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REVIEW ARTICLE

MECHANISM BASED INHIBITION OF HYDROXYSTEROID DEHYDROGENASES

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Steroid hormone action can be regulated not only at the receptor level but also by the enzymes that are responsible for the synthesis and degradation of biologically active steroids. Traditionally the pharmacological intervention of steroid hormone action has focused on the development of steroidal and nonsteroidal hormone receptor agonists and antagonists with appropriate pharmacokinetics. Recently, the development of selective inhibitors/inactivators of steroid metabolizing enzymes has gained momentum. This review will concentrate on the development of mechanism-based inhibitors for one class of steroid hormone transforming enzymes, the hydroxysteroid dehydrogenases.

KEY WORDS: Hydroxysteroid dehydrogenases, tight-binding inhibitors, suicide substrates, steroid heterocycles, Michael acceptors, oxiranes.

1. INTRODUCTION

The hydroxysteroid dehydrogenases (HSDs) belong to a group of pyridine nucleotide-dependent enzymes which catalyze the oxidoreduction of alcohols and carbonyls (Figure 1) in a positional and stereospecific manner on the steroid nucleus or side chain.[1] These enzymes play pivotal roles in the regulation of steroid action by catalyzing the synthesis and degradation of active steroid hormones. Due to their actions HSDs have the ability to alter processes mediated through steroid hormone action and represent important drug targets.

Members of the hydroxysteroid dehydrogenase family which will be considered in this review are those that represent potential therapeutic targets, and the bacterial enzymes that serve as models for their eukaryotic counterparts. The mammalian

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Abbreviations used: HSD, hydroxysteroid dehydrogenase; 3α-HSD, 3α-hydroxysteroid dehydrogenase [3α-hydroxysteroid:NAD(P)$^+$ oxidoreductase, EC 1.1.1.50]; 3(17)β-HSD, 3(17)β-hydroxysteroid dehydrogenase [3(or 17)β-hydroxysteroid:NADP$^+$ oxidoreductase, EC 1.1.1.51]; 3β-HSD/KSI, sequential enzyme activities catalyzed by 3β-hydroxy-Δ^5-steroid dehydrogenase (EC 1.1.1.145) and 3-keto-Δ^5-steroid isomerase (EC 5.3.3.1); 11β-HSD, 11β-hydroxysteroid dehydrogenase [11β-hydroxysteroid:NADP$^+$ 11-oxidoreductase, EC 1.1.1.146]; 3α,20β-HSD, 3α,20β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* dual activities catalyzed by [(20*R*)-17α20,21-tri-hydroxysteroid:NAD$^+$ oxidoreductase (EC 1.1.1.53)]; 17β-HSD or 17β,20α-HSD, 17β-hydroxysteroid dehydrogenase [estradiol-17β:NAD$^+$ 17-oxidoreductase, EC 1.1.1.62]; and 20α-HSD, 20α-hydroxysteroid dehydrogenase [20α-hydroxysteroid oxidoreductase (EC 1.1.1.149)].

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FIGURE 1 Generalized reaction catalyzed by hydroxysteroid dehydrogenases. R_1 and R_2 are components of either the steroid nucleus and/or side-chain.

enzymes of interest include 3α-, 3β-, 11β-, 17β- and 20α-HSDs. The reactions catalyzed by these enzymes and the therapeutic uses of inhibitors or inactivators of these enzymes are described below.

In androgen target tissues 3α-HSD catalyzes the reduction of the potent androgen 5α-dihydrotestosterone to the inactive androgen, 5α-androstan-3α,17β-diol,[2-4] and can terminate androgen action in these tissues (Figure 2). Inhibitors or inactivators of 3α-HSD could potentiate androgen action and would be useful in the treatment of some androgen insufficiencies.

In all endocrine tissues 3β-HSD catalyzes the oxidation of pregnenolone to 5-pregnene-3,20-dione and is the rate limiting step in the conversion of prenenolone to progesterone.[5] Selective blockade of the ovarian and placental activities would be expected to demonstrate contra-gestational activity by causing an acute cessation of progesterone synthesis which would lead to the termination of pregnancy.[6,7]

Circulating levels of glucocorticoids (cortisol in humans and corticosterone in rats) exceed the level of aldosterone. These steroids would act as mineralocorticoids in the kidney if it were not for the action of renal 11β-HSD, which catalyzes their oxidation to the corresponding inactive 11-ketosteroids.[8-10] Thus inactivators targeting kidney 11β-HSD have the potential to act as hypertensive agents.

17β-HSD catalyzes the reduction of 4-androstene-3,17-dione to testosterone in the testis,[11,12] and the reduction of estrone to 17β-estradiol in the placenta and ovary.[13,14] In both instances a less active steroid is converted to a more potent hormone by the action of 17β-HSD. Thus, androgen and estrogen dependent processes (such as androgen or estrogen dependent tumor growth) could be attenuated by inactivators of 17β-HSD.

20α-HSD can terminate the action of progesterone by catalyzing the reduction of progesterone to its inactive metabolite 20α-hydroxyprogesterone.[15-17] Since ovarian or placental progesterone production is required for the maintenance of pregnancy, 20α-HSD inhibitors or inactivators could act as gestation maintaining agents.

While each reaction noted involves the interconversion of a single alcohol/ketone functionality, these alterations dramatically alter steroid hormone potency. The direction of the physiological reactions are believed to be as indicated. With two exceptions, 3β-HSD/Δ^5-ketosteroid isomerase (3β-HSD/KSI) and 11β-HSD, carbonyl reduction appears to be the dominant trend. Discussion of 3β-HSD inactivators is complicated by reports on the purification,[18-20] cloning and expression of 3β-HSD/KSI,[21,22] which indicates a single protein catalyzes both the dehydrogenation and isomerization reactions. Because these activities are coupled, the isomerization reaction may well bias both the direction of the dehydrogenase reaction and the mechanism of enzyme inactivation. In this review we will consider mammalian microsomal 3β-HSD/KSI as a single protein; however, we will discuss only those compounds that directly effect the 3β-HSD activity.

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2. PROPERTIES OF HYDROXYSTEROID DEHYDROGENASES

Since this review will focus on the rational development of mechanism-based HSD inhibitors/inactivators it is important to summarize our knowledge of the properties of enzymes that belong to this family.

The following HSD's have been purified to homogeneity from the mammalian sources indicated: 3α-HSD [rat liver,[23,24] rat brain,[25]] 3β-HSD/KSI [rat adrenal,[18] and human placenta[20]]. 11β-HSD [rat liver[26]], 17β,20α-HSD [porcine testis[11,12] and human placenta[13,14]], and 20α-HSD [rat ovary[16,17]]. With the exception of 20α-HSD from rat ovary, these enzymes have been cloned and sequenced from at least one of the sources indicated.[27-30] Of the sequences that exist there is no peptide homology across members of the family. Bacterial isoforms which were purified prior to their eukaryotic counterparts; 3(17)β-HSD from *Pseudomonas testosteroni*[31-33] and 3α,20β-HSD from *Streptomyces hydrogenans*[34] also warrant consideration since they have been used routinely in the evaluation of HSD inactivators. A list of pertinent mammalian HSDs and their properties are given in Table I. The table lists a few salient points requiring consideration in inhibitor/inactivator design. First, isozymic forms of an HSD may exist in the target tissue, e.g. microsomal versus soluble isoforms. Second, several HSDs may have dual enzyme activity which may make the dehydrogenase a less attractive drug target, e.g., human placental 17β,20α-HSD.[35,36] Third, at least one member of the HSD family, 3β-HSD/KSI catalyzes two separate reactions.[20-22] Failure to consider the distribution of isozymes and associated activities of a target HSD could result in inappropriate specificity, or inadvertent loss of desired secondary (dual) enzyme activity.

In considering the rational development of mechanism-based inhibitors for HSDs it is also important to consider the catalytic and kinetic mechanisms that drive these reactions.

3. THE CATALYTIC MECHANISM OF HYDROXYSTEROID DEHYDROGENASES

HSDs catalyze the reversible oxidoreduction of alcohol and ketone functionalities. *In vitro*, the direction of the reaction, and the ratio of the steroid products and reactants at equilibrium are affected by pH and nucleotide concentration.[1] Like alcohol dehydrogenase, HSDs are believed to catalyze the direct transfer of a hydride ion from the C-4 position of the pyridine nucleotide to the acceptor carbonyl.[37,38] Moreover, the hydride ion is transferred with a high degree of stereospecificity from either the A-or B-face of the pyridine ring resulting in the transfer of either the pro(*R*)- or pro(*S*)- hydrogen respectively. The stereochemistry of hydride transfer has been defined for many HSDs.[1,11,39-42] It was noted by Akhtar[43] that mammalian steroid dehydrogenases which transfer hydrogen to the A-face of the steroid molecule use type-B stereochemistry, while those transferring hydrogen to the B-face of the steroid molecule use type-A stereochemistry.

A systematic study of pyridine nucleotide-dependent steroid double bond reduction led to the proposal of a general mechanism of olefin and carbonyl group reduction.[44,45] As applied to both functionalities the mechanism involves compulsory polarization of the double bond to facilitate hydride transfer. In the case of an olefin, hydride transfer

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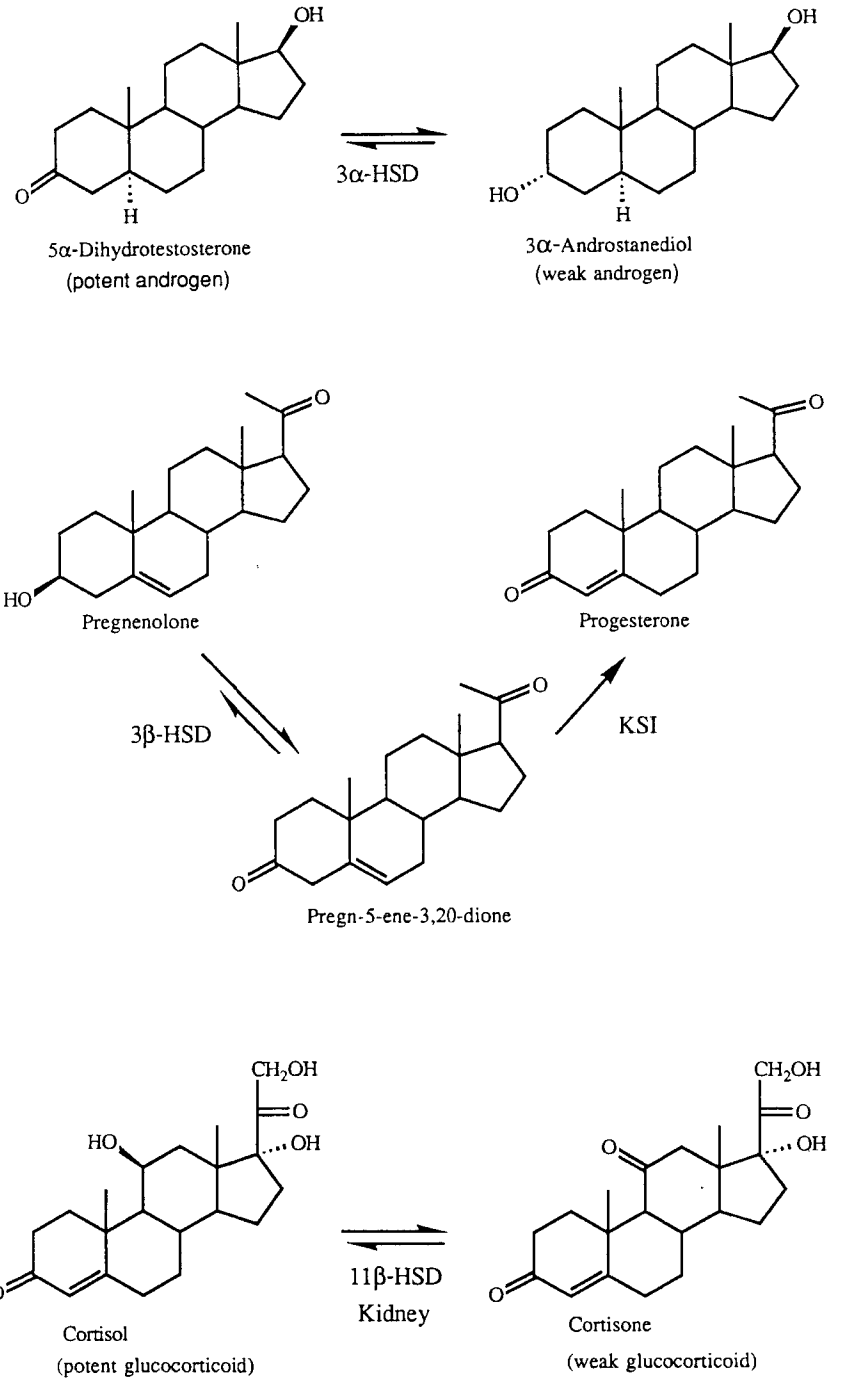

