INHIBITION OF PYRIDINE-NUCLEOTIDE-DEPENDENT ENZYMES BY PYRAZOLES. SYNTHESIS AND ENZYMOLOGY OF A NOVEL A-RING PYRAZOLE STEROID

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ABSTRACT

A novel A-ring pyrazole steroid, 2,3-bisaza-A-nor-1,5(10)-estradien-17β-ol (3), was synthesized as a potential inhibitor of steroidal NAD(P)H-dependent oxidoreductases. Compound 3 proved to be a potent inhibitor of 3(17)β-hydroxysteroid dehydrogenase (from *P. testosteroni*) exhibiting a K_i of 90±20 nM. The activities of 3α,20β-hydroxysteroid dehydrogenase (from *S. hydrogenans*), steroid-5α-reductase (from rat prostate), and 3α-hydroxysteroid dehydrogenase (from rat liver) were unaffected by pyrazole 3. Dead end inhibition studies indicate an ordered binding of cofactor prior to substrate or pyrazole inhibitor.

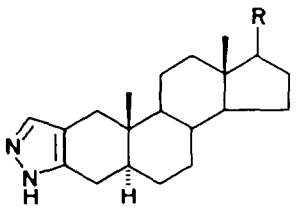
INTRODUCTION

Pyrazole, an extremely potent inhibitor of liver alcohol dehydrogenase (LAD), has been shown to form a tightly associated ternary complex with the enzyme (E) and NAD^+ cofactor (1). Spectrophotometric and titrimetric studies indicate that formation of this complex is accompanied by the release of one proton. The implication is that pyrazole coordinates to the active-site zinc of the E•NAD^+ complex, thereby displacing a zinc-bound water ligand. Proton loss from the pyrazole results in a delocalized pyrazole anion, which is further stabilized through its coordination to zinc and most importantly through its electrostatic interaction with the positively charged cofactor. X-ray analysis of the ternary complex confirms that one pyrazole nitrogen coordinates to the zinc while the second pyrazole nitrogen is in close proximity to C-4 of the nicotinamide (2). Recent ^{15}N-NMR studies suggest that the pyrazole is covalently bound to C-4 of the cofactor (3). The pK_a of the pyrazole in the ternary complex, E•NAD^+•pyrazole, is less than 4 (estimated to be 2.4), whereas the pK_a of E•pyrazole

is greater than 10 (4). This dramatic perturbation of pK_a is further evidence of the strong interaction between the pyrazole and NAD^+.

Our interest in manipulating steroid biosynthesis has led us to investigate an extension of this concept to NAD(P)H-dependent steroid oxidoreductases using steroidal pyrazoles as potential inhibitors. This report describes the synthesis of a novel A-ring pyrazole steroid and its effect on 3(17)β-hydroxysteroid dehydrogenase, 3α,20β-hydroxysteroid dehydrogenase, 3α-hydroxysteroid dehydrogenase, and steroid-5α-reductase.

Steroids with pyrazole rings fused to the A-ring, particularly at the 2,3-position (*e.g.*, 1) or the 3,4-position (*e.g.*, 2) were initially prepared in the early 1960's and examined as potential anabolic/nonandrogenic steroids (5). Compound 1, R = β-OH, α-Me (Stanozolol,USP), is marketed as such. A-ring pyrazole steroids such as 3 represent a novel class of compounds. Thus compound 3 was synthesized and its inhibitory potency towards several pyridine-nucleotide-dependent enzymes was determined.



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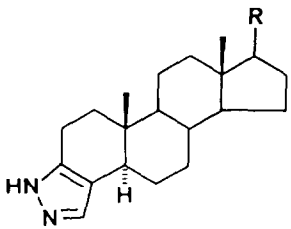

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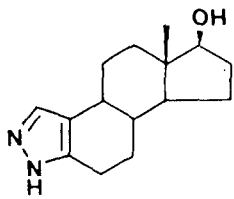

