BIOORGANIC CHEMISTRY 19, 245–260 (1991)

3-Phosphinic Acid and 3-Phosphonic Acid Steroids as Inhibitors of Steroid 5α-Reductase: Species Comparison and Mechanistic Studies

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Received December 20, 1990

3-Phosphinic acid and 3-phosphonic acid steroids are presented as a new class of steroid 5α-reductase (SR) inhibitors. Representative compounds have been studied as inhibitors of prostatic SR from human, rat, and cynomolgus monkey (*Macaca fascicularis*). The most potent of the phosphinic acid inhibitors of the human enzyme activity demonstrated nanomolar inhibition constants, while the affinities of the phosphonic acids were diminished. Comparison of compound potency revealed interspecies variability with the best correlation on K_i/K_m for the two primate SRs. Results from dead-end and multiple inhibition analyses with 17β-(*N*,*N*-diisopropylcarbamoyl)androsta-3,5-diene-3-phosphinic acid (**2a**) and 17β-(*N*,*N*-diisopropylcarbamoyl)androsta-3-5-diene-3-phosphonic acid (**3a**) were consistent with the preferential binding of these compounds to an enzyme–$NADP^+$ complex. The pH profiles of solubilized rat liver SR inhibition by **2a** and **3a** indicated preferential binding of the phosphinic and phosphonic acids as the anion and the monoanion, respectively.

Inhibition of steroid 5α-reductase (SR; EC 1.3.1.30) has been proposed as a means of attenuating 5α-dihydrotestosterone (DHT)-mediated pharmacological disorders such as benign prostatic hypertrophy (BPH) (*1*, *2*). Toward this objective, several classes of compounds have been identified as SR inhibitors. The 6-methylene progesterone and testosterone analogues (*3*, *4*) and 4-diazo steroids (*5*, *6*) are time-dependent, presumably mechanism-based, inhibitors of SR activity. Reversible inhibition has been demonstrated with differing compound classes including the 3-oxo-4-aza steroids (*7*, *8*) and steroidal 3-carboxylic acids (*9–12*). Of these latter classes the 3-oxo-4-aza steroids, exemplified by MK-906 (finasteride) which is currently undergoing clinical trials for BPH (*13*, *14*), are competitive versus the steroid substrate testosterone (T) (*15*) while the steroidal acrylates **1** and A-ring aromatic 3-carboxylic acids **4** demonstrate uncompetitive kinetic patterns versus both substrates, T and NADPH (*9–11*, *16*, *17*).

Kinetic analysis of liver and prostatic SRs are consistent with the ordered binding of substrates and release of products from the enzyme surface (*18–20*) where the nicotinamide dinucleotide cofactor is involved in both the frontside

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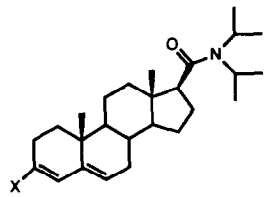

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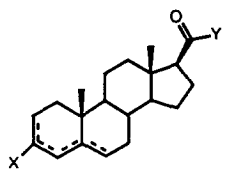
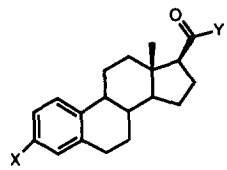
1a = - CO_2H
2a = - PHO_2H
3a = - PO_3H_2



X

1 = - CO_2H
2 = - PHO_2H
3 = - PO_3H_2

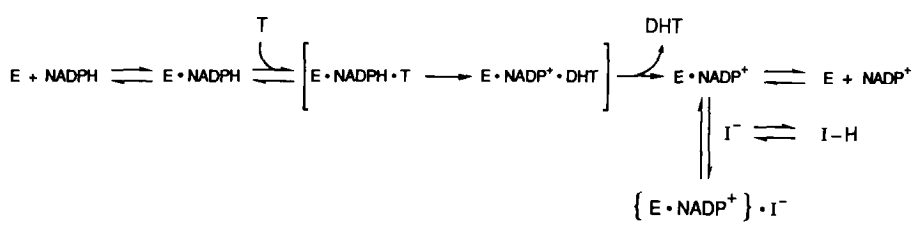


X

4 = - CO_2H
5 = - PHO_2H
6 = - PO_3H_2

(enzyme–NADPH) and backside (enzyme–$NADP^+$) binary complexes (Scheme I). The inhibition patterns with **1** and **4**, supplemented and supported by the results from double-inhibition and filtration binding experiments, have been interpreted to result from the preferential association of the steroidal 3-carboxylic acids to the SR–$NADP^+$ binary complex (*9–11, 16, 17*). Studies of the pH dependence on the SR inhibition by **1a** supports the proposal that favorable electrostatic interaction between the enzyme–$NADP^+$ complex and the deprotonated steroid 3-carboxylic acids contributes to the potent inhibition and the proposed mechanism of enzyme–inhibitor interaction (*16*).

Extrapolation of our strategy employing anionic olefinic and aromatic steroids as stable analogues of the proposed enol(ate) intermediate in the SR-catalyzed T to DHT conversion has involved the preparation and evaluation of steroidal unsaturated 3-phosphinic acids (**2** and **5**) and 3-phosphonic acids (**3** and **6**) as potential inhibitors of SR in which the carboxyl group of compounds **1** and **4** is



SCHEME I. Inhibition of steroid 5α-reductase by steroidal 3-phosphinic, 3-phosphonic, and 3-carboxylic acids. E, enzyme; T, testosterone; DHT, 5α-dihydrotesteroine; I-H, inhibitor, I^-, anionic inhibitor.

replaced by phosphorous acid moieties. Here, we report that these steroidal 3-phosphinic and 3-phosphonic acids are new classes of potent inhibitors of prostatic SRs from several species. Rat and human prostatic SRs have been used for routine evaluation. To determine whether a non-human primate might provide an alternative biochemical and pharmacological model to the canine (*21*) or rodent (*22*), selected compounds also were evaluated with prostatic SR from the cynomolgus monkey (*Macaca fascicularis*). The proposed kinetic mechanism of SR inhibition through ternary complex formation of steroidal phosphinic and steroidal phosphonic acids with enzyme and $NADP^+$ is supported by dead-end and multiple-inhibition studies, while the ionization states of enzyme-bound inhibitors **2a** and **3a** have been elucidated through pH profiles of SR inhibition.

EXPERIMENTAL PROCEDURES

General Methods.