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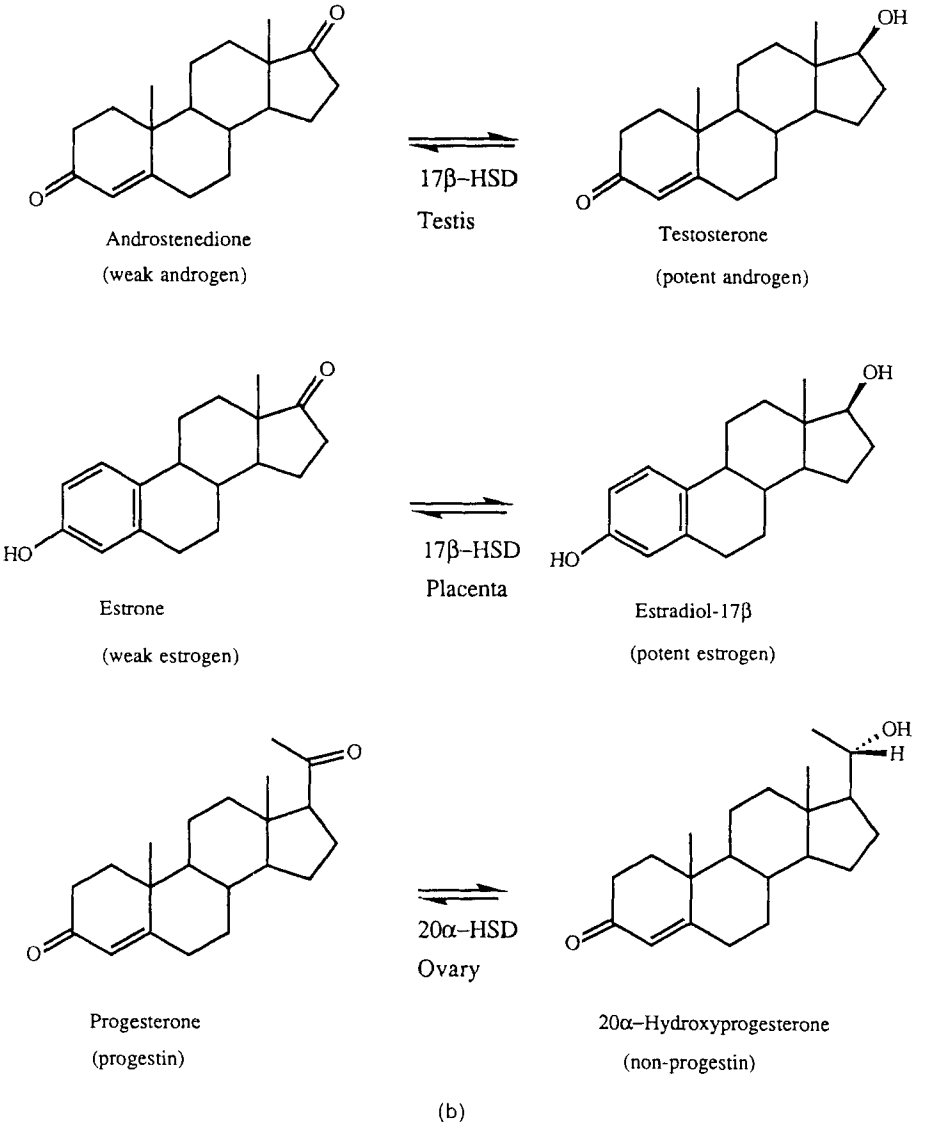

(b)

FIGURE 2 (a & b) Specific reactions catalyzed by mammalian hydroxysteroid dehydrogenases.

is directed by the most stable carbonium ion (Markovnikov addition). In the case of a carbonyl group, there is polarization of the carbonyl oxygen prior to direct hydride transfer from the reduced pyridine nucleotide. It is uncertain whether this polarization involves the formation of a formal or partial carbonium ion[46] (Figure 3). This mechanism is similar to that described for alcohol dehydrogenase in which a zinc atom is responsible for polarization of the carbonyl group.[47,48] Examination of homogenous 3(17)β-HSD from *Pseudomonas testosteroni*[44] and 17β,20α-HSD from human

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TABLE I
Some well characterized mammalian hydroxysteroid dehydrogenases

Enzyme	Source	Cofactor	Mol. Wt.	Pure	Location	Monomer	Cloned	Comments
3α-HSD	Rat Liver	$NAD(P)^+$	37,000[a]	Y	Cytosol	Y	Y[b]	Inhibited by NSAIDs
	Rat Brain	$NAD(P)^+$	34,000[c]	Y	Cytosol	Y	N	Inhibited by NSAIDs
	Rat Prostate	$NAD(P)^+$	34,000[d]	N	Cytosol	Y	N	Inhibited by NSAIDs[c]
3β-HSD/KSI	Human Placenta	NAD^+	42,000[f]	Y	Microsomal	Y	Y[g]	Dehydrogenase & Isomerase
	Bovine Adrenal	NAD^+	42,000[h]	Y	Microsomal	Y	N	Dehydrogenase only
	Bovine Ovary	NAD^+	42,000	Y		Y	Y[i]	
	Rat Testis	NAD^+		N[j]	Microsomal	Y	N	Dehydrogenase & Isomerase
	Rat Adrenal	NAD^+	42,000[k]	Y	Microsomal	Dimer	N	
	Rat Ovary	NAD^+	42,000[l]	Y		Y	Y	
11β-HSD	Rat Liver	$NAD(P)^+$	34,000[m]	Y	Microsomal	Y	Y[n]	No separate 11-ketoreductase
17β-HSD	Human Placenta	$NAD(P)^+$	36,000[o]	Y	Cytosol	Dimer[p]	Y[q]	Dual 17β,20α-HSD[r,t]
	Porcine Testis	$NAD(P)^+$	34,000[s]	Y	Microsomal	Y	N	
	Rat Testis	$NAD(P)^+$	180,000[t]	N	Microsomal			
20α-HSD	Rat Ovary	$NAD(P)^+$	36,000[u]	Y	Cytosol	Dimer	N	

[a]Penning, *et al.*, (1984) *Biochem. J.*, **222**, 601. [b]Pawlowski, *et al.*, (1991) *J. Biol. Chem.*, **266**, 8820. [c]Penning, *et al.*, (1985) *J. Biol. Chem.*, **260**, 15266. [d]Taurog, *et al.*, (1975) *Biochemistry*, **14**, 810. [e]Smithgall and Penning, (1985) *Biochem. Pharmacol.*, **34**, 831. [f]Thomas, *et al.*, (1988) *J. Steroid Biochem.*, **31**, 785. [g]Luu-The, *et al.*, (1989) *Mol. Endocrinology*, **3**, 1310. [h]Hiwatashi, *et al.*, (1985) *J. Biochem.*, **98**, 1519. [i]Zhao *et al.*, (1989) *FEBS Lett.*, **259**, 153. [j]Ishi-Ohba, *et al.*, (1986) *J. Steroid Biochem.*, **25**, 550. [k]Ishi-Ohba, *et al.*, (1986) *J. Steroid Biochem.*, **24**, 753. [l]Zhao, *et al.*, (1991) *J. Biol. Chem.*, **266**, 583. [m]Lakshmi and Monder, (1988) *Endocrinology*, **123**. [n]Agarwal, *et al.*, (1989) *J. Biol. Chem.*, **264**, 18939. [o]Jarabak and Sack, (1969) *Biochemistry*, **8**, 2203. [p]Jarabak and Sweat, (1971) *Biochemistry*, **10**, 3831. [q]Luu-The, *et al.*, (1989) *Mol. Endocrinology*, **3**, 1301. [r]Strickler, *et al.*, (1981) *J. Biol. Chem.*, **256**, 316. [s]Inano and Tamaoki, (1986) *Steroids*, **48**, 3. [t]Bogovich and Payne, (1980) *J. Biol. Chem.*, **255**, 5552. [u]Pongsawasdi and Anderson, (1984) *Biochem. Biophys. Acta.*, **799**, 51.

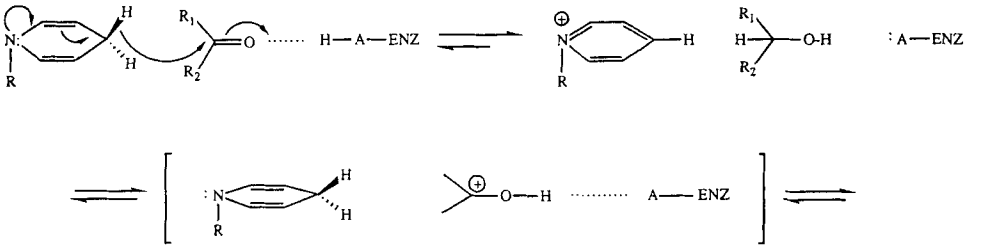

FIGURE 3 Catalytic mechanism for hydroxysteroid dehydrogenases. ENZ = enzyme, A–H = general acid at the enzyme active site, R_1 and R_2 = components of either the steroid nucleus or side chain. Polarization of the acceptor carbonyl with the formation of a formal carbonium ion is shown in parenthesis. (After Bloxham *et al.*,[46] reproduced with permission from *Steroids*).

placenta[50] for the presence of zinc by atomic absorption spectroscopy indicates that as a class HSD's are not metalloenzymes. Thus, HSD catalyzed carbonyl reduction requires an active site amino acid which can function as a general acid, while alcohol oxidation requires an amino acid which functions as a general base. The same amino acid may serve both functions. Studies have shown that histidine may function as the genral acid and base at the active sites of 3(17)β-HSD from *Pseudomonas testosteroni*[49] and human placenta 17β,20α-HSD.[50,51]

Methods of exploiting this catalytic mechanism in the design of mechanism-based inhibitors for HSDs will be discussed later.

4. THE KINETIC MECHANISM OF HYDROXYSTEROID DEHYDROGENASES

As a consequence of the direct hydride transfer between pyridine nucleotide and steroid substrate all HSDs have sequential bi-substrate kinetic mechanisms. These mechanisms can be random, in which either substrate can bind first to form a binary complex, or they can be compulsory ordered mechanisms in which only one substrate can bind to free enzyme to form a binary complex. Preferentially ordered mechanisms represent a limiting case of the random mechanism in which the flux of substrate through one enzyme-substrate binary complex greatly exceeds the flux through the alternative binary complex (Figure 4).

