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Steroid 5α-Reductase: Comparative Study of Mechanism of Inhibition by Nonsteroids ONO-3805 and LY191704

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Two nonsteroids, ONO-3805 and LY191704, were evaluated as inhibitors of the human and rat 5α-reductases (5αR). ONO-3805 was prepared in a 12-step convergent synthesis. This compound is a potent inhibitor of the human and rat 5αRs, with more potent inhibition seen against the rat enzymes. The inhibition patterns of this compound were best fit to an uncompetitive model which suggests binding in a ternary complex with enzyme and $NADP^+$. Apparent K_i values of 27, 31, 1, and 0.5 nM versus testosterone were obtained with human type 1, human type 2, rat type 1, and rat type 2 5αR, respectively. Multiple inhibition studies with ONO-3805 and $NADP^+$ support synergistic binding of these two inhibitors with all isozymes. LY191704 was also evaluated as an inhibitor of the human and rat 5αRs. This compound is a selective, competitive inhibitor of human type 1 5αR. Poor inhibition was observed with human type 2 and rat types 1 and 2 5αR. © 1996 Academic Press, Inc.

INTRODUCTION

Dihydrotestosterone (DHT)[1] rather than testosterone (T) is considered the primary androgenic mediator in the development of benign prostatic hyperplasia (BPH), male pattern baldness, acne, and hirsutism (*1,2*). As a consequence, considerable effort has been devoted toward the design of inhibitors of steroid 5α-reductase (5αR), the enzyme which catalyzes the conversion of T to DHT. This enzyme is found in the androgen-responsive tissues such as prostate, seminal vesicle, epididymis, and skin where DHT rather than T is the active androgen (*3–7*). Two isozymes of the enzyme are known, which are localized in different tissues (*7–10*). The ability to tissue-specifically inhibit the isozymes of 5αR has heightened interest in this enzyme as a therapeutic target.

Various steroidal and nonsteroidal inhibitors of 5αR have been reported, and several are now in human clinical study. Steroidal compounds in the Δ^1-3-oxo-4-aza series such as finasteride (Fig. 1) are potent, competitive, mechanism-based inactivators of the enzyme (*11, 12*). Finasteride, a selective inhibitor of the human type 2 5αR, reduces serum DHT by ~70% and produces beneficial effects in men with BPH (*13*). The 3-carboxy-androstadienes (Fig. 1) were designed to mimic the

[1] Abbreviations used: DHT, dihydrotestosterone; T, testosterone; 5αR, 5α-reductase; BPH, benign prostatic hyperplasia.

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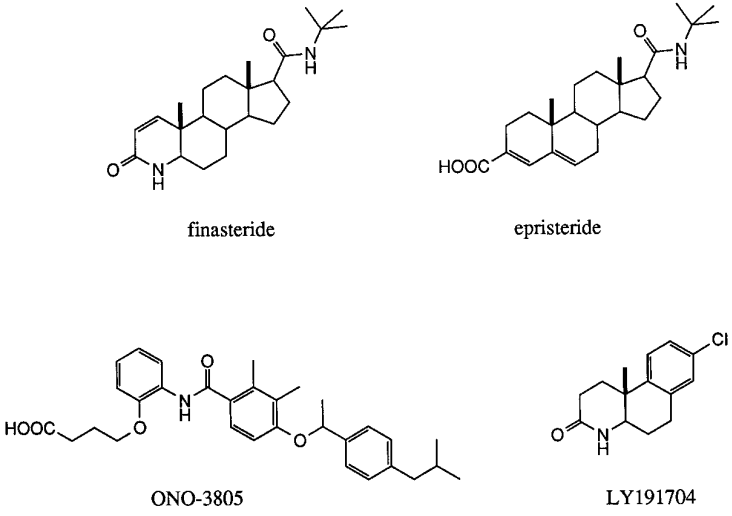


FIG. 1. Inhibitors of 5αR.

enolate transition state and display uncompetitive kinetics indicating binding to E:NADP$^+$ complex (Fig. 2) (*14*). In human clinical trials, epristeride produces similar effects on serum DHT as finasteride, albeit at higher doses (*15*). Long-term clinical effects of epristeride are not yet known.

