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Active-Site-Directed Inactivation of Aromatase from Human Placental Microsomes by Brominated Androgen Derivatives†

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ABSTRACT: Several brominated androgen derivatives were tested for their ability to inactivate microsomal aromatase from term human placenta. In the experimental protocol, the microsomal homogenate was incubated either with androstenedione or a brominated derivative of androstenedione (16α-bromo-6-ketoandrostenedione, 16α-bromoandrostenedione, 7α-(3′-bromoacetoxypropyl)androstenedione, 6α-bromoandrostenedione, or 6β-bromoandrostenedione) and reduced nicotinamide adenine dinucleotide phosphate in a nitrogen saturated buffer composed of glycerol, ethylenediaminetetraacetic acid, and dithiothreitol in tris(hydroxymethyl)aminomethane hydrochloride (pH 7.4) under nitrogen at 4 °C with shaking. After the incubation period, the microsomes were recovered by centrifugation and washed once before determining aromatase specific activity. The brominated androgen derivatives which inactivated aromatase were 7α-(3′-bromoacetoxypropyl)androstenedione and 6α-bromoandrostenedione. The structures of 6α- and 6β-bromoandrostenedione were unequivocally established by single crystal x-ray diffraction techniques. The extent of the enzyme inactivation by 6α-bromoandrostenedione was linearly proportional to the logarithm of its concentration. The evidence that this inactivation occurs at the aromatase active site is that androstenedione, when coincubated with 6α-bromoandrostenedione, protected aromatase from this inactivation. Progesterone provided much less protection than androstenedione. Furthermore, both 6α- and 6β-bromoandrostenedione are competitive inhibitors of androstenedione aromatization, as determined by a Lineweaver–Burk plot, and 6α-bromoandrostenedione gives the same type I cytochrome P-450 binding spectrum with placental microsomes as androstenedione. These data suggest that 6α-bromoandrostenedione is effective as an active-site-directed inhibitor of placental microsomal aromatase.

Estrogen synthetase, or aromatase, catalyzes the final step in the biosynthetic sequence from cholesterol to the estrogens. Studies of estrogen biosynthesis, utilizing the microsomal fraction of human term placenta (Ryan, 1959) as the source of the enzyme, have led to proposals that (1) androgen aromatization requires 3 mol of both molecular oxygen and NADPH and the participation of cytochrome P-450 (Thompson and Siiteri, 1974a, b), (2) androgens are converted directly to estrogens at a single active site without free intermediates (Hollander, 1962; Osawa and Shibata, 1973; Kelly et al., 1975), and (3) several different active sites are available to accommodate the various androgen substrates (Meigs and Ryan, 1971; Bellino and Osawa, 1974; Zachariah and Juchau, 1975; Canick and Ryan, 1975). Further definitive studies into the physical characteristics and macromolecular structure of aromatase have been limited by the instability of the enzyme and its refractoriness toward solubilization. To circumvent the problem of enzyme instability during solubilization and purification procedures, we searched for an affinity labeling reagent which, when obtained in a radioactive form, would permit the localization of the enzyme by radioactive detection rather than enzyme activity. This would also permit studies

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of the amino acid environment of the aromatase active site(s). We describe here the initial steps in the acquisition of an affinity labeling reagent for aromatase by identifying an active-site-directed brominated androgen derivative which irreversibly inactivates aromatase.

Materials and Methods

Steroid Substrates. The drastic alkali treatment (Osawa and Spaeth, 1971) was used to prepare 1β-^{3}H-androstenedione[1] (6.5 Ci/mol) from 1β,2β-^{3}H-androstenedione (New England Nuclear). The following steroids were gifts of Dr. A. J. Solo of the Department of Medicinal Chemistry, State University of New York, Buffalo, N.Y.: 7α-(3′-bromoacetoxypropyl)androstenedione and 6-bromoandrostenedione. Androstenedione was obtained from Preparations Laboratories, Inc., Huntington Station, N.Y.

The effectiveness of 6-bromoandrostenedione as an irreversible inactivator of aromatase was observed early in this study. Subsequent characterization of this compound using NMR showed it to be an approximately 1:1 mixture of the 6α and 6β isomers. These isomers could not be separated by thin-layer chromatography (Burnett and Kirk, 1973) using Eastman Chromagram silica gel sheet 13181 with fluorescent indicator and developed in cyclohexane–ethyl acetate (2:1, v/v). Characterization of the stereoisomers has not been rigorously established and significantly different characteristics have been reported (Djerassi et al., 1950; Wittstruck et al., 1963; Devis and Beauloye, 1972; Burnett and Kirk, 1973). Therefore we synthesized and purified each isomer and determined total structures by single crystal diffraction methods.