To date the kinetic mechanisms of seven HSDs have been determined, those displaying sequential ordered mechanisms include: *Pseudomonas* 3α-HSD,[52] rat and murine liver 3α-HSD,[42,53] *Pseudomonas* 3(17)β-HSD,[44] bovine adrenal 3β-HSD/KSI,[54] rat ovarian 20α-HSD[17] and *Streptomyces hydrogenans* 3β,20α-HSD.[55,56] In each case pyridine nucleotide binds first and leaves last. By contrast, 17β,20α-HSD of human placenta displays a random mechanism.[57] While most HSDs have ordered sequential kinetic mechanisms, it is unclear whether these mechanisms reflect compulsory or preferentially ordered pathways, although Skalhegg[52] indicates a compulsory ordered mechanism for *Pseudomonas* 3α-HSD.

Understanding the kinetic mechanism of a target HSD offers insights into the development and evaluation of HSD inhibitors and inactivators. For dehydrogenases with a compulsory ordered mechanism in which pyridine nucleotide binds first

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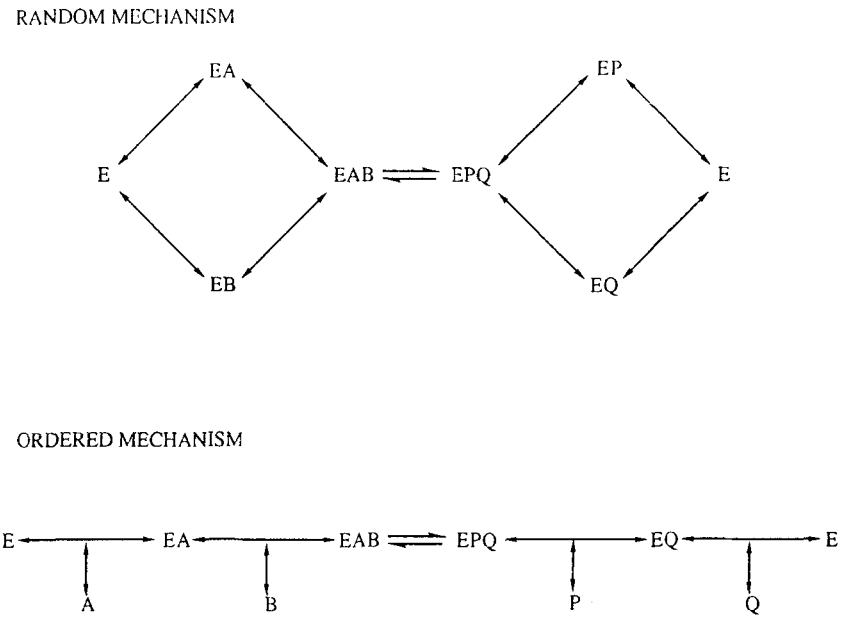

FIGURE 4 Kinetic mechanisms for hydroxysteroid dehydrogenases. E = enzyme, A = $NAD(P)^+$, B = steroid substrate, P = steroid product and Q = NAD(P)H.

and leaves last, it would be anticipated that the species subjected to inhibition or inactivation by a steroid analog would be the binary E·NAD(P)(H) complex. For dehydrogenases displaying a random mechanism, the species subjected to inhibition or inactivation by a steroid analog could be either E or E·NAD(P)(H). The resultant binary and ternary complexes may differ in their dissociation constants for an inhibitor, and may have variable susceptibility to alkylation by an enzyme inactivator.

Kinetic mechanism may also be important in considering the design of transition state analogs. If these compounds are bi-substrate analogs their ability to form tight binding complexes with their target enzyme may be diminished by the presence of either substrate if the enzyme has a random mechanism, or by NAD(P)(H) if the enzyme has an ordered mechanism. Some tight binding inhibitors of 3β-HSD have been proposed to act as transition state or reaction intermediate analogs.

5. CLASSES OF ENZYME INHIBITORS

Compounds which inhibit HSDs have the potential to regulate steroid hormone action, and fall into one of three classes: (a) selective reversible inhibitors, (b) affinity alkylators, (c) mechanism-based inhibitors.

The first class of agents relies on binding affinity for their selectivity. This class of agents excludes substrate access to the catalytic site by direct competition. Such

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compounds are effective therapeutics as long as appropriate concentrations can be maintained. Simple competitive inhibitors which fit into this category will not be discussed in this review.

The second class of compounds, affinity alkylators (generally substrate or competitive inhibitor analogs) contain a nucleophilic acceptor, and undergo reactions which result in the covalent modification of the active site of the target enzyme. Like reversible inhibitors, they rely on affinity for their specificity. Archetypical HSD affinity alkylators are the bromoacetoxy-steroids.[58-61]

The interaction of an affinity alkylator with its target enzyme is shown in Figure 5 (Eqn. 1). This simplified scheme does not account for the complication introduced by kinetic mechanism or the presence of competing substrate. One advantage to affinity alkylators is a potentially long duration of action, since *de-novo* protein synthesis is presumably required for the restoration of enzyme activity. However, because affinity alkylators are introduced as reactive molecules, they can interact with low molecular weight nucleophiles or cellular macromolecules prior to reaching their target, and therefore have diminished specificity. While such compounds are useful for active site mapping their high reactivity with extraneous nucleophiles renders them ineffective as therapeutics.

The third class of compounds which can block the action of HSDs and thereby regulate steroid hormone action are mechanism-based inhibitors. Loosely defined, mechanism-based inhibitors encompass all compounds which exploit some component of the target enzyme's catalytic mechanism to promote enzyme inhibition or inactivation. Under this definition tight-binding enzyme inhibitors (reaction intermediate and transition state analogs) can be considered to have a mechanism-based action. For a compound to be considered an analog of an intermediate or transition state its structure must resemble that reaction intermediate on the catalytic path of the target enzyme. Unlike inactivators which covalently modify their target enzyme, these compounds can produce stoichiometric pseudo-irreversible inhibition due to their slow rate of dissociation and can effect V_{max} (Figure 5 (Eqn. 2)). Although no true transition state analogs have been described for HSDs, several tight-binding inhibitors have been developed which might be categorized as intermediate state analogs.

A more stringently defined class of enzyme-activated compounds, suicide substrates, requires target enzyme activation of a latent precursor to yield a reactive species which subsequently will inactivate the target enzyme. Suicide inactivation must meet several criteria: (1) The compound must be converted by the enzyme from an innocuous form to a reactive alkylator. (2) The compound must cause a time-dependent loss of enzyme activity which is generally pseudo-first order with respect to enzyme. (3) The inactivation must display saturation kinetics. (4) The inactivation of target enzyme must occur prior to the release of the reactive alkylating species. (5) The target enzyme is covalently modified by the activated compound. (6) The inactivation process is slowed by the presence of competing substrate.

Compounds fitting these criteria were originally described by Helmkamp *et al.*,[62] and Endo *et al.*,[63] as inactivators of β-hydroxydecanoylthioester dehydrase. Suicide substrates have also been termed "k_{cat} inhibitors" and "Trojan horse reagents"[64] . . . The properties of suicide substrates have been the subject of several reviews.[64-69] In this review we will make the distinction between: suicide substrates and enzyme-generated affinity alkylators (compounds that require enzymatic conversion to a reactive species, but are released prior to the inactivation event). Another class of

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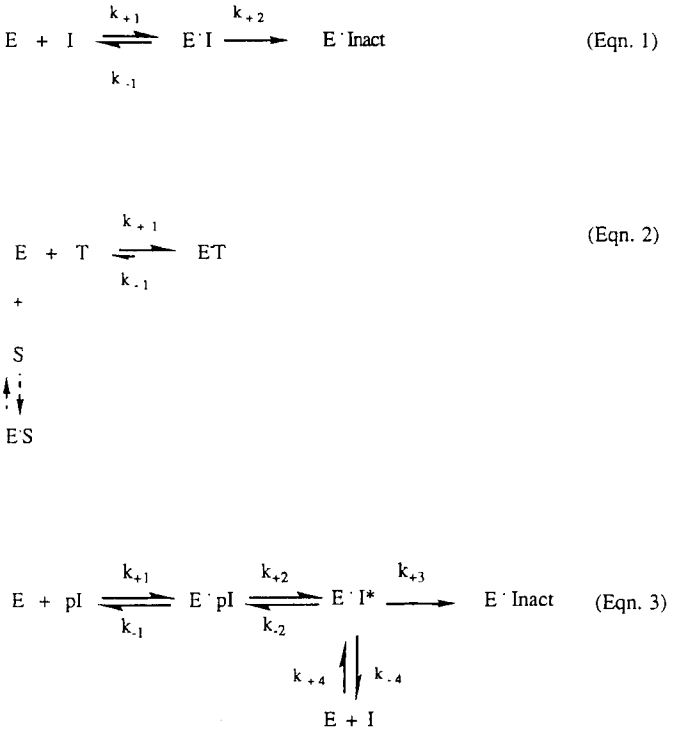

FIGURE 5 Simple kinetic schemes which depict modes of hydroxysteroid dehydrogenase inhibition or inactivation. Inactivation by an affinity alkylator (Eqn. (1)), inhibition by a tight-binding inhibitor (T) where (S) is substrate (Eqn. (2)), inactivation by a suicide substrate where pI = proinhibitor and I = enzyme-generated inactivator (Eqn. (3)).

compounds that satisfy many of the criteria ascribed to suicide substrates are enzyme-activated inactivators (compounds which are activated to a reactive species and cause enzyme inactivation without formal turnover).

Data generated with enzyme inactivators has been evaluated by the method of Kitz and Wilson,[70] which assumes a simple model of enzyme inactivation as shown in Figure 5 (Eqn. 3) where k_{+3} (covalent bond formation) is regarded as the rate-limiting step. In determining the efficiency of a suicide substrate a conceptual value frequently quoted is the partition-coefficient or partition-ratio introduced by Walsh.[68] This ratio is a measure of the number of inactivation events relative to the number of latent inactivator molecules turned over. It is expressed formally as k_{cat}/k_{inact}. Ideally this number should be as close to 1.0 as possible, so that for each molecule of inactivator turned over one molecule of enzyme is inactivated. A consequence of a low partition ratio is that is may be exceedingly difficult to detect substrate turnover prior to enzyme inactivation unless high concentrations of enzyme are used. When this ratio exceeds 1.0 a portion of the enzyme generated alkylator is released into solution before inactivating the enzyme, i.e., the k_{-4} term is significant. When a high partition-ratio exists an enzyme generated affinity-labeling sitution is created in which it is possible

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for the released alkylator to alkylate both the target enzyme and other molecules. Under these conditions selectivity built into the latent compound can be lost.

In the case of suicide inactivation, kinetic mechanism may influence the size of the partition ratio. Since HSD·NAD(P)(H) complexes can be refractory to inactivation by alkylation,[58–60] those HSDs with a random order of product release can form significant amounts of E·I which will lead to inactivated enzyme (Figure 6). In contrast, HSDs with ordered mechanisms all have pyridine nucleotide binding first and leaving last, thus inactivator dissociates from E·NAD(P)(H) limiting the potential for active site alkylation. Together these arguments imply that HSDs with an ordered mechanism of product release will tend to have higher partition coefficients than their random counter parts.

Despite these considerations, suicide substrates and enzyme-activated inactivators have advantages over reversible inhibitors and affinity alkylators when employed *in vivo*. First, since the inactivators are introduced in an innocuous form, they are more likely to alkylate their target enzyme rather than other macromolecules. Second, the requirement for activation by the target enzyme provides them with an increased specificity over reversible inhibitors and affinity labeling agents. Third, like affinity alkylators, these compounds covalently modify their target enzyme and they should have a long duration of action which can only be overcome by *de-novo* protein synthesis. Fourth, HSD inactivators should have poor specificity for steroid hormone receptors, therefore their effects can be immediately overcome by administration of receptor agonist/antagonists. In contrast, the complete reversal of a receptor ligand's action will be hindered by its presence after the administration of an appropriate antagonist.

