Clues to the development of mechanism-based inactivators of 3α-hydroxysteroid dehydrogenase: comparison of steroidal and nonsteroidal Michael acceptors and epoxides

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A series of steroidal and nonsteroidal Michael acceptors that represent reaction products for 3α-hydroxysteroid dehydrogenase were synthesized and evaluated as potential enzyme-generated inactivators. Introduction of exocyclic olefins either at C-2 or C-6 produced inhibitors with high affinity for the enzyme (0.05 to 5.0 μM). However, despite this affinity, none of these compounds produced time-dependent inactivation of the enzyme. By contrast, analogs based on 1-phenyl-2-propen-1-one were stoichiometric inactivators of the enzyme and ease of turnover of the parent latent Michael acceptor depended on the presence of an electron-withdrawing substituent at the para *position. A series of steroidal and nonsteroidal epoxides in which the oxiranyl oxygen could be substituted for the 3-ketone (the acceptor carbonyl of a steroid substrate) were also synthesized and evaluated as potential mechanism-based inactivators. Steroidal 2α,3α-, and 3α,4α-epoxides as well as 3α- and 3β-spiroepoxides did not bind to the enzyme and were unable to cause enzyme inactivation in either the presence or absence of pyridine nucleotide. In contrast, nitrostyrene oxides produced time-dependent inactivation, the rate of which was governed by the presence of an electron withdrawing group at the* para *position. These data indicate that the design of mechanism-based inactivators for 3α-hydroxysteroid dehydrogenase requires the incorporation of electron-withdrawing groups adjacent to the latent enzyme-activated group and, as a result, the turnover and/or reactivity of these compounds is increased. Moreover, these compounds can be modeled on nonsteroids.* (*Steroids* **56**:420–427, 1991)

Keywords: steroids; 3α-hydroxysteroid dehydrogenase; epoxides; Michael acceptors; nitroepoxides; nitroacetophenones

Introduction

3α-Hydroxysteroid dehydrogenase (3α-HSD; EC 1.1.1.50) in endocrine tissues is a drug target since the enzyme will catalyze the NAD(P)H-dependent reduction of 5α-dihydrotestosterone (a potent androgen) to 3α-androstanediol (a weak androgen).[1–3] The components of the reaction mechanism involve direct hydride transfer from position 4 of the pyridine moiety of the dinucleotide to the acceptor carbonyl at C-3.[4] The hydride transfer, as in other oxidoreductases, is presumably facilitated by compulsory polarization of the acceptor carbonyl by an appropriate acidic amino acid at the active site. This polarization may involve formal protonation to give a partial carbonium ion (Figure 1).[5–7]

Two features of this mechanism can be exploited for the development of mechanism-based inactivators: the ability of the enzyme to catalyze the interconversion of alcohols and ketones and polarization of the acceptor carbonyl. The ability of hydroxysteroid dehydroge-

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Received October 9, 1990; accepted March 9, 1991.

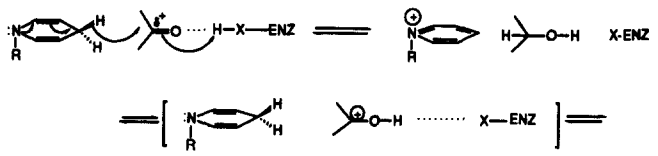


Figure 1 Presumptive catalytic mechanism for HSDs. ENZ, enzyme; X-H, acidic amino acid. A formal carbonium ion is shown in square brackets, which in the mechanism shown may be represented as a partial positive charge. (Modified from Bloxham et al.[7])

nases (HSDs) to catalyze the oxidation of α,β-unsaturated steroidal alcohols to α,β-unsaturated steroidal ketones (Michael acceptors) has been exploited repeatedly to prepare mechanism-based inactivators of 17β-, 3α,20β-, and 3β-HSDs.[8-11] In each instance, the enzyme-generated ketone alkylates the active site by Michael addition. Attempts to exploit the polarization of the acceptor carbonyl could focus on replacing it with either an epoxide or cyclopropyl ring. 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. To date there have been no reports of steroidal epoxides or cyclopropanes acting as inactivators of an HSD.