Among the many other inhibitors of 5αR, there are two major series of nonsteroidal inhibitors: the aryl(oxy)butanoate and benzoquinolinone series. ONO-3805 (Fig. 1) was selected for study as the prototype aryl(oxy)butanoate since it is the earliest and best described in the literature (*16–20*). Although chemical synthesis and enzymological studies are only tersely disclosed, a number of *in vivo* studies have been published. ONO-3805 has been shown to be effective in reducing prostate size in

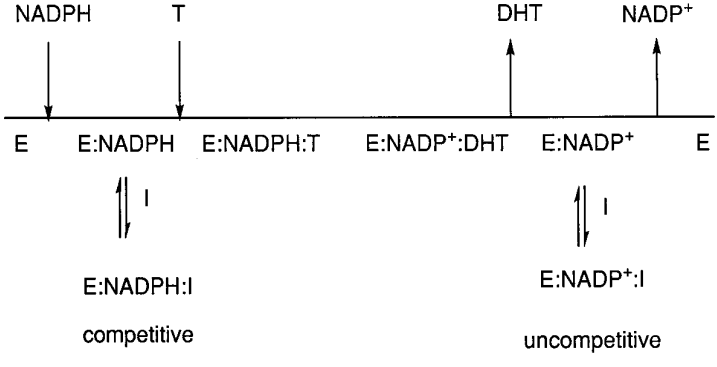


FIG. 2. Comparison of competitive and uncompetitive inhibitors of 5αR.

rats (*21, 22*). Clinical studies in humans are reportedly underway, although recent results are not available (*23, 24*). There are a host of other inhibitors in this structural class including inhibitors from Fujisawa (FK-143) (*25–29*), Kyowa Hakko Kogyo (KF18678) (*30*), and Pfizer (*31, 32*).

The second major nonsteroidal series is the benzoquinolinones, of which LY191704 (Fig. 1) is the prototype (*33, 34*). Although LY191704 is the first example of a selective human type 1 5αR nonsteroidal inhibitor, the *in vivo* evaluation of this compound has not yet been reported.

As a continuation of our studies on the mechanism of various inhibitor classes of 5αR, we present results on the mechanism of the nonsteroids ONO-3805 and LY191704. Furthermore, we describe here a synthesis of ONO-3805 which reveals details not available previously (*17*).

EXPERIMENTAL PROCEDURES

[7-^{3}H]Testosterone was purchased from New England Nuclear. Aquasol 2 and Flo-Scint II were obtained from New England Nuclear and Radiomatic Instruments, respectively. All other chemicals were obtained from Sigma Chemical Co. or Aldrich Chemicals. Column chromatography was performed on E. Merck silica gel 60 (70–230 mesh) or grade 62 (60–200 mesh). Mass spectra were obtained with a Varian MAT 731 instrument and were consistent with proposed structures for all chemical intermediates (Scheme 1).

Chemical Synthesis

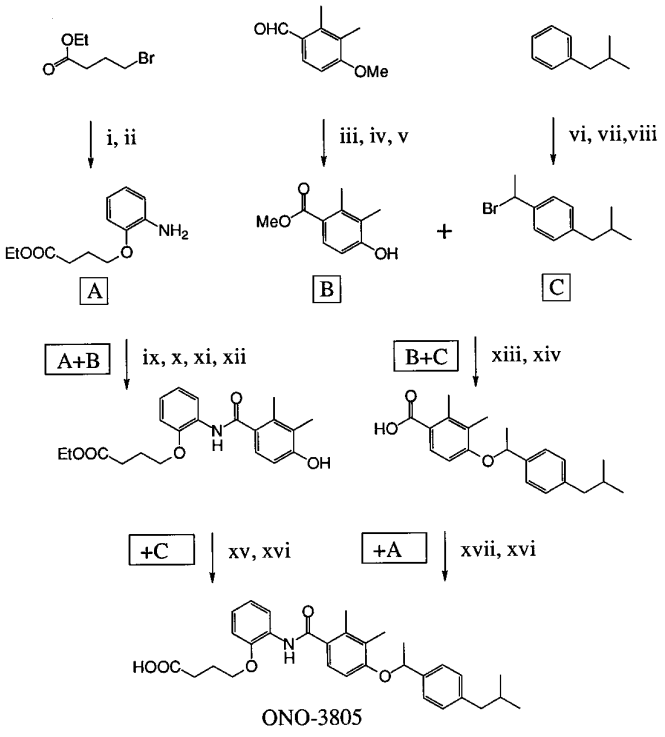
*Synthon **A***

Ethyl 4-(2-nitrophenoxy)butyrate. To a stirred solution of 2-nitrophenol (1.4 g, 10 mmol) and ethyl 4-bromobutyrate (2.1 g, 1.57 ml, 11 mmol) in dry acetone (35 ml) was added anhydrous, ground potassium carbonate (2 g, 14.5 mmol). The resultant orange-colored mixture was then heated under a nitrogen atmosphere at gentle reflux until the color due to the phenol anion had dissipated and a yellow mixture remained. Concentration of the cooled and filtered mixture gave an oil which, on flash chromatography (silica gel; ethyl acetate/hexane or methylene chloride as eluant), yielded 2.4 g (96% yield) of product as an oil.