6. MECHANISM-BASED INHIBITORS

This review will consider enzyme-activated compounds in the broadest sense. It will examine tight binding inhibitors of HSDs, some of which may exploit components of their target enzymes catalytic mechanism (steroid pyrazoles and azasteroids). It will describe both the development of steroidal and nonsteroidal latent Michael acceptors which can act as suicide substrates or enzyme-generated affinity alkylators, and the development of steroidal and nonsteroidal epoxides which have the potential to act as enzyme-activated inactivators.

6.1 Tight-Binding Inhibitors of HSDs

The majority of tight binding inhibitors for HSDs have been described for 3β-HSD/KSI with the anticipation that these agents may act as contragestational agents. These compounds include: 2α-substituted ketones (containing nitrile and hydroxymethylene functionalities), steroid heterocycles (isoxazoles and pyrazoles) and the 4-azasteroids. Compounds from each of these groups display nanomolar affinity for their target enzyme and several display pseudo-irreversible enzyme inhibition. Recent evidence suggests that the steroid pyrazoles are believed to act as activated-ternates and the azasteroids are believed to function as intermediate-state analogs. Mechanisms have been suggested to explain the high affinity of the 2α-substituted ketones for *Pseudomonas* and mammalian 3β-HSDs, however a detailed knowledge of their mechanism is unknown.

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INFLUENCE OF KINETIC MECHANISM ON INACTIVATION OF HSDs BY SUICIDE SUBSTRATES

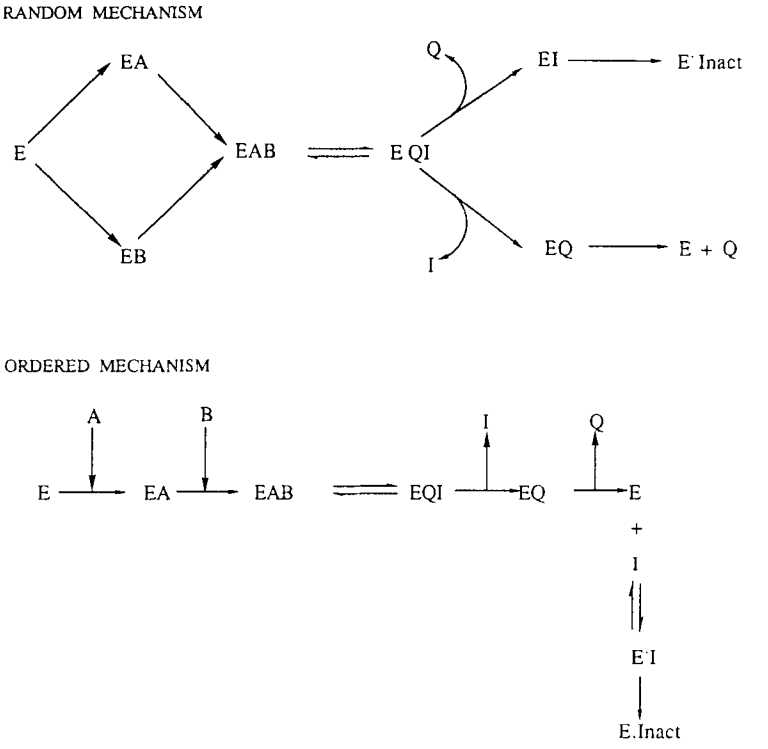

FIGURE 6 Influence of kinetic mechanism on inactivation of hydroxysteroid dehydrogenases by suicide substrates. In each case I [enzyme-generated inactivator] is substituted for P in the Cleland notation.[114-116] A = $NAD(P)^+$, B = proinactivator, Q = NAD(P)H, E = enzyme, E·Inact = Inactivated enzyme. In the ordered mechanism an abortive binary complex represented by E·I forms only after the enzyme generated inactivator is released from the central complex EQI.

6.1.1. 2α-Substituted ketones and steroid isoxazoles 2α-Cyano-4,4,17α-trimethyl-androst-5-en-17β-ol-3-one (cyanoketone) **I** and 2-hydroxymethylene-5α-androstan-17β-ol-3-one **II** (hydroxymethylene) were found to be very effective inhibitors of the 3(17)β-HSD from *Pseudomonas testosteroni*.[71] Goldman found both of these compounds were potent inhibitors of the bovine adrenal cortex 3β-HSD/KSI and that these compounds were pseudo-irreversible and acted as stoichiometric inactivators.[72,73] These compounds also produced persistent inhibition of the adrenal activity and thus promoted adrenal insufficiency.[74] These early observations sparked interest in this field and analogs of these original compounds have been studied. Of particular interest are: trilostane [(4α,5α,17β)-4,5-epoxy-3,17-dihydroxyandrost-2-ene-2-carbonitrile, enole of **VI**], epostane [(4α,5α,17β)-4,5-epoxy-3,17-dihydroxy-4,17-dimethylandrost-2-ene-2-carbonitrile, enol of **V**] and 2α-cyanoprogesterone **IV**.

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A summary of their inhibitory properties for mammalian 3β-HSD/KSI are given in Table II.

Trilostane and epostane are closely related and are potent inhibitors of the bovine adrenal and human placental 3β-HSD *in vitro*, yielding K_i values in the nanomolar range.[6] 2α-Cyanoprogesterone, an epostane analog, also potently inhibits mammalian 3β-HSD.[5] However, 2α-cyanoprogesterone demonstrates a greater specificity for the bovine corpus luteum enzyme (K_i = 15 nM) than for either the bovine adrenal (K_i = 150 nM) or human placental isoforms (K_i = 1 μM).[5] Because of this property it has been suggested that this compound, like epostane could act as an anti-gestational agent.[5]

The steroid isoxazoles (of the general type 17β-hydroxy-4,4-17α-trimethylandrost-5-en-(2,3d)-isoxazole, **III** represent the corresponding fused-ring heterocycles of the 2α-substituted cyanoketones. These compounds also act as tight binding inhibitors of 3(17)β-HSD from *Pseudomonas testosteroni* and 3β-HSD/KSI from bovine adrenal cortex.[71–73]

The similarities between 2α-cyano-ketones and the steroid isoxazoles have been recognized for some time. It has been suggested that a charge distribution over the cyanoketone or steroid isoxazole may resemble that observed in the central complex [E·NAD(P)H·Ketosteroid][75] (Figure 7). This mechanism would predict that competition would occur between the steroid inhibitor and the pyridine nucleotide cofactor, thus tight binding should not be observed in enzymes which display an ordered mechanism with pyridine nucleotide binding first. Since these compounds display tight binding to bovine adrenal 3β-HSD/KSI which appears to have an ordered kinetic mechanism,[54] this implies that an alternative mechanism of inhibition is involved.

6.1.2. Steroid pyrazoles The concept that steroidal pyrazoles could have a mechanism-based action for the inhibition of HSDs was developed by Levy and co-workers[49] and was based on the classical work of Theorell[76] which demonstrated that liver alcohol dehydrogenase is potently inhibited by the zinc chelator pyrazole. Crystallographic studies indicate the basis of pyrazole inhibition is the formation of a coordinate complex, with one pyrazole ring nitrogen coordinated to zinc while the other pyrazole nitrogen is coordinated with the C-4 of the nicotinamide ring.[77,78] Although HSDs do not contain essential metal ions at their active sites,[49,50] they employ a general acid to affect the polarization of the acceptor carbonyl (see Section 3), suggesting that pyrazole inhibition of HSDs may also be possible. Levy and co-workers[49] have developed this concept by synthesizing a series of 2,3- and 3,4-fused ring steroidal pyrazoles (**VIII-XI**). Tests indicated these compounds were effective inhibitors of *Pseudomonas* 3(17)β-HSD yieldng K_i values as low as 20 nM, (Figure 8).

Since compounds **VIII-XI** bind to the E·NAD$^+$ complex, this effectively rules out the pyrazole ring acting as pyridine-ring substitute. Chemical modification studies with diethylpyrrocarbonate have provided evidence for the involvement of an essential histidine at the enzyme's active site which may act as the general acid in catalysis.[49] Based on these observations Levy *et al.*[49] have proposed a model in which NAD$^+$, the active site histidine, and the steroid pyrazole form an activated-ternate. In this model the pyrazole ring is oriented to share the hydrogen of one of its ring nitrogens with a lone pair provided by an enzyme histidine while the lone pair of the remaining pyrazole ring nitrogen coordinates with C-4 of the nicotinamide ring (Figure 9). It is possible that an activated-ternate complex may also explain the inhibition of 3β-HSD

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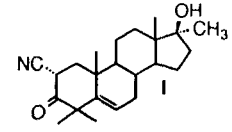
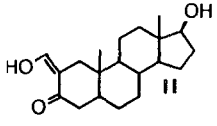
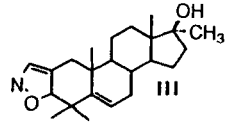
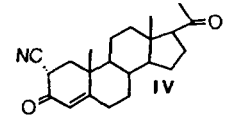
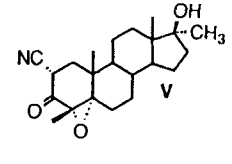
TABLE II
Tight-binding inhibitors of 3β-HSD/KSI

Compound	Enzyme 3β-HSD	IC_{50}	K_i		Enzyme KSI	IC_{50}	K_i	
	Bovine adrenal Human placenta		700 nM 3.5 nM	NC[a] NC[b]	Bovine adrenal		600 nM	C[a]
	Bovine adrenal		1,700 nM	NC[a]	Bovine adrenal		80 nM	C[a]
	Bovine adrenal Human placenta		7,100 nM 1,500 nM	NC[a] NC[b]	Bovine adenal		15,000 nM	C[a]
	Bovine adrenal Bovine corpus lutea Human placenta	120 nM 66 nM 700 nM	150 nM 15 nM 1000 nM	NC[c] C[c] NC[c]	Bovine adrenal Bovine corpus lutea Human Placenta		50 μM 70 μM N.D.	C[c]
	Human placenta		1.0 nM	C[b]				

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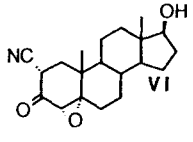
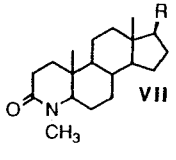
 VI	Human placenta	4.0 nM	C[b]
 VII	Bovine adrenal	30 nM	NC[d]

NC = noncompetitive inhibition, C = competitive inhibition, N.D. = not determined.

[a]Inhibition constants for bovine adrenal microsomal 3β-HSD/KSI from Goldman (1968) *Clin. Endocrinol.*, **28**, 1539. [b]Inhibition constants for human placental mitochondrial 3β-HSD, from Rabe *et al.*, (1983) *Fertil. Steril.*, **39**, 6. [c]From Sharp *et al.*, (1989) *Biochem. J.*, **230**, 587. [d]From Brandt and Levy (1989) *Biochemistry*, **28**, 140.

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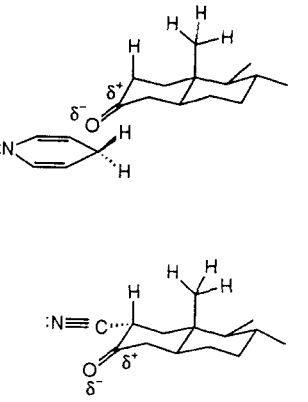

FIGURE 7 Charge distribution in 2α-cyanoketosteroids mimics that observed in the pyridine nucleotide reduction of 3-ketosteroids to 3β-hydroxysteroids.

by the 2,3-isoxazoles if a lone pair of electrons on the isoxazole oxygen fulfills the role of a lone pair on a pyrazole nitrogen.

