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INHIBITION OF STEROID 5α-REDUCTASE BY 3-NITROSTEROIDS: SYNTHESIS, MECHANISM OF INHIBITION, AND *IN VIVO* ACTIVITY.

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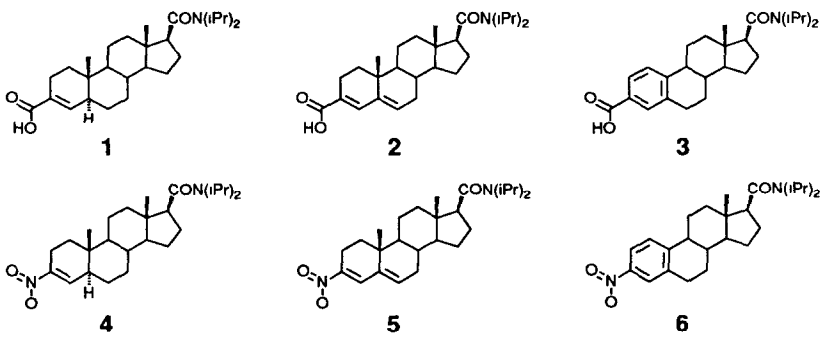
Abstract: A series of A-ring unsaturated 3-nitrosteroids has been prepared and assayed *in vitro* as inhibitors of steroid 5α-reductase. One of these compounds, 3-nitro-5α-androst-3-ene-17β-diisopropylcarboxamide (**4**), inhibits the human enzyme with a K_i of 50 nM and exhibits competitive inhibition versus testosterone. Potent oral activity of **4** was demonstrated in *cynomolgus* monkeys.

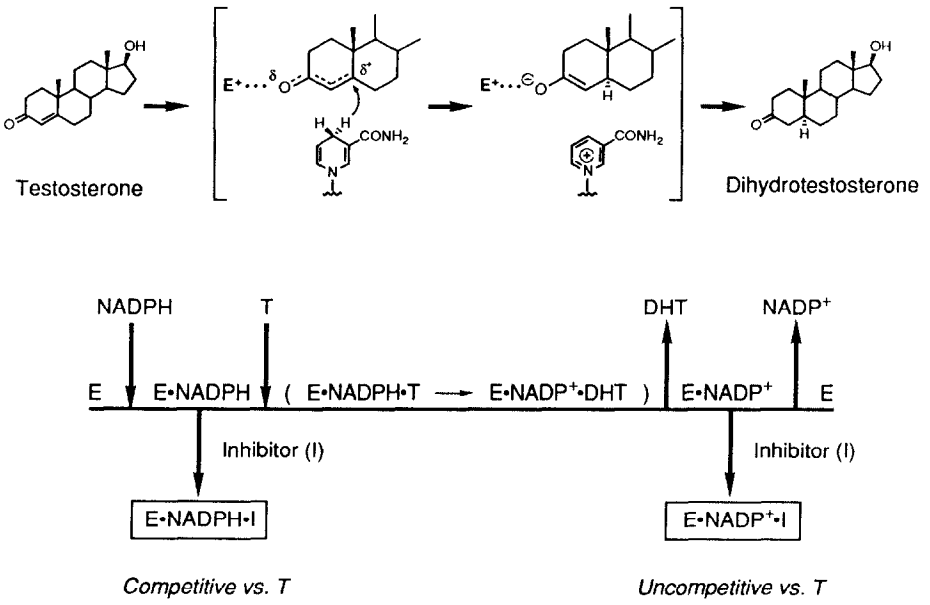
The lack of effective, non-invasive therapy for the widely occurring disease benign prostatic hyperplasia (BPH) coupled with mounting evidence that prostatic growth is supported by the androgen dihydrotestosterone (DHT), not testosterone (T), has fueled a search for potent inhibitors of steroid 5α-reductase (EC,1.3.1.30), the enzyme responsible for the biosynthesis of DHT from T.[1] Previously, we described two novel classes of steroidal-3-carboxylic acids,[2-4] exemplified by compounds **1**, **2**, and **3**, which are potent inhibitors of human prostatic steroid 5α-reductase ($K_{i,app}$ = 30 nM, 7 nM, and 20 nM, respectively) and exhibit uncompetitive (vs. T) mechanisms of inhibition.[5-7] Herein, we report the syntheses and biological evaluations of the isosteric series of 3-nitrosteroids (compounds **4-6**).