Ethyl 4-(2-aminophenoxy)butyrate. A solution of the nitro compound (1.27 g, 5.0 mmol) in ethyl acetate (15 ml) containing 5% palladium on carbon (0.20 g) was reacted in a hydrogen atmosphere (40 psi) at room temperature until hydrogen uptake ceased. The mixture was then filtered through a layer of anhydrous sodium sulfate and concentrated *in vacuo* to yield product (1.0 g) as an oil/low-melting solid.

*Synthon **B***

4-Methoxy-2,3-dimethylbenzoic acid. To a stirred solution of 4-methoxy-2,3-dimethylbenzaldehyde (4.92 g, 30 mmol) in glacial acetic acid (25 ml) at ca. 15°C was added a chromium trioxide solution (15 ml); prepared from chromium trioxide (9.9



SCHEME 1. (i) 2-Nitrophenol, K_2CO_3, Me_2CO; (ii) H_2, 5% Pd/C, EtOAc; (iii) CrO_3, HOAc; (iv) BBr_3, CH_2Cl_2; (v) MeOH, H_2SO_4; (vi) AcCl, $AlCl_3$, $C_2H_4Cl_2$; (vii) $NaBH_4$, MeOH; (viii) HBr, PhH; (ix) $PhCH_2Br$, K_2CO_3, Me_2CO; (x) NaOH, aq. MeOH; (xi) $(COCl)_2$, CH_2Cl_2; (xii) H_2, 10% Pd/C, EtOAc-HOAc; (xiii) K_2CO_3, Me_2CO; (xiv) NaOH, aq. MeOH; (xv) K_2CO_3, Me_2CO; (xvi) NaOH, then HCl; (xvii) DCC, DMAP, CH_2Cl_2.

g, 100 mmol) dissolved in water (6 ml) and then diluted with glacial acetic acid (23 ml) dropwise over 15 min. The resultant mixture was allowed to stir with ice-bath cooling until the internal temperature dropped to 15°C, and the bath removed. After stirring overnight at ambient temperatures, the mixture was diluted with water (ca. 150 ml), aged, and filtered, and the solid was washed well with water and dried; 2.2 g of product was obtained as a gray solid.

4-Hydroxy-2,3-dimethylbenzoic acid. (*35*). To a stirred suspension of the 4-methoxy acid (1.8 g, 10 mmol) in dried dichloromethane (20 ml) at −70°C was added boron tribromide (13 ml of 1 N boron tribromide in dichloromethane) dropwise over 8 min. After stirring at this temperature for 3 h the cooling bath was removed and the resultant suspension was allowed to stir overnight at ambient temperatures. The reaction mixture was added to a stirred ice–water mixture (100 ml), the layers were separated, and the aqueous layer was exhaustively extracted with dichloromethane to yield 1.15 g product as a tan solid. More compound could be obtained by concentration of the aqueous solution followed by repeated dichloromethane extractions.

Methyl 4-hydroxy-2,3-dimethylbenzoate. To a solution of the benzoic acid (1.15 g, 6.9 mmol) in dry methanol (35 ml) was added five drops of concentrated sulfuric acid (prediluted with a small amount of dry methanol) and the mixture was heated at reflux until TLC analysis indicated the absence of starting material. The methanol was removed *in vacuo*, the residue was stirred with a mixture of water–hexane and filtered, and the resultant solid was washed with water and dried to give 1.1 g product as a tan solid.

*Synthon **C***

4-Isobutylacetophenone. To a stirred solution of isobutylbenzene (13.4 g, 15.7 ml, 0.1 mol) and acetyl chloride (7.82 ml, 0.11 mol) in dried 1,2-dichloroethane (200 ml) at +3°C was added anhydrous aluminum chloride (16.0 g, 0.12 mol) in portions over 22 min. The temperature rose to +8°C during the addition and an orange color developed. After an additional hour at ice-bath temperatures, the bath was removed and the mixture allowed to stir at ambient temperature for 2 days. After cautious addition of the reaction mixture to a stirred mixture of ice, water, and methylene chloride, the mixture was allowed to warm to room temperature and separated, and the organic layer was dried over anhydrous sodium sulfate. Concentration followed by chromatography on a silica gel column using dichloromethane as eluant gave the product as a fluid liquid in 68% yield.