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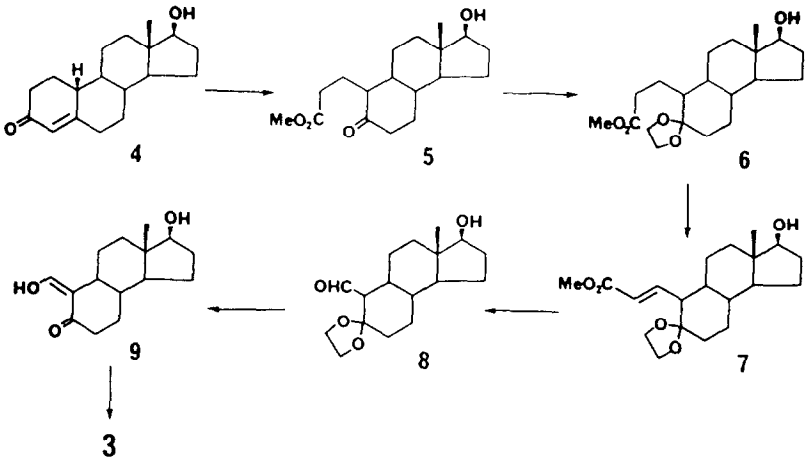
MATERIALS AND METHODS

[4-^{14}C]Testosterone (52 mCi/mmol), 5α-[1,2-^{3}H]androstan-3α,17β-diol (30 Ci/mmol), [9,11-^{3}H(N)]androsterone (53.3 Ci/mmol), and Econosolve II were purchased from New England Nuclear. 3(17)β-Hydroxysteroid dehydrogenase (EC 1.1.1.51) from *P. testosteroni*, 3α,20β-hydroxysteroid dehydrogenase (EC 1.1.1.53) from *S. hydrogenans*, testosterone, 4-androsten-3,17-dione, 5α-androstan-3α,17β-diol (ADIOL), 3-oxo-5α-androstan-17β-ol (5α-dihydrotestosterone), androsterone, 5α-androstan-3,17-dione, dithiothreitol (DTT), bovine serum albumin (BSA), NAD^+, NADH, NADP, and NADPH were obtained from Sigma Chemical Company. Radioactivity was determined using a Beckman LS-5801 scintillation counter which was calibrated for correction to DPM with Beckman ^{14}C and ^{3}H standards or a System 2000 BIOSCAN Imaging Scanner (BIOSCAN, Washington, D.C.). Analyses of enzymatic reactions were performed using either plastic-backed, silica TLC plates (Kieselgel 60 F_{254}, Merck) or prechanneled glass-backed plates with a preabsorbing region (Si250F-PA, Baker). Livers and prostates of adult male Sprague-Dawley rats were removed, rinsed in cold 20mM potassium phosphate buffer, pH 6.5, containing 320 mM sucrose and 1 mM DTT, and frozen at -80°C until used for enzyme preparation.

Chemistry

As outlined in Scheme I, 19-nortestosterone (**4**) was treated with excess ozone at -78°C (2:1 CH_2Cl_2:MeOH) to give after esterification (MeI, $NaHCO_3$, DMA, RT, 2 days) secoketoester **5** as a colorless oil in *ca.* 50% yield. Ketalization ($HOCH_2CH_2OH$, $C_6H_5CH_3$, HCl, reflux) afforded compound **6** as a viscous oil in 68% yield after chromatography. Two carbon degradation of the propionate chain was effected by selenation/elimination (6) to give unsaturated ester **7** (70%, mp 82-84°C, partial NMR: δ5.85 (d, 1H, J = 16 Hz), δ6.75 (dd, 1H, J = 10, 16 Hz)) followed by ozonolysis (2:1 CH_2Cl_2:MeOH, $NaHCO_3$, -78°C) which provided aldehyde **8** (60%). Hydrolysis of the ketal required more forcing conditions than expected; however, heating ketal **8** in 80% aqueous acetic acid at 65°C for 20 h afforded hydroxymethylene ketone **9**. Treatment with excess hydrazine hydrate in refluxing ethanol completed the synthesis, affording pyrazole **3** as white crystals (recrystallized from ethanol/water), mp 237.5-239.5°C, NMR(DMSO-d_6): δ 0.66 (s, 3H, H.18), 1.1-2.1 (m, 12H), 2.5-2.75 (m, 3H, H.6,H.9), 3.51 (m, 1H, H.17), 4.49 (d, 1H, J = 4.7 Hz, exchangeable, OH), 7.3 (bs, 1H, H.1), 12.2 (bs, 1H, exchangeable, NH). CHN: Calculated for $C_{15}H_{22}N_2O \cdot 1/8H_2O$; C-72.47, H-9.02, N-11.27. Found; C-72.44, H-9.02, N-10.89. Exact mass: Calculated for $C_{15}H_{22}N_2O$; 246.173. Found; 246.173.

SCHEME I



Enzyme Preparations and Assays

3(17)β-Hydroxysteroid dehydrogenase (from *P. testosteroni*): The commercially obtained 3(17)β-hydroxysteroid dehydrogenase was reconstituted in 20 mM potassium phosphate, pH 7.2, containing 20% glycerol and serially diluted in 10 mM phosphate, pH 7.2, containing 10 mg/mL BSA; the BSA was added to help maintain enzyme activity upon dilution. Diluted enzyme could be stored for several weeks at -20°C.