6.1.3. 4-Azasteroids Azasteroids of the general type (*N*,*N*-diethyl-4-methyl-3-oxo-4-aza-5α-androstane-17β-carboxamide **VII**) have been described as tight binding inhibitors of steroidogenic enzymes (5α-reductase) that form a Δ^3-enolate intermediate.[79,80] These compounds also inhibit 3β-HSD/KSI of rat testicular microsomes[81] and granulosa cells.[82] By donating the lone pair of electrons from the N-4 position of the azasteroid these compounds appear to mimic the Δ^5-3-dienol reaction intermediate of the isomerase reaction.[83] Based on this argument it would be predicted that the 4-azasteroids would inhibit the isomerase rather than the dehydrogenase function of 3β-HSD/KSI.

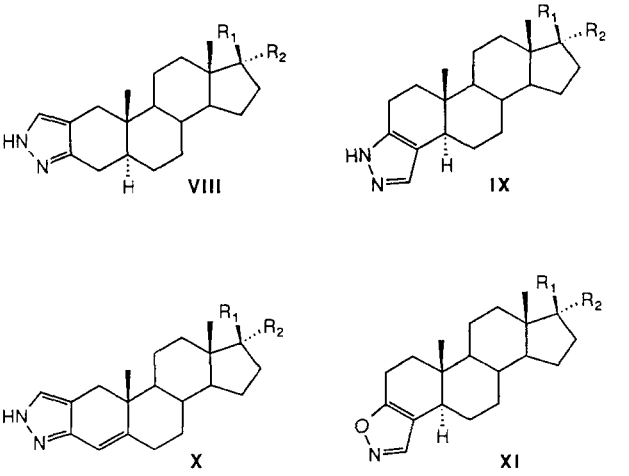

FIGURE 8 2,3- and 3,4-fused steroid pyrazoles examined as inhibitors of 3(17)β-hydroxysteroid dehydrogenase.

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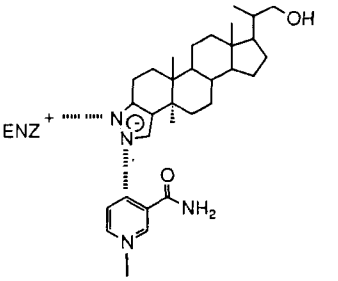

FIGURE 9 An activated-ternate formed with a 3,4-fused pyrazole during the inhibition of 3β-HSD, (Reproduced with permission from *Biochemistry*).

Brant and Levy[54] have studied the ability of 4-azasteroids to inhibit bovine adrenal 3β-HSD/KSI. It was demonstrated that azasteroids preferentially bind to the E·NAD$^+$ complex but they do not alter the ratio of Δ^5 to Δ^4 3-ketosteroids negating inhibition of the ketosteroid isomerase. By uncoupling the dehydrogenation and isomerization reactions it was shown tht the azasteroids had a much higher affinity for the dehydrogenase than the isomerase active site. Since the 3β-HSD reaction requires polarization of the acceptor carbonyl (reverse direction) it is believed that the amide carbonyl of the 4-azasteroid could act as an analog of the polarized reaction intermediate. Formation of this "intermediate-state analog" might explain the high affinity of 4-azasteroids for this enzyme.

6.2. Enzyme-Generated Inactivtors

The observed inactivation[66] of yeast alcohol dehydrogenase by allyl alcohol in the presence of NAD$^+$ and the subsequent work by Alston *et al.*,[84] and MacInnes *et al.*,[85] introduced the concept that simple allylic alcohols could effectively inactivate an alcohol dehydrogenase. This led to the concept that one method of exploiting the catalytic mechanism of HSDs was to introduce α,β-unsaturated alcohols (latent Michael acceptors) into the steroid nucleus and side chain. Upon enzymatic oxidation, this group would be converted to an α,β-unsaturated ketone (a Michael acceptor) which could ultimately lead to suicide inactivation (Figure 10).

6.2.1. Steroid latent michael acceptors as HSD inactivators. Covey's[86–88] and Robinson's[89–90] laboratories were among the first to synthesize unsubstituted steroidal latent Michael acceptors as potential suicide substrates for 3α,20β-HSD (*Streptomyces hydrogenans*), 17β,20α-HSD (human placenta), 3(17)β-HSD (*Pseudomonas testosteroni*) and 3β-HSD/KSI (bovine adrenal) (Table III).

Results with these latent Michael acceptors (**XII–XVIII**) can be summarized briefly. First, enzyme inactivation has an obligatory requirement for NAD(P)$^+$ indicating the oxidation of the latent Michael acceptor is a prerequisite for covalent modification. Second, the latent Michael acceptors are poor substrates and are oxidized at only a fraction of the utilization ratio documented for the native substrate. One exception exists to the second generalization, 16-methylene estradiol (**XVI**) which was developed as a suicide substrate for human placental 17β,20α-HSD.[88] This latent inactivator is oxidized to 16-methylene-estrone **XXVII** (α,β-unsaturated ketone) at rates approaching

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EXPLOITATION OF ENZYME MECHANISM I

ENZYME GENERATION OF MICHAEL ACCEPTORS

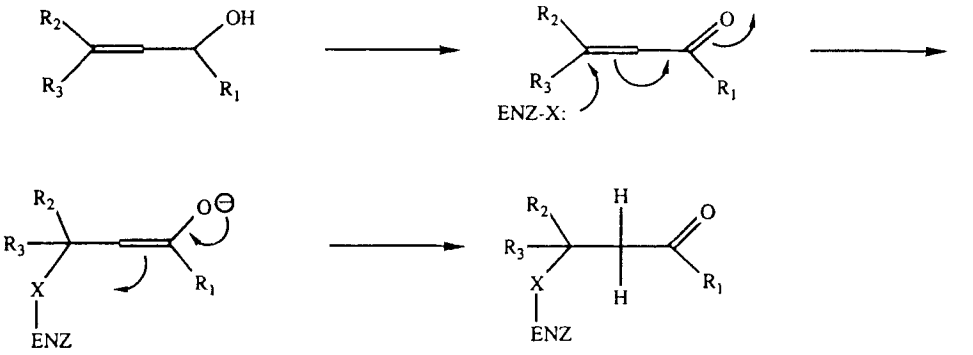

FIGURE 10 Enzyme generation of active Michael acceptors. ENZ-X = enzyme nucleophile, R_1–R_3 are components of either the steroid nucleus and/or side chain.

those of the normal substrate. However, 16-methylene estrone inactivates the enzyme rather slowly yielding a limiting $t_{1/2}$ life of 2.7 h at pH 7.0.

Studies in which the active steroidal Michael acceptors were used as HSD inactivators indicate that slow rates of enzyme inactivation are observed with $t_{1/2}$ lives ranging from 7.2–26 min. (Table IV). An exception to these findings is the inactivation of rat ovarian 20α-HSD by 1-(5α-androstan-3-one-17β-yl)-2-propen-1-one **XXIX**, where the limiting $t_{1/2}$ life is only 20 s. Unfortunately, the parent allylic alcohol was not a substrate for the enzyme.[91]

When the rates of oxidation of latent steroidal Michael acceptors are compared with the rates of inactivation achieved with the enzyme-generated compounds, it is apparent that the latent inactivators have high effective partition ratios for HSDs.

In designing a series of inactivators for rat liver 3α-HSD Penning *et al.*,[92] synthesized a group of steroid Michael acceptors with olefins exocyclic to the A- and B-rings. In order to circumvent the difficult synthesis of allylic 3α-hydroxysteroids, the more accessible steroidal ketones (Michael acceptors) were synthesized and tested first (**XXII–XXV**). Of these compounds, several displayed nanomolar affinity for 3α-HSD. However, since all the compounds failed to inactivate 3α-HSD,[92] none of the allylic alcohols were synthesized.

Studies with steroidal Michael acceptors emphasized two problems associated with this approach to inactivator design. First, latent steroid Michael acceptors are not oxidized well. Second, despite high-affinity, Michael acceptors are inefficient inactivators indicating they are not reactive enough to undergo nucleophilic attack at the active site. These observations have led to the concept that it may be necessary to alter the turnover and reactivity of the latent Michael acceptor by introducing activating groups on either side of the unsaturated alcohol.

Covey *et al.*[93] introduced the concept of adding substituents α to the acetylene of a latent Michael acceptor by synthesizing an ethoxyacetylenic alcohol [17β-[(1S)-hydroxy-3-ethoxy-2-propynyl]androst-4-en-3-one, **XXX**] which could function as a

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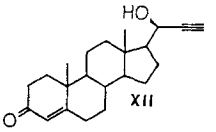
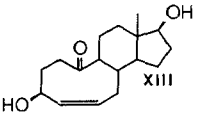
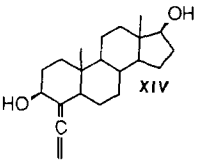
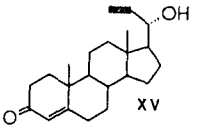
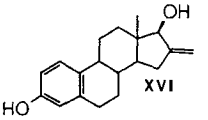
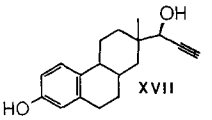
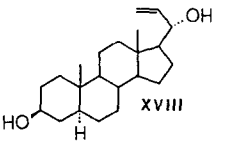
TABLE III
Comparison of latent steroidal Michael acceptors as inactivators of HSDs

Latent Michael Acceptor	Enzyme	Oxidation Turnover	K_m	V_{max} [a]	Inactivation k_{inact}	Inactivation $t_{1/2}$
 XII	3α,20β-HSD[b] Streptomyces	3.8%[c]	145 μM	63.0		16 min[d] at 10 μM
	3(17)β-HSD[e] Pseudomonas	150%[f]	N.D.	N.D.	None	
 XIII	3β-HSD[e] Bovine adrenal	Yes[g]	N.D.	N.D.	N.D.	40 min at 30 μM
 XIV	3β-HSD[h] Bovine adrenal	Yes[g]	N.D.	N.D.	N.D.	68 min at 87 μM
 XV	17β,20α-HSD[i] Human placenta	0.03%[c]	435 μM	0.04	N.D.	500 min at 50 μM
 XVI	17β,20α-HSD[j] Human placenta	108%[c]	8 μM	2.8	N.D.	60 min[d] at 100 μM
 XVII	17β,20α-HSD[k] Human placenta	0.02%	79 μM	0.008	N.D.	45 min at 40 μM
 XVIII	20α-HSD[l] Rat Ovarian	No				

[a] V_{max} is in μmoles/min/mg. [b] Values taken from Strickler *et al.*, (1980) *Biochemistry*, **19**, 4950. [c] Values are given as a percent of the utilization ration (V_{max}/K_m) observed with the normal substrate. [d] Inactivation rates reported at pH 9.2. [e] Values taken from Balasubramanian and Robinson (1981) *Biochem. Biophys. Res. Commun.*, **101**, 495. [f] Relative rates of oxidation where the rate of oxidation for the normal substrate is given as 100%. [g] Absolute rates not given. [h] Values taken from Balasubramanian *et al.*, (1982) *Steroids*, **40**, 109. [i] Values taken from Tobias *et al.*, (1982) *J. Biol. Chem.*, **257**, 2783. [j] Values taken from Thomas *et al.*, (1983) *J. Biol. Chem.*, **258**, 11500. [k] Values taken from Auchus and Covey (1986) *Biochemistry*, **25**, 7295. [l] Values taken from Ricigliano and Penning (1986) *Biochem. J.*, **240**, 717.