Melting points are uncorrected. ^1H NMR spectra were obtained in $CDCl_3$ solutions (unless otherwise noted) with Bruker AM-250 or Varian EM390 spectrometers and are reported (in part) as ppm downfield from Me_4Si with multiplicity, number of protons, and coupling constants (Hz) indicated parenthetically. Chromatography refers to flash chromatography using Kieselgel 60, 230–400 mesh silica gel. Trifluoromethyl sulfonate and 3-one 4-ene steroidal starting materials (*11*, *12*) and hypophosphorous acid (H_3PO_2, 95%) (*23*) were prepared as described.

[4 – ^{14}C]Testosterone (55 to 57 mCi/mmol) was purchased from Amersham Corp. and New England Nuclear (NEN). Econosolve II and Aquasol 2 were obtained from NEN. Other chemicals and enzymes used in the enzymatic studies were purchased from Sigma Chemical Co. or Aldrich Chemicals. Radioactivity was determined with either a Beckman LS-5801 scintillation counter calibrated to disintegrations per min (dpm) with Beckman standards or with a System 2000 Bioscan imaging scanner (Bioscan, Washington D.C.). Analysis of enzyme reactions were performed on prechanneled silica TLC plates containing a preabsorbing region (Si250F-PA, Baker).

Compound Syntheses

Dimethyl 17β-(N,N-diisopropylcarbamoyl)androsta-3,5-diene-3-phosphonate (*7*). To a solution of *N,N*-diisopropyl-3-[[(trifluoromethyl)sulfonyl]oxy]androsta-3,5-diene-17β-carboxamide (*12*) (2.39 g, 4.5 mmol) in 50 ml DMF was added triethylamine (2.6 ml, 18 mmol) and dimethyl phosphite (0.5 ml, 5 mmol). The solution was flushed with argon and tetrakis(triphenylphosphine)palladium(0) (292 mg, 0.22 mmol) was added. The reaction mixture was stirred under argon for 1 h then poured into water. The product was extracted with dichloromethane and washed with dilute HCl, aqueous sodium bicarbonate, and brine, dried over sodium sulfate, and concentrated. Chromatography (silica, 50% ethyl acetate–hexanes) afforded **7** as a white solid (1.48 g, 67%): NMR 0.78 (s, 3H), 0.93 (s, 3H), 1.13 (d, 3H, J = 8.3 Hz), 1.24 (d, 3H, J = 8.3 Hz), 1.40 (d, 3H, J = 8.3 Hz), 1.43 (d, 3H,

J = 8.3 Hz), 3.42 (m, 1H), 3.72 (d, 6H, J = 12.5 Hz), 4.24 (m, 1H), 5.84 (s, 1H), 6.80 (d, 1H, J = 23 Hz).

The following compounds were prepared by analogous procedures from the corresponding triflates: dimethyl 17β-(*N,N*-diisopropylcarbamoyl)-5α-androsta-2,4-diene-3-phosphonate, dimethyl 17β-(*N-tert*-butylcarbamoyl)androsta-3,5-diene-3-phosphonate, and methyl 17β-(*N,N*-diisopropylcarbamoyl)androsta-3,5-diene-3-methylphosphinate.

17β-(N,N,-Diisopropylcarbamoyl)androsta-3,5-diene-3-phosphonic acid (**3a**). A solution of dimethyl phosphonate **7** (245 mg, 0.5 mmol) in acetonitrile (5 ml) was purged with argon then treated with NaI (150 mg, 1 mmol) and trimethylsilyl chloride (0.13 ml, 1 mmol) for 24 h at ambient temperature. The reaction mixture was then partitioned between chloroform and water. The organic layer was washed with water, dilute HCl, brine, and aqueous sodium sulfite. Concentration afforded 232 mg of a white solid that was purified by preparative reverse-phase HPLC (Zorbax ODS, 70 : 30 MeOH : 100 mM KH_2PO_4, pH 6.5 buffer) to provide **3a** as a white crystalline solid: mp 240–243°C; NMR 0.78 (s, 3H), 0.96 (d, 3H, J = 8.3 Hz), 1.27 (d, 3H, J = 8.3 Hz), 1.37 (d, 3H, J = 8.3 Hz), 1.40 (d, 3H, J = 8.3 Hz), 3.46 (m, 1H), 4.30 (m, 1H), 5.70 (s, 1H), 6.70 (d, 1H, J = 23 Hz). Anal. ($C_{26}H_{42}PO_4N$) C,H,N.

17β-(N,N-Diisopropylcarbamoyl)androsta-2,4-diene-3-phosphonic acid (**3c**). Compound **3c** was prepared in a fashion analogous to that described for **3a** above. Compound **3c** (isolated as the monopotassium salt): mp >250°C (d). Anal. ($C_{26}H_{41}NO_4PK \cdot \frac{1}{2}H_2O$) C,H,N.

17β-(N-tert-Butylcarbamoyl)androsta-3,5-diene-3-phosphonic acid (**3b**). Compound **3b** was prepared in a similar fashion to compound **3a** except that trimethylsilyl bromide was employed in place of the *in situ* generated trimethylsilyl iodide (NaI/TMSCl). Compound **3b**: mp 241–243°C. Anal. ($C_{24}H_{38}PO_4N \cdot \frac{1}{2}H_2O$) C,H,N.

17β-(N,N-Diisopropylcarbamoyl)androsta-3,5-diene-3-methylphosphinic acid (**2g**). Compound **2g** was prepared from the methyl phosphinic ester according to the procedure described for compound **3a** and isolated as the potassium salt. Anal. ($C_{27}H_{43}NO_3PK$) C,H,N.

Monomethyl 17β-(N,N-diisopropylcarbamoyl)androsta-3,5-diene-3-phosphonic acid (**3d**). To a solution of the dimethyl phosphonate **7** (50 mg, 0.10 mmol) in 10 : 1 methanol : water (10 ml) was added solid potassium carbonate (10 mg) and the resulting mixture was rapidly stirred and heated at reflux for 17 h. The volatiles were removed *in vacuo* and the residue was dissolved in water and acidified with dilute HCl. The aqueous mixture was repeatedly washed with chloroform and the combined organic extracts were dried and evaporated to dryness. Recrystallization from methanol–water provided **3d** as a white solid: mp 212–215°C. Anal. $C_{27}H_{44}NO_4P \cdot \frac{1}{4}H_2O$) C,H,N.

