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IRREVERSIBLE INACTIVATION OF MAMMALIAN Δ^5-3β-HYDROXYSTEROID DEHYDROGENASES BY 5,10-SECOSTEROIDS. ENZYMATIC OXIDATION OF ALLENIC ALCOHOLS TO THE CORRESPONDING ALLENIC KETONES.

Venkataraman Balasubramanian and Cecil H. Robinson
Johns Hopkins University School of Medicine, Department of Pharmacology and Experimental Therapeutics, Baltimore, Maryland 21205

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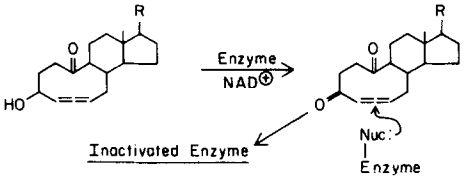
SUMMARY

Novel 4,5-allenic 3β-hydroxy-5,10-secosteroids have been synthesized by sodium borohydride reduction of the corresponding conjugated allenic 3-oxo-5,10-secosteroids. The secosteroid allenic alcohols are substrates for bovine adrenal and human placental Δ^5-3β-hydroxysteroid dehydrogenases, and the resulting electrophilic conjugated allenic ketones are shown to inactivate these dehydrogenases in a time-dependent manner. Inactivated enzyme did not recover activity after filtration through Sephadex G-25. In contrast, the secosteroid allenic alcohols were not oxidized at C-3 by the bacterial 3β(and 17β)-hydroxysteroid dehydrogenase from P. testosteroni, nor did the corresponding allenic ketones inactivate this enzyme when incubated directly.

INTRODUCTION

In continuation of our studies with secosteroids as potential irreversible inhibitors of important steroid-transforming enzymes (1), we report the preparation and evaluation of novel secosteroid allenic alcohols designed as mechanism-based inhibitors of mammalian Δ^5-3β- hydroxysteroid dehydrogenases. These enzymes are important in the biosynthesis of hormonal steroids such as progesterone and testosterone (2,3). Scheme 1 shows the rationale for the expected enzyme-induced inactivation process.



MATERIALS AND METHODS

3β(and 17β)-hydroxysteroid dehydrogenase from Pseudomonas testosteroni was purchased from P.L. Chemicals Inc., and gel filtration media were obtained from Pharmacia Fine Chemicals. [1,2-^{3}H(N)]-dehydroepiandrosterone (58.6

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Ci/mmole) was supplied by New England Nuclear and Δ^5[7(N)-^{3}H]-Pregnenolone (40.2 Ci/mmole) from Amersham. All protein assays were done with a BioRad Protein assay kit (BioRad Laboratories) using bovine serum albumin (Sigma Chemical Co.) as standard. All solvents and chemicals were analyzed reagent (J.T. Baker). All water used was double distilled in an all glass still.

Preparation of Adrenal Microsomes:

All operations described below were carried out at 4°C unless otherwise specified. Bovine adrenal glands were obtained from freshly killed animals and processed as soon as possible. Adhering fat was trimmed from the adrenal glands which were cut open and the medulla scraped off using a scalpel. The cortex was then scraped free of capsule using a razor blade and was transferred to 3 volumes of homogenizing buffer comprising 0.8mM Na_2HPO_4, 0.2mM NaH_2PO_4, 250mM sucrose and 20% Glycerol (adjusted pH = 7.00). The suspension was homogenized using a Waring Blender for 90 sec at medium speed. The homogenate was centrifuged at 1,000g for 10 min and the supernatant was then spun at 10,000g for 20 min. The pellet was discarded and the supernatant was now centrifuged at 108,000g for 1 hr. The microsomal pellets were then reconstituted in the homogenizing buffer and were frozen with liquid N_2 and stored at -80°C.

