

Cytochrome P450 3A4-catalyzed Testosterone 6 β -Hydroxylation Stereochemistry, Kinetic Deuterium Isotope Effects, and Rate-limiting Steps*[§]

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Testosterone 6 β -hydroxylation is a prototypic reaction of cytochrome P450 (P450) 3A4, the major human P450. Biomimetic reactions produced a variety of testosterone oxidation products with 6 β -hydroxylation being only a minor reaction, indicating that P450 3A4 has considerable control over the course of steroid hydroxylation because 6 β -hydroxylation is not dominant in a thermodynamically controlled oxidation of the substrate. Several isotopically labeled testosterone substrates were prepared and used to probe the catalytic mechanism of P450 3A4: (i) 2,2,4,6,6-²H₅; (ii) 6,6-²H₂; (iii) 6 α -²H; (iv) 6 β -²H; and (v) 6 β -³H testosterone. Only the 6 β -hydrogen was removed by P450 3A4 and not the 6 α , indicating that P450 3A4 abstracts hydrogen and rebounds oxygen only at the β face. Analysis of the rates of hydroxylation of 6 β -¹H-, 6 β -²H-, and 6 β -³H-labeled testosterone and application of the Northrop method yielded an apparent intrinsic kinetic deuterium isotope effect (^Dk) of 15. The deuterium isotope effects on k_{cat} and k_{cat}/K_m in non-competitive reactions were only 2–3. Some “switching” to other hydroxylations occurred because of 6 β -²H substitution. The high ^Dk value is consistent with an initial hydrogen atom abstraction reaction. Attenuation of the high ^Dk in the non-competitive experiments implies that C–H bond breaking is not a dominant rate-limiting step. Considerable attenuation of a high ^Dk value was also seen with a slower P450 3A4 reaction, the O-dealkylation of 7-benzyloxyquinoline. Thus P450 3A4 is an enzyme with regioselective flexibility but also considerable regioselectivity and stereoselectivity in product formation, not necessarily dominated by the ease of C–H bond breaking.

P450¹ enzymes are found in organisms from *Archebacter* to humans and catalyze oxidations of a great variety of chemicals

(1, 2). The human P450s are the major enzymes involved in the clearance of drugs, and P450 3A4, the most abundant P450 in liver and small intestine, is involved in the oxidation of one-half of the drugs used today (3, 4). Two crystal structures of P450 3A4 have been published recently (5, 6); however, neither has a substrate bound in a position amenable to oxidation. P450 3A4 exhibits heterotropic and homotropic cooperativity in some but not all of its reactions (4, 7–10), and several models have been proposed to explain such behavior (9, 11–15). A view frequently expressed for smaller substrates is that binding is relatively loose and the regioselectivity of oxidation of substrates is largely a function of the ease of C–H bond breaking (16, 17).

Relatively little information is available regarding what step is rate-limiting in P450 3A4 reactions. The addition of the first electron in the catalytic cycle (step 2 of Scheme 1) is apparently relatively fast in the presence of an optimal concentration of NADPH-P450 reductase (18). Only limited information has been obtained regarding steps 1 and 3–9 (Scheme 1) with P450 3A4 reactions. Without information on the extent to which chemical steps (e.g. substrate oxidation) limit catalysis, it will not be possible to understand or predict the behavior of this system.

One long-standing approach to understanding the kinetics of individual reaction steps is the application of kinetic isotope effects, particularly involving hydrogen isotopes (19, 20). The approach has been used extensively in chemical reactions and applied to numerous enzyme systems (21–26). However, the use of kinetic isotope effect methods with enzymes is subject to the complexity of these multi-step systems and the isotope effect on the chemical step can be masked by the contributions of other steps (27, 28). Kinetic isotope effect studies have been used with P450 reactions for >30 years (29–33). In principle, such experiments can sense the degree to which step 7 is rate-limiting (Scheme 1). In many of the early studies, the experimental designs were not always most appropriate for mechanistic interpretation. However, more sophisticated studies with bacterial P450 101A1 (34) and mammalian P450s (35–37) have been done. A number of theoretical treatments of P450 kinetic isotope effects were done by the Gillette group (38, 39). Although many of the expressed kinetic isotope effects cited in the literature are low and there has been a tendency to regard C–H bond breaking as generally being relatively rapid, our own work (40–45) has shown relatively high expressed kinetic isotope effects for several reactions catalyzed by mammalian P450 enzymes.

Reports of kinetic isotope effect studies with P450 3A4 reac-

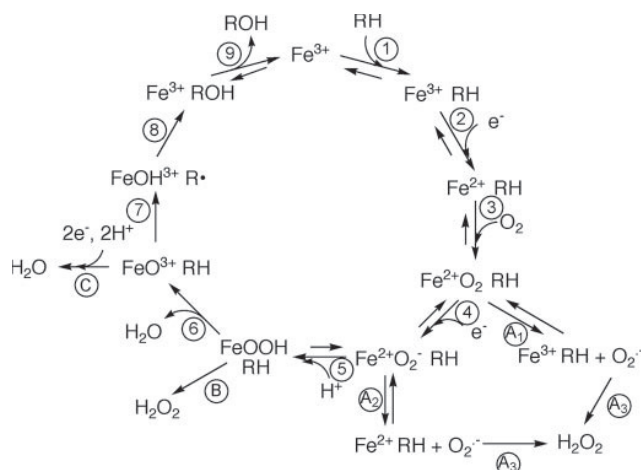
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[§] The on-line version of this article (available at <http://www.jbc.org>) contains ¹³C NMR spectrum of 6,6-*d*₂-testosterone, HPLC/UV, and LC/MS traces of products of testosterone oxidation by a manganoporphyrin complex/PhI = O system, Hill plots ($\log_{10} [v/(k_{cat} - v)]$ versus $\log_{10} [S]$) for testosterone 6 β -hydroxylation shown in Supplemental Figs. 1S–3S.

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¹ The abbreviations used are: P450, cytochrome P450; MS, mass

spectrometry; HPLC, high pressure liquid chromatography; APCI, atmospheric pressure chemical ionization; LC, liquid chromatography; HRMS, high resolution mass spectra; FAB, fast atom bombardment; NBA, 3-nitrobenzoic acid; MeOH, methanol; EtOAc, ethyl acetate; THF, tetrahydrofuran; MS, mass spectrometry.



SCHEME 1. Cytochrome P450 catalytic cycle.

tions are very limited (46). Many of the reactions are not amenable because of the nature of the substrates (*e.g.* nifedipine ring dehydrogenation (involving *N*), 17 β -estradiol 2-hydroxylation), and isotope effects in *N*-dealkylation reactions are dominated by the deprotonation event (47, 48). Testosterone 6 β -hydroxylation is a prototypic P450 3A4 reaction (49) and should be amenable to such analysis. However, the synthesis of the appropriately isotopically labeled testosterone had not been reported and, in our own experience, was not trivial.

We prepared a series of H-6 isotopically labeled forms of testosterone and used these as substrates with P450 3A4 enzyme systems. The results reveal stereospecific abstraction of the 6 β -hydrogen and a high intrinsic kinetic hydrogen isotope effect that is strongly attenuated with partial switching of oxidation to other (β) positions. The results provide a view of this enzyme that differs from some current conceptions regarding P450 3A4 selectivity and the nature of rate-limiting steps in the catalytic cycle.

EXPERIMENTAL PROCEDURES

Chemicals

All of the reagents used for synthesis and product analysis (generally from Aldrich Chemical Co., Milwaukee, WI) were of ACS grade or better unless specified otherwise. HPLC solvents were of HPLC grade (EM Science, Gibbstown, NJ). Steroids were purchased from Steraloids (Newport, RI).

Spectroscopy

All of the ^1H and ^{13}C NMR spectra were recorded on a Bruker AM 300 spectrometer operating at 300 and 75 MHz, respectively, at 27 $^\circ\text{C}$ (Bruker, Billerica, MA) in the Vanderbilt facility. Samples were prepared in CDCl_3 . UV spectra were recorded using a Cary 14/OLIS spectrophotometer (On-Line Instrument Systems, Bogart, GA). Fluorescence assays (7-hydroxyquinoline formation) were done using an OLIS RSM-1000 instrument in the steady-state mode (with a cuvette accessory and manual mixing).

HPLC/UV analysis of the testosterone oxidation products was done with an HPLC system consisting of a Hitachi L-7100 pump (Hitachi High Technologies, San Jose, CA) coupled to a ThermoFinnigan UV3000HR rapid-scanning spectrophotometer (ThermoSeparations, Piscataway, NJ).

Mass Spectrometry

Most mass spectra were obtained in the Vanderbilt facility with direct infusion of samples using a Finnigan TSQ 7000 instrument triple quadrupole mass spectrometer (ThermoFinnigan, San Jose, CA) equipped with a standard API-1 APCI source operating in the positive ion mode. N_2 was used as the sheath gas (80 p.s.i.). The vaporizer temperature was 350 $^\circ\text{C}$, and the corona current was maintained at 5 mA. The capillary was set at 200 $^\circ\text{C}$ and 34.5 V. The tube lens voltage was 83 V. Data acquisition and spectral analysis were done with Finni-

gan ICIS software on a Digital Equipment Corp. Alpha workstation. HRMS of the synthetic compounds were recorded at the University of Notre Dame Mass Spectrometry Center (South Bend, IN) using NBA as the matrix (FAB ionization).