17β-(N,N-Diisopropylcarbamoyl)androsta-3,5-diene-3-phosphinic acid (**2a**). To a solution of *N,N*-diisopropyl-3-oxo-4-androstene-17β-carboxamide (*12*) (1.0 g, 2.5 mmol) in THF (25 ml) was added 95% H_3PO_2 (*23*) (1 g). The reaction mixture was stirred at 65°C for 48 h. The volatiles were then removed *in vacuo* and the residue was dissolved in chloroform and washed with water until the aqueous wash was neutral pH. Concentration of the chloroform solution followed by recystalliza-

tion of the residue from methanol–water afforded **2a** as a white solid (1.07 g, 96%): mp 134–140°C. NMR 0.7 (s, 3H), 0.9 (s, 3H), 1.13 (d, 3H, J = 8.3 Hz), 1.25 (d, 3H, J = 8.3 Hz), 1.38 (d, 3H, J = 8.3 Hz), 1.41 (d, 3H, J = 8.3 Hz), 3.40 (m, 1H), 4.20 (m, 1H), 5.78 (s, 1H), 6.7(d, 1H, J = 25 Hz), 7.08 (d, 1H, J = 594 Hz). Anal. ($C_{26}H_{42}NO_3P$) C,H,N.

17β-(N-tert-Butylcarbamoyl)androsta-3,5-diene-3-phosphinic acid (***2b***). The title compound was prepared analogously to compound **2a**: Mp >235°C. Anal. ($C_{24}H_{38}NO_3P$) C,H,N.

17β-(N,N-Diisopropylcarbamoyl)-5α-androst-2-ene-3-phosphinic acid (***2d***). To a solution of *N,N*-diisopropyl-3-[[trifluoromethyl)sulfonyl]oxy]-5α-androst-2-ene-17β-carboxamide (200 mg, 0.375 mmol) in DMF (40 ml) was added triethylamine (0.24 ml), tetrakis(triphenylphosphine)palladium(0) (50 mg), and 95% H_3PO_2 (300 mg). The reaction mixture was stirred under argon for 3 h at ambient temperature and then poured into water. The product was extracted into dichloromethane, washed with water, dilute HCl, aqueous sodium bicarbonate, and brine, dried, and concentrated. Chromatography (silica, 80 : 20 : 2 $CHCl_3$: MeOH : H_2O) afforded **2d** as a white solid which was recrystallized from acetonitrile: mp 220–225°C. Anal.($C_{26}H_{44}NO_3P$) C,H,N.

The following compounds were prepared in an analogous fashion from the corresponding triflates: 17β-(*N,N*-diisopropylcarbamoyl)-5α-androst-3-ene-3-phosphinic acid (**2c**), mp 228–232°C, Anal. ($C_{26}H_{44}NO_3P \cdot \frac{1}{2}H_2O$) C,H,N,; 17β-(*N,N*-diisopropylcarbamoyl)androsta-2,4-diene-3-phosphinic acid (**2e**), mp 132°C, Anal. ($C_{26}H_{42}NO_3P \cdot 1.25H_2O$) C,H,N; and 17β-(*N-tert*-butylcarbamoyl)-5α-androst-2-ene-3-phosphinic acid (**2f**), mp 232–235°C, Anal. ($C_{24}H_{40}NO_3P \cdot \frac{1}{2}H_2O$) C,H,N.

Dimethyl 17β-(N,N-diisopropylcarbamoyl)esta-1,3,5(10)-triene-3-phosphonate (***8***). A mixture of *N,N*-diisopropyl-3-[[(trifluoromethyl)sulfonyl]oxy]esta-1,3,5(10)-triene-17β-carboxamide (*11*) (2 g, 3.89 mmol), dimethyl phosphite (700 mg), *N*-methylmorpholine (560 mg), and tetrakis(triphenylphosphine)palladium(0) (150 mg) in acetonitrile (25 ml) was heated at 70°C overnight. The volatiles were then removed *in vacuo*. The residue was dissolved in ethyl acetate, washed with dilute HCl, aqueous sodium bicarbonate, and brine, dried, and concentrated. The residue was chromatographed (silica, 50% ethyl acetate–hexanes) to yield **8** as an off-white solid (1.4 g, 78%). NMR 0.80 (s, 3H), 1.16 (d, 3H, J = 6.6 Hz), 1.26 (d, 3H, J = 6.6 Hz), 1.40 (d, 3H, J = 6.6 Hz), 1.43 (d, 3H, J = 6.6 Hz), 2.35 (m, 3H), 2.7 (m, 1H), 2.9 (m 1H), 3.43 (m, 1H), 3.75 (d, 6H, J = 11 Hz), 4.25 (m, 1H), 7.3–7.7 (m, 3H).

Dimethyl 17β-(*N-tert*-butylcarbamoyl)esta-1,3,5(10)-triene-3-phosphonate was prepared in an analogous fashion.

17β-(N,N-Diisopropylcarbamoyl)esta-1,3,5(10)-triene-3-phosphonic acid (***6a***). Dimethyl phosphonate **8** (100 mg, 0.24 mmol) was dissolved in a mixture of 2 ml acetic acid and 2 ml 48% aqueous HBr and heated at 80°C for 3 days. The volatiles were then removed *in vacuo*. The residue was dissolved in methanol, eluted through a bed of activated carbon, and then slowly diluted with water to provide **6a** as a white precipitate which was collected and dried: mp 215–218°C. NMR ($CDCl_3$–CD_3OD) 0.79 (s, 3H), 1.19 (d, 3H, J = 6.6 Hz), 1.30 (d, 3H, J = 6.6 Hz), 1.40 (d, 3H, J = 6.6 Hz), 1.42 (d, 3H, J = 6.6 Hz), 2.35 (m, 3H), 2.7 (m, 1H), 2.9

(m 1H), 3.49 (m, 1H), 4.35 (m, 1H), 7.3–7.6 (m, 3H). Anal. ($C_{25}H_{38}NO_4P \cdot \frac{1}{2}H_2O$) C,H,N.

17β-(N-tert-Butylcarbamoyl)estra-1,3,5(10)-triene-3-phosphonic acid (**6b**). The dimethyl phosphonate of **6b** was treated with TMSBr as described for the preparation of **6a** to provide **6b** as a white solid: mp 215–217°C. Anal. ($C_{23}H_{34}NO_4P \cdot \frac{3}{4}H_2O$) C,H,N.