1-(4-Isobutyl)phenylethanol. To a stirred solution of the acetophenone (1.76 g, 10 mmol) in dry methanol (25 ml) at +5°C was added sodium borohydride (0.38 g, 10 mmol) and the resultant mixture was stirred in an ice bath for 10 min. The bath was then removed, and the reaction mixture was stirred at ambient temperatures for 2 h. After removal of the methanol *in vacuo* the residue was covered with methylene chloride (50 ml) and treated with water. When both layers cleared, the organic layer was separated and dried over anhydrous sodium sulfate. Concentration of the filtered solution yielded 1.74 g of product as a clear liquid.

1-(4-Isobutylphenyl)bromoethane. Anhydrous hydrogen bromide was gently bubbled into an ice-cooled stirred solution of the phenylethanol (0.78 g, 4.38 mmol) in dried benzene (30 ml) for 15 min. After an additional 10 min at ice-bath temperatures, the resultant two-phase mixture was stirred at ambient temperatures for 2 h, dry nitrogen was then bubbled through the mixture to displace the excess hydrogen bromide, the mixture was allowed to settle, and the benzene layer was carefully decanted from the water droplets. Removal of the benzene *in vacuo* yielded the product (1.03 g) as a clear, pale-yellow oil that darkened with time.

*Coupling of **A** + **B***

Methyl 4-benzyloxy-2,3-dimethylbenzoate. A mixture of **B** (1.8 g, 10 mmol), benzyl bromide (1.31 ml, 11 mmol) and anhydrous ground potassium carbonate (5.0 g, 36 mmol) in dry acetone (50 ml) was heated and worked up as for **A.** Flash chromatography on silica gel (using 15% ethyl acetate–hexane as eluant) of the residue thus obtained gave 2.1 g of product as a thick oil which solidified on standing.

4-Benzyloxy-2,3-dimethylbenzoic acid. A mixture of the benzyloxy ester (1.51 g, 5.6 mmol), water (4.2 ml), 2.5 N sodium hydroxide solution (7.0 ml, 17.5 mmol),

and methanol (120 ml) was heated at gentle simmer under a nitrogen atmosphere until TLC analysis showed the absence of ester. The methanol was removed *in vacuo*, the residue was stirred with water (150 ml), and the resulting suspension was treated dropwise with 2 N hydrochloric acid (11.2 ml) and aged. Filtration followed by water washing and drying gave 1.39 g of product as a white solid.

Ethyl 4-(2-(4-benzyloxy-2,3-dimethylbenzoylamino)butanoate. To a suspension of the acid (0.256 g, 1.0 mmol) in anhydrous dichloromethane (5.0 ml) at room temperature was added oxalyl chloride (2.0 ml, 23 mmol) dropwise over 2 min. After 1.5 h the volatiles were removed *in vacuo* to yield a crust. This crust was dissolved in dichloromethane (2 ml) and added dropwise to a stirred ice-cold mixture of **A** (0.245 g, 1.1 mmol) and dry pyridine (1.0 ml) in dichloromethane (15 ml) over 1 min. After 30 min the ice bath was removed and the mixture was stirred at ambient temperatures overnight. The mixture was then transferred to a separatory funnel with dichloromethane and washed with 0.5 N hydrochloric acid (2×) and water, dried (sodium sulfate), filtered, and concentrated to 0.47 g of product.

Ethyl 4-(2-(4-hydroxy-2,3-dimethylbenzoylamino)-phenoxy)butanoate. A solution of the benzylated coupling product (0.23 g, 0.5 mmol) in ethyl acetate (20 ml) containing one small drop of glacial acetic acid and 10% palladium on carbon (0.2 g) was reduced under a hydrogen atmosphere (40 psi, room temperature) until no benzyl ether remained by TLC. The filtered and concentrated mixture was then flash chromatographed on silica gel (15–35% ethyl acetate/hexane eluant) to give 0.163 g of product as a thick oil which solidified on standing.

*Coupling of **B** + **C***

Methyl 4-(1-(4-isobutylphenyl)ethoxy)-2,3-dimethyl-benzoate. A stirred mixture of **B** (0.75 g, 4.14 mmol), **C** (1.0 g, 4.14 mmol), and anhydrous, finely ground potassium carbonate (0.75 g, 5.4 mmol) in anhydrous acetone (30 ml) was heated at reflux for 25 h, cooled, and filtered. Concentration of the filtrate followed by flash chromatography on silica gel (5% ethyl acetate/hexane as eluant) gave 0.92 g of product as a pale yellow oil.