Syntheses

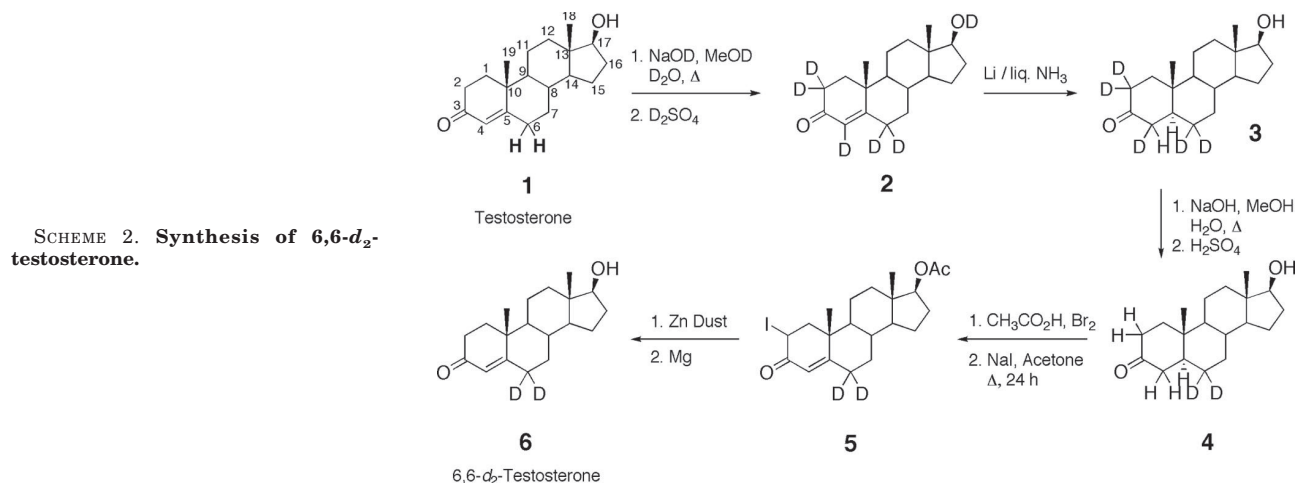
2,2,4,6,6-*d*₅-Testosterone (Scheme 2) (2, 50)—Cleaned pieces of Na^0 (8.5 g) that had been cut, scraped, weighed, and dipped into deuterated MeOH (50 ml) solution until shiny were added to a chilled 150-ml solution of deuterated MeOH (under N_2 , on ice) in ~ 1 -g portions (Scheme 2). When all of the Na^0 had reacted, 100 ml of D_2O was added. The solution was allowed to cool to room temperature, and 12.7 g of unlabeled testosterone (*d*₀) was added. The solution was stirred while heating under reflux under N_2 for 24 h. The solvent was removed *in vacuo*, and 150 ml of D_2O was added. The suspension was cooled on ice and made acidic with concentrated D_2SO_4 . The suspension was extracted with EtOAc (3×250 ml), and the organic phase was dried over MgSO_4 and concentrated to dryness (40 $^\circ\text{C}$). The isotopic purity was checked by MS, and the reaction was repeated with the crude solid, if necessary, until the isotopic purity was $\geq 99.5\%$. The crude solid was recrystallized from EtOAc/hexane or aqueous acetone (D_2O) or purified by silica chromatography (EtOAc: CH_2Cl_2 , 1:1, v/v) to yield 2,2,4,6,6-*d*₅-testosterone (compound 2) (11.9 g, 92% yield, and 99.5% isotopic enrichment as judged by ^1H NMR) (50, 51): ^1H NMR (CDCl_3) δ 0.77 (s, 3H, CH_3), 0.93–1.1 (m, 3H), 1.2 (s, 3H, CH_3), 1.20–2.35 (m, 13H), 3.7 (m, 1H, CHOH); ^{13}C NMR (CDCl_3) δ 11.5 (C-18), 17.8 (C-19), 21.0 (C-11) 23.7 (C-15), 30.9 (C-16), 31.9 (C-7), 36.0 (C-8), 36.1 (C-1), 36.8 (C-16), 43.2 (C-13), 50.9 (C-14), 54.3 (C-9), 82.1 (C-17), 171.8 (C-5), 200.1 (C-3); UV (MeOH) λ_{max} 240 nm; HRMS (FAB, NBA) calculated for $\text{C}_{19}\text{H}_{24}\text{D}_5\text{O}_2$, 294.2481; found, 294.2465 [$\text{M} + \text{H}$] $^+$.

6,6-*d*₂-Testosterone (Scheme 2) (6)

6,6-*d*₂-Dihydrotestosterone (Compound 4) (52, 53)— NH_3 gas was liquified using a dry ice/acetone condenser and bath (500 ml) at -78 $^\circ\text{C}$. Li^0 (0.9 g) was added and stirred for 10 min at -78 $^\circ\text{C}$. 2,2,4,6,6-*d*₅-Testosterone (compound 2) (9 g) (see above) was dissolved in 250 ml of toluene/THF (1:1, v/v), and this solution was chilled at -78 $^\circ\text{C}$ before addition (in one portion) to the Li/NH_3 solution. The reaction was stirred for 30 min at -78 $^\circ\text{C}$. Solid NH_4Cl was carefully added until the blue color was discharged, yielding a white suspension. The solution was allowed to slowly warm to room temperature with evaporation of the NH_3 . A solution of 10% aqueous HCl (300 ml) was then added. The pH was checked with litmus paper, and if necessary, the solution was made acidic by the addition of concentrated HCl. The aqueous layer was extracted with EtOAc (3×250 ml). The combined organic layers were washed with brine (100 ml) and then dried over MgSO_4 . The solvent was removed *in vacuo*, leaving a sticky yellow solid. The solid was triturated with ether (30 ml) to facilitate removal of higher boiling point solvents, which afforded ~ 6 g of a light yellow solid. The mother liquor was concentrated to afford an additional 3 g of solid. MeOH (250 ml) was added to 7 g of the crude [2,2,4,6,6-*d*₅]-4,5-dihydrotestosterone product (compound 3) and titrated with 25–30 ml of 20% NaOH (w/v) until the solution was slightly turbid. The solution was heated at reflux overnight. The MeOH and H_2O were removed *in vacuo*, and 100 ml H_2O was added to the solid. The suspension was acidified with conc. H_2SO_4 , and the product was extracted with EtOAc (3×200 ml), washed with H_2O and then brine (50 ml), and then dried over MgSO_4 . The crude product (7 g of yellow solid) was purified on silica using CH_2Cl_2 :EtOAc (9:1, v/v) to yield 6,6-*d*₂-dihydrotestosterone (compound 4) (4.0 g, 44% yield, and 99.5% isotopic purity) (52–54): ^1H NMR (CDCl_3) δ 0.60–0.73 (m, 1H), 0.74 (s, 3H), 0.75–0.99 (m, 3H), 1.0 (s, 3H), 1.05–1.90 (m, 12H), 1.95–2.31 (m, 6H), 3.61 (t, 1H); HRMS (FAB, NBA) calculated for $\text{C}_{19}\text{H}_{29}\text{D}_2\text{O}_2$, 293.2450; found, 293.2447 [$\text{M} + \text{H}$] $^+$.

2,4-Dibromo-4,5-dihydrotestosterone-17-acetate (55)—A solution of 2.0 g of 6,6-*d*₂-4,5-dihydrotestosterone (compound 4) was dissolved in glacial $\text{CH}_3\text{CO}_2\text{H}$ (200 ml), and a solution of 1 ml of Br_2 in 15 ml of glacial $\text{CH}_3\text{CO}_2\text{H}$ was added. The solution was stirred at room temperature for 12 h. HBr (48% in $\text{CH}_3\text{CO}_2\text{H}$, 2 ml) was added to yield the 2,2-dibromo derivative, and the solution was stirred for an additional 1.5 h to afford conversion to the 2,4-isomer. The product was precipitated by the addition of 2 liters of ice-cold H_2O . The precipitate was collected by filtration, washed with H_2O , dried *in vacuo* with a dessicator and vacuum pump, and used directly in the next step (3.2 g). The solid could be further purified by repeated recrystallization from aqueous acetone and then from cyclohexane (55).

6,6-*d*₂-17-Acetoxytestosterone (56)—2,4-Dibromo-17-acetoxy-2,4-dihydrotestosterone (2.5 g) was dissolved in 300 ml of acetone. NaI (6.0 g)



SCHEME 2. Synthesis of 6,6-d₂-testosterone.

was added, and the solution was heated at reflux for 1.5 h. The resulting NaBr was removed by filtration while the solution was hot. The solution was heated at reflux overnight, yielding a strong iodine color. Most of the acetone was removed *in vacuo* (45 °C) to leave ~10 ml of residual material. H₂O (150 ml) was added, and Na₂SO₃ was added until the iodine color was discharged. The solution was extracted into EtOAc (2 × 200 ml), washed with brine, dried over MgSO₄, concentrated *in vacuo*, and dried *in vacuo* (vacuum pump) to afford a yellowish crude solid (compound 5) (~2 g), which was used directly in the next step.

The crude 2-iodo-6,6-d₂-17-acetoxytestosterone (2 g) was dissolved in MeOH (120 ml). Zinc dust (5.0 g) was added, and the resulting suspension was stirred overnight. The reaction mixture was heated at 50 °C for ~1.5 h until reaction was complete as indicated by TLC. The zinc was removed by filtration through a plug of sand on cotton, and the MeOH was removed *in vacuo* (45 °C). The product was dried *in vacuo*, affording a yellowish solid, which was purified by chromatography on silica (CH₂Cl₂:EtOAc, 95:5, v/v) to yield 6,6-d₂-17-acetoxytestosterone (56): 450 mg, 26%, 98.8% (d₂) isotopic enrichment; ¹H NMR (CDCl₃) δ 0.81 (s, 3H, CH₃), 0.85–1.15 (m, 4H), 1.16 (s, 3H, CH₃), 1.20–1.95 (s, 9H), 2.0 (s, 3H, COCH₃), 2.10–2.40 (m, 4H), 3.7 (m, 1H), 5.71 (s, 1H); ¹³C NMR (CDCl₃) δ 12.4 (C-18), 17.8 (C-19), 20.9 (C-11), 21.6 (COCH₃), 23.9 (C-15), 31.9 (C-16), 33.1 (C-7), 34.3 (C-6), 35.8 (C-2), 36.1 (C-8), 37.0 (C-1), 39.0 (C-16), 42.9 (C-13), 50.6 (C-14), 54.1 (C-14), 82.9 (C-17), 124.4 (C-4), 171.6 (C-5), 199.9 (C-3); HRMS (FAB, NBA) calculated for C₂₁H₂₆D₂O₃, 333.2399; found, 333.2378 2447 ([M + H]⁺).

6,6-d₂-Testosterone (Compound 6) (57)—Mg⁰ (26 mg, 2 eq for reaction) was added to MeOH (15 ml) under an N₂ atmosphere, and the solution was stirred until the metal completely dissolved (~8 h). 6,6-d₂-17-Acetoxytestosterone (175 mg) was dissolved in 5 ml of THF and added to the solution. The reaction was stirred overnight and then reduced to one-half of the volume under an N₂ stream. Ice-cold H₂O (100 ml) was added, and the mixture was brought to pH 7 by the addition of 5% HCl. The solution was extracted with EtOAc (2 × 100 ml), and the organic phase was washed with brine (50 ml), dried over MgSO₄, and concentrated *in vacuo*. Ether (50 ml) was added to the resulting oil and then removed *in vacuo* to facilitate the removal of the EtOAc. Approximately 0.15 g of crude solid was obtained. The product 6,6-d₂-testosterone (compound 6) was separated (TLC, 1.0-mm silica plates, CH₂Cl₂:EtOAc/3:1, v/v) and recrystallized from acetone (57): 75 mg, 50% yield, 98.4% (d₂) isotopic enrichment; ¹H NMR (CDCl₃) δ 0.77 (s, 3H, CH₃), 0.93–1.15 (m, 4H), 1.17 (s, 3H, CH₃), 1.20–2.35 (m, 14H), 3.66 (m, 1H, CHOH), 5.71 (s, 1H, vinylic); ¹³C NMR (CDCl₃) (see Fig. 1S): δ 11.5 (C-18), 17.8 (C-19), 21.0 (C-11), 23.7 (C-15), 30.9 (C-16), 31.9 (C-7), 34.4 (C-2), 36.0 (C-8), 36.1 (C-1), 36.8 (C-12), 39.1 (C-10), 43.2 (C-13), 50.9 (C-14), 54.3 (C-9), 82.1 (C-17), 124.3 (C-4), 171.8 (C-5), 200.1 (C-3); UV (MeOH) λ_{max} 240 nm; HRMS (FAB, NBA) calculated for C₁₉H₂₇D₂O₂, 291.2293; found, 291.2302 ([M + H]⁺).