17β-(N,N-Diisopropylcarbamoyl)estra-1,3,5(10)-triene-3-phosphinic acid (**5a**). A mixture of *N,N*-diisopropyl-3-[[trifluoromethyl)sulfonyl]oxy]estra-1,3,5(10)-triene-17β-carboxamine (150 mg, 0.285 mmol), triethylamine (84 μl, 0.63 mmol), DMF (3 ml), tetrakis(triphenylphosphine)palladium(0) (13 mg, 0.009 mmol), 1,3-bis(diphenylphosphino)propane (3.7 mg, 0.009 mmol) and 95% H_3PO_2 (42 mg) was heated at 90°C under argon for 6 h. The reaction mixture was cooled, diluted with ice–water, and extracted with dichloromethane. The organic extract was washed with dilute HCl, aqueous sodium bicarbonate, and brine, dried, and concentrated. The residual dark oil was chromatographed (C18 reverse-phase silica, 70:30 MeOH:20 mM, pH 6.6, phosphate buffer) to afford after acidification (pH 3) of the concentrated eluent, a beige solid. Trituration with acetonitrile afforded **5a** as an off-white solid: mp 230–234°C. NMR 0.8 (s, 3H), 1.2 (d, 3H, J = 8.2 Hz), 1.3 (d, 3H, J = 8.2 Hz), 1.4 (dd, 6H, J = 8.2, 8.2 Hz), 3.5 (m, 1H), 4.36 (m, 1H), 7.5 (d, 1H, J = 613 Hz), 7.5 (br m, 3H). Anal. ($C_{25}H_{38}NO_3P$) C,H,N.

17β-(*N-tert*-Butylcarbamoyl)estra-1,3,5(10)-triene-3-phosphinic acid (**5b**) was prepared in an analogous fashion: mp 172–176°C. Anal. ($C_{23}H_{34}NO_3P \cdot \frac{1}{2}H_2O$) C,H,N.

Enzymology

SR preparations. Microsomes from human, dog, and rat prostatic tissues containing SR activities were prepared as previously described (*15, 16*). Microsomes from prostates of cynomolgus monkeys (*M. fascicularis*) were prepared as for the human tissues. The protocol for solubilization of SR from rat liver has been detailed (*20*). Typically, 0.8 to 2μg protein of the solubilized liver preparation was used in the standard activity assay as described below.

Enzymatic assay for SR activity. SR activity was determined by following the conversion of T to 5α-reduced steroids, represented by the sum of DHT and androstanediol (ADIOL). [^{14}C]T in ethanol was deposited in test tubes and concentrated to dryness in a SAVANT Speed-Vac evaporator. Buffer and NADPH were added and each tube was equilibrated to assay temperature. Except as noted for individual experiments, a cofactor regenerating system ($NADP^+ \rightarrow NADPH$) consisting of 1 mM glucose 6-phosphate and 0.5 units/ml glucose-6-phosphate dehydrogenase was included in each assay. The reaction was initiated by addition of an aliquot of enzyme preparation to a final volume of 0.5 ml. All assays with the human and non-human primate (cynomolgus) enzymes were carried out in 50 mM sodium citrate, pH 5.0; determinations of liver SR activities were in 20 mM sodium phosphate buffer, pH 7.5. Assays with solubilized liver SR were conducted at 30°C while those with microsomal associated enzymes were incubated at 37°C. The reaction was stopped by addition of 4 ml ethyl acetate containing 0.2 μmol of T, DHT, ADIOL, and androstenedione each as carriers and markers. Isolated

steroids were separated by TLC, developing twice with chloroform: acetone (9 : 1) and the relative content of radiolabel in the substrate (T) and the products (DHT plus ADIOL) was determined for each lane using a BIOSCAN imaging scanner (*16*). Typically, the specific activities of SR in microsomes from dog and monkey prostates at 1.0 and 1.2 μM T, respectively, and 400 μM NADPH were 83–160 pmol/(h · mg) and 0.7–1.4 nmol/(h · mg), respectively. With 1.0 μM, T and 200 μM NADPH at 30°C and pH 6.5 the specific activity of the solubilized liver SR was estimated to be 3.6 nmol · (min · mg)$^{-1}$. The apparent K_m values for T at 400 μM NADPH with human, cynomolgus, rat, and dog prostatic SRs were 4.5 μM (pH 5.0), 0.5 μM (pH 5.0), 1.2 μM (pH 6.6) and 2.8 μM (pH 6.6), respectively. The pH of the assays for enzyme activity from different species corresponded to that of maximal enzyme velocity.

Inhibition studies. Potential steroidal inhibitors in ethanol were added to the assay tubes with substrate (T) and the contents were evaporated to dryness. All other procedures were the same as outlined for the SR activity assay. Inhibition constants ($K_{i,app}$) were estimated by Dixon analysis (*24*) with initial substrate concentrations of 1.0 μM T and 200 to 400 μM NADPH. With the steroidal inhibitors, the cofactor regenerating system was included to eliminate inhibition by $NADP^+$; no regenerating system was added in the $NADP^+$ product inhibition studies.

When used in the double-inhibition experiments, $NADP^+$ was added with the incubation buffer. The concentrations of substrates in the double inhibition studies were held constant with [T] = 1.0 μM and [NADPH] = 25μM. All other procedures for enzyme inhibition were the same as previously outlined (*16, 20*). Data analyses were performed as described below.

Dependence of SR inhibition by ***2a*** *and* ***3a*** *on pH.* Enzyme activities were determined in constant ionic strength (μ = 0.02 M) buffer solutions consisting of succinic acid, imidazole, and diethanolamine (*25*). Effects of pH upon the potency of SR inhibition by **2a** and **3a** were determined at constant concentration (T (1 μM) and NADPH (20 μM) in the presence of the cofactor regenerating system. Apparent inhibition constants were determined (*24*) at each pH; further data analysis was performed as described below.