4-(1-(4-Isobutylphenyl)ethoxy)-2,3-dimethylbenzoic acid. A stirred mixture of the resultant ester from above (0.41 g, 1.2 mmol) in methanol (25 ml), water (1 ml), and 2.5 N sodium hydroxide solution (1.3 ml, 3.25 mmol) was simmered under a nitrogen atmosphere until TLC analysis showed the absence of ester (ca. 48 h). The cooled solution was concentrated *in vacuo*, the resultant crust was stirred with water (75 ml), and the solution was filtered and acidified with 2 N hydrochloric acid. After standing for an hour, the solid was filtered, washed with water, and dried to give 0.37 g of product as a pale, cream-colored solid.

*Coupling of **C** to [**A** + **B**]*

Ethyl 4-(2-(4-(1-(4-isobutylphenyl)ethoxy)-2,3-dimethylbenzoylamino)phenoxy)-butanoate. A mixture of [**A + B**] (0.536 g, 1.44 mmol), **C** (0.40 g, 1.66 mmol), and anhydrous ground potassium carbonate (0.6 g) in acetone (40 ml) was heated and worked up as described above. Flash chromatography on silica gel (10–20% ethyl

acetate/hexane as eluant) of the residue thus obtained gave product (0.72 g) identical to that obtained from the coupling of **A** to [**B + C**].

ONO-3805: 4-(2-(4-(1-(4-Isobutylphenyl)ethoxy)-2,3-dimethylbenzoylamino)phenoxy)-butanoic acid. To a stirred, ice-cooled solution of the above ester (0.72 g, 1.35 mmol) in methanol (35 ml) was added water (seven drops) and 2.5 N sodium hydroxide solution (1.3 ml, 3.3 mmol) dropwise. After 5 min the bath was removed and the mixture was stirred under a nitrogen atmosphere at ambient temperatures until TLC analysis showed no ester (ethyl or methyl, from ester exchange) remained. The methanol was removed *in vacuo* (<25°C), the residue was dissolved in water (90 ml) and filtered, and the filtrate was acidified dropwise with 2 N hydrochloric acid. The resultant precipitate was filtered, washed well with water, and dried to give 0.66 g of product as a waxy solid; mass spectral data and TLC mobility were consistent with published data (*17*). *Anal.* Calcd for $C_{31}H_{37}NO_5$ (503.64): C, 73.93; H, 7.41; N, 2.78. Found: C, 74.09; H, 7.63; N, 2.61.

Coupling of **A** *to* [***B*** + ***C***]

Ethyl 4-(2-(4-(1-(4-isobutylphenyl)ethoxy)-2,3-dimethylbenzoylamino)phenoxy) butanoate. To a stirred mixture of [**B + C**] (0.082 g, 0.25 mmol) and **A** (0.11 g, 0.49 mmol) in dried dichloromethane (5 ml) at room temperature was added *N*,*N*,-dimethylaminopyridine (0.032g, 0.26 mmol) followed by *N*,*N*′-dicyclohexylcarbodiimide (0.069 g, 0.33 mmol), and the mixture was covered with a nitrogen atmosphere. A precipitate of *N*,*N*′-dicyclohexylurea appeared within 5 min. After stirring overnight the mixture was filtered, the filtrate was concentrated, the residue was triturated with small amounts of ether and dichloromethane separately, and the combined ether/dichloromethane extracts were concentrated. Chromatography on a predeveloped silica gel preparative plate using 22% ethyl acetate/hexane as eluant yielded 0.11 g of ONO-3805 ethyl ester as a clear oil.

Steroid 5α-R Assays

Enzyme assays were carried out as described previously (*7*) using baculovirus-expressed human 5α-Rs (*36*) and COS cell-expressed rat 5α-Rs (*37*). Assays of human and rat type 1 5α-R were conducted in 40 mM succinate/imidazole/diethanolamine buffer (SID) (*38*), pH 6.5, while 40 mM SID, pH 5.5, was used to assay the type 2 5α-Rs. NADPH was routinely used at a final concentration of 0.5 mM.

Inhibition Studies

Stock solutions of ONO-3805 and LY191704 were prepared in ethanol and diluted to the appropriate concentration in 10% ethanol. In these studies the final concentration of ethanol was less than 2.5%. IC_{50} values were determined at K_m concentrations of T using a five-point titration ranging in concentration from 0.1 to 1000 nM for type 1 5α-R or from 1 to 10,000 nM for type 2 5α-R.

Data Analysis

Kinetic data were fit to Eqs. [1]–[4] using nonlinear regression analysis. *S* reflects the concentration of T and *I* represents inhibitor concentration. K_i and K_{ii} are the kinetic constants for competitive (Eq. [2]) and uncompetitive inhibitors (Eq. [3]),