Preparation of 6β-Bromoandrostenedione. Androstenedione (2.5 mmol) and *N*-bromosuccinimide (3.25 mmol) were heated in 50 ml of anhydrous carbon tetrachloride for 18 h with refluxing in the dark. The mixture was cooled to room temperature, the precipitates were filtered off, and the solution was condensed to give 594 mg (65% yield) of 6β-bromoandrostenedione, mp 153–155 °C. The NMR examination showed that the product had no detectable amount of the 6α isomer. Recrystallizations from ethanol gave an analytical sample of 6β-bromoandrostenedione as colorless needles: mp 158–160 °C dec (lit. 162–164 °C dec, Burnett and Kirk, 1973; 187–190 °C dec, Devis and Beauloye, 1972); λ_{max} (EtOH) 247 nm (ϵ 14 300) (246.5 nm (ϵ 12 150), Burnett and Kirk, 1973; 252 nm (ϵ 19 280), Devis and Beauloye, 1972); ν_{max} (KBr) 1735, 1678, 1608 cm^{-1}; NMR (60 MHz, $CDCl_3$) δ 0.98 (3 H, s, 18-CH_3), 1.57 (3 H, s, 19-CH_3), 5.05 (1 H, m, 6α-H), 5.95 (1 H, s, 4-H) (4.93 for 6α-H, Wittstruck et al., 1963; 0.97, 1.55, 5.04, 5.93, Burnett and Kirk, 1973). Anal. Calcd for $C_{19}H_{25}BrO_2$: C, 62.45; H, 6.80; Br, 21.9. Found: C, 62.81; H, 6.83; Br, 22.9.

Preparation of 6α-Bromoandrostenedione. 6β-Bromoandrostenedione (0.136 mmol) was heated at 45 °C for 1 h in 12 ml of methanol containing a trace of hydrochloric acid and the solvent was evaporated under nitrogen to dryness. The NMR examination showed the residue to be approximately a 1:1 mixture of the 6α and 6β isomers. Burnett and Kirk (1973) reported that the isomers could not be separated by chromatography on silica gel. However, the front running portion of the thin-layer chromatogram (Uniplate Silica Gel GF, 500 μm) of the residue developed in cyclohexane–ethyl acetate (2:1, v/v) gave a 6α isomer enriched fraction. Recrystallizations of the fraction from methylene chloride–ethyl ether gave an analytical sample (5.0 mg, 0.014 mmol) of 6α-bromoandrostenedione as colorless short needles: mp 171–173 °C dec (175–177 °C dec, Devis and Beauloye, 1972; Djerassi et al., 1950), λ_{max} (EtOH) 237.5 (ϵ 13 400) (240 nm (ϵ 17 050), Devis and Beauloye, 1972); ν_{max} (KBr) 1730, 1660, 1610 cm^{-1}; NMR (60 MHz, $CDCl_3$), δ 0.92 (3 H, s, 18-CH_3), 1.25 (3 H, s, 19-CH_3), 4.85 (1 H, m, 6β-H), 6.45 (1 H, d, $J_{4,6\beta}$ = 2 Hz, 4-H) (5.07 for 6β-H, Wittstruck et al., 1963; 0.91, 1.25, 4.85, 6.43, Burnett and Kirk, 1973). Recrystallization from ethanol gave mp 175–177 °C dec. Anal. Calcd for $C_{19}H_{25}BrO_2$: C, 62.45; H, 6.80. Found: C, 62.51; H, 6.98.

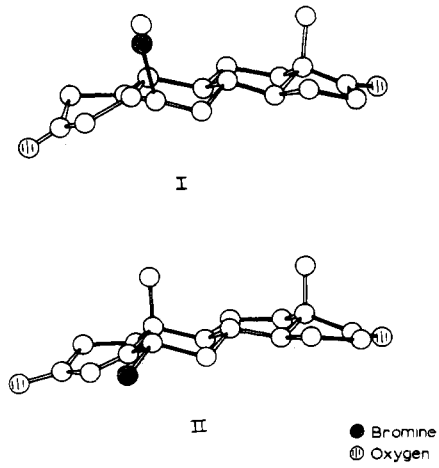

FIGURE 1: Perspective views of 6β-bromoandrostenedione (I) and 6α-bromoandrostenedione (II).

Structure Determination of 6β- and 6α-Bromoandrostenedione by Single Crystal X-Ray Diffraction Techniques. Suitable single crystals were grown from ethanol solution. The structure was solved by straightforward application of the heavy atom method (Lipson and Cochran, 1966) and refined to a reliability index (*R*) of 6.1% for 994 observed reflections of 6β-bromoandrostenedione and 6.5% for 844 observed reflections of the 6α isomer. The average standard deviations in nonhydrogen bond distances and angles are ±0.013 Å and ±0.7°, respectively. Perspective views of both molecules are compared in Figure 1. Values for bonds and angles in the steroid nucleus are not significantly different for the stereoisomers. The conformation about the C(5)–C(6) bonds in each isomer is illustrated in Figure 2. The B-ring torsion angles, given in Table I, show that the B ring of the 6β isomer has a distorted chair conformation that is significantly flattened with average angles of 50.9°, whereas the 6α isomer has an ideal chair conformation with average angles of 55.6°. The flattening of the B ring in the 6β isomer is apparently due to the 1,3-diaxial interaction between the bromine and the 19-methyl group. Detailed crystal and structure data will be published elsewhere (Strong, Hazel, Duax, and Osawa, in preparation).