Data analysis. Dead-end inhibition data were fit to appropriate rate equations with the COMP, NONCOMP, and UNCOMP programs described by Cleland (*26*). Linear data evaluations were fit to the equation $y = mx + b$ using the LINE program. Data from the pH dependence of inhibition by the steroidal phosphinic acid **2a** was evaluated, with the HBBELL program, by Eq. [1]

$$\log(1/K_{i,app}) = \log [C/(1 + K/H)], \qquad [1]$$

where K represents the group whose ionization causes a decrease in inhibition potency, H is the hydrogen ion concentration, and C is the pH independent value for $1/K_{i,app}$. The pH dependence of SR inhibition by steroidal phosphonic acid **3a** was evaluated by Eq. [2] which describes a parameter that decreases at high (or low) pH, but levels to a new value:

$$\log(1/K_{i,app}) = \log\{(YL + YH(K/H))/(1 + K/H)\}, \qquad [2]$$

in which YL and YH are the values of $1/K_{i,app}$ at low and high pH, respectively, Double inhibition experiments at constant substrate concentrations were analyzed with Eq. 3 (*27, 28*)

$$v_i = v_o/[1 + I/K_I + J/K_J + IJ(\beta K_I K_J)]. \quad [3]$$

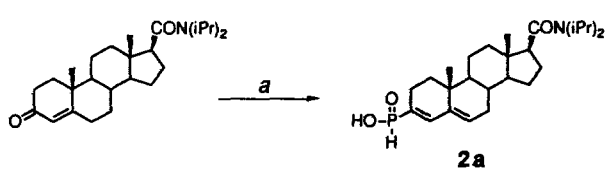
For this equation, I and J are the concentrations of the two variable inhibitors, v_o and v_i are the velocities in the absence and in the presence of compounds I (**2a**) and J ($NADP^+$) whose apparent dissociation constants are K_I and K_J, respectively, and β is an experimentally derived term that represents the degree of binding cooperativity between the two inhibitors. When the value of β is less than unity ($\beta < 1$), association of the two inhibitors to enzyme is considered to be synergistic. Curve fitting of experimental data to Eqs. [2] and [3] was accomplished with a nonlinear regression procedure using the Marquardt algorithm (*29*) in the SAS statistical analysis software package (SAS Institute, Inc., Cary NC). In the figures, displayed points are experimental values and the curves are computer calculated best fits. The statistical criteria suggested by Cleland (*26*) were employed in determining the best fit of model to the experimental data.

RESULTS AND DISCUSSION

Preparation of 3-Phosphinic (2 and 5) and 3-Phosphonic Acid Steroids (3 and 6)

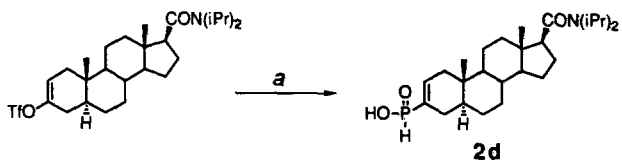
Preparation of the 3,5-dienyl-3-phosphinic acids **2a** and **2b** was accomplished in a single step from the previously described androst-4-en-3-ones following the protocol of Zeches (*30*). In this procedure, carbon–phosphorus bond formation is effected via a nucleophilic 1,2-addition of hypophosphorous acid to the A-ring enone. The resulting 4-ene-3-hydroxy-3-phosphinic acids dehydrate under the reaction conditions to yield the dienyl phosphinates directly (Scheme II).

Several attempts to apply the Zeches procedure to the preparation of monoene phosphinic acids or to the preparation of phosphonic acid derivatives were unsuccessful. Access to these analogues was achieved through the development of a new methodology for the synthesis of α,β-unsaturated phosphinates (e.g., **2d**, Scheme III) and phosphonates (e.g., **7**, Scheme IV) from readily available alkenyl



2a

SCHEME II. (*a*) 95% H_3PO_2, THF, 65°C.



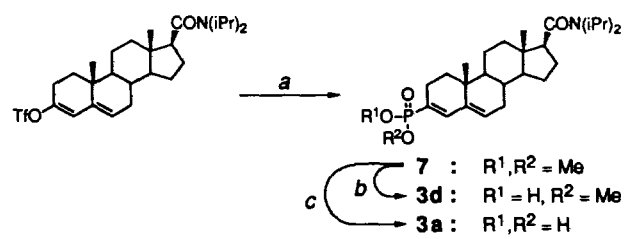
SCHEME III. (*a*) 95% H_3PO_2, Et_3N, $Pd(PPh_3)_4$, DMF, 25°C.

triflates by means of palladium(0)-catalyzed coupling with hypophosphorous acid or dialkylphosphites, respectively (*23*). This method was also extended to the synthesis of aryl phosphinates (e.g., **5a**) and phosphonates (e.g., **6a**) from aryl triflates (Scheme V).

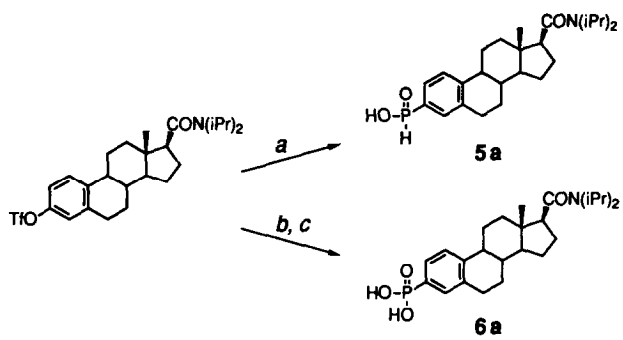
The alkenyl and aryl dimethyl phosphonate esters prepared in this manner were hydrolyzed by conventional means. Mild basic hydrolysis with potassium carbonate provided the monomethyl phosphonic acids (e.g., **3d**) while treatment with trimethylsilyl iodide or HBr/acetic acid served to remove both phosphonate methyls to provide the diacids (e.g., **3a**). For compounds possessing a *tert*-butyl amide D-ring appendage, trimethylsilyl bromide was required to effect selective double demethylation without concomitant destruction of the amide.

Inhibition of Prostatic SRs by Steroidal 3-Phosphinic and Steroidal 3-Phosphonic Acids

Isosteric replacement of the carboxylic acid in the SR 3-carboxy steroid inhibitors (**1** and **4**) resulted in the identification of a new class of compounds, the steroidal 3-phosphinic acids (**2** and **5**), demonstrating potent enzyme inhibition (Table 1). Of the compounds in Table 1, the olefinic 3-phosphinic acid **2a** was the best inhibitor of the human prostatic SR with an apparent inhibition constant of 7 nM. The decreased inhibitory potency of the phosphonic acids (**3a**, **3b**, **6a**, and **6b**)



SCHEME IV. (*a*) $(MeO)_2P(O)H$, Et_3N, $Pd(PPh_3)_4$, DMF, 25°C; (*b*) K_2CO_3, MeOH, H_2O, reflux; (*c*) TMSCl, NaI, CH_3CN, 25°C.



