with DOC for both mineralocorticoid receptor binding affinity and *in vivo* bioactivity (Table 3, Fig. 8). In contrast, 18-acetylenic DOC exhibited weak mineralocorticoid receptor binding affinity and no sodium retention in the Kagaway bioassay. This compound did not exhibit androgen receptor binding activity. 11β-Hydroxy 18-acetylenic progesterone was much less effective in rat adrenal preparations than in bovine mitochondrial preparations [15], while 18-acetylenic DOC exhibited 6.5-fold lower K_i values in rat glomerulosa cytosolic preparations than in bovine glomerulosa cell cultures [65–67]. The species differences in enzyme kinetics of these steroidal probes may well reflect the similarity in product formation (both corticosterone and aldosterone) observed for bovine CY*P*450 11β-isozymes, while these corticoids are separate products for the rat adrenal 11β-isozymes [68]. The product spectrum of the human 11β-isozymes corresponds more closely with those of rat than of the bovine [68]. In primate adrenal mitochondrial preparations, 18-acetylenic DOC selectively inhibited only hydroxylations at C_{18} of corticosterone during aldosterone biosynthesis (Scheme 3) [69]. Infusion of sodium-depleted monkeys with 18-acetylenic DOC caused a prolonged suppression of serum aldosterone levels without altering cortisol serum levels [70].

The acetylenic analogs of androstenedione (Plomestane) and deoxycorticosterone (18-acetylenic DOC) represent the first rationally designed, enzyme-activated inhibitors of aromatase [10, 71–73], and aldosterone synthase [14, 15, 26, 28]. These compounds exhibited classical criteria for mechanism-based enzyme inactivation. They achieved selective enzyme inactivation by being pseudo-substrates for the specific CY*P*450 that normally hydroxylates the angular methyl group of the sterol nucleus (Schemes 1 and 2). The mechanism for enzyme inactivation remains unclear, but presumably the targeted CY*P*450 monooxygenase would deliver activated oxygen to the acetylenic bond to form the transient reactive oxirene that could covalently bind to the respective enzyme. The low nanomolar K_i values of these inhibitors make them the most potent known steroidal inactivators of their respective targeted enzymes. The selective pharmacological synergism demonstrated by prolonged suppression of hormonal levels in animals or man is indicative of their therapeutic potential.

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REFERENCES

1. Walsh C.: Scope and mechanism of enzymatic monooxygenation reactions. *Ann. Rpt. Med. Chem.* **15** (1980) 207–216.
2. Weinstein I., Jeffrey A. M., Leffler S., Pulkrabek P., Yamasaki H. and Grunberger D.: Interactions between polycyclic aromatic hydrocarbons and cellular macromolecules. In *Polycyclic Hydrocarbons and Cancer: Environment, Chemistry and Cell Biology* (Edited by H. V. Gelboin and P. O. P. Ts'o). Academic Press, New York (1978) Vol. 2, pp. 1–36.
3. Parke D. V.: In *Enzyme Induction* (Edited by D. V. Parke). Plenum Press, New York (1975) pp. 115–148.
4. Hodgson E. and Tate L. G.: Cytochrome *P*450 interactions. In *Insecticide Biochemistry and Physiology* (Edited by C. F. Wilkinson). Plenum Press, New York (1976) pp. 115–143.
5. Fellig J., Barnes J. R., Rachlin A. I., O'Brien J. P. and Focella A.: Substituted phenyl 2-propynyl ethers as carbamate synergists. *J. Agric. Food Chem.* **18** (1970) 78–80.
6. Waxman D. J.: Interactions of hepatic cytochromes *P*-450 with steroid hormones: regioselectivity and stereospecificity of steroid metabolism and hormonal regulation of rat *P*450 enzyme metabolism and hormonal regulation of rat *P*-450 enzyme expression. *Biochem. Pharmac.* **37** (1988) 71–84.