6α-d₁-Testosterone (14)

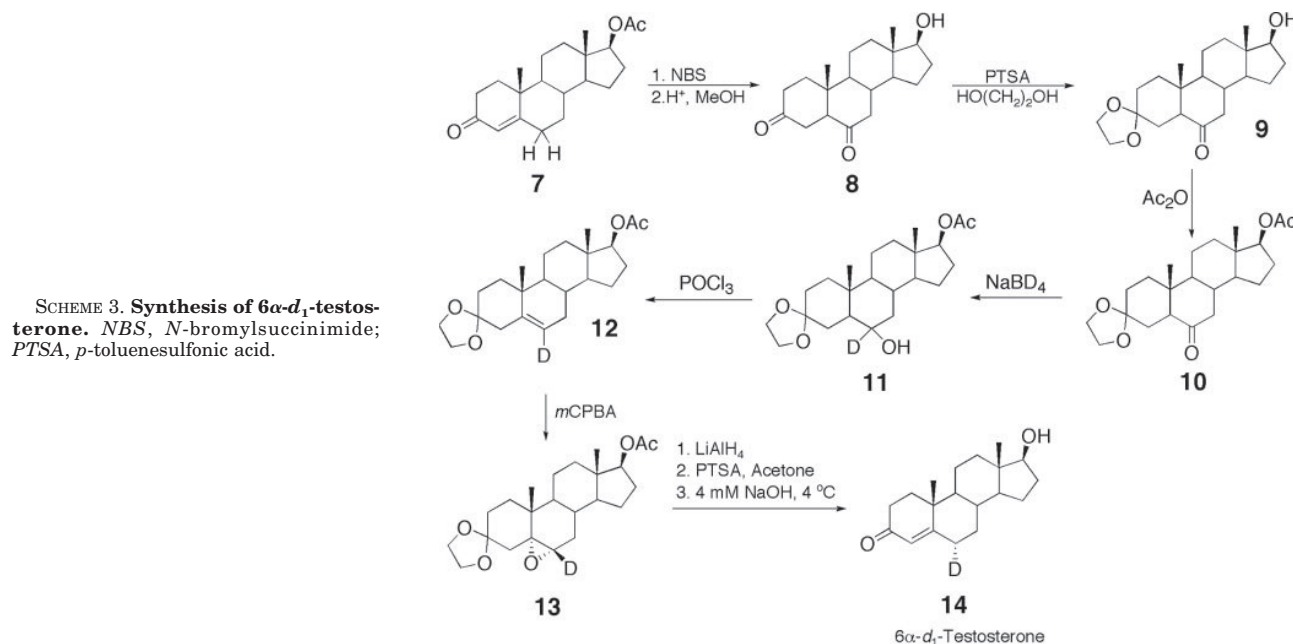
17-Acetoxytestosterone (Compound 7) (58)—Testosterone (compound 1, 8.0 g) (Scheme 3) was dissolved in pyridine (50 ml) and acetic anhydride (50 ml). 4-Dimethylaminopyridine (250 mg) was added, and the reaction mixture was stirred overnight at 5 °C. Ice-cold H₂O (1.5 liters) was slowly added to the reaction mixture while the suspension was stirred vigorously. The solid was collected by filtration and washed with H₂O (2 × 150 ml) with brief sonication of the suspension with each

wash. The solid was collected by filtration again and dissolved in EtOAc (150 ml). The solution was washed with brine, dried over MgSO₄, concentrated, and dried *in vacuo*. The solid was dissolved in a minimal amount of hot EtOAc, and hexane was added (~125 ml). The solution was heated at reflux, and the solvent was slowly removed *in vacuo* (50 °C). As soon as crystals began to form, the flask was removed and cooled to room temperature. The crystals were collected by filtration and washed with a small portion of hexane to remove the remaining yellow color. The white solid (compound 7, 8.7 g, 94%) was dried using a vacuum pump (and, if necessary, can be recrystallized from EtOAc/hexane): ¹H NMR (CDCl₃) δ 0.81 (s, 3H, CH₃), 0.85–1.15 (m, 4H), 1.16 (s, 3H, CH₃), 1.20–1.95 (s, 9H), 2.0 (s, 3H, COCH₃), 2.10–2.40 (m, 6H), 3.7 (m, 1H, CHOH), 5.71 (s, 1H, vinylic); ¹³C NMR (CDCl₃) δ 12.4 (C-18), 17.8 (C-19), 20.9 (C-11), 21.6 (COCH₃), 23.9 (C-15), 31.9 (C-16), 33.1 (C-7), 34.3 (C-6), 35.8 (C-2), 36.1 (C-8), 37.0 (C-1), 39.0 (C-16), 42.9 (C-13), 50.6 (C-14), 54.1 (C-14), 82.9 (C-17), 124.4 (C-4), 171.6 (C-5), 199.9 (C-3); MS (APCI) *m/z* 331.2 (100, [M + H]⁺).

6-Oxo-4,5-dihydrotestosterone (Compound 8) (59, 60)—*N*-Bromosuccinimide (7 g) and benzoyl peroxide (250 mg) were added to a solution of 17-acetoxytestosterone (compound 7, 8 g) in CCl₄ (300 ml). The solution was heated under vigorous reflux for 30 min and then cooled. Insoluble material was removed by filtration through filter paper. The filtrate was washed sequentially with H₂O (300 ml), 5% NaHCO₃ (w/v, 300 ml), H₂O (300 ml), and brine (300 ml). The organic layer was dried over MgSO₄, filtered, and concentrated *in vacuo* at 40 °C. The residue was dried *in vacuo* (vacuum pump) and then triturated with hexane. The product was collected by filtration and dried, affording crude 6-bromo-17-acetoxytestosterone (8 g, 88%). The product was dissolved in MeOH (250 ml) containing 11 ml of concentrated HCl. The reaction was heated under reflux for 3 h, and the solvent was removed *in vacuo*. The resulting solid was triturated with H₂O (2 × 100 ml) and collected by filtration. The wet solid was dissolved in CH₂Cl₂ (150 ml), washed with H₂O (2 × 100 ml), dried over MgSO₄, and concentrated *in vacuo*. The residue was triturated with ether (2 × 100 ml) and dried *in vacuo* to afford 4.5 g (75% yield) of 6-oxo-4,5-dihydrotestosterone (compound 8): ¹H NMR (CDCl₃) δ 0.76 (s, 3H, CH₃), 0.95 (s, 3H, CH₃), 1.15–2.58 (m, 21H), 3.84 (t, 1H, CHOH); MS (APCI) of 2,4-dinitrophenylhydrazine derivative: *m/z* 663.22 (40, [M + H]⁺), disubstituted dinitrophenylhydrazine derivative of 6-oxo-4,5-dihydrotestosterone, *m/z* 483.31 (100, [M + H]⁺), monosubstituted dinitrophenylhydrazine derivative of 6-oxo-4,5-dihydrotestosterone (61).

3,3-Dioxolane-6-oxo-4,5-dihydrotestosterone (Compound 9) (60, 62)—6-Oxo-4,5-dihydrotestosterone (compound 8, 1.04 g) was heated in benzene (65 ml) under reflux with ethylene glycol (314 mg, 5.1 mmol, 1.1 eq) in the presence of a catalytic amount of *p*-toluenesulfonic acid (55 mg) for 15 min using a Dean-Stark trap for azeotropic removal of H₂O from benzene. The organic phase was then washed with saturated NaHCO₃ (25 ml), H₂O (25 ml), and brine (25 ml) and then dried over MgSO₄. The solvent was removed *in vacuo*, and ether was added to facilitate drying, affording 3,3-dioxolane-6-oxo-4,5-dihydrotestosterone (compound 9, 1.5 g, 80%), which was used directly in the next step (MS (APCI): *m/z* 349.11 (100, [M + H]⁺)).

3,3-Dioxolane-6-oxo-17-acetoxy-4,5-dihydrotestosterone (10)—3,3-Dioxolane-6-oxo-4,5-dihydrotestosterone (1.0 g) was added to a chilled solution (5 °C) of pyridine (15 ml), acetic anhydride (10 ml), and 4-dimethylaminopyridine (0.03 g). The solution was stirred overnight at 5 °C



and then poured into ice-cold H₂O (500 ml). The precipitate was removed by filtration, washed with cold H₂O (2 × 100 ml), and extracted into ether (100 ml). The organic layers were washed with H₂O (30 ml) and then brine (30 ml), dried over MgSO₄, and concentrated *in vacuo*. The 3,3-dioxolane-6-oxo-17-acetoxy-4,5-dihydrotestosterone (10, 1.5 g, 76%) was dried *in vacuo* and immediately used in the next step (MS (APCI): *m/z* 391.12 (100, [M + H]⁺)).