Preparation of 16α-Bromoandrostenedione. A standard solution of Jones reagent was prepared by dissolving 0.534 g of chromic trioxide in 0.46 ml of sulfuric acid diluted to 2 ml with water (Djerassi et al., 1956). A mixture of 16α-bromo-3β-hydroxy-5-androsten-17-one (1.65 mmol) (Glazier, 1962) and Jones reagent (0.55 ml) in 81 ml of acetone was allowed to stand in an ice bath for 3 min. The mixture was poured into 400 ml of ice-water. The precipitates were collected by fil-

[1] Abbreviations used: androstenedione, 4-androstene-3,17-dione; progesterone, 4-pregnene-3,20-dione; NMR, nuclear magnetic resonance; Tris-HCl, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; NADPH, reduced nicotinamide adenine dinucleotide phosphate; "S" buffer, nitrogen saturated buffer of 20% glycerol (v/v), 0.5 mM EDTA, 0.05 M Tris-HCl (pH 7.5); SD, standard deviation.

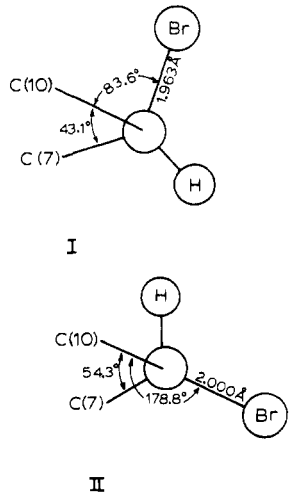

FIGURE 2: Conformation around C(5)-C(6) of 6β-bromoandrostenedione (I) and 6α-bromoandrostenedione (II).

tration, washed with water, and dried over phosphorus pentoxide to give 1.47 mmol of crude 16α-bromo-5-androstene-3,17-dione. After recrystallization from ethanol, 1.30 mmol of the unconjugated ketone was treated with 0.25 mmol of *p*-toluenesulfonic acid monohydrate in 13 ml of acetone for 17 h at room temperature. Ether (30 ml) and water (10 ml) were added and the reaction mixture was neutralized with a dilute sodium bicarbonate solution. The aqueous phase was extracted with ether and the combined ethereal solution was washed with saturated sodium chloride solution, dried over anhydrous sodium sulfate, and evaporated to dryness. The residue was dissolved in methylene chloride and the solution was condensed to give 1.29 mmol of colorless crude material of 16α-bromoandrostenedione. Recrystallizations from ethyl acetate gave analytically pure samples: mp 172–174 °C; ν_{max} (KBr) 1748, 1665, 1618 cm^{-1} (172–174 °C; 1750, 1665, 1645 cm^{-1}, Glazier, 1962); NMR (60 MHz, $CDCl_3$) δ 0.97 (3 H, s, 18-CH_3), 1.23 (3 H, s, 19-CH_3), 4.51 (1 H, m, 16β-H), 5.77 (1 H, s, 4-H).

Preparation of 16α-Bromo-4-androstene-3,6,17-trione. A mixture of 16α-bromo-3β-hydroxy-5-androsten-17-one (0.654 mmol) and Jones reagent (0.7 ml) in 3 ml of acetone was stirred in an ice bath for 30 min. Glacial acetic acid (0.4 ml) was added and the mixture was stirred for an additional 30 min. The mixture was poured into ice–water and extracted with ethyl acetate. Evaporation of the extract gave 0.3 g of crude solid material. Two recrystallizations from ethyl acetate–heptane, thin-layer chromatography on a silica gel plate in benzene–ethyl acetate (4:1 v/v), and a third recrystallization from ethyl acetate–heptane afforded an analytical sample (0.12 mmol) of 16α-bromo-4-androstene-3,6,17-trione: mp 195–197 °C; λ_{max} (EtOH) 250 nm (ε 11 900); ν_{max} (KBr) 1748, 1674, 1604 cm^{-1}; NMR (60 MHz, $CDCl_3$) δ 1.01 (3 H, s, 18-CH_3), 1.24 (3 H, s, 19-CH_3), 4.67 (1 H, m, 16β-H), 6.33 (1 H, s, 4-H). Anal. Calcd for $C_{19}H_{23}BrO_3$: C, 60.16; H, 6.11. Found: C, 59.78; H, 6.18.

Procedure for Aromatase Inactivation. Lyophilized microsomes were prepared from full term human placenta as described previously (Bellino and Osawa, 1974). Dissolved air was replaced by nitrogen in a glycerol-containing buffer (20% glycerol (v/v), 0.5 mM EDTA, 0.05 M Tris-HCl (pH 7.5)) by three alternating cycles of vacuum and nitrogen pressure (10 psi) applied to the buffer at room temperature (20–22 °C). Dithiothreitol (1 mM) was added to the nitrogen saturated glycerol-containing buffer ("S" buffer) to improve the stability of microsomal aromatase. "S" buffer was prepared fresh daily and kept at 4 °C until required.