7. Wood A. W., Ryan D. E., Thomas P. E. and Levin W.: Regio- and stereoselective metabolism of two C_{19} steroids by five highly purified and reconstituted rat hepatic cytochrome *P*-450 isozymes. *J. Biol. Chem.* **258** (1983) 8839–8847.
8. Cole M. P., Jones C. T. A. and Dodd I. D. H.: A new antiestrogenic agent in late breast cancer. *Br. J. Cancer* **25** (1971) 270–275.
9. Santen R. J., Santner S., Davis B., Veldhuis J., Samojlik E. and Ruby E.: Aminoglutethimide inhibits extraglandular estrogen production in postmenopausal women with breast carcinoma. *J. Clin. Endocrinol. Metab.* **47** (1978) 1257–1265.
10. Metcalf B. W., Wright C. L., Burkhart J. P. and Johnston J. O.: Substrate-induced inactivation of aromatase by allenic and acetylenic steroids. *J. Am. Chem. Soc.* **103** (1981) 3221–3222.
11. Lauber M. and Muller J.: Purification and characterization of two distinct forms of rat adrenal cytochrome $P\text{-}450_{11\beta}$: functional and structural aspects. *Archs. Biochem. Biophys.* **274** (1989) 109–119.
12. Imai M., Shimada H., Okada Y., Matsushima-Hibiya Y., Ogishima T. and Ishimura Y.: Molecular cloning of a cDNA encoding aldosterone synthase cytochrome *P*-450 in rat adrenal cortex. *FEBS Letts.* **263** (1990) 299–302.
13. Matsukawa N., Nonaka Y., Ying Z., Higaki J., Ogihara T. and Okamoto M.: Molecular cloning and expression of cDNA encoding rat aldosterone synthase: variants of cytochrome $P\text{-}450_{11\beta}$. *Biochem. Biophys. Res. Commun.* **169** (1990) 245–252.
14. Johnston J. O., Wright C. L., Holbert G. W. and Metcalf B. W.: A time-dependent inhibitor of aldosterone biosynthesis. *Endocrinology* **116** (1985) Abst. 451, p. 113.
15. Johnston J. O., Wright C. L. and Holbert G. W.: Acetylenic deoxycorticosterone selectively inhibits aldosterone biosynthesis via inactivation of C_{18} methyl oxidation. *Endocrinology* **126** (1990) Abst. 7, p. 26.
16. Guengerich F. P.: Covalent binding to apoprotein is a major fate of heme in a variety of reactions in which cytochrome *P*-450 is destroyed. *Biochem. Biophys. Res. Commun.* **138** (1986) 193–198.
17. Sjoerdsma A.: Suicide inhibitors as potential drugs. *Clin. Pharmac. Ther.* **30** (1981) 3–22.

18. Ortiz de Montellano P. R., Kunze K. L., Yost G. S. and Mico B. A.: Self-catalyzed destruction of cytochrome *P*-450: covalent binding of ethynyl sterols to prosthetic heme. *Proc. Natn. Acad. Sci. U.S.A.* **76** (1979) 746–749.
19. White I. H. and Muller-Eberhard U.: Decreased liver cytochrome *P*-450 and haem. *Biochem. J.* **166** (1977) 57–64.
20. Botelho L. H., Ryan D. E. and Levin W.: Amino acid compositions and partial amino acid sequences of the three highly purified forms of liver microsomal cytochrome *P*-450 from rats treated with polychlorinated biphenyls, phenobarbital, or 3-methylcholanthrene. *J. Biol. Chem.* **254** (1979) 5635–5640.
21. Ortiz de Montellano P. R. and Kunze K. L.: Self-catalyzed Inactivation of hepatic cytochrome *P*-450 by ethynyl substrates. *J. Biol. Chem.* **255** (1980) 5578–5585.