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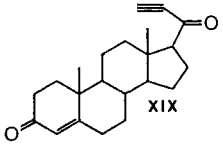
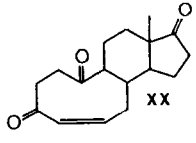
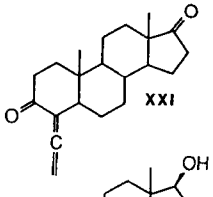
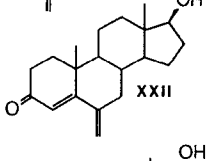
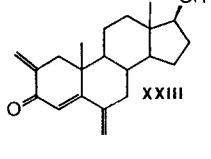
TABLE IV
Comparison of steroidal active Michael acceptors as inactivators for HSDs

Active Michael Acceptor	Enzyme	Reversible Inhibition K_i	Inact.	Irreversible Inhibition K_i	k_{+3}	$t_{1/2}$
 XIX	3α,20β-HSD Streptomyces	N.D.	Yes	40 μM	1.8×10^{-2} s^{-1}[b]	
 XX	3(17)β-HSD[c] Pseudomonas	N.D.	No			
	3β-HSD[c] Bovine Adrenal	N.D.	Yes[d]	N.D.	N.D.	
 XXI	3β-HSD[e] Bovine Adrenal	2.6 μM C	Yes	N.D.	N.D.	26 min at 82 μM
 XXII	3α-HSD[f] Rat Liver	0.44 μM C	No	–	–	–
 XXIII	3α-HSD[f] Rat Liver	4.4 μM C	No	–	–	–

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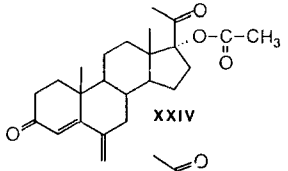
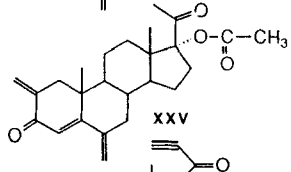
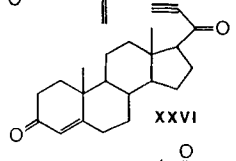
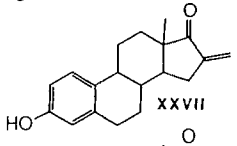
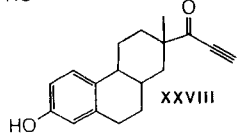
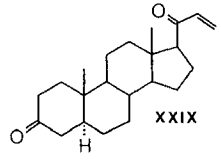
 XXIV	3α-HSD[f] Rat Liver	0.05 μM C	No	–	–	–
 XXV	3α-HSD[f] Rat Liver	4.4 μM C	No	–	–	–
 XXVI	17β,20α-HSD[g] Human Placenta	N.D.	Yes	190 μM	$9.4 \times 10^{-2} s^{-1}$[b]	
 XXVII	17β,20α-HSD[h] Human Placenta	N.D.	Yes			7.2 min[b] at 25 μM
 XXVIII	17β,20α-HSD[i] Human Placenta	N.D.	Yes	2.8 μM		limiting $t_{1/2}$[j] 12 min
 XXIX	20α-HSD[j] Rat Ovary	2.0 μM C	Yes	2.0 μM		20 s at sat.

N.D. = not determined. C = competitive inhibition. [a]Values taken from Strickler *et al.*, (1980) *Biochemistry*, **19**, 4950. [b]Determined at pH 9.2; values at pH 7.0 gave slower inactivation rates. [c]Values taken from Balasubramanian and Robinson (1981) *Biochem. Biophys. Res. Commun.*, **101**, 495. [d]Enzyme activity was lost over 45 min but no kinetic constants were determined. [e]Values taken from Balasubramanian *et al.*, (1982) *Steroids*, **40**, 109. [f]Values taken from Penning *et al.*, (1991) *Steroids*, In press. [g]Values taken from Tobias *et al.*, (1982) *J. Biol. Chem.*, **257**, 2783. [h]Values taken from Thomas *et al.*, (1983) *J. Biol. Chem.*, **258**, 11500. [i]Values taken from Auchus and Covey (1986) *Biochemistry*, **25**, 7295. [j]Values taken from Ricigliano and Penning (1986) *Biochem. J.*, **240**, 717.

suicide substrate for *Streptomyces hydrogenans* 3α,20β-HSD (Figure 11). Introduction of this electron donating group would predict that hydride transfer to and from the acceptor carbonyl would be slowed, and that the ethoxyacetylenic ketone would be less reactive in solution. As predicted ethoxylation was found to reduce the V_{max} of oxidation by 10-fold, but the K_m was reduced to 70-fold giving the compound an increased utilization ratio. Furthermore, ethoxylation reduced the reactivity of the acetylenic ketone **XXXI** in solution, permitting inactivation in the presence of 1 mM glutathione. Overall, **XXX** is an efficient inactivator of *Streptomyces hydrogenans* 3α,20β-HSD, yielding a $t_{1/2}$ of less than 10 s at saturation. Apparently, the success of the ethoxyacetylenic alcohol is due to an optimization of two factors, turnover and reactivity.

A priori it may be predicted that introduction of an electron withdrawing group to the unsaturated bond of a latent Michael acceptor would lead to an increase in the reactivity of the resultant enzyme generated α,β-unsaturated ketone. By contrast introduction of an electron withdrawing group geminal to the hydroxyl group of a latent Michael acceptor promotes turnover by facilitating the deprotonation event that occurs during oxidation; it also increases the reactivity of the enzyme generated Michael acceptor (Figure 12). Moreover, electron-withdrawing groups introduced adjacent to the hydroxyl group leave the electrophilic carbon sterically unhindered and more accessible to nucleophilic attack.

Studies on the effects of electron withdrawing groups on the turnover and reactivity of latent and activated steroidal Michael acceptors have been limited. Work by Covey and co-workers[94] has shown that a strong electron withdrawing group added to the unsaturated bond of a latent Michael acceptor can promote direct nucleophilic attack. The introduction of a trifluoromethyl group to a triple bond of a secosteroid propargylic alcohol **XXXII** resulted in the formation of a potent affinity alkylator for human placental 17β-HSD which did not require oxidation of the alcohol for enzyme inactivation.[44] This result may have been anticipated based upon the electron-withdrawing effects of the trifluoromethyl group and the factors which affect Michael addition reactions.[95]

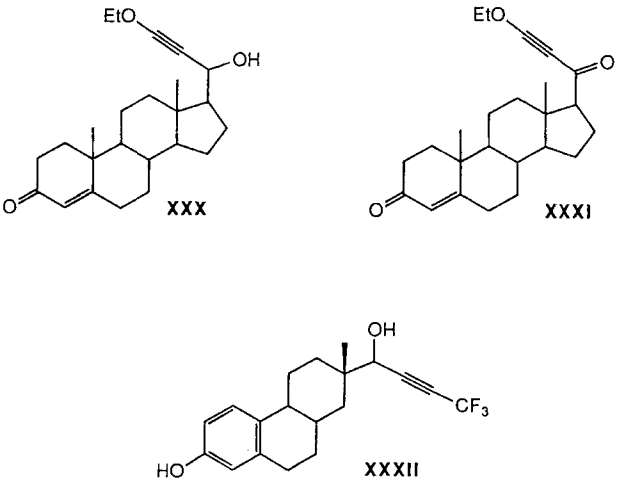


FIGURE 11 Steroid latent and active Michael acceptors containing electron withdrawing and donating substituents.

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INFLUENCE OF ELECTRON WITHDRAWING SUBSTITUENTS ON LATENT AND ACTIVATED MICHAEL ACCEPTORS

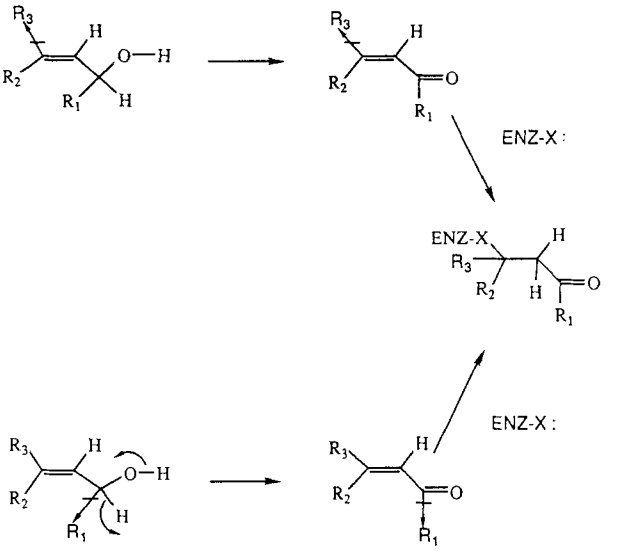

FIGURE 12 Influence of electron-withdrawing substituents on latent and activated Michael acceptors. ENZ-X = enzyme nucleophile, R_1–R_3 = components of either the steroid nucleus and/or side chain.

Attempts to inactivate HSDs by introducing halogen or cyano substituents adjacent to α,β-unsturated alcohols are complicated since a number of modes of enzyme inactivation are possible.[96] For example, introduction of a cyano group α to an α,β-unsaturated ketone could produce enzyme inactivation by Michael addition, Michael addition to an isomerized ketenimine, or direct S_N2 displacement of the cyano group. Examination of 6α-cyano-pregn-4-ene-3,20-dione (6-cyanoprogesterone) as an inactivator of rat liver 3α-HSD failed to yield inactivated enzyme.[96] By contrast, 6β-bromo-androst-4-en-3-one-17-acetate will inactivate 3α-HSD, although it is uncertain whether Michael addition or nucleophilic displacement of the bromine occurs. In this instance the instability of the corresponding 3α-alcohol precluded its evaluation as a suicide substrate.[96] However, 6β-bromo-androst-4-en-3-one-17-acetate is a substrate for 3α-HSD implying that the Δ^4-3-ketone system is strongly affected by this substituent.

Overall, these findings imply tht the effectiveness of steroid based Michael acceptors are not limited by their affinity for their target enzymes (e.g., affinity of 2,6-dimethylene pregnanes for 3α-HSD is in the nanomolar range, see Table IV), but by the ability of their latent precursors to act as substrates, and by the ability of the Michael acceptors to act as alkylators. Introduction of modifying groups into the latent and activated steroid Michael acceptors may provide significant improvements. The introduction of such groups has occurred on a limited scale and has not been pursued systematically.

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One reason for the lack of progress in the design of suicide substrates for HSDs is that the rigid confines of the steroid nucleus poses severe limitations on the nature of the Michael acceptor and electron withdrawing or donating groups that can be introduced. The work of Ringold[97,98] which demonstrated that multiple points of interaction between an HSD and a steroid substrate contribute to overall binding affinity, argues that an intact steroid nucleus represents the appropriate parent compound for the introduction of latent groups. Faced with these limitations several groups have elected to synthesize secosteroids to accomodate latent and active Michael acceptors (see Table III and Table IV). While the use of secosteroids circumvents the problems associated with the rigid confines of the steroid nucleus, they have not successfully addressed the issue of poor turnover or reactivity. Overall the use of secosteroids has not yielded significant gains in enzyme inactivation.