SCHEME V. (*a*) 95% H_3PO_2, Et_3N, $Pd(PPh_3)_4$, dppp, DMF, 90°C; (*b*) $(MeO)_2$ P(O) H, MeMorph, $Pd(PPh_3)_4$, CH_3CN, 70°C; (*c*) HBr, HOAc, 80°C.

may result from increased bulk of the 3-substituent leading to a steric intolerance for binding to enzyme—a conclusion further supported by the loss of inhibitory potency with the dialkyl phosphinic acid **2g** and the phosphinic acid methyl ester **3d**. In constrast, the inhibition potency of rat prostatic SR for both classes of 3-

TABLE 1

Inhibition of Prostatic Steroid 5α-Reductase Activities by Steroidal 3-Phosphinic and Steroidal 3-Phosphonic Acids

Compound	X	Other functionality	Y	Apparent inhibition constants $[K_{i,app}, nM(K_{i,app}/K_m)]^{a,b}$ Human	Rat	Primate
1a	$-CO_2H$	Δ3,5	$-N(isopropyl)_2$	7 (0.002)	30 (0.03)	<1 (<0.002)
1b	$-CO_2H$	Δ3,5	-NH(*tert*-butyl)	25 (0.006)	23 (0.02)	<1 (<0.002)
2a	$-PHO_2H$	Δ3,5	$-N(isopropyl)_2$	7 (0.002)	160 (0.1)	4 (0.008)
2b	$-PHO_2H$	Δ3,5	-NH(*tert*-butyl)	35 (0.008)	250 (0.2)	5 (0.01)
2c	$-PHO_2H$	Δ3;5α-H	$-N(isopropyl)_2$	25 (0.006)	100 (0.08)	5 (0.01)
2d	$-PHO_2H$	Δ2;5α-H	$-N(isopropyl)_2$	10 (0.002)	160 (0.1)	3 (0.006)
2e	$-PHO_2H$	Δ2,4	$-N(isopropyl)_2$	30 (0.007)	440 (0.4)	9 (0.018)
2f	$-PHO_2H$	Δ2;5α-H	-NH(*tert*-butyl)	130 (0.03)	310 (0.3)	ND (ND)[c]
2g	$-P(CH_3)O_2H$	Δ3,5	$-N(isopropyl)_2$	600 (0.1)	>5000 (>4)	100 (0.2)
3a	$-PO_3H_2$	Δ3,5	$-N(isopropyl)_2$	25 (0.006)	200 (0.2)	6 (0.012)
3b	$-PO_3H_2$	Δ3,5	-NH(*tert*-butyl)	190 (0.04)	350 (0.3)	ND (ND)
3c	$-PO_3H_2$	Δ2,4	$-N(isopropyl)_2$	160 (0.04)	620 (0.5)	ND (ND)
3d	$-PO(OH)(OCH_3)$	Δ3,5	$-N(isopropyl)_2$	>2000 (>0.4)	>5000 (>4)	ND (ND)
4a	$-CO_2H$		$-N(isopropyl)_2$	20 (0.003)	360 (0.3)	<1 (<0.002)
4b	$-CO_2H$		-NH(*tert*-butyl)	43 (0.01)	150 (0.1)	1 (0.003)
5a	$-PHO_2H$		$-N(isopropyl)_2$	13 (0.003)	2100 (2)	10 (0.020)
5b	$-PHO_2H$		-NH(*tert*-butyl)	230 (0.05)	3200 (3)	ND (ND)
6a	$-PO_3H_2$		$-N(isopropyl)_2$	50 (0.01)	>5000 (>4)	5 (0.01)
6b	$-PO_3H_2$		-NH(*tert*-butyl)	300 (0.07)	>5000 (>4)	ND (ND)

[a] Assays were conducted with microsomal prostatic preparations as described under Experimental Procedures. Inhibitor concentrations were varied from 0.5 to 5000 nM. Data for inhibition of rat and human SR activities with compounds **1** (*9, 10, 16, 20*) and **4** (*11, 17*) have been reported previously and are presented only for comparison. Values listed under "primate" refer to inhibition of the cynomolgus monkey prostatic SR activity.

[b] Ratios of the apparent inhibition constants ($K_{i,app}$) for each compound to the K_m for T are presented in parenthesis.

[c] Inhibition not determined (ND).

phosphosteroids is consistently weaker than that demonstrated by the steroidal 3-carboxylic acids. The differential inhibition potencies for SR from human and rat has been recognized previously with steroidal A-ring aryl carboxylic acids, **4** (*11, 17*). Additional species differences have been found with canine SR where none of the steroidal 3-carboxylic acids of **1a** ($K_{i,app}$ = 1.3 μM for dog SR), **1b** ($K_{i,app}$ = 1.0 μM), **4a** ($K_{i,app}$ = 4.0 μM) or **4b** ($K_{i,app}$ = 7.5 μM) approach the nanomolar inhibition potency observed for the human or rat enzymes, and similar differences between species have been reported for the 3-oxo-4-aza steroids (*15*).

Direct comparisons of the data summarized in Table 1 show that equally potent inhibition of human SR is achieved with the steroidal 3-phosphinic acids (**2a**, **2b**, **5a**, and **5b**) as that observed previously with their steroidal acrylate (**1a** and **1b**) and 3-carboxy A-ring aryl steroid (**4a** and **4b**) partners. For this, the enzyme must be able to accommodate the tetrahedral geometry of the phosphorous acid as well as the planar geometry of the carbon acid. Since inhibition potency of the rat activity with compounds of the two series does not follow this trend, the binding domain of rodent enzyme which accommodates the 3-substituent must be considerably more restrictive than that within human SR.

In anticipation of the *in vivo* biochemical and pharmacological assessment of these compounds, it was felt that one criterion for species selection might be potency of enzyme inhibition comparable to that found for the human SR. Based on this consideration for the steroidal acrylates **1**, the rat, but not the dog, might be regarded as an appropriate model species; however, divergence of inhibition potency between human and rat SRs with the 3-phosphosteroids and the aromatic 3-carboxylic acids posed a limitation to the utility of rodents with these compounds. In such a model, poorer enzyme inhibition as suggested in Table I for the rat enzyme, might not result in DHT suppression representative of the compound's true potential to modulate prostatic growth in man. For this reason, inhibition of prostatic SR from a non-human primate, the cynomolgus monkey (*M. fascicularis*), was undertaken.