TABLE I: Torsion Angles for the B Ring of 6β-Bromoandrostenedione (I) and 6α-Bromoandrostenedione (II).

Torsion Angles	I	II
C(10)-C(5)-C(6)-C(7)	−43.1	−54.3
C(5)-C(6)-C(7)-C(8)	49.4	57.7
C(6)-C(7)-C(8)-C(9)	−60.1	−57.3
C(7)-C(8)-C(9)-C(10)	62.2	57.9
C(8)-C(9)-C(10)-C(5)	−49.9	−55.0
C(9)-C(10)-C(5)-C(6)	41.1	51.5
Average torsion angle	50.9	55.6

Lyophilized microsomes were homogenized in "S" buffer (1 mg of microsomes/ml) and 10-ml aliquots were added to light-shielded Erlenmeyer flasks containing the appropriate quantity of androgen or androgen derivative in 0.1 ml of ethanol and 2 mg of NADPH in a total volume of 11 ml. The flasks containing the incubation mixture were flushed with nitrogen and the contents were incubated under 10 psi nitrogen with shaking. After the appropriate incubation period, the microsomes were recovered from the incubation medium by centrifugation at 200 000*g* (MSE SS-75 ultracentrifuge) for 30 min, washed once by an equivalent centrifugation in 0.067 M phosphate buffer (pH 7.4), and resuspended in 2 ml of the same buffer. The incubation and recovery of microsomes were performed at 4 °C. Aliquots were removed for aromatase activity determination and protein assay. The steroid substrate remaining and products formed during the incubation were obtained by organic extraction of the incubation medium recovered from the first centrifugation.

Aromatase Assay. An aliquot of the recovered microsomal homogenate was incubated with [1β-^{3}H,4-^{14}C]androstenedione (10^5 dpm ^{3}H; 2000 dpm ^{14}C; 7 nmol) and 1 mg of NADPH in a total volume of 2 ml for 6 min at 37 °C with shaking in air. The reaction was terminated with 0.3 ml of 10% trichloroacetic acid and the terminated reaction mixture was applied to a column (1.1 × 30 cm) containing a bottom layer (9 cm) of washed Amberlite XAD-2 resin (Rohm and Haas Co.) and a top layer (9 cm) of granulated activated charcoal. The column was eluted with distilled water (35 ml, 5 ml/min). The first 4 ml (void volume) was discarded and the next 30 ml collected. The amount of tritiated water in the effluent, which is proportional to the amount of estrogen produced during the incubation (Osawa, 1973), was measured by adding 10 ml of Scinti-Verse (Fisher Scientific) to a 3-ml aliquot of the column effluent and counting the gel in a Packard scintillation counter. The complete absence of ^{14}C counts above background in all determinations showed that steroid leakage through the column was negligible. The tritium disintegrations per minute were calculated from the counts per minute data using a counting efficiency graph obtained from a set of variably quenched radioactive standards.

The number of picomoles of estrogen formed was calculated from the disintegrations per minute of tritiated water produced during the incubation using the [1β-^{3}H]androstenedione specific activity corrected for the tritium distribution (75% is at the 1β position; Osawa and Spaeth, 1971). The aromatase specific activity for each incubation was calculated by dividing the number of picomoles of estrogen produced per minute by the protein concentration. The protein concentration was determined by the procedure of Lowry et al. (1951) after dis-

solving 0.1 ml of the microsomal homogenate in 0.5 ml of a 0.5 N NaOH–1.2% sodium deoxycholate solution. Bovine serum albumin was treated in the same way and used as the standard.

The experiments to determine the initial velocity of androstenedione aromatization in the presence of the bromoandrogens were performed by incubating 6α- or 6β-bromoandrostenedione (0, 20.5, 82.1 nM) with [1β-^{3}H,4-^{14}C]androstenedione (4.7×10^3 ^{3}H dpm/pmol and 0.47×10^3 ^{3}H dpm/pmol), 1 mg of NADPH, and 5 mg of lyophilized microsomes for 20 s at 37 °C in 2 ml with shaking. The incubation buffer was 0.067 M phosphate buffer (pH 7.4) for one experiment and "S" buffer in another. The tritiated water produced during the incubation was quantitated as previously described. Control incubations showed that the aromatization reaction was linear during the incubation period. The resulting data were plotted in a Lineweaver–Burk graph and a linear fit to each data set obtained at a constant bromoandrogen concentration (five data points per bromoandrogen concentration) was obtained by least-squares analysis.

Steroid Recovery from Incubation Mixture. An aliquot of the incubation medium recovered from the first centrifugation step was extracted three times with an equal volume of ethyl acetate. The organic extracts were combined, washed once with water, and evaporated under a stream of nitrogen. Ethanol was added to the residue which was applied to a thin-layer chromatogram (Eastman chromagram sheet 13181–silica gel with fluorescent indicator) and developed in cyclohexane–ethyl acetate (2:1, v/v). The R_f of the steroid substrate and any degradation products extracted from the incubation mixture was compared with the R_f of the substrate by visualization under ultraviolet light.