6.2.2. Nonsteroidal latent michael acceptors as HSD inactivators. An alternative approach to the development of suicide substrates for HSDs would be to replace the steroid nucleus with a nonsteroid. This discussion will be limited to nonsteroid latent and activated Michael acceptors that have been developed for 3α-HSD. While several nonsteroidal substrates have been described for rat liver 3α-HSD,[23] 4-nitrobenzaldehyde and 4-nitroacetophenone were considered the most promising because: (1) the compounds have utilization ratios within an order of magnitude of that reported for steroidal substrates, (2) substituents at the para-position are subject to the electron withdrawing action of the nitro-group which could increase turnover and/or reactivity, (3) Michael acceptors can be introduced so that the reaction intermediate that occurs during nucleophilic addition is stabilized by conjugation with the aromatic ring system, and (4) potential inactivators based upon these compounds are readily prepared compared to their steroidal counterparts. 1-(4-Nitrophenyl)-2-propen-1-ol **XXXIII** and 1-(4-nitrophenyl)-2-propyn-1-ol **XXXIV** (Table V) represent the first nonsteroidal latent Michael acceptors of HSDs introduced into the literature.[99] Both compounds irreversibly inactivate purified rat liver 3α-HSD in a time and concentration dependent manner, only in the presence of $NAD(P)^+$. Analysis of the pseudo-first order inactivation data gave a K_d of 1.2 mM for the allylic alcohol and a $t_{1/2}$ life for the enzyme of less than 10 s at saturation. The corresponding acetylenic alcohol give a K_d of 1.5 mM and a $t_{1/2}$ life for the enzyme of 9.9 min at saturation. Although the absolute stereochemistry of the substrate utilized was not determined the alcohols were oxidized stereoselectively by 3α-HSD yielding a K_m of 2.0 mM and V_{max} of 0.58 μmoles/min/mg for the allylic alcohol, and a K_m of 0.75 mM and a V_{max} of 0.29 μmoles/min/mg for the acetylenic alcohol. The alcohols, 1-(4-nitrophenyl)-2-propen-1-ol and 1-(4-nitrophenyl)-2-propyn-1-ol are oxidized by 3α-HSD to the corresponding α,β-unsaturated ketones, 1-(4-nitrophenyl)-2-propen-1-one **XXXVI** and 1-(4-nitrophenyl)-2-propyn-1-one **XXXVII**. Compounds **XXXVI** and **XXXVII** stoichiometrically and irreversibly inactivate 3α-HSD in the absence of NAD^+ at a rate too rapid to accurately measure ($t_{1/2} < 10$ s, $K_d < 2\,\mu$M).

It was hypothesized that the efficiency of enzyme inactivation was related to an increase in the electropositivity of the enzyme-generated Michael acceptors, due to the presence of an electron withdrawing (*p*-nitrophenyl) group at C-1. Comparable studies with 1-phenyl-2-propen-1-one **XXXVIII** reveal that this compound is also an excellent inactivator, yielding a $K_i = 17\,\mu$M and a $t_{1/2}$ of 1 s at saturation (Table V).[92] These data imply that the presence of the electrophilic phenyl group is sufficient for effective enzyme inactivation.

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In these compounds the *p*-nitrophenyl group has a marked effect on the efficiency of oxidation of the parent alcohols. The k_{cat} observed for the unsubstituted 1-phenyl-2-propen-1-ol **XXXV** is 2 orders of magnitude lower than that for 1-(4-nitrophenyl)-2-propen-1-ol **XXXIII** (Table V). This implies that either the abstraction of the hydroxylic proton or loss of the hydride ion at C-1 is facilitated by the *p*-nitrophenyl ring. These findings may be best explained by a mechanism in which hydride transfer precedes proton abstraction. Destabilization of the intermediate carbonium ion by the *p*-nitrophenyl ring would facilitate loss of the hydroxylic proton generating the conjugated ketone, and speed up the overall reaction. Compounds containing an unsubstituted phenyl group would by contrast yield a tertiary carbonium ion in which the charge could be stabilized as a phenonium ion over the ring system. This delocalization of charge would slow the deprotonation event and retard the overall reaction (Figure 13).

In summary, these findings suggest that the reactivity of Michael acceptors can be increased by the introduction of a phenyl ring at C-1. Turnover of the latent Michael acceptor can be increased if the phenyl group is replaced by a more powerful electron withdrawing *p*-nitrophenyl group. This group destabilizes the intermediate carbonium ion, which in turn facilitates abstraction of the hydroxylic proton.

Since the nonsteroidal compounds described are relatively simple it is possible that they could be oxidized by several HSDs causing non-specific enzyme inactivation. A screen of two aliphatic alcohol dehydrogenases and five HSDs indicate that the aromatic allylic and acetylenic alcohols are oxidized only by rat liver 3α-HSD and the isofunctional enzyme from *Pseudomonas testosteroni*.[99] However, the aromatic allylic alcohol showed a distinct selectivity for the rat liver enzyme which was inactivated 30 × faster. Although the enzyme generated aromatic ketones could inactivate all the oxidoreductases examined there was a distinct preference for 3α-HSD.

Calculation of the effective partition ratios for the allylic and acetylenic alcohols (k_{cat}/k_{inact}) gave values of 5.3 and 141 respectively. Despite these low ratios, these compounds appear to inactivate 3α-HSD by a release and return mechanism. This might be anticipated since 3α-HSD has an ordered kinetic mechanism.[42] However, it also raises the issue of where these compounds ultimately bind. Several lines of evidence suggest that these compounds covalently modify the $NAD(P)^+$ binding site.[100]

The successful use of aromatic latent Michael acceptors to inactivate 3α-HSDs implies that the use of nonsteroids may represent a general method of developing inactivators for other HSDs. Although the steroid specificity of many HSDs has been examined in detail, few of the enzymes have been examined for their ability to employ nonsteroidal substrates. Those enzymes which have been examined are generally from liver or bacterial sources, rather than steroid hormone responsive tissues. Examples of HSDs known to use non-steroidal compounds as substrates include *Pseudomonas testosteroni* 3α-HSD,[98,101,102] *Pseudomonas testosteroni* 3(17)β-HSD,[99,101,102] *Streptomyces hydrogenans* 3α,20β-HSD,[103] rat liver 3α-HSD,[23] and guinea-pig liver and testis 17β-HSD.[104–107] Compounds containing a partial steroid nucleus have also been shown to be substrates for placental 17β-HSD.[108] In addition numerous nonsteroids have been shown to act as competitive inhibitors of HSDs. For example, diethylstilbestrol, *trans* and *cis*-clomiphene and metyrapone all inhibit placental 17β-HSD;[13] prostatic 3α(β)-HSD is inhibited by dicyclohexene derivatives[109] and rat liver 3α-HSD is inhibited by nonsteroidal anti-inflammatory drugs.[23] These compounds could be used as leads to develop nonsteroidal suicide substrates for these enzymes.

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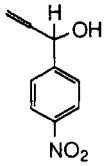
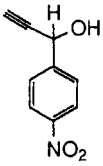
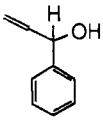
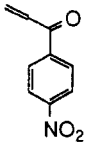
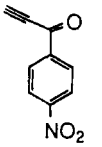
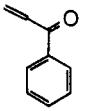
TABLE V
Examination of nonsteroidal *p*-nitrophenyl derivatives as inactivators of 3α-HSD

Compound	Turnover[a] K_m (mM)	V_{max} (μmol/min/mg)	V_{max}/K_m (μmol/min/mg) mM^{-1}	k_{cat} (min^{-1})
 XXXIII	2.0 ± 0.9[b]	0.58 ± 0.14[b]	2.9	19.72
 XXXIV	0.75 ± 0.05[b]	0.29 ± 0.01[b]	0.38	9.85
 XXXV	2.16 ± 0.36	0.0053 ± 0.0006	0.0024	0.18

Compound	Enzyme Inhibition: Reversible IC_{50}	Reversible K_i	Irreversible K_i	Irreversible $t_{1/2}$
 XXXVI	3.0 μM	4.0 μM[c]	Stoichiometric Inactivator	< 1s
 XXXVII	6.0 μM	8.0 μM[c]	Stoichiometric Inactivator	< 1s
 XXXVIII	91 μM	N.D.	17 μM	< 1s

[a]The kinetic constants were derived using mixtures of the racemic alcohols. 3α-HSD will oxidize only one of the stereoisomers in the mixture. The configuration of the isomer oxidized has not been determined. [b]Values taken from Ricigliano and Penning (1989) *Biochem. J.*, **262**, 139. [c]K_i values for nonsteroids were obtained from Dixon analysis. These compounds appear to be competitive inhibitors of NAD^+ binding. (Data reproduced with permission from *Steroids*).

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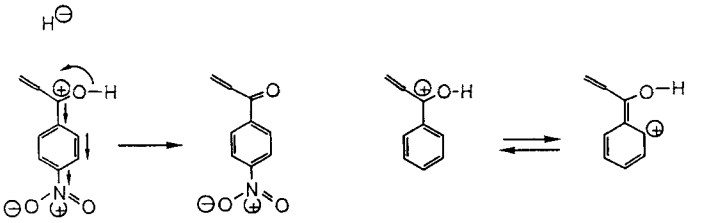

FIGURE 13 Influence of the *p*-nitrophenyl ring on proton abstraction. The *p*-nitrophenyl ring destabilizes the intermediate carbonium ion facilitating proton abstraction, while the phenyl ring stabilizes the intermediate carbonium ion as a phenonium ion (Reproduced with permission from *Steroids*).

6.3. Epoxides As Enzyme-Activated Inactivators of HSDs

The presumptive catalytic mechanism for HSDs involves compulsory polarization of the acceptor carbonyl to facilitate hydride transfer. This portion of the enzyme mechanism can be exploited by replacing the acceptor carbonyl with either an epoxide or cyclopropyl ring to form an enzyme-activated inactivator. Protonation of the oxiranyl oxygen or bridge head carbon at the active site, would increase ring strain and facilitate nucleophilic attack at a ring carbon (Figure 14). Work by Bloxham *et al.*[46] with epoxide inhibitors of two aliphatic alcohol (lactate and β-hydroxybutyrate) dehydrogenases demonstrates the formation of such protonated epoxide species, however, no subsequent enzyme inactivation was noted.

6.3.1. Steroid epoxides. A number of steroid epoxides have been examined as inactivators of rat liver 3α-HSD[92,96] [2α,3α-epoxy-5α-androstan-17-one, 3α,4α-epoxy-5α-androstan-17-one, 3α-spiro-epoxy-5α-pregnan-20β-ol and 3β-spiro-epoxy-5α-pregnan-20β-ol] and rat ovarian 20α-HSD[96] [(20*R*)-20,21-epoxy-5α-pregnan-3β-ol and (20*S*)-20,21-epoxy-5α-pregnan-3β-ol, **XXXIX–XLIV**] (Figure 15).

Examination of the data suggests that as a group these compounds possess low affinity for their target enzymes. Furthermore, time-dependent inactivation was not observed with any of these compounds either in the presence or absence of NAD(P)(H). The low affinity of the steroidal epoxides and their inability to inactivate their target enzymes is unrelated to their stereochemistry since both epimers of the C-3 spiro-epoxides and the C-20 side chain epoxides were examined and were equally ineffective in binding to their target enzymes. These data imply that some other property of the epoxides is responsible for their poor affinity (steric bulk) and/or low reactivity (no polarization of the oxiranyl oxygen). To date no studies have been described which examine the ability of cyclopropyl- or thioranyl-steroids to act as enzyme-activated inactivators of HSDs.

6.3.2. Nonsteroidal epoxides. The ability of latent nonsteroidal Michael acceptors to act as efficient substrates of rat liver 3α-HSD results from the electron withdrawing properties of the *p*-nitrophenyl ring. By placing the *p*-nitrophenyl ring adjacent to an epoxide this would increase the chance of nucleophilic attack. The electrophilicity of *p*-nitrophenyl-epoxides would be enhanced further by polarization of the oxiranyl oxygen by the general acid at the HSD active site. This reasoning led to the examination of 3- and 4-nitrostyrene epoxides **XLV–XLVI** as epoxide inactivators of 3α-HSD.

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EXPLOITATION OF ENZYME MECHANISM II

POLARIZATION OF CARBONYL

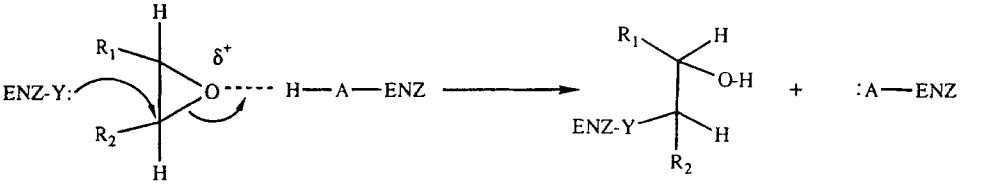
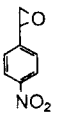


FIGURE 14 Inactivation of hydroxysteroid dehydrogenases by steroid epoxides. ENZ-Y = enzyme nucleophile, R_1 and R_2 are components of either the steroid nucleus or side chain, H-A-ENZ = general acid at the active site of an HSD.