Enzyme activity was determined by following the conversion of testosterone to 4-androsten-3,17-dione. [^{14}C] Testosterone in ethanol was deposited in test tubes and concentrated to dryness in a SAVANT Speed-Vac. To each tube was added NAD^+ and buffer. The reaction was initiated by introduction of the enzyme (5-10 x 10^{-6} units** in 10-50 μL), to give a final reaction volume of 0.5 mL 10 mM sodium pyrophosphate buffer, pH 8.9. After incubation of the tubes at 25°C for 10 min, the reaction was terminated by the addition of 4 mL of ethyl acetate and 0.15 μmol each of testosterone and 4-androsten-3,17-dione as carrier. The organic layer was removed to a second test tube and the solvent was evaporated in a Speed Vac. The residue was redissolved in 20-30 μL chloroform, spotted on 20 x 20 cm plastic-backed silica gel TLC plates, and developed with acetone:chloroform (1:9). The regions of the plates containing testosterone and 4-androsten-3,17-dione, as located by UV, were cut out, soaked in 2 mL ethyl acetate, and counted in 10 mL of Econosolve II. The percent of recovered radiolabel converted to product was calculated, from which enzyme activity was determined. No more than 12% of the substrate was consumed in any incubation. With this assay, the specific activity of the 3(17)β-hydroxysteroid dehydrogenase was 70 units/mg protein.

3α,20β-Hydroxysteroid dehydrogenase (from *S. hydrogenans*): An aliquot of the enzyme solution provided as an ammonium sulfate suspension was pelleted by centrifugation. The residue was dissolved in 100 mM sodium phosphate, pH 7.5, containing 10 mg/mL BSA, and was serially diluted with the same buffer immediately prior to use. Assays were performed as described above for the 3(17)β-hydroxysteroid dehydrogenase except that 5α-[1,2-^{3}H] androstan-3α,17β-diol was used as the substrate, and unlabeled ADIOL and 5α-dihydrotestosterone (product) were added as the carriers following the ethyl acetate quench. The residue from the reaction was spotted on prechanneled silica gel plates (Baker) and developed as above. Radiochemical content of the individual steroids was determined on a BIOSCAN Imaging Scanner. Enzyme activity was calculated from the percent of recovered radiolabel converted to 5α-dihydrotestosterone; the specific activity was determined to be 0.5 units/mg protein.

3α-Hydroxysteroid dehydrogenase (from rat liver): 3α-Hydroxysteroid dehydrogenase from rat liver was prepared by the method of Penning *et al* (7) through the ammonium sulfate precipitation. The resulting precipitate was resuspended in 30 mL buffer containing 1mM EDTA, 1 mM DTT, and 10 mM Tris, pH 8.6. The solution was dialyzed three times against the suspension buffer, and a fourth time against the same buffer containing 20% glycerol. Activity was determined as described for the 3(17)β-hydroxysteroid dehydrogenase, except that [9,11-^{3}H(N)] androsterone and $NADP^+$ were used as substrates, and that unlabeled androsterone and 5α-androstan-3,17-dione were added during the quench. The activity of this enzyme preparation was 0.03 units/mg protein.

Steroid 5α-reductase (from rat prostate): Rat prostatic microsomes were prepared as described by Blohm *et al* (8). Activity was determined as previously reported (8) except that the incubations were run for 20 min at 37°C and the reaction was quenched with 4 mL ethyl acetate, as described above. This preparation had a specific activity of 54 pmol per mg protein per hour.

Inhibition experiments: Potential steroidal inhibitors in ethanol were added to the test tubes along with the steroid substrate, and the contents were evaporated to dryness. All cofactors were added following addition of the incubation buffer; typically, 5-10 x 10^{-6} units of enzyme activity were used per each assay. All other procedures were as described above for the individual assays.

Kinetic analysis: Kinetic data were analyzed using the computer programs described by Cleland (9).