22. Ortiz de Motellano P. R. and Kunze K. L.: Occurrence of a 1,2 shift during enzymatic and chemical oxidation of a terminal acetylene. *J. Am. Chem. Soc.* **255** (1980) 7373–7375.
23. Johnston J. O., Wright C. L. and Metcalf B. W.: Biochemical and endocrine properties of a mechanism-based inhibitor of aromatase. *Endocrinology* **115** (1985) 776–785.
24. Burkhart J. P., Weintraub P. M., Wright C. L. and Johnston J. O.: Novel sialylated steroids as aromatase inhibitors. *Steroids* **45** (1985) 357–374.
25. Johnston J. O., Wright C. L., Holbert G. W. and Benson H. D.: Enzyme inactivation by potential metabolites of an aromatase-activated inhibitor (MDL 18,962). *J. Enzyme Inhibition* **4** (1990) 137–142.
26. Holbert G. W., Johnston J. O. and Metcalf B. W.: Synthesis of 11β-hydroxy-18-ethynylprogesterone: an inhibitor of aldosterone biosynthesis. *Tetrahedron Lett.* **26** (1985) 1137–1140.
27. Rubottom G. M., Vazquez M. A. and Pelegrina D. R.: Peracid oxidation of trimethylsilyl enol ethers-facile α-hydroxylation procedure. *Tetrahedron Lett.* (1974) 4319–4322.
28. Metcalf B. W. and Johnston J. O.: 18-Substituted pregn-4-ene-3,20-diones. U.S. Patent 4,293,548, October 6, 1981.
29. Johnston J. O. and Holbert G. W.: Method of treatment using 18-cyano-progesterone derivatives. U. S. Patent 4,954,491, September 4, 1990.
30. Brodie A. M. H., Garrett W. M., Hendrickson J. R., Tsai-Morris C., Marcotte P. A. and Robinson C. H.: Inactivation of aromatase *in vitro* by 4-hydroxy-4-androstene-3,17-dione and 4-acetoxy-4-androstene-3,17-dione and sustained effects *in vivo*. *Steroids* **38** (1981) 693–702.
31. Covey D. F. and Hood W. F.: Aromatase enzyme catalysis is involved in the potent inhibition of estrogen biosynthesis caused by 4-acetoxy and 4-hydroxy-4-androstene-3,17-dione. *Molec. Pharmac.* **21** (1982) 173–180.
32. Henderson D., Norbisrath G. and Kerb U.: 1-Methyl-1,4-androstadiene-3,17-dione (SH489): characterization of an irreversible inhibitor of estrogen biosynthesis. *J. Steroid Biochem.* **24** (1986) 303–306.
33. Burnett R. D. and Kirk D. N.: Some observations on the preparation of 2-hydroxy-steroid-4-en-3-ones. *J. Chem. Soc. Perkin Trans.* **I** (1973) 1830–1836.
34. Johnston J. O., Wright C. L., Burkhart J. P. and Peet N. P., Biological characterization of A-ring steroids. *J. Steroid Biochem. Molec. Biol.* **44** (1993) 623–631.
35. Bradford M. M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72** (1976) 248–254.
36. Kagawa C. M., Shipley E. G. and Meyer R. K.: A biological method for determining small quantities of sodium retaining substances. *Proc. Soc. Exp. Biol. Med.* **80** (1952) 281–285.
37. Morris D. J. and Souness G. W.: The 11β-OHSD inhibitor, carbenoxolone, enhances Na retention by aldosterone and 11-deoxycorticosterone. *Am. J. Physiol. (Renal Fluid Electrolyte Physiol. 27)* **258** (1990) F756–F759.
38. Boxenbaum H., Cramer M. B., Elstum L. T., Heck K., Keeley F. J. and Taylor C. H.: A double-blind, random cross-over tolerance study of oral MDL 18,962 in normal male volunteers (Unpublished Data). Clinical Research Department, Marion Merrell Dow Inc., Kansas City, MO (1991).