6α-d₁-Testosterone (Compound 14) (Scheme 3) (63–65)—3,3-Dioxolane-6-oxo-17-acetyl-4,5-dihydrotestosterone (compound 10, 1.5 g) in THF (15 ml) was added to a stirred solution of THF (50 ml) and deuterated MeOH (50 ml) containing NaBD₄ (250 mg) at 0 °C. The reaction was stirred for 3 h at 4 °C and then warmed to room temperature. A saturated solution of NH₄Cl in D₂O (5 ml) was then added. The solvents were removed *in vacuo* at 35 °C, and D₂O (50 ml) was added. The aqueous suspension was extracted into EtOAc (2 × 50 ml), and the organic layers were combined and washed with brine in D₂O (10 ml), dried over MgSO₄, and filtered. Solvent was removed *in vacuo* (40 °C) to afford 1.2 g of solid 3,3-dioxolane-6-d₁-6-hydroxy-17-acetoxy-4,5-dihydrotestosterone (compound 11). POCl₃ (189 mg, 115 microliters, 1.5 eq, 1.52 mmol) was added slowly to a chilled solution of the above product (0.4 g, 1.0 mmol) in pyridine (10 ml) at 0 °C. The reaction was stirred overnight at 4 °C, and then 100 ml of chilled D₂O was added. The solid was collected by filtration, washed with D₂O, and extracted into ether (2 × 100 ml). The ether solution was washed with brine (D₂O/NaCl) and dried over MgSO₄. The organic layer was concentrated *in vacuo* at 30 °C. The residual pyridine was removed *in vacuo* using a vacuum pump. Ether was added and removed *in vacuo* several times to facilitate removal of pyridine, affording crude 3,3-dioxolane-6-d₁-17-acetoxy-5-androstene (compound 12, 150 mg), which was immediately mixed with 3-chloroperbenzoic acid (120 mg) in 10 ml of CH₂Cl₂ and stirred for 3 h. The reaction was quenched with 1 ml of saturated Na₂S₂O₃, washed with H₂O, and dried over MgSO₄. The solvent was removed *in vacuo* affording a residue (150 mg), which was then added to a solution of LiAlH₄ (60 mg) in THF (10 ml). The reaction was heated at reflux for 1 h and then quenched by the careful addition of 1% NaOH (w/v, 10 ml). The product was extracted into ether (2 × 50 ml), washed with brine, and dried over MgSO₄. The solvent was removed *in vacuo*. The residue was dissolved in acetone (10 ml) containing *p*-toluenesulfonic acid (10 mg) and stirred overnight at room temperature. The solvent was reduced to one-half of the volume followed by the addition of H₂O (10 ml). The product was extracted into EtOAc (2 × 20 ml), washed with brine, and dried over MgSO₄, and the solvent was removed *in vacuo*. The residue was dissolved in a mixture of THF (1 ml), MeOH (2 ml), and H₂O (1 ml). The reaction was initiated by the addition of 20 mM NaOH (1.5 ml) with a final NaOH concentration of 4 mM. The reaction was stirred for 7 days at 4 °C. The organic solvents were removed under a stream of N₂. H₂O (10 ml) was added, and the product was extracted into EtOAc (2 × 20 ml). The combined organic layers were washed with brine and dried over MgSO₄, and the solvent was removed *in vacuo* to

afford a residue. Ether was added to facilitate the removal of solvent in further *in vacuo* treatment. The product 14 (5 mg) was then purified via preparative TLC (silica, CH₂Cl₂:EtOAc, 1:1, v/v): ¹H NMR (CDCl₃): δ 0.77 (s, 3H), 0.93–1.15, 1.17 (s, 3H), 1.20–2.35 (m, 15H), (m, 4H), 3.66 (m, 1H), 5.71 (s, 1H); ¹³C NMR (CDCl₃) δ 11.5 (C-18), 17.8 (C-19), 21.0 (C-11), 23.7 (C-15), 30.9 (C-16), 31.9 (C-7), 34.4 (C-2), 36.0 (C-8), 36.1 (C-1), 36.8 (C-12), 39.1 (C-10), 43.2 (C-13), 50.9 (C-14), 54.3 (C-9), 82.1 (C-17), 124.3 (C-4), 171.8 (C-5), 200.1 (C-3); UV (MeOH) λ_{max} 240 nm; HRMS (FAB, NBA) calculated for C₁₉H₂₈D₁O₂, 290.2215; found, 290.2226 ([M + H]⁺).

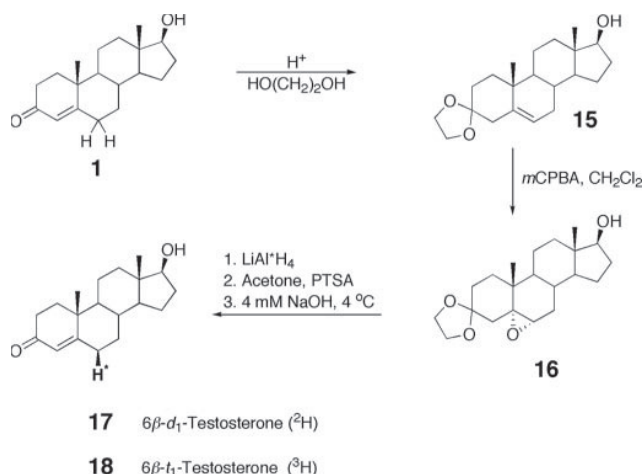
6β-d₁-Testosterone (14) (Scheme 4)

3,3-Diethylenedioxyandrostane-5α-17β-ol (Compound 15) (66)—Testosterone (compound 1, 3.0 g), ethylene glycol (2.25 g), and *p*-toluenesulfonic acid (40 mg) were dissolved in benzene (100 ml) and heated under reflux for 48 h with continuous azeotropic removal of H₂O using a Dean-Stark trap (Scheme 4). The mixture was cooled, diluted with EtOAc (60 ml), and washed with saturated NaHCO₃ and then brine (30 ml). The organic layer was dried over MgSO₄ and reduced *in vacuo*. The residue was recrystallized from EtOAc/hexane affording 3,3-diethylenedioxyandrostane-5α-17β-ol (compound 15, 2.0 g).

5α,6α-Epoxy-3,3-diethylenedioxyandrostane-5α-17β-ol (Compound 16) (64, 67)—NaHCO₃ (1.2 g) was added to a solution of 3,3-diethylenedioxyandrostane-5α-17β-ol (compound 15, 2.0 g) dissolved in CH₂Cl₂ (100 ml) followed by 3-chloroperbenzoic acid (3 g). The reaction was stirred for 1.5 h and then washed sequentially with saturated Na₂SO₃ (50 ml), H₂O (50 ml), and brine (50 ml). The organic layer was dried over MgSO₄, and the solvent was removed *in vacuo*. The solid was purified by chromatography on silica (CH₂Cl₂:EtOAc, 1:1, v/v) to yield 5α,6α-epoxy-3,3-diethylenedioxyandrostane-5α-17β-ol (compound 16, 700 mg, 40%).

6β-d₁-3,3-diethylenedioxyandrostane-5α-17β-diol (68)—5α,6α-Epoxy-3,3-diethylenedioxyandrostane-5α-17β-ol (compound 16, 250 mg) was added to a solution of LiAlD₄ (25 mg) in THF (25 ml), and the solution was heated at reflux under N₂ for 1.5 h. The reaction was reduced to one-half of the volume under a stream of N₂ and quenched by the careful addition of aqueous 1% NaOH (w/v). The mixture was diluted with H₂O (30 ml) and extracted with ether (2 × 75 ml). The combined organic layers were washed with brine (25 ml), dried over MgSO₄, and reduced *in vacuo* to afford 6β-d₁-3,3-diethylenedioxyandrostane-5α-17β-diol (200 mg).

6β-d₁-Testosterone (Compound 17) (Scheme 4) (68)—6β-d₁-3,3-Diethylenedioxyandrostane-5α-17β-diol (200 mg) was dissolved in acetone (20 ml) containing *p*-toluenesulfonic acid (20 mg) and stirred overnight. The reaction volume was reduced to 5 ml under a stream of N₂. H₂O (20 ml) was added, and the aqueous solution was extracted with benzene (2 × 25 ml). The organic layer was washed with saturated NaHCO₃, (10 ml), H₂O (10 ml) and brine (10 ml), dried over MgSO₄, and concentrated



SCHEME 4. Synthesis of 6β-d₁-testosterone and 6β-t₁-testosterone. PTSA, *p*-toluenesulfonic acid.

in vacuo. The residue was dissolved in THF (1.5 ml) and added to a solution of 4 mM aqueous NaOH (1.5 ml) and MeOH (1 ml). The reaction was stirred at 5 °C for 7 days or until reaction was complete as determined by TLC. The volume was reduced to one-half under a stream of N₂. H₂O (20 ml) was added, and the solution was extracted into EtOAc (2 × 50 ml). The combined organic layers were washed with brine, dried over MgSO₄, and concentrated under an N₂ stream affording a crude solid. The solid was purified via preparative TLC (silica, 1 mm, CH₂Cl₂:EtOAc, 1:1, v/v) yielding 6β-d₁-testosterone (compound 17, 50 mg, 30% yield): ¹H NMR (CDCl₃) δ 0.77 (s, 3H, CH₃), 0.93–0.93 (m, 4H), 1.17 (s, 3H, CH₃), 1.25–2.35 (m, 15H), 3.66 (m, 1H, CHOH), 5.71 (s, 1H, vinylic); ¹³C NMR (CDCl₃) δ 11.5 (C-18), 17.8 (C-19), 21.0 (C-11), 23.7 (C-15), 30.9 (C-16), 31.9 (C-7), 34.4 (C-2), 36.0 (C-8), 36.2 (C-1), 36.8 (C-12), 39.1 (C-10), 43.2 (C-13), 50.9 (C-14), 54.3 (C-9), 82.1 (C-17), 124.3 (C-4), 171.8 (C-5), 200.1 (C-3); UV (MeOH) λ_{max} 240 nm; HRMS (FAB, NBA) calculated for C₁₉H₂₈D₁O₂, 290.2215; found, 290.2230 [M + H]⁺.

Other Syntheses

6β-t₁-Testosterone (Compound 18) (Scheme 4) (68)—5α,6α-Epoxy-3,3-diethylenedioxyandrostane-5α-17β-ol (compound 16) was prepared as above and submitted to American Radiolabeled Chemicals (St. Louis, MO) for tritium labeling with LiAlT₄ using the procedure described (see above). The resulting 6β-t₁-3,3-diethylenedioxyandrostane-5α-17β-diol (<1 mg) was diluted with unlabeled 3,3-diethylenedioxyandrostane-5α-17β-diol (200 mg) and converted to 6β-t₁-testosterone (compound 18) in the same manner as described for 6β-d₁-testosterone (compound 17). The chemical (>99%) and radiochemical (>99%) purity of 6β-t₁-testosterone was confirmed, and the specific radioactivity (44 mCi mmol⁻¹) was determined by HPLC using a UV detector (A₂₄₄) connected in tandem with a β-RAM Flow-through System Model 2 flow counter (IN/US Systems, Pine Brook, NJ) using a 6:1 ratio (v/v) of Flow-Scint II scintillant (Packard, Meriden, CT) to mobile phase.

7-d₂-Benzyloxyquinoline (69, 70)—7-Hydroxyquinoline (250 mg) was dissolved in dry *N,N*-dimethylformamide (15 ml) followed by the addition of K₂CO₃ (3 eq). The mixture was stirred for 1 h before the addition of [methylene-d₂]benzyl bromide (Aldrich). The reaction was stirred for 72 h, poured into ice-cold H₂O (50 ml), and extracted with EtOAc (2 × 50 ml). The combined organic layers were washed with H₂O and brine, dried over MgSO₄, and concentrated *in vacuo*. The solid was chromatographed on a silica column (EtOAc/hexane, 1:1, v/v) to yield the product (150 mg ¹H NMR (CDCl₃) δ 7.14–7.51 (m, 8H, phenyl, CHCHN & CHCOCD₂, CHCOCD₂), 7.71 (d, 1H, CCHCHCO), 8.09 (d, 1H, CHCHCCH), 8.84 (s, 1H, CHNCCH); MS (APCI): *m/z* 238.02 (100, [M + H]⁺).