TABLE VI
Nitrophenyl-epoxide inactivators for 3α-HSD

	Enzyme inhibition				
	Reversible		Irreversible		
Compound	IC_{50}	K_i	K_i	k_{+2}	$t_{1/2}$
 XLV [a]	250 μM	85 μM[b]	1 mM	$8.3 \times 10^{-3} s^{-1}$	83 s
 XLVI [a]	300 μM	133 μM[b]	≫ 20 mM	Curved Rates	

[a] The chirality of the nonsteroid epoxides is unknown.

The compounds were moderate inhibitors yielding K_i values for the E·NAD$^+$ complex of 85–133 μM. 4-Nitrostyrene oxide rapidly inactivated 3α-HSD in the absence of pyridine nucleotide with a $t_{1/2}$ of 83 s at saturation and a K_i = 1.0 mM. By contrast 3-nitrostyrene oxide is far less reactive as an inactivator requiring a concentration of 20 mM to achieve greater than 80% loss of enzyme activity (Table VI).

Several lines of evidence argue that 4-nitrostyrene oxide acts in a specific manner on 3α-HSD. Firstly, both NAD$^+$ and compounds which compete for the steroid binding site (i.e., indomethacin) protect the enzyme from inactivation. Secondly, 4-nitrostyrene oxide is a competitive inhibitor of androsterone, and a noncompetitive inhibitor against nucleotide. This inhibition is consistent with the ordered kinetic mechanism for the enzyme[42] and implies that 4-nitrostyrene oxide will bind at the steroid site. Thirdly, the related analog 3-nitrostyrene oxide is far less active as an inactivator implying that the influence of the electron withdrawing group is essential for enzyme inactivation. Together, these data argue that a specific E·4-Nitrostyrene-oxide complex is responsible for inactivation. However, no unambiguous evidence for

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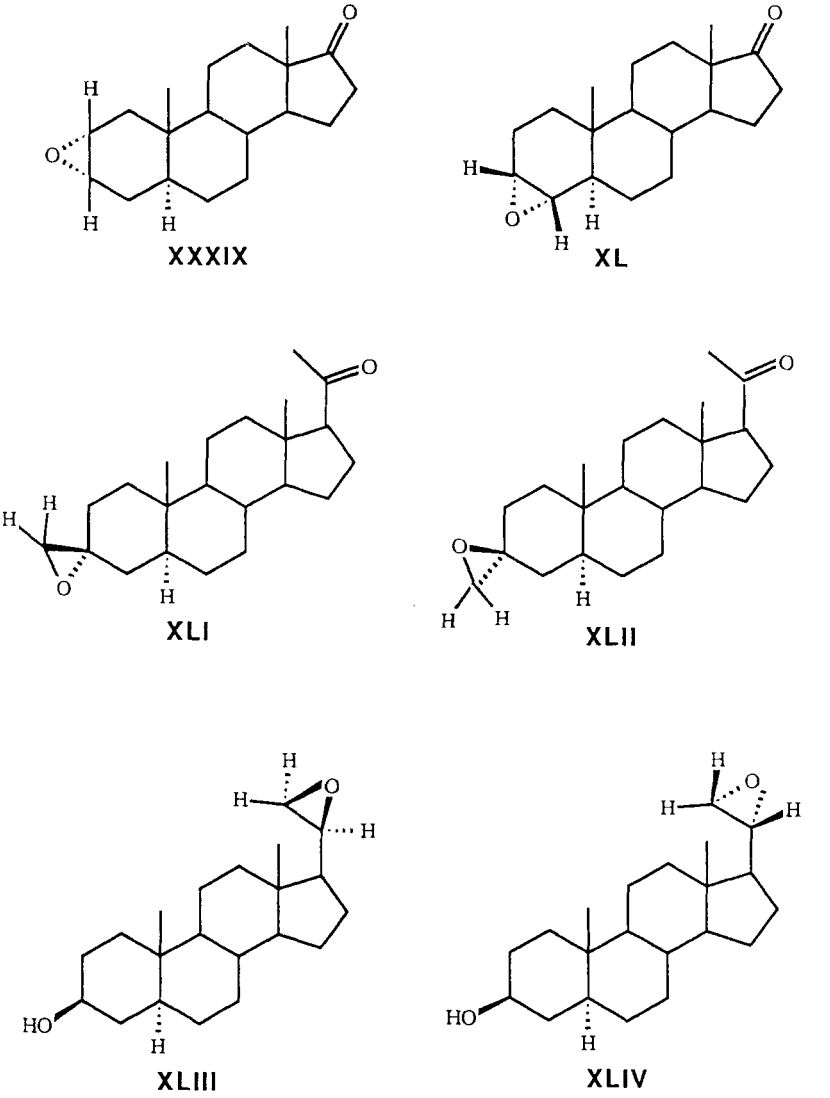

FIGURE 15 Steroid epoxides examined as inactivators of hydroxysteroid dehydrogenases.

a mechanism-based action for these compounds has been presented. The activated epoxide has not been trapped and there is no evidence that inactivation is stereoselective or pH dependent.

6.3.3. Design of epoxides as enzyme-activated inactivators of HSDs. The results presented question the use of epoxides as mechanism-based inactivators of HSDs. The assumption made is that by mimicry of the acceptor carbonyl the epoxide ring could be activated at the enzyme active site. While the position of the oxygen atom in epoxides and carbonyls is similar, the carbon-oxygen bonds of the epoxide ring are

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like those of an alcohol and result from the combination of sp^3 orbitals. Moreover, the oxiranyl carbons already bear a hydrogen atom and therefore the epoxides may more closely resemble substrate alcohols rather than ketones. Assuming that the epoxide ring more closely resembles an alcohol rather than a carbonyl, alternative mechanisms of epoxide activation can be described involving the partial abstraction of a hydride ion by $NAD(P)^+$.

7. MECHANISM-BASED COMPOUNDS THAT ARE EFFECTIVE AT BLOCKING HSD ACTION EITHER IN CELL CULTURE OR *IN VIVO*

A considerable effort has gone into the rational design of mechanism-based compounds for HSDs. Compounds that have demonstrated biological activity beyond the enzyme level either by being effective in cell culture or by being effective in whole animals constitutes the success that has been achieved.

Testing of the 3α-HSD nonsteroidal mechanism-based inactivators in primary cultures of neonatal rat Leydig cells indicated that the *p*-nitrophenyl allylic alcohol **XXXIII** could produce a total blockade in the metabolism of 5α-dihydrotestosterone to 3α-androstanediol, (IC_{50} = 200 μM).[110] It is noteworthy that after a single dose of the allylic alcohol (**XXXIII**) the effects on Leydig cells persisted even after refeeding the cells, implying that these agents irreversibly inhibited their target enzyme. These findings suggest that these *p*-nitrophenyl compounds offer promising leads to agents that may potentiate androgen action.

Successful inhibition of 3β-HSD/KSI has been achieved with 2α-cyanosteroid inhibitors. Studies with 2α-cyanoprogesterone **IV** show that at concentrations of 1.0 μM synthesis of progesterone can be blocked in both normal (cytotrophoblast) and transformed (JEG) human placental cells.[75] The ability to block progesterone production in JEG cells is particularly impressive since these cells produce elevated amounts of human chorionic gonadotropin in an uncontrolled manner, which in turn stimulates progesterone production. The effects of 2α-cyanoprogesterone on progesterone biosynthesis is reversible in both cell types and can be washed out on refeeding the cells. This is consistent with the reversible nature of enzyme inhibition observed with these agents.

Two other 2α-cyanosteroids (trilostane and epostane **VI** and **V**) developed earlier by Sterling-Wintrop have great therapeutic potential. These compounds were shown to have comparable inhibitory properties on the adrenal 3β-HSD/KSI.[6] However, *in vivo* they show marked selectivity in their effects.[6] Trilostane shows 10-fold selectivity for blocking aldosterone biosynthesis and can be used as a hypotensive agent. Thus, trilostane will inhibit furosemide-induced hyperaldosterism in rats.[111] By contrast, epostane shows marked selectivity as an interceptive or contragestational agent in rats and rhesus monkeys.[112] In human clinical trials epostane has been shown to effectively terminate early pregnancy through an abrupt decrease in plasma progesterone levels.[7] This observation is in agreement with the proposition that the *in vivo* effects of epostane are mediated through the inhibition of 3β-HSD/KSI. It has been concluded that epostane taken orally is an effective and non-invasive method for the termination of unwanted early prenancy and could offer a challenge to RU486.

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8. SUMMARY

Several features of the HSD catalytic mechanism have been exploited to develop mechanism-based inhibitors and inactivators. These include formation of activated-ternates with the general acid involved in catalysis (steroid pyrazoles), the development of intermediate state analogs (azasteroids), synthesis of potential suicide substrates in the form of latent and activated Michael acceptors and the synthesis of oxiranes as enzyme-activated inactivators.

Compounds that have progressed into the clinic include the 2α-cyanoketones (epostane and trilostane) which block 3β-HSD/KSI and for which no formal mechanism of tight-binding is accepted. It is conceivable that like the steroid pyrazoles they can form an activated-ternate. No serious attempt has been made to synthesize a mono- or bi-substrate transition state analog for an HSD reaction.

Although synthesis of latent and activated steroid Michael acceptors has been accomplished by several groups none have the desired turnover and reactivity to be good candidates as therapeutic agents. It is possible that these shortcomings might be solved by the introduction of appropriately placed modifying groups (e.g. electron withdrawing and donating groups). Success has been achieved with an ethoxyacetylenic alcohol developed as a suicide substrate for 3α,20β-HSD from *Streptomyces hydrogenans*, but these ideas have not progressed to the design of inactivators for mammalian HSDs.

The issue of whether it is possible to synthesize a suicide substrate with a low partition ratio for HSDs has been a concern. Many HSDs display ordered kinetic mechanisms with pyridine nucleotide binding first and leaving last. For these HSDs binary E·I (enzyme-inactivator) complexes may not form as the result of substrate turnover. This implies that enzyme-generated inactivators will be released prior to inactivation. Further, irrespective of kinetic mechanism all enzyme generated HSD inactivtors described to date inactivate by a release and return mechanism and therefore these compounds really fall into the category of enzyme-generated affinity alkylators. In every instance pyridine nucleotide protects the target enzyme from inactivation by the enzyme-generated inactivators suggesting that the alkylators prefer to covalently modify free enzyme. These observations raise two important issues: loss of selectivity of the latent compound and the ultimate site that is alkylated. For enzymes with ordered mechanisms, alkylation may occur at the steroid site via an abortive binary complex for which no analogous E·Steroid complex forms during the enzyme catalyzed reaction. Alternatively, alkylation may occur at the nucleotide binding site as has been proposed for the nonsteroidal Michael acceptors for 3α-HSD,[100] or it may occur at a third site.

9. CONCLUDING STATEMENT

Much needs to be done in designing effective inactivators of HSDs. This field is ripe for new developments and some future directions have been outlined. Conceiving novel approaches to the rational design of mechanism-based inactivtors for HSDs will require more extensive knowledge of the active sites of these enzymes; The interactions that are essential for catalysis, binding substrate analogs and inhibitors needs to be documented. More powerful techniques such as NMR[113] can be used to identify the amino acids involved in forming covalent adducts with active site-directed agents.

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These techniques, in combination with sources of cloned and over expressed enzymes may rapidly provide some of the necessary data to develop effective inhibitors of this important family of enzymes.

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