Solubilization of the microsomal Δ^5-3-β-hydroxysteroid dehydrogenase was carried out using a modification of the published procedure (4). Routinely, the microsomes were thawed and diluted with the homogenizing buffer containing 1 Molar sodium chloride (ie. 20mg protein in 5ml buffer 1M in NaCl) at 0°C. The microsomal suspension was vortexed occasionally and kept at 0°C for 2 hr, and was then stored at -20°C overnight. It was then spun at 108,000g for 2 hr. All of the dehydrogenase activity appeared in the supernatant, which was used as such for all enzyme assays and inhibition studies. The supernatant which was clear initially, stayed clear for at least a week and then gradually turned cloudy without loss of Δ^5-3-β-hydroxysteroid dehydrogenase activity.

3β-Hydroxysteroid Dehydrogenase Assays.

A spectrophotometric assay was generally used for the bacterial enzyme from P. testosteroni and enzyme activity was determined as described (5) at 30°C in a temperature-controlled Gilford spectrophotometer using testosterone as substrate.

The Δ^5-3β-hydroxysteroid dehydrogenase activity of solubilized adrenal microsomes was determined by using a radiochemical assay. The assay involved measurement of the rate of formation of Δ^4-androstene-3,17-dione from dehydroepiandrosterone (DHEA) in the presence of enzyme and NAD. In a total volume of 1ml was contained: 910ul phosphate buffer (0.1M, pH=7), 40ul NAD (5 x 10^{-6}M), 30ul solubilized adrenal microsomes (30μg) and 2 to 8μg dehydroepiandrosterone/[1,2-^{3}H(N)]- dehydroepiandrosterone 58.6Ci/mM in 20ul methanol. The incubation was carried out at 25°C in a Dubnoff metabolic shaking incubator. The reaction was initiated by the addition of substrate and aliquots were withdrawn every minute over an 8-min period during the linear rate period of 10 min. The aliquots were quenched with 3ml methylene chloride, mixed thoroughly in a vortex mixer (1 min) and centrifuged in a clinical Beckman centrifuge. The aqueous layer, along with the precipitated protein, was discarded; and unlabelled substrate and product were added to the organic phase. The organic phase was evaporated to dryness under nitrogen, and the residue thus obtained was dissolved using 2 x 100ul portions of methylene chloride. The combined solution was put on a 19 channel, 20 x 20cm Whatman Analytical TLC precoated plate, [LK5D, Linear K, silica gel, 80°A 250u thickness]. The plates were developed with Hexane-Chloroform-Acetone

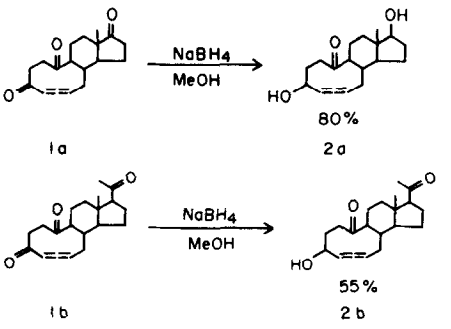


Fig. 1 SYNTHESIS OF ALLENIC ALCOHOL

4:3:1 in a pre-equilibrated tank, dried with a hot air dryer, sprayed with 10 sulphuric acid in methanol, and the bands were visualized by heating the plate carefully with a heat gun. The bands corresponding to the product and substrate were scraped out and transferred to scintillation vials containing 10ml of ACS^{R} (Aqueous Liquid Scintillant, Amersham). Scintillation counting was carried out on a Beckman LS7000 microprocessor-controlled liquid scintillation counter with a tritium counting efficiency of 60%. Using the Beckman tritium standard, a quench curve was generated with varying amounts of 10% sulphuric acid in methanol as quenching agent. Appropriate corrections were made in the counts of actual samples whenever necessary. Routinely, the sample quenching was marginal and the counting efficiency was over 98% of that of the instrument tritium efficiency.

Irreversible Inhibition:

Inactivation of the Δ^5-3β-hydroxysteroid dehydrogenase was carried out by incubating the solubilized adrenal microsomes with inhibitor in phosphate buffer (pH=7) in the presence of NAD at 25°C along with proper controls. In order to vary inhibitor concentration in such incubations the total volume of incubation medium was varied by varying the amount of phosphate buffer without changing the amounts of NAD, enzyme and inhibitor (40ul NAD, $5x10^{-6}$M; 200ul enzyme = 259μg; 2μg inhibitor). At various time intervals (every 10 min over 60 min and at 90 min) appropriate aliquots were drawn from preincubations and assayed for residual Δ^5-3β-hydroxysteroid dehydrogenase activity, using the radiochemical assay. A substrate/inhibitor ratio of 20 was attained in the assays by the above procedure.