Detection of Cytochrome P-450 and Determination of Steroid Binding Spectra. The procedure of Thompson and Siiteri (1974b) was employed to detect a CO–dithionite minus CO peak absorbance at 450 nm in a placental microsomal suspension. The steroid binding spectra were obtained in the following manner. The baseline was determined by scanning a microsomal suspension (13.6 mg of protein/ml) in the sample and reference cuvettes from 350 to 500 nm using an Aminco DW-2 spectrophotometer. Then the steroid, dissolved in ethanol, was added to the sample cuvette (33 μM steroid, 1% ethanol (v/v) final concentrations) and the spectrum was obtained. The arithmetic difference between the spectrum obtained with the steroid in the sample cuvette and the baseline was the binding spectrum for that steroid. The microsomal suspension used in this determination was prepared as described by Thompson and Siiteri (1974a) and stored frozen at −96 °C in phosphate buffer (0.067 M, pH 7.4) for several weeks before use.

Results

Aromatase Inactivation. Several concentrations of androstenedione and various brominated derivatives of androstenedione were incubated with placental microsomes, under the conditions described in Materials and Methods, for either a short period of time ($t \leq 10$ min: the time required to mix substrate with microsomal incubation system, transfer the contents of up to eight flasks to centrifuge tubes, and initiate high-speed centrifugation) or overnight (approximately 17 h). The aromatase specific activity was measured in each case after the microsomes were recovered and washed. The results shown in Table II give the percent of the recovered aromatase specific activity after incubations with the bromoandrogens relative to the control incubation (containing androstenedione as substrate).

Microsomes recovered from the 16α-bromo-, 16α-bromo-6-keto-, and 6β-bromoandrostenedione incubations retained 80 to 95% of the specific activity of the androstenedione incubated (control) microsomes. On the other hand, 7α-(3′-bromoacetoxypropyl)androstenedione, 6-bromoandrostenedione (ca. 1:1 mixture of α and β isomers), and 6α-bromoandrostenedione were effective in significantly reducing the aromatase specific activity relative to the control. A considerable amount of aromatase inactivation caused by 6α-bromoandrostenedione occurred during the short incubation period of approximately 10 min. No evidence of estrogen formation was obtained in the 17-h incubation with androstenedione under this incubation condition.

Although 6α-bromoandrostenedione is the most active compound examined, the aromatase inactivation appears to be incomplete since 10 to 12% of the enzyme activity remains after 17 h incubations with 260 and 520 nmol amounts of the bromoandrogen. We sought to determine if this residual activity represents a portion of microsomal aromatase that is protected in some way from the inactivating effect or if some other microsomal enzyme activity becomes apparent in the aromatase assay when microsomal aromatase is completely inactivated. Placental microsomes were preincubated with 273 nmol of 6α-bromoandrostenedione for 17 h at 4 °C, recovered, and washed as described and then assayed for aromatase activity by two independent methods: one using [4-^{14}C]androstenedione as substrate and isolating estrogens and the other using [1β-^{3}H,4-^{14}C]androstenedione and assayed by tritiated water liberated. The amount of [^{14}C]estrone and [^{14}C]-17β-estradiol produced during the incubation was measured after organic solvent extraction and purification by thin-layer chromatography (Bellino and Osawa, 1974) and countercurrent distribution analysis (Spaeth and Osawa, 1974). [^{3}H]-Estrone and [^{3}H]-17β-estradiol were added to the incubation mixture before organic extraction to correct for the loss in the procedures. The aromatase specific activity measured by [^{14}C]estrogen production agreed with the tritiated water measurement. Therefore, the tritiated water production in the aromatase assay using microsomes preincubated at 4 °C with 260 or 520 nmol of 6α-bromoandrostenedione for 17 h is due to residual aromatase activity.

The stability of the steroid substrates during the incubation period, shown in Table III, was measured by thin-layer chromatographic detection of the remaining substrate and any degradation products extracted from the incubation medium after the microsomes were separated from the medium. We ascertained that the substrate degradation is caused by the incubation rather than the extraction or chromatographic procedures by extracting and chromatographing substrate recovered at zero time. All brominated androgen derivatives were stable when incubated in buffer alone (Tris-HCl, pH 7.4, or phosphate, pH 7.4) for at least 24 h at 4 °C. With the addition of placental microsomes to the buffer, 7α-(3′-bromoacetoxypropyl)androstenedione was rapidly altered to a more polar compound. The androgen derivatives with bromine at C(6) or C(16) were unstable when incubated in "S" buffer alone. Dithiothreitol was identified as the constituent of "S" buffer which caused the partial conversion of 6-bromoandrostenedione to a more polar compound after a 17-h incubation at 4 °C.