39. Wright K., Collins D. C. and Preedy J. R. K.: The use of specific radioimmunoassays to determine the renal clearance rates of estrone and 17β-estradiol during the menstrual cycle. *J. Clin. Endocrinol. Metab.* **39** (1978) 1084–1091.
40. RS/1 computer programs. BBN Software Products Corporation, Cambridge, MA.
41. Cornish-Bowden A.: A simple graphical method for determining the inhibition constants for mixed, uncompetitive and noncompetitive inhibition. *Biochem. J.* **137** (1974) 143–144.
42. Lineweaver H. and Burk D.: The determination of enzyme dissociation constants. *J. Am. Chem. Soc.* **56** (1934) 658–666.
43. Duggleby R. G.: Regression analysis on nonlinear Arrhenius plots: an empiricial model and a computer program. *Comput. Biol. Med.* **14** (1984) 447–455.
44. Kitz R. and Wilson I. B.: Esters of methanesulfonic acid as irreversible inhibitors of acetylcholinesterase. *J. Biol. Chem.* **237** (1962) 3245–3249.
45. Gibaldi M. and Perrier D.: *Pharmacokinetics*. Marcel Dekker Inc., New York (1982) 2nd edition.
46. Johnston J. O. and Metcalf B. W.: Aromatase: a target enzyme in breast cancer. In *Novel Approaches to Cancer Chemotherpy* (Edited by S. S. Prasad). Academic Press Inc., New York (1984) pp. 307–328.
47. Lesuisse D., Gourvest J. F., Hartmann C., Tric B., Benslimane O., Philibert D. and Vevert J. P.: Synthesis and evaluation of a new series of mechanism-based aromatase inhibitors. *J. Med. Chem.* **35** (1992) 1588–1597.
48. di Salle E., Ornati G., Giudici D., Lassus M., Evans T. R. J. and Coombes R. C.: Exemestane (FCE 24304), a new steroidal aromatase inhibitor. *J. Steroid Biochem. Molec. Biol.* **43** (1992) 137–143.
49. Funder J. W., Feldman D. and Edelman I. S.: The roles of plasma binding and receptor specificity in the mineralocorticoid action of aldosterone. *Endocrinology* **92** (1973) 994–1004.
50. Grekin R. J. and Sider R. S.: Aldosterone receptor assay in rat kidney cytosol. *J. Steroid Biochem.* **13** (1980) 835–837.
51. Cole P. A. and Robinson C. H.: Mechanism and inhibition of cytochrome *P*-450 aromatase. *J. Med. Chem.* **33** (1990) 2934–2942.
52. Wright J. N. and Akhtar M.: Studies on estrogen biosynthesis using radioactive and stable isotopes. *Steroids* **55** (1990) 142–151.
53. Oh S. S. and Robinson C. H.: Mechanism of human placental aromatase: A new active site Model. *J. Steroid Biochem. Molec. Biol.* **44** (1993) 389–397.
54. Bruggemeier R. W.: (Unpublished data). Presented at the *Third International Aromatase Conference*. Basic and Clinical Aspects of Aromatase, Bologna, Italy, June 14–17, 1992.
55. Johnston J. O., Wright C. L. and Metcalf B. W.: Time-dependent inhibition of aromatase in trophoblastic tumor cells in tissue culture. *J. Steroid Biochem.* **20** (1984) 1221–1226.
56. Johnston J. O., Wright C. L. and Shumaker R. C.: Human trophoblast xenografts in athymic mice: a model for peripheral aromatization. *J. Steroid Biochem.* **33** (1989) 521–529.
57. Longcope C., Femino A. and Johnston J. O.: Inhibition of peripheral aromatase in baboons by an enzyme-activated aromatase inhibitor (MDL 18,962). *Endocrinology* **122** (1988) 2007–2011.
58. Johnston J. O.: Biological characterization of 10-(2-propynyl)estr-4-ene-3,17-dione (MDL 18,962), an enzyme-activated inhibitor of aromatase. *Steroids* **50** (1987) 105–120.