We describe the synthesis and evaluation of steroidal and nonsteroidal Michael acceptors, and steroidal and nonsteroidal epoxides as potential mechanism-based inactivators for the homogeneous rat liver 3α-HSD. A common theme observed is that enzyme inactivation can only be achieved if the alkylating functionality (Michael acceptor or epoxide) is made more reactive by the introduction of an adjacent electron-withdrawing group.

Experimental

Materials

All unlabeled steroids were purchased from Steraloids Inc. (Wilton, NH, USA). *m*-Chloroperoxybenzoic acid (*m*CPBA) was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). *m*CPBA was treated with phosphate buffer, pH 7.5, before use.[12] 3- and 4-Nitrostyrene were obtained from Pfaltz and Bauer Inc. (Waterbury, CT, USA).

Silica gel (60 A pore size, 75- to 150-μm particles), 20 × 20 cm × 1000 μm fluorescent silica plates type 02011, and fluorescent silica plates 2 × 10 cm type 02521 were purchased from Analtech Inc. (Newark, DE, USA). All other reagents were ACS grade or better.

Nuclear magnetic resonance (NMR) spectra were recorded on a Varian EM-360 (60 MHz) or Brucker WH-360 MHz (360 MHz) spectrometer in $CDCl_3$ relative to tetramethylsilane. NMR data are reported as follows: δ (ppm) (solvent); chemical shift (peak type, integration, coupling constant in Hz, and proton assignment). Abbreviations used in reporting NMR data: s, singlet; d, doublet; m, multiplet; q, quartet; ppm, parts per million; and J, a coupling constant in Hz. Infrared (IR) spectra were recorded in $CHCl_3$ with a Perkin-Elmer 521 IR spectrometer equipped with an extended range interchange. Absorption bands are reported in cm^{-1}. The appearance of absorption bands are described by the following abbreviations: s, strong; m, medium; w, weak; and b, broad. UV/vis spectra were recorded on a Beckman DU-7. Melting points were recorded on a Fischer Johns melting point apparatus and were not corrected.

Synthesis of steroid Michael acceptors

17β-Hydroxy-6-methyleneandrost-4-en-3-one (I) and 17β-hydroxy-2,6-dimethyleneandrost-4-en-3-one (II). Reaction of testosterone with paraformaldehyde and BF_3-DMSO as described in ref. 13 provided compounds **I** and **II**, which could be separated by flash chromatography on silica in toluene/acetonitrile (10:3, v/v). Compound **I** was the major product and compound **II** was the minor product. Spectral data agreed with literature assignments, as did the melting points.[13]

17α-Acetoxy-6-methylenepregn-4-ene-3,20-dione (III) and 17α-acetoxy-2,6-dimethylenepregn-4-ene-3,20-dione (IV). Reaction of 17α-acetoxy-pregn-4-ene-3,20-dione with paraformaldehyde and BF_3-DMSO as described above provided **III** and **IV**, which were again separated by flash chromatography in toluene/acetonitrile (10:2, v/v). Compound **III**, the major product, gave IR, C=O, 1,727 s, and 1,661 s; C=C, 2,936 s, 1,610 m; NMR δ (ppm) ($CDCl_3$), 0.65 (s, 3H, C-18 methyl), 1.06 (s, 3H, C-19 methyl), 2.01 (s, 3H, C-21 methyl), 2.08 (s, 3H, —CO—CH_3), 4.88 (d, 1H, vinyl), 5.02 (d, 1H, vinyl), 5.88 (s, 1H, vinyl). UV λ_{max} = 257 nm, ε = 16,450 cm^{-1} M^{-1}.