7-d₁-Benzyloxyquinoline (69–75)—LiAlD₄ (3 g, 71.4 mmol) was added to a solution of benzaldehyde (18 g, 170 mmol) in 150 ml of ether. The reaction was stirred at room temperature for 3 h and then carefully quenched with a saturated solution of Rochelle's salt (potassium-sodium tartrate) in D₂O. The organic layer was then washed with brine, dried with MgSO₄, and concentrated *in vacuo*. The residual liquid was then distilled at 205–206 °C (760 torr). The resulting d₁-benzyl alcohol (5 g, 46 mmol) was cooled to 0 °C and brominated by the careful addition of PBr₃ (2.2 ml, 23 mmol). The mixture was stirred at 110 °C for 1.5 h and then stirred overnight at room temperature. The reaction was cooled to 0 °C, diluted with H₂O, and extracted into ether. The ether

was removed *in vacuo*, and the oil was used directly in the next step.

NaH (200 mg, 8.6 mmol) was added to dry *N,N*-dimethylformamide (15 ml) on ice followed by 7-hydroxyquinoline (500 mg, 3.8 mmol). The solution was warmed to room temperature and then stirred for 1 h before the addition of α-d₁-benzyl bromide (1.3 g, 7.6 mmol) (see above). The reaction was stirred for 72 h, poured into ice-cold H₂O (100 ml), and extracted with EtOAc (2 × 50 ml). The combined organic layers were washed with H₂O and then brine, dried over MgSO₄, and concentrated *in vacuo*. The resulting solid was chromatographed on a silica column (EtOAc:CH₂Cl₂, 1:1, v/v) to yield the product 7-d₁-benzyloxyquinoline (250 mg, 56% yield): ¹H NMR (CDCl₃) δ 5.22 (s, 1H, CHD), 7.14–7.51 (m, 8H, phenyl, CHCHN and CHCOCD₂, CHCOCD₂), 7.71 (d, 1H, CCHCHCO), 8.09 (d, 1H, CHCHCCH), 8.84 (s, 1H, CHNCCH); MS (APCI): *m/z* 237.02 (100, [M + H]⁺).

Assays

Testosterone Hydroxylation Assays—The P450 3A4 enzyme reactions involved either “bicistronic” membranes prepared from *Escherichia coli* cells in which both P450 3A4 and human NADPH-P450 reductase were expressed, using 30 pmol of P450 (76), Baculosomes® (microsomes isolated from baculovirus-infected insect cells expressing P450 3A4 plus excess NADPH-P450 reductase from Invitrogen) (4 pmol), or a reconstituted P450 3A4 system consisting of 31 pmol of P450 3A4 (12), 62 pmol of recombinant rat NADPH-P450 reductase (77), 62 pmol of human cytochrome b₅ (45), and a phospholipid/cholesterol mixture (78) in a final volume of 500 microliters of 100 mM potassium phosphate buffer (pH 7.4). All of the testosterone substrates were dissolved in MeOH as stock solutions and were added to enzymes with a final MeOH concentration of <2% (v/v) with final testosterone concentrations of 4–200 μM. Compounds were preincubated with the enzyme and buffer at 37 °C for 3 min, and reactions were initiated by the addition of an NADPH-generating system (79). Reactions proceeded for 8 min and were terminated by the addition of 1.3 ml of CH₂Cl₂ and 1.0 ml of 0.3 M NaCl with mixing in a vortex device. The internal standard corticosterone (10 microliters of a 50 μM solution in MeOH) was added after quenching. The layers were separated by centrifugation, a 2.0-ml aliquot of the organic phase was removed, and the solvent was removed under N₂. The dried samples were redissolved in 30 microliters of MeOH immediately prior to injection, and 20 microliters was injected onto the HPLC column. Most of the testosterone assays involved analysis of hydroxytestosterone products by reversed-phase HPLC coupled to a UV detector (244 nm). A 4.6 × 150 mm Prodigy octadecylsilane (C₁₈) HPLC column (3 μm, Phenomenex, Torrance, CA) was used with a flow rate of 1.5 ml min⁻¹. The mobile phase was 24% CH₃CN in H₂O (v/v) for a 0–5.5-min elapsed time followed by a linear gradient increasing to 62% CH₃CN (v/v) from 5.5 to 12 min and holding at 62% CH₃CN (v/v) to 26 min. The identities of the products were established by co-chromatography with authentic standards or, in the case of 1β-hydroxytestosterone, by LC-NMR and LC/MS (80).

Oxidation of Testosterone by Biomimetic Models—A mixture of testosterone (3.5 mM), PhI = O (9 mM) (81), and either Fe or Mn 5,10,15,20-tetraphenyl-21H,23H-phosphine (285 μM) (82) was reacted in CH₂Cl₂ at room temperature for 30 min. The CH₂Cl₂ solution was washed with H₂O, and an aliquot of the organic layer was concentrated and analyzed by HPLC/UV and HPLC/MS in the manner described above.

Stereoselectivity Experiments—Incubations were done with the substrate testosterone (d₀, 6-d_{1a}, 6-d_{1b}, 6, 6-d₂, or 2,2,4,6,6-d₅) at a concentration of 500 μM with the baculovirus-based enzyme system. The 6β-hydroxytestosterone HPLC fraction was collected, extracted into CH₂Cl₂, dried, dissolved in 100 μl of MeOH, and analyzed by MS.

Intermolecular Competitive Isotope Effect Assays—Incubations were done with a 1:1 mixture of d₀-6,6-d₂-testosterone at a (total) concentration of 100 μM with the baculovirus-based enzyme system. The 6β-hydroxytestosterone HPLC fraction was collected, extracted, dried, dissolved in 100 μl of MeOH, and analyzed by MS.

Tritium Isotope Effects—P450 3A4 (baculovirus-infected insect microsomes, 10 pmol of P450) in 100 mM potassium phosphate buffer (pH 7.4) was incubated with 500 μM 6β-t₁-testosterone (44 mCi mmol⁻¹) in a final volume of 500 μl with an NADPH-generating system for 8 min at 37 °C (79). The products were extracted and analyzed by HPLC as described above with the column monitored in-line with a β-RAM Flow-through System Model 2 flow counter using a 6:1 ratio (v/v) of Flow-Scint II scintillant to mobile phase.

The remaining aqueous layer (from the incubation) was extracted three times with ether (3 × 1 ml) with separation of the layers by centrifugation each time (2000 × g, 10 min). An aliquot (0.6 ml) of aqueous phase was removed and counted in 4 ml of scintillation mixture

(Scintiverse II, Fisher, Fair Lawn, NJ) using a Beckman LS-6500 scintillation counter operating in the automatic disintegrations/min calculation mode to establish the amount of tritium released from 6 β -*t*-testosterone as $^3\text{H}_2\text{O}$.

Non-Competitive Kinetic Isotope Effects (Testosterone)—The assays were done as described above with varying concentrations of d_0 , 6,6- d_2 , or 2,2,4,6,6- d_5 testosterone, and the products were analyzed by HPLC/UV.

Non-competitive Kinetic Isotope Effects (7- d_1 -Benzyloxyquinoline)—The non-competitive d_2 - and d_0 -benzyloxyquinoline assays were done using continuous fluorescence measurements of the released 7-hydroxyquinoline. The excitation and emission wavelengths were 410 and 510 nm, respectively. In a typical reaction (3 ml), a cuvette contained 100 mM potassium phosphate buffer (pH 7.4), 96 pmol of bicistronic P450 3A4 membranes prepared from *E. coli*, and 0–250 μM benzyloxyquinoline substrate (added from stock MeOH, $\leq 2\%$ MeOH (v/v) in enzyme reaction). The reaction was preincubated at 37 °C and initiated by the addition of an NADPH-generating system (79). The rate of 7-hydroxyquinoline release was monitored fluorometrically for 180 s.

Intramolecular Non-competitive Isotope Effects (7- d_1 -Benzyloxyquinoline)—Reactions were done as described above with the substrate 7- d_1 -benzyloxyquinoline at a concentration of 200 μM with the *E. coli* membrane-based enzyme system. A typical reaction (1 ml) included 100 mM potassium phosphate buffer (pH 7.4), 8.3 pmol of *E. coli* derived P450 3A4/NADPH reductase, and 200 μM 7- d_1 -benzyloxyquinoline substrate (added from stock MeOH, $\leq 2\%$ MeOH (v/v) in enzyme reaction). The reaction was preincubated at 37 °C and initiated by the addition of an NADPH-generating system (79). After 8 min, the reaction was quenched and the benzaldehyde that was formed was converted to the 2,4-dinitrophenylhydrazone by the addition of 200 μl of 0.1% 2,4-dinitrophenylhydrazine (w/v) in 6 M HCl. Hexane (2 ml) was added, and the samples were mixed using a vortex device and then with a rotating wheel for 30 min. The samples were dried and dissolved in 100 μl of MeOH immediately prior to injection (20 microliters) on a 4.6 $\times$ 150 mm Prodigy octadecylsilane (C_{18}) HPLC column (3 μm , Phenomenex, Torrance, CA) connected to the mass spectrometer (flow rate of 1.5 ml min^{-1}). The (isocratic) mobile phase was 75% CH_3CN in H_2O (v/v). The isotopic ratio of the derivatized product was established by LC/MS (APCI source), operating in the negative ion mode. N_2 was used as both the sheath gas (80 p.s.i.) and auxiliary gas (10 p.s.i.). The vaporizer temperature was set to 400 °C, and the corona current was maintained at 5 mA. The capillary was set at 200 °C and –20 V, and the tube lens voltage was set to –83 V.

RESULTS

Regioselectivity of Testosterone Oxidations Catalyzed by Biomimetic Metalloporphyrin Systems—To address the issue of whether the selectivity of P450 3A4 reaction is dominated by the lability of C–H bonds, the P450 3A4 reaction was compared with reactions done with relatively simple biomimetic models, Fe and Mn (*meso*)-tetraphenylporphine/ $\text{PhI} = \text{O}$ systems (83, 84). Reactions were done under limited turnover conditions, with testosterone in excess. Many products were obtained in both systems, with the manganese system yielding more products (see Supplementary Fig. 2S). At least six (manganese-derived) products had the expected $[\text{M} + \text{H}]^+$ ion (m/z 305) for hydroxytestosterone (plus at least two that appear to correspond to a dehydrotestosterone, no products appear to be ketotestosterones). The peak at t_R 7.6 min (Supplementary Fig. 2S, parts B and E) corresponds to 6 β -hydroxytestosterone, which is not a major peak. The other products did not match retention times of our other available standards. This biomimetic system has little steric control, and we conclude that the 6 β - and other major hydroxytestosterone products are not the result of inherent C–H bond lability.