RESULTS AND DISCUSSION

To determine the activity and specificity of A-ring pyrazole **3** as a potential inhibitor of steroid biosynthetic enzymes which utilize similar substrates and cofactors, we have investigated the ability of the A-ring pyrazole (**3**) to inhibit steroid 5α-reductase from rat prostatic microsomes, 3α-hydroxysteroid dehydrogenase from rat liver cytosol, 3(17)β-hydroxysteroid dehydrogenase from *P. testosteroni*, and 3α,20β-hydroxysteroid dehydrogenase from *S. hydrogenans*. To determine the inhibitory potency of compound **3**, a radiochemical assay for each activity was developed in which the steroidal product was isolated and quantified.[***] Using these procedures, the 3(17)β-hydroxysteroid dehydrogenase uniquely was proven to be inhibited by compound **3**; the activity of the other three enzymes is not affected at elevated concentrations (1-5 μM) which approach the solubility limits of the A-ring pyrazole in the reaction media. The potent inhibition of this 3(17)β-hydroxysteroid dehydrogenase of broad substrate specificity by pyrazole **3** is characterized by a K_i of 90±20 nM (Figure **1A**) in contrast to the K_m for testosterone of 700±45 nM (as determined by initial velocity data that were analyzed according to a sequential mechanism, data not shown). Independently, we have determined that testosterone is soluble to concentrations greater than 30 μM, thereby indicating that the measured Michaelis constant represents a saturation of the enzyme within the defined experimental conditions.

To gain insight as to the order of binding of substrates, and hence the relative order of binding of NAD^+ and the inhibitor **3** to 3(17)β-hydroxysteroid dehydrogenase, several dead end inhibition experiments were performed in which the steroidal pyrazole **3** and NADH were used as testosterone and NAD^+ analogues respectively. The patterns obtained for compound **3** versus testosterone and NAD^+, Figure **1**, were found to be competitive and uncompetitive, respectively, while those obtained for NADH versus testosterone and NAD^+ (patterns not shown) were noncompetitive and competitive, respectively. Such patterns are consistent with an ordered association to

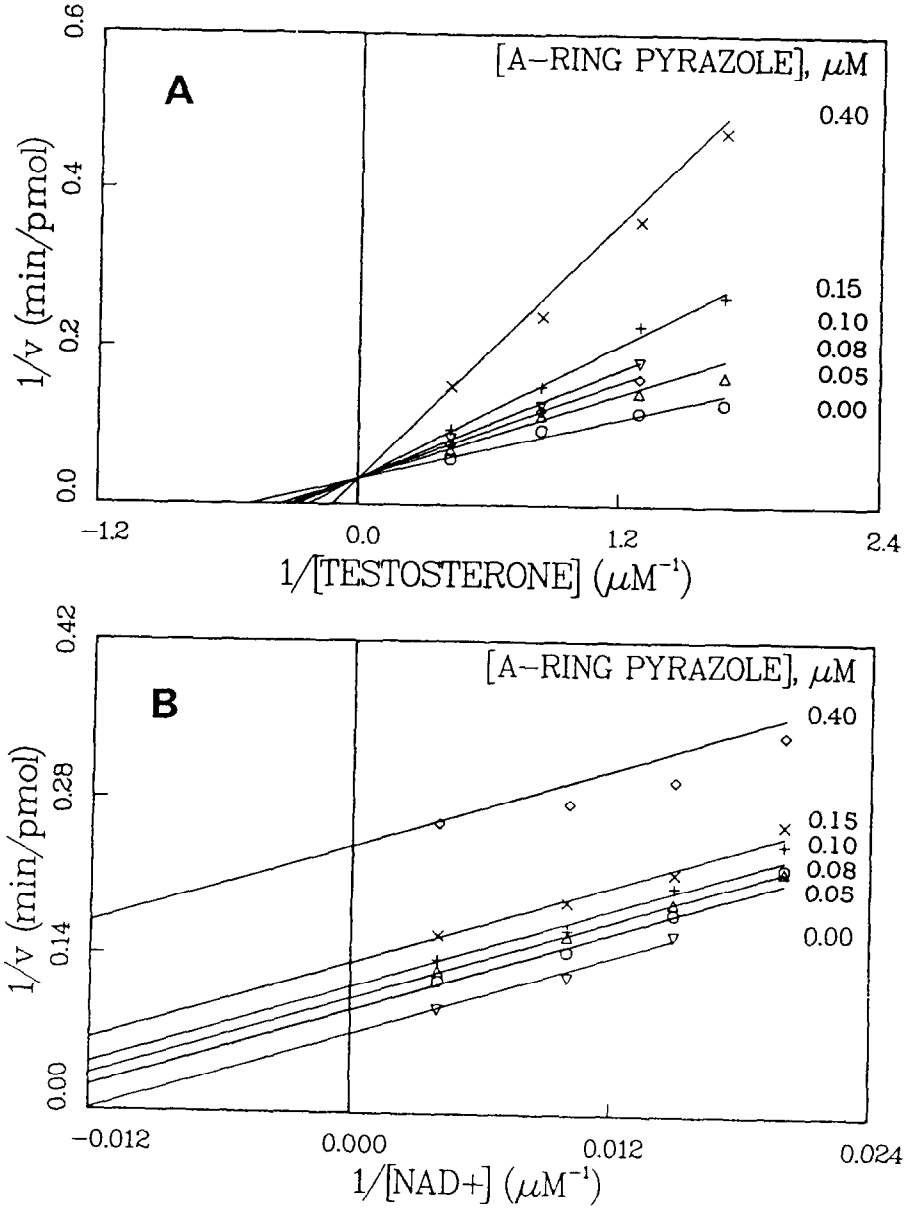
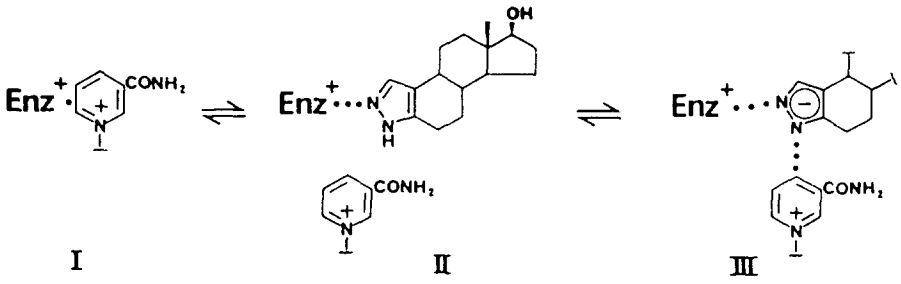