Steroid 5α-reductase is an NADPH-dependent enzyme which follows an ordered sequential kinetic mechanism with NADPH binding first followed by T (Figure 1). After catalysis, DHT is released followed by NADP+. Radiolabeling studies support a direct, stereospecific transfer of hydride from the

dihydronicotinamide to C-5 of T leading, speculatively, to an enolate intermediate which is ultimately protonated in the active site to yield DHT.[5] Acrylate **1** was designed[2] as a mimic of this putative enolate intermediate, a species presumed to approximate the steroidal component of the transition state. Central to this original design was the maintenance of: (1) the A-ring geometry, determined by the 5α-ring fusion and the C-3/C-4 unsaturation, and (2) the negative charge of the oxyanion, supplied by the anionic carboxylate group. Eventually, structure-activity studies[3] revealed that the exact nature of the A-ring unsaturation was less critical for inhibitory activity (e.g., compound **2**) than the electrostatic properties of the C-3 substituent and that similar inhibitor potency could be obtained by presentation of the carboxylic acid on an A-ring aromatic nucleus (compound **3**).[4] On the other hand, uncharged carboxylic acid derivatives (esters, amides, alcohols, and the protonated form of the acid) were relatively inactive.[6]

Figure 1. Chemical and Kinetic Mechanisms of Steroid 5α-Reductase.



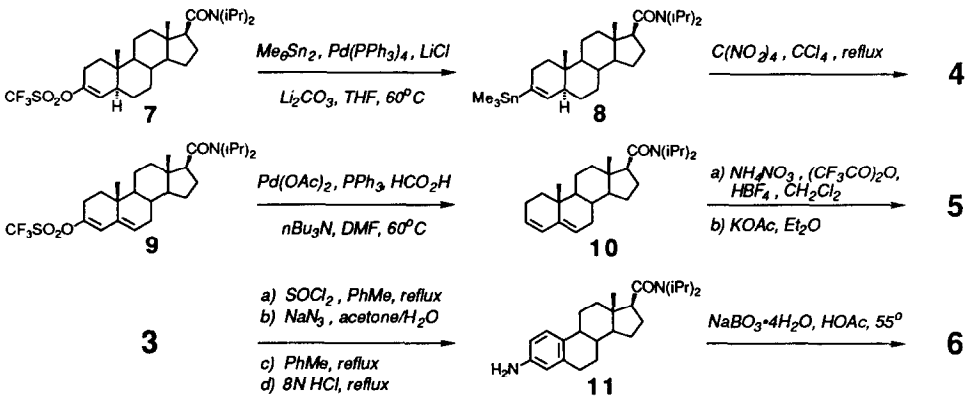
The apparent overriding importance of the negative charge led to an extension of this design strategy and the exploitation of phosphinic and phosphonic acids,[8] and sulfonic acids[9] as carboxylate (and presumably enolate) surrogates. These analogs also proved to be potent inhibitors of human steroid 5α-reductase and in the case of the phosphorus compounds (sulfonates were not characterized), the inhibition was also shown to be uncompetitive versus T -- an observation consistent with the preferential formation of inhibitor ternary complexes with enzyme and $NADP^+$.

Our hypothesis that anionic inhibitors preferentially bind to the enzyme-$NADP^+$ complex due to an electrostatic interaction between the inhibitor and positively-charged nicotinamide[10] was supported by the

finding that the uncharged 4-aza-3-one series of A-ring lactam steroid 5α-reductase inhibitors reported by Merck[11] were competitive versus T (i.e., selectively bind to the enzyme-NADPH complex). However, steric differences between the acrylate and 4-aza inhibitor classes, as well as different orientations of A-ring hydrogen-bonding sites, could not be discounted as the principle causes for the observed differences in mechanisms. The nitro group is a unique isostere for the deprotonated carboxylate anion, possessing essentially identical size, geometry, and pair of electron-rich oxygens, yet lacking the formal negative charge at the physiologically relevant pH. Therefore, as an interesting variation of our original design which could provide further insight into the above distinctions, we prepared the series of 3-nitrosteroids **4**-**6** as neutral isosteric analogs of the carboxylic acids **1**-**3** (Scheme1).