Syntheses of Testosterone Labeled with Hydrogen Isotopes—Because a regioselective synthesis of 6,6- d_2 -testosterone had not been reported, limitations existed for studying kinetic deuterium isotope effects for P450-catalyzed testosterone 6 β -hydroxylation. Previous literature had reported an isotope effect with a microsomal system using a deuterium-labeled testosterone (30). However, in that and other studies (30, 75, 85, 86), the testosterone analogs were deuterium-labeled at multiple sites containing various isotopic purities at each position. In addition,

the studies were done with a microsomal mixture of enzymes and not a specific P450. Therefore, a dideuterated testosterone labeled exclusively at the C-6 position (isotopic purity $\geq 99\%$) was needed to study the true isotope effects at this site.

In the synthesis of 6,6- d_2 -testosterone (compound 6) (Scheme 2), protons at the 2,2,4,6- and 6-positions were exchanged with solvent deuterium to afford 2,2,4,6,6- d_5 -testosterone (compound 2) in 99.5% isotopic purity (50). Compound 2 was converted to 3 via Li/liquid. NH_3 reduction and the enolizable deuteriums at positions 2 and 4 were back-exchanged to afford 6,6- d_2 -4,5-dihydrotestosterone (compound 4). Compound 4 was dibrominated at the kinetically favored 2,2-positions, and the product was rearranged to the thermodynamically stable 2,4-dibromo product (87). The 2,4-dibromo compound was iodinated at the 2- and 4-positions, and the iodine at the 4-position was eliminated to afford the α,β -unsaturated ketone (compound 5). Zinc reduction and deblocking the 17-acetoxy group afforded 6,6- d_2 -testosterone (compound 6).

In the synthesis of 6 α - d_1 -testosterone (Scheme 3), 17-acetoxystosterone (compound 7), was selectively brominated to give 6 β -bromo-17-acetoxystosterone, which upon treatment with acid yielded 6-oxo-4,5-dihydrotestosterone (compound 8). Selective blocking with a hemiketal at the more reactive 3-position gave compound 9, which was then blocked at the 17-position with an acetyl and reduced with NaBD_4 to give 11. Treatment with POCl_3 and elimination afforded the deuterated 5,6-ene 12, which was epoxidized to 13, separable as a single diastereomer. Treatment with LiAlD_4 resulted in hydroxylation at the 5-position as well as inversion of configuration of the deuterium at the 6-position. Deblocking of the ketal afforded the respective ketone, which underwent elimination under mild basic conditions to afford 6 α - d_1 -testosterone (compound 14). 6 β - d_1 -Testosterone (compound 17) was prepared as described (68) (Scheme 4), and 6 β - t_1 -testosterone (compound 18) was prepared in an analogous manner by the reduction of compound 16 with LiAlT_4 .

Stereoselectivity Experiments—The stereoselectivity of hydrogen abstraction by P450 3A4 was analyzed using 6 α - d_1 - and 6 β - d_1 -testosterone to probe the stereochemical preference of the H-abstraction step in the P450 cycle (step 7 of Scheme 1). The substrates 6 α - d_1 -, 6 β - d_1 -, and 6,6- d_2 -testosterone were used in separate reactions with P450 3A4 derived from the baculovirus microsomal system. The 6 β -hydroxytestosterone showed m/z values ($[\text{M} + \text{H}]^+$) of 306, 305, and 306, respectively (Fig. 1). Only the β -hydrogen was abstracted. We conclude that the C–H bond cleavage as well as the rebound step are stereoselective (β) processes (Scheme 5).

Competitive Deuterium Isotope Effects—The competitive deuterium isotope effect provides information on the “commitment to catalysis,” *i.e.* the extent to which a substrate is able to move in and out of the active site. The competitive effect for the deuterated substrate is also needed to calculate the intrinsic isotope effect (see below). A competitive deuterium isotope effect of 1.7 ± 0.2 on testosterone 6 β -hydroxylation was calculated using a 1:1 mixture of d_0 and 6,6- d_2 -testosterone (baculovirus microsomal system) (ratio of d_0 6 β -hydroxytestosterone formed/ d_1 6 β -hydroxytestosterone formed).

Estimation of Tritium Isotope Effects—The intrinsic isotope effect (^Dk)² provides valuable information on the “true” isotope effect of the C–H bond-breaking step, in that it is a measure of the contribution of step 7 of Scheme 1. The intrinsic isotope effect is needed to properly evaluate other kinetic deuterium isotope effects in the context of contribution to rate-limiting

² The conventions used for kinetic hydrogen isotope effects are ^Dk = intrinsic kinetic deuterium isotope effect, $^D V = ^Hk_{\text{cat}}/^Dk_{\text{cat}}$, and $^D(V/K) = (^Hk_{\text{cat}}/^H K_m)/(^Dk_{\text{cat}}/^D K_m)$ (see Refs. 27, 28).

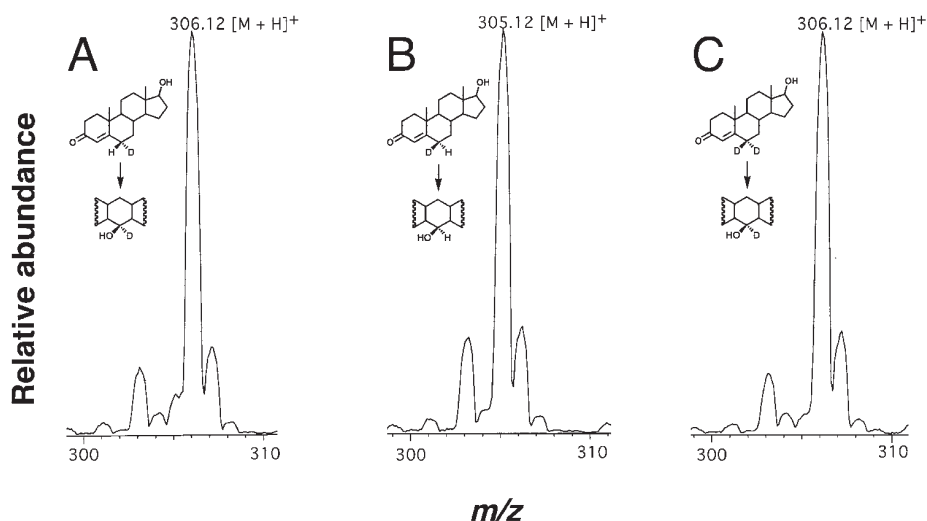
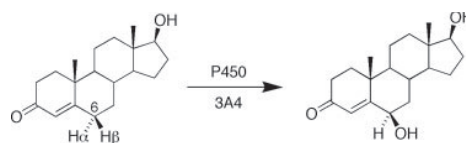


FIG. 1. **Stereoselectivity of hydrogen abstraction by P450 3A4.** The substrates used in each incubation (with a baculovirus-derived microsomal system) are shown along with the molecular ion region in the mass spectra of the product 6 β -hydroxytestosterone. The [M + H]⁺ ions indicate retention, loss, and retention of the substrate deuterium atom in parts A, B, and C, respectively.



SCHEME 5. **Stereoselective abstraction and rebound at the 6 β -hydrogen of testosterone.**

steps (21, 27, 28). An intramolecular non-competitive kinetic deuterium isotope effect (intrinsic) would, in principle, allow for the selection of breaking a C–H bond *versus* a C–D bond at the C-6 position. However, the H-6 hydrogens are non-equivalent (see above). Therefore, we used the method of Northrop (21, 27, 28) in which deuterium and tritium isotope effects are compared using the Swain-Scott relationship. k_H/k_T (formally defined as $^T(V/K)$) (27) was estimated at 3.5 ± 0.1 from the experimental values of $v_{T-H_2O} = 22.4 \pm 0.8 \text{ min}^{-1}$ and $v_{6\beta-OH} = 77.5 \pm 2.5 \text{ min}^{-1}$. The intrinsic isotope effect ^Dk was estimated using $k_H/k_D = 1.7 \pm 0.2$ ($= ^D(V/K)$ (27)), the result from the analogous deuterium experiment (see above). The parameter y in the expression shown in Equation 1,

$$y = \frac{(k_H/k_D) - 1}{(k_H/k_T) - 1} \quad (\text{Eq. 1})$$

is 0.28 ($1.7 - 1/3.5 - 1 = 0.7/2.5 = 0.28$), which yields an intrinsic isotope effect value of 15.4 using Northrop's tables (21).

Non-Competitive Enzyme Assays—The intermolecular non-competitive isotope effects were determined and can be used to determine to what extent a particular step in the P450 catalytic cycle is rate-limiting, or at least partially rate-limiting, with knowledge of the intrinsic isotope effect (see above). These isotope effects were experimentally assessed by determining the rates of hydroxylation of d_0 -testosterone (compound 1), 2,2,4,6,6- d_5 -testosterone (compound 2), and 6,6- d_2 -testosterone (compound 6) (Figs. 2 and 3).

Testosterone (compound 1) yielded four major hydroxylation products formed in the rank order (main product first) 6 β > 2 β > 15 β –1 β (Fig. 2 and Table I). This order remained the same with different P450 3A4 systems, and the kinetic isotope effects were similar (Table II).³

$^D V$ values for 6 β -hydroxytestosterone formation from two deuterated substrates (2 and 6) were 3.2 and 1.5, respectively (Table I). We initially postulated that the isotope effect from 6 β -hydroxylation for 2 and 6 might be the same; however, 2 β -hydroxytestosterone formation showed a $^D V$ of 6.7 with 2 as the substrate. Because an isotope effect for compound 2 is observed at the 6-position, the compound may “switch” to the 2-position, which exhibits a larger isotope effect, and then revert back to the 6-position (37). In addition, the switching explanation would also hold for the inverse isotope effects observed for the 1 β - and 15 β -hydroxytestosterone where an increase of these products relative to non-deuterated testosterone is observed.

Cooperativity was determined using the Hill equation ($v = k_{\text{cat}} \times S/(S + S_{50})$) or $\log_{10} [v/(k_{\text{cat}} - v)]$ versus $\log_{10} [S]$ with the slope being n_{app} for 6 β -hydroxytestosterone formation with all of the deuterated substrates (Table II and Supplementary Fig. 3S). Positive homotropic cooperativity was observed with all of the deuterated and non-deuterated testosterone substrates. A small but reproducible decrease in the n value was observed in comparing results from the deuterated with the non-deuterated substrates (Table II and also see Supplementary Data).