Of the two active compounds from Table II, 6α-bromoandrostenedione and 7α-(3′-bromoacetoxypropyl)androstenedione, 6α-bromoandrostenedione was chosen for further study

TABLE II: Aromatase Activity Remaining after Incubation with Brominated Androgen Derivatives.[a]

Steroid Substrates[b] Amount:	520 nmol		260 nmol		130 nmol	
Incubation Time, t:	10 min	17 h	10 min	17 h	10 min	17 h
16α-Bromo-6-ketoandrostenedione	80% (1)					
16α-Bromoandrostenedione	95% (2)	88% (1)				
7α-(3′-Bromoacetoxypropyl)androstenedione		37% (2)				
6-Bromoandrostenedione		39.4% (7)	63% (1)	48% (2)		
6β-Bromoandrostenedione		91% (1)	95% (1)			
6α-Bromoandrostenedione	45% (1)	12% (2)	33.5% (2)	10% (1)	48% (3)	31% (2)

[a] The steroid substrate was incubated with placental microsomes at 4 °C in the amount indicated as described in Materials and Methods. After the incubation period, the microsomes were recovered and washed, and the aromatase specific activity was determined. The aromatase specific activities in microsomes incubated with androstenedione for 10 min at 4 °C were 160.3 pmol of estrogen/min per mg of protein and 144.6 pmol of estrogen/min per mg of protein for two batches of placental microsomes used in these studies. After 17 h of incubation at 4 °C in "S" buffer, the specific activities decreased to 66% of the above values when no substrate was included in the incubate (eight determinations) and 71% when androstenedione was the substrate (nine determinations). The number of determinations is given in parentheses. [b] The percent of aromatase specific activity in microsomes recovered from incubations containing the indicated steroid relative to a control incubation of the same duration containing 520 nmol of androstenedione.

since it is a more stable compound under this incubation condition. Figure 3 shows that the extent of aromatase inactivation is linearly proportional to the logarithm of the concentration of 6α-bromoandrostenedione. This proportionality is observed for both the short term (≤10 min) and 17-h incubations. Figure 4 shows that a natural substrate for aromatase, androstenedione, protects the enzyme from the inactivating effect of 6α-bromoandrostenedione. The degree of protection is proportional to the molar ratio of androstenedione to 6α-bromoandrostenedione. The specificity of androgen protection of aromatase from 6α-bromoandrostenedione inactivation was tested by measuring the ability of a nonsubstrate steroid, progesterone, to protect the enzyme. The amount of aromatase specific activity recovered from 17-h incubations with 6α-bromoandrostenedione alone or in the presence of an eightfold molar excess of androstenedione or progesterone relative to a control incubation with androstenedione (540 nmol) was: (a) 6α-bromoandrostenedione (68 nmol), 36%; (b) 6α-bromoandrostenedione (68 nmol) and androstenedione (540 nmol), 80%; (c) 6α-bromoandrostenedione (68 nmol) and progesterone (540 nmol), 48%.

Inhibition of Androstenedione Aromatization. To determine if 6α- and 6β-bromoandrostenedione go to the same aromatase active site as androstenedione, the effect of these bromoandrogens on the initial velocity of androstenedione aromatization was measured in phosphate buffer and in "S" buffer. The data were analyzed by the $1/v$ vs. $1/S$ (Lineweaver-Burk) plot. The linear least-squares fit to the data indicates that these compounds are competitive inhibitors of androstenedione aromatization in phosphate buffer (apparent V_{max}, K_M, and K_I for both bromoandrogens are 43.4 pmol/min per mg of protein, 29.9 nM and 67.6 ± 1.3 (SD) nM, respectively) and in "S" buffer (apparent V_{max}, K_M, and K_I for both bromoandrogens are 37 pmol/min per mg of protein, 34.4 nM, and 52.2 nM, respectively).

Cytochrome P-450 Binding Spectra. Thompson and Siiteri (1974b) obtained experimental evidence to support their propositions that the mechanism of aromatization requires the participation of cytochrome P-450 and that essentially all of the cytochrome P-450 detected in human term placental microsomes is involved in aromatization. Furthermore, they found that only substrates, intermediates, and inhibitors of aromatization give a type I binding spectra.

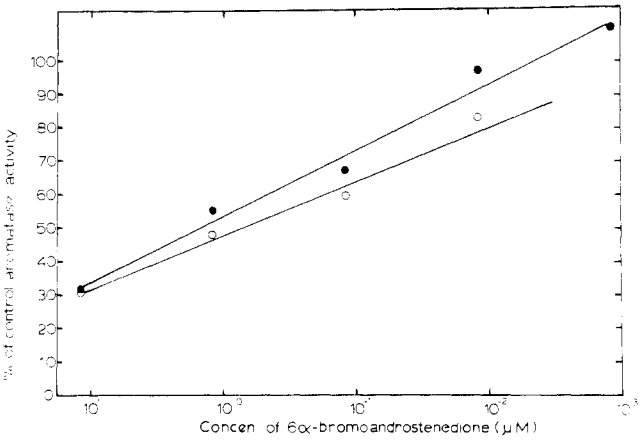

FIGURE 3: Aromatase activity remaining after incubation with 6α-bromoandrostenedione. Several concentrations of 6α-bromoandrostenedione were incubated for the indicated duration with homogenized placental microsomes at 4 °C which were recovered, washed, and assayed for aromatase specific activity as described in Materials and Methods. The aromatase specific activity recovered from a similar incubation with 520 nmol of androstenedione is the control (100%) activity. The incubation times are (●) $t \leq 10$ min and (○) $t = 17$ h.