59. Cramer M. B., Heck K., Taylor C. H., Johnston J. O. and McLees B. D.: Effects of an i.v. aromatase inhibitor on serum estrogen concentrations in normal males. *Clin. Pharmac. Ther.* **49** (1991) 148 (Abst. P1–101).
60. Komor J. and Müller J.: Effects of prolonged infusion of potassium chloride, adrenocorticotrophin or angiotensin II upon serum aldosterone concentration and the conversion of corticosterone to aldosterone in rats. *Acta Endocr. (Copenh.)* **90** (1979) 680–691.
61. Müller J., Hofstetter L., Schwendener-Canlas P., Brunner D. B. and Lund E.-G.: Role of the renin–angiotensin system in the regulation of late steps in aldosterone biosynthesis by sodium intake of potassium-deficient rats. *Endocrinology* **115** (1984) 350–356.
62. Viger A., Coustal S., Perard S. and Marquet A.: Synthesis of new inhibitors of aldosterone biosynthesis. *Tetrahedron* **44** (1988) 1127–1134.
63. Viger A., Coustal S., Perard S., Chappe B. and Marquet A.: Synthesis and activity of new inhibitors of aldosterone biosynthesis. *J. Steroid Biochem.* **30** (1988) 469–472.

64. Viger A., Coustal S., Perard S., Piffeteau A. and Marquet A.: 18-Substituted progesterone derivatives as inhibitors of aldosterone biosynthesis. *J. Steroid Biochem.* **33** (1989) 119–124.
65. Yamakita N., Chiou S. and Gomez-Sanchez C. E.: Inhibition of aldosterone biosynthesis by 18-ethynyl-deoxycorticosterone. *Endocrinology* **129** (1991) 2361–2366.
66. Gomez-Sanchez C. E., Chiou S. and Yamakita N.: 18-Ethynyl-deoxycorticosterone inhibition of steroid production is different in freshly isolated compared to cultured calf zona glomerulosa cells. *J. Steroid Biochem. Molec. Biol.* **46** (1993) 805–810.
67. Johnston J. O., Wright C. L. and Holbert G. W.: Acetylenic substrate analogs as probes of kinetically distinct adrenal P_{450} hydroxylases. *Endocrinology* **124** (1989) Abst. 417, p. 127.
68. Müller J.: Final steps of aldosterone biosynthesis: Molecular solution of a physiological problem. *J. Steroid Biochem. Molec. Biol.* **45** (1993) 153–159.
69. Johnston J. O., Wright C. L., Brohnke R. A. and Kastner P. R.: Inhibition of aldosterone biosynthesis in primates by 18-acetylenic deoxycorticosterone. *Endocrinology* **128** (1991) Abst. 670, p. 198.
70. Kastner P. R., Brohnke R. A., Wright C. L. and Johnston J. O.: Selective inhibition of aldosterone synthesis by MDL 19,347 (18-acetylenic deoxycorticosterone) in sodium-depleted monkeys. *Physiologist* **34** (1991) 252 (Abst. 38.18).
71. Covey D. F., Parikh V. D. and Chien W. W.: 19-Acetylenic, 19-hydroxy-androst-4-en-3,17-diones. Potential suicide substrates of estrogen biosynthesis. *Tetrahedron Lett.* (1979) 2105-2108.
72. Covey D. F., Hood W. F. and Parikh V. D.: 10β-Propynyl-substituted steroids. Mechanism-based enzyme-activated irreversible inhibitors of estrogen biosynthesis. *J. Biol. Chem.* **256** (1981) 1076–1079.
73. Marcotte P. A. and Robinson C. H.: Synthesis and evaluation of 10β-substituted 4-estrene-3,17-diones as inhibitors of human placental microsomal aromatase. *Steroids* **39** (1982) 325–344.