Deuterium Isotope Effects for P450 3A4-catalyzed 7-Benzoyloxyquinoline O-Debenzylation—Testosterone 6 β -hydroxylation is one of the fastest reactions catalyzed by P450 3A4 (4). The possibility was considered that the large attenuation of the intrinsic isotope effect might be an anomaly of that system. A slower reaction of P450 3A4, the O-debenzylation of 7-benzoyloxyquinoline, was also considered (Scheme 6).

The k_{cat} measured for the reaction with d_0 substrate was 3.2 min^{-1} with the *E. coli* bicistronic membrane system. Analysis of the benzaldehyde formed from the d_1 substrate yielded an apparent intrinsic ^Dk of 8.6 ± 0.4 ($n = 3$). This value has the caveat that the methylene carbon oxidized is prochiral, but the ^Dk is necessarily ≥ 8.6 .

Non-competitive intermolecular isotope experiments comparing the rates of the O-debenzylation of d_0 and d_2 substrates

the baculovirus-based microsomes. The baculovirus-derived system has a large excess of the reductase expressed, which apparently facilitates the higher rates. A simplistic explanation for the pattern is that reduction is more rate-limiting in the systems with lower rates of product formation, and therefore, the observed kinetic isotope effect is suppressed.

³ A comparison of the $^D V$ and $^D(V/K)$ values for d_2 -testosterone in Table II indicates a pattern for lower values in the systems other than

FIG. 2. HPLC/UV chromatogram of products derived from d_0 , $6,6-d_2$ - and $2,2,4,6,6-d_5$ -testosterone following incubation with P450 3A4. The identities of the products (obtained using a baculovirus-derived microsomal system) are shown with the indicated structures. The peak at t_R 16 min is the internal standard cortexolone.

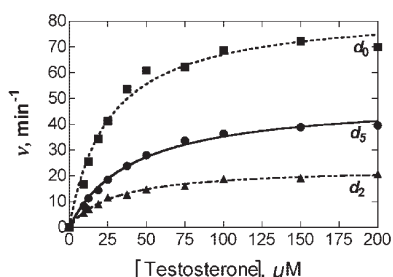
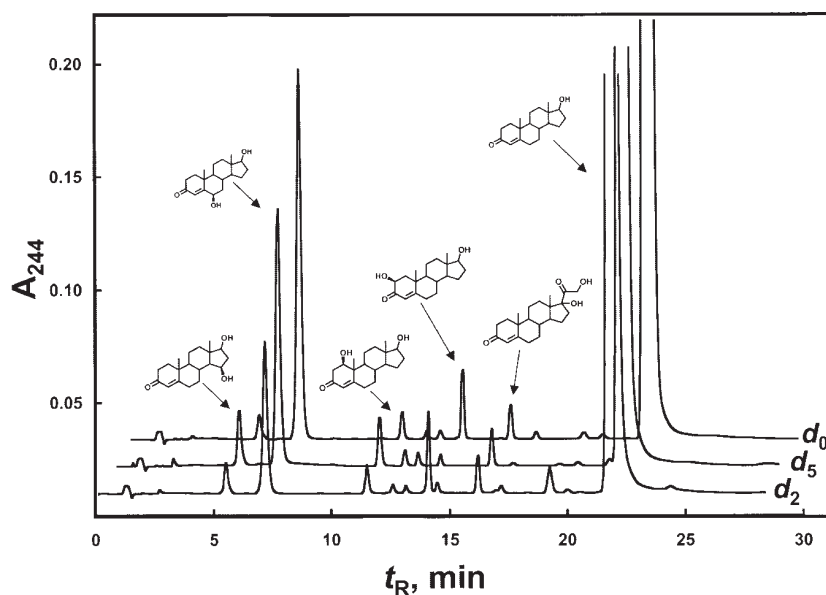


FIG. 3. Rates of d_0 , $6,6-d_2$ - and $2,2,4,6,6-d_5$ -testosterone 6β -hydroxylation by P450 3A4. A baculovirus-derived microsomal system was used here. See Table I for parameters.

yielded $^D V = 2.6 \pm 0.1$ and $^D(V/K) = 3.0 \pm 0.3$ (from the parameters $^H k_{cat} = 3.20 \pm 0.07 \text{ min}^{-1}$, $^H K_m = 14 \pm 1.1 \mu\text{M}$, $^D k_{cat} = 1.2 \pm 0.04 \text{ min}^{-1}$, and $^D K_m = 21.5 \pm 1.6 \mu\text{M}$). Although the intrinsic $^D k$ may be underestimated in these experiments, the results clearly show strong attenuation of the isotope effect in the noncompetitive intermolecular experiments.

DISCUSSION

P450 3A4 is one of the oxidases of greatest interest because of its practical significance, the inherent interest in its very broad catalytic selectivity, and the enzyme cooperativity (4), but relatively few experimental mechanistic studies have been reported. One problem has been the unavailability of appropriately labeled substrates for conducting kinetic isotope effect studies. Readily labeled substrates can be prepared for *N*-dealkylation reactions but have caveats in that C–H bond cleavage can involve deprotonation instead of homolytic cleavage (step 7 of Scheme 1) (47). Testosterone 6β -hydroxylation is a classic P450 3A4 reaction (4, 49, 88) and has one of the highest reported rates. Previously, syntheses of testosterone substituted with isotopes of hydrogen at the 6-position have been reported (30, 33, 86, 89) but all are either multiply substituted or partially labeled. We were able to circumvent labeling problems using a new strategy, which should be applicable to labeling other 3-keto- Δ^4 steroids with hydrogen isotopes at C-6. We established that both hydrogen abstraction and rebound are stereoselective, that cleavage of the 6β C–H bond is characterized by a high intrinsic primary kinetic isotope ef-

fect,⁴ and that the observed non-competitive deuterium kinetic isotope effect is low, indicative of a low contribution of C–H bond breaking as rate-limiting. Together the results provide a view of P450 3A4 different from that often presented in the literature about this enzyme, *i.e.* a protein with a large cavity in which substrates have considerable freedom to move and the oxidation reactions are dominated primarily by the inherent chemical reactivity at individual atoms (16, 17).

Two crystal structures of P450 3A4 have appeared recently (5, 6). However, only one of these has a substrate present, progesterone, and the molecule is not bound in a position in which it can be oxidized (5). However, the crystal structures have provided evidence that P450 3A4 has a large cavity available for substrate binding. As mentioned earlier, one view of the active site of P450 3A4 is that binding is relatively loose and the regioselectivity of oxidations is dominated by the ease of oxidation at individual atoms (16, 17). However, the results of the experiments with the relatively unselective metalloporphyrin biomimetic models argue against this view. Although the allylic C-6 atom might be expected to be more readily oxidized than the methyls and methylenes, oxidation was relatively non-selective (Supplementary Fig. 2S). Thus we conclude that structural features of substrates, or at least steroids, are important in docking with residues in P450 3A4 to influence the regioselectivity and stereoselectivity of oxidation.

Bacterial P450 101A1 has been reported to abstract either the 5-*endo*- or 5-*exo*- hydrogen of *d*-camphor (34). With P450 101A1, the rebound step occurred almost exclusively to the *exo* position. We found that only the 6β -hydrogen of testosterone was abstracted by P450 3A4 (Fig. 1). The rebound step is also very selective. Only traces of product eluting at the position of standard 6α -hydroxytestosterone (near 15β -hydroxytestosterone) were detected. Of interest is the report by White *et al.* (91) that another relatively "non-selective" mammalian P450, rabbit P450 2B4, abstracts the pro-*S* hydrogen of phenylethane with a 4:1 selectivity (and rebounds to either face).

The existence of a very high intrinsic kinetic deuterium isotope effect that is masked in other experimental settings is reminiscent of the work of Miwa *et al.* (92) with some rat liver

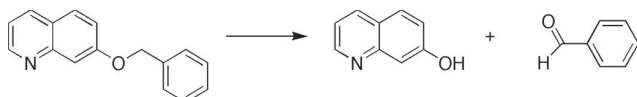
⁴ We have not examined potential secondary isotope effects, which tend to be small (90). In principle, a careful comparison of reactions with $6,6-d_2$ - and $6\beta-d_1$ -testosterone substrates might reveal a (geminal) secondary kinetic isotope effect.

TABLE I
 Kinetic parameters for testosterone hydroxylation products (baculovirus-based system)

Product and deuterated substrate	k_{cat}	K_m	k_{cat}/K_m	$^D V$	$^D(V/K)$
	min^{-1}	μM	$\text{min}^{-1}\mu\text{M}^{-1}$		
6 β -Hydroxy					
d_0 -Testosterone	78 ± 3	23 ± 2	3.4 ± 0.4		
d_2 -Testosterone	25 ± 1	20 ± 1	1.3 ± 0.1	3.2 ± 0.1	2.7 ± 0.1
d_5 -Testosterone	53 ± 2	35 ± 3	1.5 ± 0.2	1.5 ± 0.1	2.3 ± 0.2
2 β -Hydroxy					
d_0 -Testosterone	14 ± 1	44 ± 4	0.3 ± 0.1		
d_2 -Testosterone	12 ± 1	32 ± 3	0.4 ± 0.1	1.1 ± 0.1	0.8 ± 0.1
d_5 -Testosterone	2.0 ± 0.1	41 ± 4	0.1 ± 0.1	6.7 ± 0.3	6.1 ± 0.9
15 β -Hydroxy					
d_0 -Testosterone	7.1 ± 0.2	32 ± 3	0.2 ± 0.1		
d_2 -Testosterone	6.4 ± 0.2	13 ± 1	0.5 ± 0.1	1.1 ± 0.1	0.5 ± 0.1
d_5 -Testosterone	14.2 ± 0.5	24 ± 3	0.6 ± 0.1	0.5 ± 0.1	0.4 ± 0.1
1 β -Hydroxy					
d_0 -Testosterone	7.1 ± 0.3	17 ± 2	0.6 ± 0.1		
d_2 -Testosterone	6.9 ± 0.3	7.7 ± 1.2	0.9 ± 0.1	1.0 ± 0.1	0.5 ± 0.1
d_5 -Testosterone	13.6 ± 0.6	15 ± 2	0.9 ± 0.1	0.5 ± 0.1	0.4 ± 0.1

 TABLE II
 Kinetic parameters obtained for 6 β -hydroxylation of deuterated testosterone substrates with P450 3A4 systems

System and deuterated substrate	k_{cat}	K_m	k_{cat}/K_m	$^D V$	$^D(V/K)$	n
	min^{-1}	μM	$\text{min}^{-1}\mu\text{M}^{-1}$			
Bicistronic <i>E. coli</i> 3A4 membranes						
d_0 -Testosterone	15.3 ± 0.2	54 ± 1	0.29 ± 0.01			1.39 ± 0.04
d_2 -Testosterone	8.6 ± 0.3	64 ± 6	0.14 ± 0.01	1.8 ± 0.1	2.1 ± 0.2	1.22 ± 0.05
d_5 -Testosterone	12.1 ± 0.3	69 ± 4	0.18 ± 0.06	1.3 ± 0.0	1.6 ± 0.1	1.15 ± 0.07
Human liver microsomes						
d_0 -Testosterone	9.9 ± 0.4	145 ± 10	0.07 ± 0.01			1.42 ± 0.10
d_2 -Testosterone	6.9 ± 0.2	141 ± 10	0.05 ± 0.04	1.4 ± 0.1	1.4 ± 0.2	1.18 ± 0.07
Reconstituted 3A4 system						
d_0 -Testosterone	14.5 ± 0.3	65 ± 2	0.22 ± 0.01			2.16 ± 0.14
d_2 -Testosterone	10.7 ± 0.3	100 ± 6	0.11 ± 0.01	1.4 ± 0.1	2.1 ± 0.2	1.64 ± 0.11
Baculovirus insect cell microsomes						
d_0 -Testosterone	78 ± 3	23 ± 2	3.4 ± 0.4			1.36 ± 0.02
d_2 -Testosterone	25 ± 1	20 ± 1	1.3 ± 0.1	3.2 ± 0.1	2.7 ± 0.4	1.04 ± 0.04
d_5 -Testosterone	53 ± 2	35 ± 3	1.5 ± 0.2	1.5 ± 0.1	2.3 ± 0.1	1.06 ± 0.04



SCHEME 6. O-Debenzylation of 7-benzyloxyquinone.