We detected a 450-nm peak from a CO-dithionite minus CO difference spectrum using an extensively washed preparation of placental microsomes, indicative of the presence of cytochrome P-450 in our preparation. The natural substrate, androstenedione, and the brominated androgen derivative which inactivates aromatase, 6α-bromoandrostenedione, both gave typical cytochrome P-450 type I binding spectra when incubated with placental microsomes under the conditions described in Materials and Methods.

Discussion

The search for an affinity label of aromatase in human placental microsomes produced two candidate compounds. The first, and most promising, is 6α-bromoandrostenedione, which is shown here to inactivate aromatase to an extent proportional to the logarithm of its concentration. Androstenedione, a natural substrate of aromatase, provides more protection from inactivation by 6α-bromoandrostenedione than progesterone, indicating that the enzyme inactivation probably occurs at the

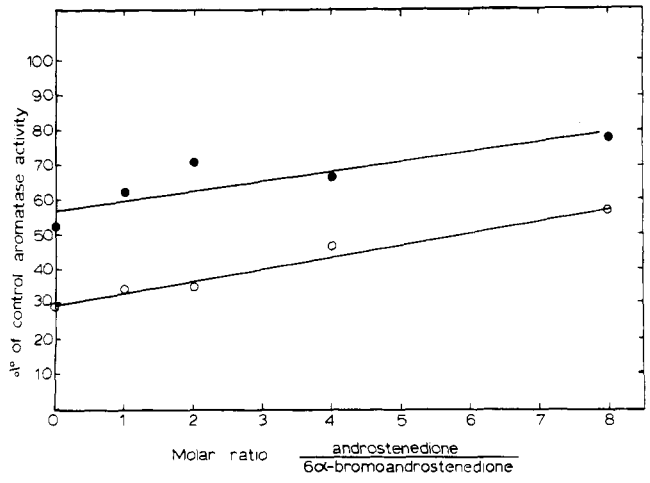

FIGURE 4: Protection of aromatase by androstenedione. Various amounts of androstenedione and 137 nmol of 6α-bromoandrostenedione were coincubated in the indicated molar ratios for the indicated duration with homogenized placental microsomes at 4 °C which were recovered, washed, and assayed for aromatase specific activity as described in Materials and Methods. The control activity and incubation times are the same as given in Figure 3.

active site. More direct evidence that 6α-bromoandrostenedione goes to the same aromatase active site as androstenedione is twofold. First, 6α-bromoandrostenedione acts as a competitive inhibitor of androstenedione aromatization. Second, a type I cytochrome P-450 binding spectrum is observed for both androstenedione and 6α-bromoandrostenedione. The regiospecificity of the inactivation reaction is suggested by the ineffectiveness of the 16α-bromoandrogens. A stereospecificity of the inactivation is suggested by the inability of 6β-bromoandrostenedione to inactivate the enzyme even though it competitively inhibits androstenedione aromatization in phosphate buffer and in "S" buffer to an extent equal to that of the 6α isomer. However, as discussed later, this stereospecificity of inactivation may be due, at least in part, to the relative instability of the 6β isomer in "S" buffer. The 6α-bromoandrostenedione inactivation of aromatase proceeds very rapidly under these experimental conditions since the inactivation reaction is 50 to 90% complete within the 10-min incubation period at 4 °C. One puzzling feature of this inactivation is that 10 to 15% of the enzyme activity remains after 17-h incubations with 260 and 520 nmol of 6α-bromoandrostenedione at 4 °C. The observations that (a) about 70% of this aromatase inactivation occurs within the 10-min incubation period (see Table II) and that (b) a large majority of the 6α substrate remains intact after a 20-min incubation period (see Table III) suggest that depletion of the available 6α substrate is not primarily responsible for this residual aromatase activity. Rather it appears as if this minor fraction of the microsomal enzyme is protected in some manner from the inactivating effects of the 6α isomer. We have no direct data to support the hypothesis that the inhibitor is covalently attached to the enzyme. The synthesis of [^{14}C]- and [^{3}H]-6α-bromoandrostenedione, currently in progress, will allow that determination to be made.