The synthesis of nitro **4** was carried out in two steps from the previously reported[3] enol triflate **7**. Palladium-catalyzed coupling of the triflate with hexamethylditin according to the procedure of Wulff[12] afforded alkenyl stannane **8** in 70% yield. Treatment of the stannane with tetranitromethane in refluxing carbon tetrachloride as described by Corey[13] provided the desired nitrosteroid, **4**[14] (45%). Although dienyl stannane formation from the corresponding dienyl triflate (**9**) proceeded without incident, subsequent attempts to isolate dienyl nitro **5** from reactions of the stannane and tetranitromethane were unsuccessful. An equally direct and successful approach to nitro **5** began with reduction of the dienyl triflate under the Cacchi conditions[15] to produce diene **10** in 88% yield. Treatment of **10** with an *in situ* generated source of trifluoroacetyl nitrate[16] and subsequent treatment with potassium acetate then afforded dienyl nitro **5** in 65% yield. The A-ring aryl nitro **6** was prepared from the carboxylic acid **3** in two steps. First, amine **11** was generated quantitatively via a Curtius rearrangement sequence. Sodium perborate oxidation[17] of the amine then provided the aryl nitro **6** in 41% yield.

Scheme 1. *Synthesis of 3-Nitrosteroid Inhibitors.*

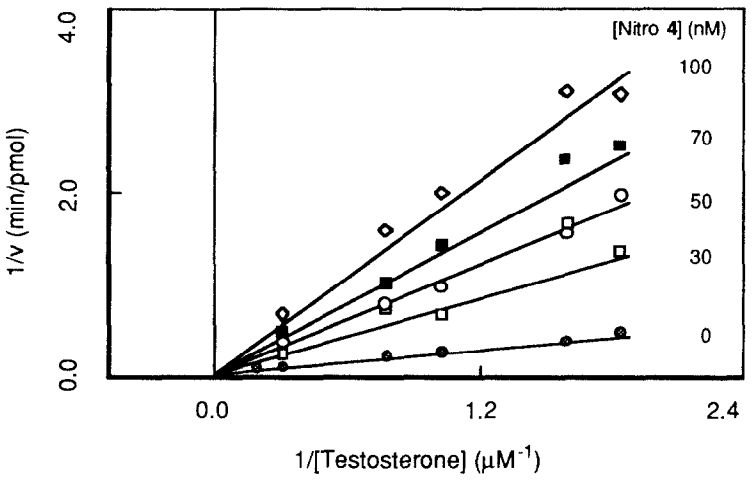


Nitro **4** proved to be a potent *in vitro* inhibitor of human steroid 5α-reductase exhibiting an apparent inhibition constant of 50 nM.[18] In contrast, the activities of nitro compounds **5** and **6** deviated remarkably from that observed for the corresponding acidic inhibitor series. Dienyl nitro **5** possessed an affinity for the

human enzyme over an order of magnitude weaker than **4**, having an apparent K_i of 590 nM, and aryl nitro **6** demonstrated no significant inhibition at concentrations up to 5 μM. Furthermore, unlike carboxylates **1-3**, none of the three nitro compounds examined showed any detectable inhibition of rat steroid 5α-reductase at micromolar concentrations.

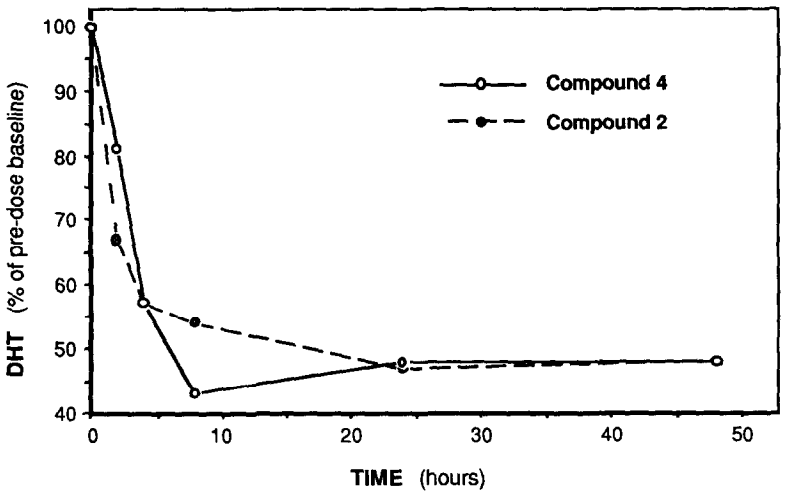
Most notably, compound **4** selectively associated to the enzyme-NADPH complex (demonstrating competitive dead-end inhibition kinetics versus testosterone, Figure 2), thus supporting our supposition that charge complementarity between the inhibitor and cofactor (resulting in net neutrality) directs the binding of the anionic inhibitors to the E-NADP$^+$ complex and the neutral inhibitors to the E-NADPH complex. The divergence of SAR for the carboxylates and nitros is presumably a manifestation of their binding to two different enzyme forms (binary complexes), each form likely presenting a uniquely shaped and charged steroidal binding site.