Preliminary assessment demonstrated that the pH maximum (pH 5.5) for prostatic SR activity derived from the cynomolgus monkey more closely resembled that of the human (pH 5.0) than that for the dog or rat (6.6 to 7.0). Compounds representing the better human enzyme inhibitors were found to be most potent inhibitors ($K_{i,app} \leq$ 10 nM) of the monkey SR. Yet, not all compounds are excellent inhibitors of the monkey SR as exemplified by the methyl phosphinic acid **2g**, a relatively poor inhibitor of both human and non-human primate SRs. While a more complete set of comparative data with the 3-phosphosteroids was precluded by limited availability of monkey prostatic tissue, data with steroidal acrylates having a range of inhibition potency covering three orders of magnitude supports the correlation of inhibitor ordering between the two primate enzymes (unpublished results).

An even better correlation is observed for the ratio between the apparent inhibition constants ($K_{i,app}$) and Michaelis constants (K_m) for T on the human and non-human primate SRs as compared to the ratio with the rat enzyme (Table 1). With known species differences for K_m of T representing variable substrate affinity that

must be overcome for effective blockage of catalysis by inhibitors, the values for $K_{i,app}/K_m$ present a rank ordering of SR binding affinity which is normalized for intrinsic affinity of steroid substrate. This ranking provides a measurement that could be more relevant to the predictive *in vivo* efficacy of inhibitors than that of the inhibition constant alone. Since the absolute level of inhibition potency of the better inhibitors on the human enzyme demonstrate a tight association to SR from cynomolgus monkey and since the values for the $K_{i,app}/K_m$ ratios of the two primate activities show excellent agreement, the *Macaca* would seem to provide an appropriate *in vivo* model for continuing compound evaluation.

Mechanism of SR Inhibition by the 3-Phosphinic and 3-Phosphonic Acid Steroids

The kinetic mechanisms of SR inhibition by 17β-(*N*,*N*-diisopropylcarbamoyl)androsta-3,5-diene-3-phosphinic acid (**2a**) and 17β-(*N*,*N*-diisopropylcarbamoyl)androsta-3,5-diene-3-phosphonic acid (**3a**) have been further investigated with solubilized rat liver enzyme (*16, 17*). Data with the phosphinic acid **2a** was best evaluated with a linear uncompetitive model versus both T (Fig. 1; K_m = 1.5 ± 0.3; K_{ii} = 37 ± 5 nM) and NADPH (K_m = 12 ± 3 μM; K_{ii} = 70 ± 10 nM); the steroidal phosphonic acid **3a** also was an uncompetitive inhibitor versus T (K_m = 1.0 ± 2 μM; K_{ii} = 84 ± 5 nM). No change in the patterns of inhibition was seen when

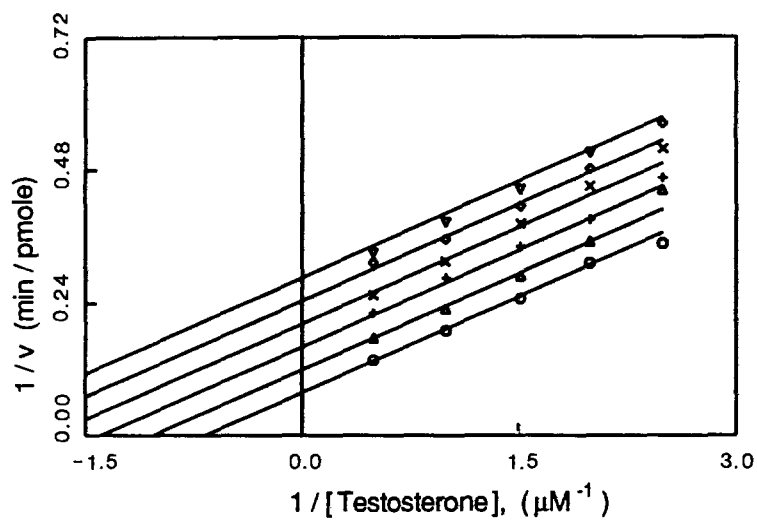


FIG. 1. Dead-end inhibition pattern of compound 17β-(*N*,*N*-diisopropylcarbamoyl)androsta-3,5-diene-3-phosphinic acid (**2a**) versus testosterone. Solubilized rat liver SR was assayed at pH 6.0 in the presence of 200 μM NADPH and the cofactor regenerating system with T as variable (0.4 to 3.5 μM) substrate; the concentrations of **2a** were 0 (o), 20 (Δ), 40 (+), 60 (x), 80 (◇), and 100 (▽) nM. Data was analyzed with the UNCOMP program (*24*) to give K_m = 1.5 ± 0.3 and K_{ii} = 37 ± 5 nM.

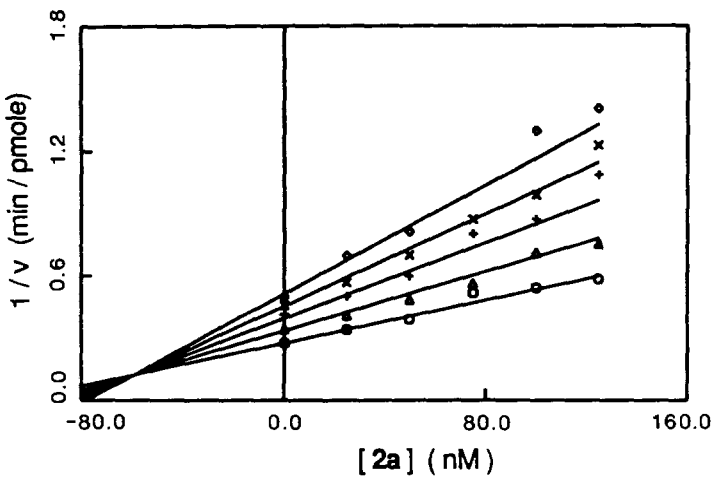


FIG. 2. Multiple inhibition analysis of SR with compound **2a** and $NADP^+$. SR activity was determined with the solubilized rat liver preparation in the presence of 1 μM T and 25 μM NADPH plus variable concentrations of $NADP^+$ without the regenerating system plus compound **2a** at pH 6.5. The concentrations of **2a** were set at 0, 20, 40, 70, 100, and 125 nM; the curves represent concentrations of $NADP^+$ at 0 (o), 25 (Δ), 50 (+), 75 (x), and 100 (◇) μM. The data was fit to Eq. [3] to give apparent inhibition constants for **2a** (K_I = 110 ± 10 nM) and $NADP^+$ (K_J = 120 ± 20 μM) and β = 0.5 ± 0.1.

determined at either pH 6.0 or pH 7.5. Within the preferentially ordered kinetic model that has been proposed for this enzyme in which the nucleotide cofactors bind to free enzyme (*20*), the dead-end inhibition plots are consistent with the association of the 3-phosphosteroid inhibitors to an enzyme–$NADP^+$ complex as depicted in Scheme I. Support for this mechanism of interaction is the synergistic inhibition of SR activity between compound **2a** and $NADP^+$. As shown in Fig. 2, the binding of oxidized cofactor and **2a** is cooperative with the experimental value of β = 0.5 ± 0.1; consequently, inhibition of SR by **2a** is enhanced by the presence of $NADP^+$. These conclusions are analogous to those that have been drawn for inhibitors **1** and **4**, the steroidal acrylates (*16*) and the A-ring aromatic acids (*17*), respectively.