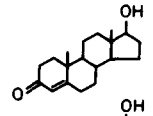
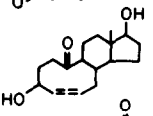
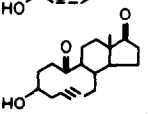
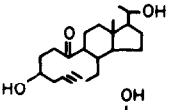
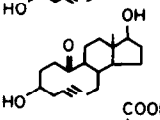
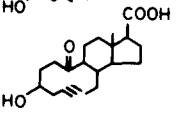
Synthesis of Allenic Alcohols:

(4R)-5,10-Secoestra-4,5-diene-3β,17β-diol-10-one (1b; Fig. 1)

To a stirred solution of (4R)-5,10-secoestra-4,5-diene-3,10,17-trione (1a; 100mg) in methanol (10ml) was added sodium borohydride (32mg). After stirring for 1 min, the reaction mixture was poured into water and extracted with CH_2Cl_2. The organic phase was washed with water, dried (Na_2SO_4)and evaporated in vacuo and the residue was chromatographed on silica gel using CH_2Cl_2. Elution with 10% acetone in CH_2Cl_2 gave the allenic alcohol (80mg) which upon further purification by HPLC (C-18 reverse phase column; 30% aqueous methanol as eluant) gave the pure allenic alcohol (1b; 60mg) mp: 178-180° (methanol-benzene); Mass Spectrum m/e 290(M^+), 272, 254, 213, 197, IR ($CHCl_3$) ν_{max} 3600, 3300(OH), 1960(C=C=C-), 1710cm^{-1} (C=O); NMR (360

Table I

Evaluation of secosteroids as substrates for 3β(17β)-hydroxysteroid dehydrogenase (P. Testosteroni).

SUBSTRATE	RELATIVE VELOCITY OF OXIDATION	AMOUNT OF NADH PRODUCED PER EQUIVALENT OF STEROID
	100	1
	150	1
	0	0
	0	0
	150	1
	0	0

All assays were done spectrophotometrically as described in text. Final concentration of steroids in the assay was 30μM.
In all cases oxidation occurred only at the 17 position.

MHz, $CDCl_3$) δ 5.28 and 5.00 (m,-CH=C=CH), 4.2 (m, 3-CH-OH), 3.65 (t, 17-CH OH), 0.82(s, 18-CH_3). Anal. Calculated for $C_{18}H_{26}O_3$. C, 74.44; H,9.03 Found: C, 74.27; H, 9.11.

(4R)-5,10-Seco-19-norpregna-4,5-dien-3β-ol-10,20-dione (2b; Fig. 1)

Under the same conditions (4R)-5,10-secopregna-4,5-diene-3,10,20-trione (2a) gave as the major product (58% yield) the desired allenic alcohol (2b), mp 140-141°C (benzene-acetone); Mass Spectrum m/e 316(M^+), 298, 270, 255, 213, 199, IR ($CHCl_3$) ν_{max} 3600(OH), 1960(-C=C=C-), 1700cm^{-1}(C=O); NMR (220 MHz, $CDCl_3$) δ 4.76 and 4.5 (m,-CH=C=CH), 3.7 (m, CHOH), 1.88 (s,-$COCH_3$), 0.68 (s, 18CH_3) ppm. Anal. Calculated for $C_{20}H_{28}O_3$: C, 75.91; H, 8.92 Found: C, 75.62; H, 9.07.