FIGURE 1. Inhibition of 3(17)β-hydroxysteroid dehydrogenase.
A: *Competitive pattern of pyrazole* 3 *vs.* testosterone.
B: Uncompetitive pattern of pyrazole 3 *vs.* NAD^+.

the enzyme (10) in which NAD^+ binds prior to testosterone and the pyrazole inhibitor. These conclusions are in agreement with those obtained from isotope exchange experiments which have suggested a preferentially ordered mechanism for substrate binding (11).

The binding of NAD^+ prior to pyrazole 3 suggests that the potent inhibition induced by the A-ring pyrazole results from its interaction with an enzyme-NAD^+ complex to form a ternary complex. Although we have not demonstrated the involvement of a deprotonation event, we hypothesize that this complex is analogous to that formed upon the inhibition of LAD (1) by pyrazole in the presence, but not absence, of NAD^+. By analogy then, our proposed mechanism of inhibition of 3(17)β-hydroxysteroid dehydrogenase by compound 3 is depicted in Scheme II. According to this hypothesis, the A-ring pyrazole becomes associated with the preformed enzyme-NAD^+ complex (I). Coordination of one pyrazole nitrogen to an enzyme-bound electrophile (Enz^+) could facilitate formation of a pyrazole anion (III), which would further be stabilized within the ternary complex by an ionic or covalent interaction with the oxidized cofactor.

SCHEME II



It is interesting to note that compound 3 is a potent inhibitor of only one of four likely enzyme targets which were investigated. This specificity may suggest that only in 3(17)β-hydroxysteroid dehydrogenase are the enzyme active site, NAD^+, and pyrazole 3 able to gain the proper juxtaposition for tight binding to occur. Thus, spatial considerations--which, *a priori*, are often unpredictable--will determine if favorable binding energetics between enzyme and pyrazole result in a strong association. Even with this restriction, the inhibition of pyridine-nucleotide-dependent oxido-reductases by pyrazole analogues of substrates or products should prove to be applicable to other targets of interest.

NOTES

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** One unit of enzyme activity is defined as the conversion to product of 1 μmol of steroid per minute.

*** Although the latter three enzymes theoretically are more easily assayed by following the increase in absorbance at 340 nm due to generation of NADH, the corresponding extinction coefficient (ϵ = 6200 M^{-1} cm^{-1}) and the low values for the Michaelis constants of the steroidal substrates (less than 4 μM) preclude an adequate absorbance increase for accurate determination of initial velocity data at substrate concentrations near K_m values. The radiochemical assays also eliminate potential problems associated with the introduction of co-solvents, a technique often used to increase steroid solubility and thereby allow for the employment of absorbance assays. The inappropriate use of spectrophotometric assays under these conditions can result in substrate saturation of solvent being mistaken for enzyme saturation. Such results would yield inadvertently high estimates for the Michaelis constants.

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