Dithiothreitol, used in this study to partially stabilize microsomal aromatase during the incubation period, is somewhat reactive with both isomers of 6-bromoandrostenedione and less than half of the starting material remained after the 17-h incubation period. The 6β isomer is a considerably more reactive electrophile than the 6α isomer. In addition to this differential

TABLE III: Steroid Substrate Stability.[a]

Steroid Substrate	Approx. % Decomposition At 22 °C	At 4 °C
Androstenedione	0 (24 h)	0 (24 h)
16α-Bromoandrostenedione	0 (24 h)[b]	
16α-Bromo-6-ketoandrostenedione	50 (4 h)	
7α-(3′-Bromoacetoxypropyl)androstenedione	90 (0.5 h)[b]	90 (17 h)
6-Bromoandrostenedione		75 (17 h)
6β-Bromoandrostenedione		70 (20 min);[c] 75 (17 h)
6α-Bromoandrostenedione		<10 (20 min);[c] 65 (17 h)

[a] The steroid substrates (520 nmol) and products formed during the incubation with "S" buffer and microsomes were recovered from the incubation mixtures via organic extraction of the first supernatant, as described in Materials and Methods. The incubation times are given in parentheses. The relative proportions of remaining substrates and polar products were estimated from developed thin-layer chromatograms through visual observation using ultraviolet irradiation. In every case where a product spot appears it was more polar than the substrate. The substrate R_f was checked by running the standard substrate on the same chromatogram. [b] The incubation mixture consisted of steroid substrate, Tris-HCl (pH 7.4) buffer, and microsomes. [c] The incubation mixture consisted of steroid substrate, "S" buffer, and microsomes. At the indicated time, an aliquot was removed and the substrate and products were recovered from the incubation mixture by organic extraction.

substrate stability, dithiothreitol may increase the selectivity of aromatase toward inactivation by the 6α isomer. The enzyme active site is apparently equally accommodative to both isomers since they both competitively inhibit androstenedione aromatization with the same apparent K_I. If the enzyme was equally receptive to inactivation by both isomers, one would expect to find in Table II that the aromatase inactivation in the incubation containing 520 nmol of the 6β isomer was at least equivalent to that found with 130 nmol of the 6α isomer (due to the 25 to 30% 6β substrate that survives in the presence of dithiothreitol). This was not observed. Furthermore, the extent of aromatase inactivation in a mixture of the two isomers, 6-bromoandrostenedione, was intermediate to that found when the two isomers were incubated individually. This finding probably reflects a partial protection of aromatase by the 6β component since this component apparently is capable of reversibly binding to the active site and it is present in significant, though greatly reduced, amounts throughout the 17-h incubation.

The other promising compound is 7α-(3′-bromoacetoxypropyl)androstenedione. Although this compound was not investigated as thoroughly as 6α-bromoandrostenedione due to its instability when incubated with placental microsomes, it is worthy of further study.

Acknowledgments

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Collagenase Enzymes from *Clostridium*: Characterization of Individual Enzymes†

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ABSTRACT: Four collagenases have been purified to apparent homogeneity from extracts of *Clostridium histolyticum* and partially characterized. The four purified enzymes are devoid of hydrolytic activity against casein and the synthetic substrate, benzoylarginine naphthylamide, but all retain activity against native collagen. The enzymes are initially separated by isoelectric focusing where three of the enzymes show distinct isoelectric points: collagenase I = 5.50, collagenase II = 5.65, and collagenases IIIa and IIIb = 5.90–6.00. Collagenases IIIa and IIIb can be subsequently separated on diethylaminoethylcellulose. The four purified enzymes show single bands upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Calibration of the molecular weights on the basis of migration distance shows a marked dependence on gel porosity. At high acrylamide concentration, collagenases I, II, and IIIa appear to converge to a limiting molecular weight ≃81 000, while collagenase IIIb has a distinctly lower value ≃72 000. The similarity between these molecular weight values and those derived from the sedimentation and diffusion coefficients of the native enzyme indicates that each collagenase is a single polypeptide chain. All of the collagenases have comparable catalytic activities against a series of natural and synthetic substrates and are immunologically cross-reactive. Although all four enzymes are evident upon initial electrofocusing of the crude extract, it is possible that the multiplicity of forms is, at least in part, a consequence of lysis following initial secretion from the cell.

Collagenolytic enzymes are among the many proteinases released extracellularly into the culture media of *Clostridium histolyticum.* One extensively studied collagenolytic enzyme, collagenase A, is known to have a high degree of substrate specificity, requiring the amino acid sequence Pro-*X*-Gly-Pro-*Y*, where *X* and *Y* can be any amino acid (Gallop et al., 1957; Harper and Kang, 1970; Seifter and Harper, 1971). The enzyme cleaves the bond between the *X* and Gly to release a Gly-NH_2 terminus. The strict substrate requirement of this enzyme and collagenases, in general, renders them powerful tools in the structural elucidation of collagen-like proteins (Seifter and Harper, 1971). However, purification of collagenolytic enzymes has been complicated by the presence in the starting materials of less specific proteases with physical and chemical characteristics similar to those of collagenases (Keller and Mandl, 1963; Kono, 1968; Mitchell, 1968; Peterkofsky and Diegelmann, 1971).

Two to six separate enzymes with collagenolytic activity have been noted in crude extracts and chromatographically

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