Figure 2. *Dead-End Inhibition of Human Steroid 5α-Reductase by Nitro* ***4****.*[18]



Finally, with the aim of developing useful therapeutics, nitrosteroid **4** was examined *in vivo* for its ability to lower circulating plasma DHT levels in adult male *cynomolgus* monkeys.[21] Figure 3 shows the result of a preliminary trial using five animals. Following a single oral dose of 0.1 mg per kg body weight, a 57% mean maximum decrease of the pre-dose baseline DHT level was observed after 4 hours. At 48 hours post-dose, DHT levels remained depressed to nearly the same extent. This observed profile of plasma DHT depression was not significantly different than that observed for compound **2** even though nitro **4** exhibited a several-fold weaker inhibition of *cynomolgus* steroid 5α-reductase *in vitro* than carboxylate **2** (K_i = 4 nM and <1 nM, respectively).

Figure 3. *Primate Plasma DHT Concentration Following a Single Oral Dose (0.1 mg/kg).*[22]



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10. Although the required proximity of the cofactor and steroid A-ring makes the cationic nicotinamide the most obvious charge-complementary partner for the carboxylate, we cannot exclude the possible presence of some other active-site cation or electrophile, such as the putative proton donor, which could coordinate to the carboxylate only in the E-$NADP^+$ complex.[6]

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14. Compound **4**: mp, 194-197°C; partial NMR ($CDCl_3$), δ 0.77 (s, 3H, C-18), 0.78 (s, 3H, C-19), 6.98 (s, 1H, C-4). Compound **5**: mp, 115-117°C; partial NMR ($CDCl_3$), δ 0.83 (s, 3H, C-18), 0.97 (s, 3H, C-19), 6.12 (br s, 1H, C-6), 7.45 (s, 1H, C-4). Compound **6**: mp, 168-169°C; partial NMR ($CDCl_3$), δ 0.82 (s, 3H, C-18), 7.4 (d, J=8, 1H), 7.94 (s, 1H), 7.96 (d, J=8, 1H). Compounds **4**-**6** provided satisfactory elemental analyses and mass spectra.

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18 Evaluation of steroid 5α-reductase activity was carried out with microsomal rat and human prostatic enzyme preparations as previously described.[2,5] Apparent inhibition constants ($K_{i,app}$) of test compounds were determined by the method of Dixon[19] as oulined.[2,5] Data for the dead-end inhibition analysis of compound **4** with human prostatic steroid 5α-reductase activity was obtained from incubations at 37°C in 50 mM citrate buffer, pH 6.0, at 600 μM NADPH with varying concentrations of testosterone (0.54, 0.63, 0.97, 1.3, 3.3, 5.2 μM) and compound **4** (0 to 150 nM). The data was fit using the COMP program described by Cleland[20] to the equation : $v=V_mA/\{K_m[1+(I/K_{is})]+A\}$ where K_m is the apparent Michaelis constant for testosterone, A and I are the concentrations of testosterone and inhibitor, respectively, K_{is} is the inhibition constant, and V_m is the maximal velocity. Kinetic constants derived from the fit were K_m=5.4±0.5 μM and K_{is}=16±1 nM.

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21. A more detailed discussion of the use of this animal model in the *in vivo* assessment of steroid 5α-reductase inhibitors will be presented elsewhere.

22. DHT concentration was measured by a highly specific radioimmunoassay (RIA) following solid phase extraction of plasma and separation of androgens by HPLC.