RESULTS AND DISCUSSION

Preliminary investigations of the allenic alcohols 2a and 2b, and related acetylenic alcohols as substrates for 3β-hydroxysteroid dehydrogenases were carried out with the readily available bacterial enzyme from P. testosteroni. The results are summarized in Table 1, and show that none of the 3β-hydroxy

secosteroids is oxidized by the enzyme at C-3. In contrast, the 17β-hydroxy group, if present, undergoes rapid oxidation at rates faster than the natural 17β-hydroxy substrate, testosterone. Incubation of the secosteroid allenic ketones (100uM) with the enzyme in the absence or presence of NAD in phosphate buffer of pH=7 at 30°C for several hours did not induce loss of activity. It was decided also to assess the secosteroid allenic alcohols (2a,2b) as substrates for the membrane-bound Δ^5-3β-hydroxysteroid dehydrogenases from bovine adrenals and human placenta. Recently, a convenient procedure (using 1M NaCl in homogenizing buffer) for solubilization of 3β-hydroxysteroid dehydrogenases from rat adrenals and testis has been reported (4). We have found that both the beef adrenal and human placental Δ^5-3 -hydroxysteroid dehydrogenases can be solubilized using the above procedure. Optimal conditions required 1M sodium chloride in 5ml of solubilizing buffer per 20mg of microsomal protein. We note that Δ^5-3-ketosteroid isomerase activity was solubilized along with the dehydrogenases.

Studies with the solubilized mammalian Δ^5-3β-hydroxysteroid dehydrogenases gave results in sharp contrast to those with the P. testosteroni enzyme. Both secosteroid allenic alcohols (2a and 2b), as shown by spectrophotometric assays, proved to be substrates for the mammalian enzymes and complete oxidation of the 3β-hydroxyl group occurred. Tlc analysis of the reaction mixture showed formation of the corresponding allenic ketones and their partial release into solution. On prolonged incubation (eg. 30 min after completion of oxidation at the C-3 position) enzyme activity was completely lost. Similarly, incubation of the solubilized adrenal microsomes directly with the secosteroid allenic ketone (1a) in the presence of NAD resulted in complete loss of activity within 45 min. Control enzyme preparations showed no loss of dehydrogenase activity during these time periods.

Competitive inhibition studies, using a radiochemical assay with DHEA as substrate, showed that both the allenic alcohols (2a and 2b) were good

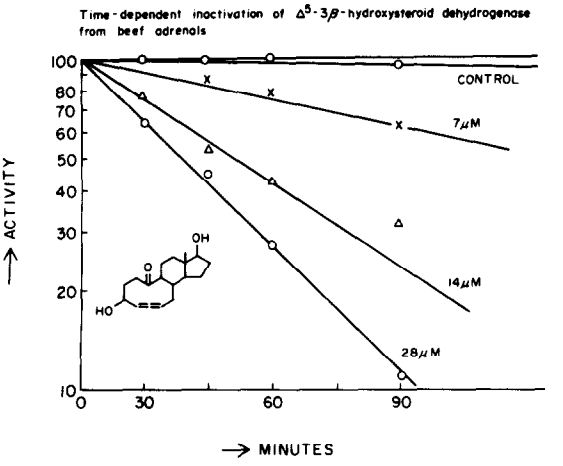


Fig 2 All assays were carried out at 25°C in phosphate buffer (pH=7). After preincubation with various concentrations of inhibitors, aliquots were assayed for residual enzyme activity as detailed in text using 7×10^{-6} M DHEA.

inhibitors of the adrenal enzyme (DHEA, Km = 0.7 μM; 1b, Ki = 0.7 μM; 2b, Ki = 0.4 μM). Preincubation of the enzyme with either of these allenic alcohols resulted in loss of enzyme activity as shown by subsequent assay with DHEA after dilution. The loss of activity found to be time-dependent and followed pseudo first-order kinetics (figure 2). The inactivation was also found to be irreversible as shown by Sephadex gel filtration. Whereas the control retained 80% of its activity after gel filtration, the inactivated enzyme did not recover its activity after identical treatment. We believe these studies represent the first example of the use of αβ,βγ-allenic alcohols to provoke enzyme-generated inactivation of hydroxysteroid dehydrogenases. The contrasting susceptibility of bacterial and mammalian dehydrogenases to inhibition by these compounds suggests that they may be useful tools in studies of steroid hormone biosynthesis.

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