The pH profile for SR inhibition by steroidal 3-carboxylic acid **1a** has been shown to be a bell-shaped curve in which one protonation event (pK_b = 7.5 ± 0.2), arising from an enzyme-$NADP^+$-associated base, and one deprotonation event (pK_a = 4.7 ± 0.2), the carboxylic acid functionality of the inhibitor, are required for maximal enzyme inhibition(*16*). This interpretation would require that other 3-substituted steroidal inhibitors with varying acidities would demonstrate displaced pK_a events relative to that observed for **1a**. Consistent with this concept is the pH profile of SR inhibition by the steroidal phosphinic acid **2a** (Fig. 3a) which shows no low pH ionization, but maintains the high pH inflection (pK = 7.6 ± 0.2). The acidity of the phosphinic acid of **2a** is estimated to be at least 100-times

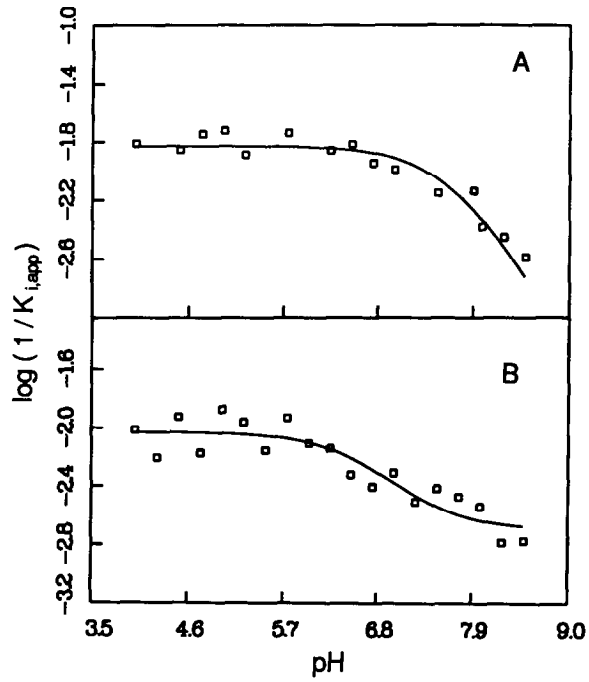


FIG. 3. Dependence of SR inhibition by **2a** and **3a** on pH. Solubilized SR was evaluated in the presence of 1 μM T, 200 μM NADPH, the cofactor regenerating system, and variable concentrations of 17β-(*N*,*N*-diisopropylcarbamoyl)androsta-3,5-diene-3-phosphinic acid (**3a**). Values for the apparent inhibition constants ($K_{i,app}$ in units of nanomolar concentrations) were determined for each compound described under Experimental Procedures. The curve presented in A for the steroidal olefinic 3-phosphinic acid **2a** was evaluated with equation 1 to give an inflection point at 7.6 ± 0.2; data presented in B for the steroidal olefinic 3-phosphonic acid **3a** was fit to Eq. [2] with an inflection at 6.6 ± 0.2.

greater (pK < 2.5) than that of the carboxylate of **1a** (pK = 4.8 ± 0.2) and therefore should be anionic throughout the pH range over which enzyme activity could be evaluated (*31*). Hence, with **2a** no commensurate break at low pH is observed.

Data for the phosphonic acid **3a** is presented in Fig. 3b. Again, inhibition is not dependent on a low pH event such as that originating from the carboxylic acid of **1a**. Rather, this profile evaluated with Eq. [3] shows that enzyme inhibition by **3a** has a maximum (1/YL = $K_{i,app}$ = 110 ± 20 nM) and minimum (*1*/YH = $K_{i,app}$ = 500 ± 70 nM) potency over the pH range about a single ionizable group (pK = 6.6 ± 0.2). The first ionization of an organic phosphonic acid (pK < 2.0) occurs at lower pH than comparable phosphinic acids, while the second leading to the dianion falls in the neutral pH range (pK = 6.5 to 7.0) (*31*). The break observed in the pH profile of SR inhibition by **3a** thereby must arise from the second phosphonic acid ionization—where the monoanionic steroidal phosphonate has a fivefold greater affinity for the enzyme than does its dianionic form.

Conclusion

As novel inhibitors of SR, the steroidal acids (**2** and **5**) and steroidal 3-phosphonic acids (**3** and **6**) could prove to be effective therapy for disorders mediated by DHT. Synergistic inhibition of SR by these compounds and $NADP^+$—leading to their preferential binding to the enzyme-$NADP^+$ complex—may prove advantageous over compounds that inhibit via complex formation with enzyme-NADPH since relief of enzyme inhibition *in vivo* through the build up of the steroid substrate, termed "metabolic resistance," is thought to be more effective with compounds that bind to the same enzyme form as does the substrate than with compounds that block catalysis at a kinetically isolated site (*32*). Key toward our ultimate objective of identifying SR inhibitors that would be most effective in man is the use of appropriate animal models for *in vivo* evaluation that would be most predictive of human biochemical and pharamacological response. While recent evidence suggests that interspecies variability of SR inhibition may derive from differences in primary protein structures and amino acid sequences (*33*), a better understanding of this phenomenon must await further experimentation. Nevertheless, one model for *in vivo* inhibitor evaluations that our studies suggest would be the non-human primate. In this regard, the pharmacological assessment of selected SR inhibitors is ongoing; results of these studies will be reported upon their completion.

ACKNOWLEDGMENTS

We greatly appreciate the efforts of Dr. H. Hughes and Dr. M. Landi, Department of Laboratory Animal Sciences, SmithKline Beecham, for providing cynomolgus monkey prostates, and Dr. M. Soloway, Department of Urology, University of Tennessee at Memphis, for providing samples of human prostatic tissues. Elemental analyses were performed by Ms. E. Reich, Department of Analytical Chemistry, SmithKline Beecham.

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