P450s (P450s 1A1 and 2B1). Intrinsic deuterium isotope effects of 12.8–14.0 for 7-ethoxycoumarin *O*-deethylation were calculated using the Northrop approach (21, 27, 28) that we applied here. The observed non-competitive intermolecular deuterium isotope effects for rat P450 1A1 and P450 2B1 were 1.9 and 3.8, respectively (92). We have also reported high (and unattenuated) intrinsic isotope effects (of 14–15) for the *O*-demethylation of 4-nitroanisole by rabbit P450 1A2 (44). The high intrinsic isotope effects are evidence for a radical recombination pathway, as first proposed by Groves *et al.* (93), *i.e.* a stepwise pathway. These very high deuterium isotope effects, including those presented here for testosterone 6 β -hydroxylation, raise the possibility that the hydrogen abstraction process may involve quantum mechanical tunneling (94, 95). Tunneling is a distinct possibility, and one issue is whether the Swain-Scott relationship used in the application of the Northrop method (21, 27, 28) (Equation 1) is valid. However, the literature indicates that tunneling should result in only a relatively small perturbation of the semi-classical relationship between deuterium and tritium isotope effects (94, 96). We are interested in the question of whether tunneling is involved in these P450 3A4 reactions, but the goal of preparing substrates with the appropriate labels (very high deuterium substitution of the same atom containing tritium) (94) has not been achieved.

The application of kinetic isotope effect approaches to the

issue of P450 mechanisms is not new and extends back to at least 1967 (29–32). However, many of these studies employed a variety of experimental designs including competitive and non-competitive intramolecular and intermolecular isotope effects and the interpretation of many of these studies is not obvious. Northrop (27) raised the issue of the difficulty in interpreting isotope effect results in the absence of knowledge about the intrinsic isotope effect. In our work on the P450 3A4-catalyzed 6 β -hydroxylation, we demonstrated a large intrinsic isotope effect (of 15) and considerable attenuation in the non-competitive intermolecular experiments (values of 2–3) (Tables and II). This strong attenuation of the isotope effect indicates that other steps in the catalytic cycle make major rate-limiting contributions. We also found low isotope effects ($^D(V/K)$) for testosterone 6 β -hydroxylation in competitive intermolecular experiments (1.7).

The issue can be raised that testosterone 6 β -hydroxylation is a relatively rapid reaction for P450 3A4 and that the C–H bond-breaking step may be more rate-limiting in slower reactions. We have not established an intrinsic deuterium isotope effect for the slower testosterone 2 β -hydroxylation (labeling this site may be unrealistic because of the potential for solvent exchange). Comparison of the rates of 2 β -hydroxylation of 6,6- d_2 - and 2,2,4,6,6- d_5 -testosterone yields values of $^D V = 6.7$ and $^D(V/K) = 6.1$. If the intrinsic isotope effect for 2 β -hydroxylation is the same as for 6 β -hydroxylation (*i.e.* 15), the isotope effect is still attenuated and implies that C–H bond breaking is not fully rate-limiting but further discernment may not be possible for testosterone 2 β -hydroxylation. The literature contains at least one example of a low isotope effect for a slower P450 3A4 reaction ($k_{\text{cat}} = 5.8 \text{ min}^{-1}$) (97); benzylic hydroxylation of the

drug ezlopitant by P450 3A4 yielded $^D V \sim ^D(V/K) = 1.25$ (46). We examined a model P450 3A4 reaction, the *O*-debenzylation of 7-benzyloxyquinoline (98), with a k_{cat} of 3.2 min^{-1} under our conditions. The isotope effect for the *O*-debenzylation of the d_1 substrate was 8.6. The use of this value as an estimate of $^D k$ has caveats regarding the prochirality of the benzylic methylene, and this issue must be considered. However, the non-competitive intermolecular parameters we estimated were $^D V = 2.6$ and $^D(V/K) = 3.0$. Thus the attenuated isotope effect for testosterone 6 β -hydroxylation does not appear to be an anomaly and is seen for other P450 3A4 reactions, both fast and slow. The point should also be made that similar testosterone 6 β -hydroxylation isotope effects were observed for various P450 3A4 systems regardless of the reaction rate in the system, which is a function of the concentration of NADPH-P450 reductase, lipid environment, and so on (Table II).

Evidence has been presented that C–H bond breaking (step 7 of Scheme 1) is not rate-limiting. If step 7 is not particularly rate-limiting, what is? Preliminary measurements indicate that the rate of testosterone binding (step 1 of Scheme 1) is not limiting.⁵ Independent evidence against slow substrate and product release can also be obtained from the lack of attenuation of the intermolecular non-competitive $^D(V/K)$ in a competitive experiment (see above). We have already provided evidence that step 2 of Scheme 1 is not rate-limiting, at least with testosterone as a substrate (18). Other unpublished studies in this laboratory indicate that step 3 (O_2 binding) is rapid (and that the FeO_2^{2+} complex is rather unstable). Thus the remaining possibilities are steps 4, 5, and 6 or variations thereof. Another point to make is that rates of abortive processes (e.g. steps A, B, and C of Scheme 1) can influence overall steady-state kinetic parameters, as we have demonstrated in simulations of rabbit P450 1A2 reactions (44). Another issue is why, if steps 4, 5, or 6 are the rate-limiting steps, all of the P450 3A4 reactions do not occur at the same rate. One possible explanation is that the substrate influences these rates, in that the rate of reduction of ferric P450 3A4 is influenced by the substrate (18). However, we do not have other specific evidence to address this hypothesis.

Another observation in our work is the apparent sigmoidicity of the plots of v versus S (e.g. Fig. 2 and Supplementary Fig. 3S). We have reported such behavior previously and established that the phenomenon was not the result of artifacts such as depletion of substrate within individual reactions (7, 12). Others have also reported such homotropic cooperativity and presented a variety of models as explanations (9, 11–15). The cooperativity can be observed in analysis of substrate binding using UV-visible spectroscopy (11, 12). One of the more popular current explanations for cooperativity is the compression of two (or more?) substrates into the active site to reduce the freedom of motion of the substrate (9, 11–15, 99, 100). In such models, the apparent cooperativity is the result of the overlap of parameters for the binding of multiple substrates (9, 14, 101) including the n value (the slope) from a fit to a Hill plot ($v = k_{\text{cat}} \times S_{50}/(S_{50} + [S])$; $\log_{10} [v/k_{\text{cat}} - v]$ versus $\log_{10} [S]$). We observed a reproducible decrease in the n value for P450 3A4-catalyzed testosterone 6 β -hydroxylation because of deuterium substitution in all of the P450 3A4 systems we used (Table II and also see Supplementary Fig. 3S). The decrease is reminiscent of the decrease in n we observed upon the addition of ligands that induce heterotropic cooperativity (7, 12).⁶ We do not have an explanation for this phenomenon, but we do interpret the result as an indication that homotropic cooperativity is more

complex than can be explained in a thermodynamic static model of P450 3A4-ligand interaction. Possible explanations may include the separate roles of substrate in promoting heme reduction and oxygen activation through induction of conformational changes, which might involve interaction distinct from those directly related to substrate hydroxylation. Further discussion is speculative at this point, but the consistency of these and other results does imply that changing the rates of oxidation for a substrate influences the cooperativity (Table II).⁷

In conclusion, we have developed definitive syntheses of Δ^4 -3-keto steroids labeled with hydrogen isotopes at the 6-position and used these reagents to evaluate kinetic hydrogen isotope effects for testosterone 6 β -hydroxylation, a classic reaction of P450 3A4. We found stereoselective hydrogen abstraction and rebound at the β face. Together the deuterium and tritium results indicate a very high intrinsic kinetic isotope effect for testosterone 6 β -hydroxylation, which is very consistent with a mechanism involving hydrogen atom abstraction by a P450 ferryl oxygen species (FeO^{3+}), whether this is high- or low-spin (102). The strong attenuation of this isotope effect in the non-competitive intermolecular experiments (Tables I, II) indicates that the C–H bond-breaking step is not very rate-limiting for the reaction. Similar results were observed with a slower reaction catalyzed by P450 3A4, the *O*-debenzylation of 7-benzyloxyquinoline, i.e. low values of $^D V$ and $^D(V/K)$ even though the apparent intrinsic isotope effect was high. These results may have additional support in published work with P450 3A4 and the drug ezlopitant (46). Overall, our work presents a view of P450 3A4 that differs from many of the current conceptions. The C–H bond-breaking step appears to be less rate-limiting in P450 3A4 reactions than with several reactions catalyzed by other mammalian P450s, e.g. P450s 1A2 (42, 44), 2A6 (45), 2E1 (41, 103), and 2D6 (43). These results, coupled with the very selective stereochemistry of hydrogen abstraction (Fig. 1), yield a paradigm of P450 3A4 that has a very large active site (5, 6) but considerable features of P450-substrate interaction. Overall rates of catalysis are influenced by late steps in P450 3A4 oxygen activation, which is probably influenced by substrate interactions, and not only by the chemical lability of substrates.

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⁷ We have fit the results to the Hill plot in an empirical manner. This is not necessarily the most mechanistic description; however, alternative methods include description of the behavior with models using separate dissociation constants and other factors (9, 12, 14, 101). Fits of our data to such models would also yield different parameters.

⁵ E. Isin and F. P. Guengerich, unpublished results.

⁶ Graphs of linear transformation of the results are included in Supplementary Data.

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