Compound **IV**, the minor product, gave IR, C=O, 1,717 s, and 1,654 s; C=C 2,925 s, 1,605 s. NMR δ (ppm) ($CDCl_3$), 0.65 (s, 3H, C-18 methyl), 0.96 (s, 3H, C-19 methyl), 1.99 (s, 3H, C-21 methyl), 2.06 (s, 3H, —CO—CH_3), 4.89 (d, 1H, vinyl), 5.02 (d, 1H, vinyl), 5.11 (d, 1H, vinyl), 5.88 (d, 1H, vinyl), 6.00 (s, 1H, vinyl). UV λ_{max} = 254 nm, ε = 10,207 cm^{-1} M^{-1}.

Synthesis of nonsteroidal latent and active Michael acceptors

1-(4-Nitrophenyl)-2-propen-1-ol (V), 1-(4-nitrophenyl)-2-propyn-1-ol (VI), 1-(4-nitrophenyl)-2-propen-1-one (VII), and 1-(4-nitrophenyl)-2-propyn-1-one (VIII). The synthesis of these racemic compounds has been described elsewhere.[14] No attempt has been made to separate the stereoisomers.

1-Phenyl-2-propen-1-ol (IX) and 1-phenyl-2-propen-1-one (X). The synthesis of these compounds was performed as described for compounds **V** through **VIII** using benzaldehyde and vinyl magnesium bromide. Compound **IX** gave NMR δ(ppm) ($CDCl_3$) 2.2 (s, 1H, broad —OH $[^2H]_2O$ exchangeable), 5.18 (m, 2H, broad CH=CH_2), 5.30 (m, 1H, C*H*—CH=), 5.90 (m, 1H, broad C*H*=CH_2), 7.30 (m, 5H, aromatic). Compound

X gave NMR δ (ppm) ($CDCl_3$) 5.80–6.00 (dd, 1H, vinyl CH=CH_2, $J_{2,3}$ *cis* 20 Hz), 6.25 to 6.5 (dd, 1H, vinyl CH=CH_2, $J_{2,3}$ *trans* 32 Hz), 7.24 (m, 1H, vinyl C*H*=CH_2), 7.3 to 8.3 (m, 5H, aromatic).

Synthesis of steroidal epoxides

2α,3α-Epoxy-5α-androstan-17-one (XI) and 3α,4α-epoxy-5α-androstan-17-one (XII). Compound **XI** was synthesized from 3β-hydroxy-5α-androstan-17-one as described by Fajkos and Sorm.[15] Compound **XII** was synthesized from androst-4-ene-3,17-dione by reduction to 5α-androst-3-en-17-one[16] and converted to the epoxide as described by Fajkos and Sorm.[15] Spectral assignments and melting points agreed with published values for these compounds.

3α-Spirooxiranyl-5α-pregnan-20β-ol (XIII) and 3β-spirooxiranyl-5α-pregnan-20β-ol (XIV). The synthesis and characterization of the pure 3α-spiroepoxide and the stereochemically enriched 3β-spiroepoxide (2 : 1 mixture 3β : 3α-spiroepoxides) have been described.[17]

Synthesis of nonsteroidal epoxides

3-Nitrostyrene oxide (XV) and 4-nitrostyrene oxide (XVI). The appropriate nitrostyrene (1 g) was suspended in 30 ml methylene chloride (CaH_2 distilled). *m*CPBA (3 g) was added, and the reaction was stirred for 5 hours at room temperature. The reaction was poured into 100 ml 0.2 M NaOH and the product was extracted with methylene chloride. Pooled extracts were washed with 0.2 M NaOH followed by saturated NaCl and dried over Na_2SO_4. Concentration gave 0.8 g of white crystals of 4-nitrostyrene oxide (mp, 30 to 35 C) or 0.4 g of yellow crystals of 3-nitrostyrene oxide (mp, 75 to 78 C). With either oxide no attempt was made to resolve the stereoisomers. Compound **XV** gave NMR δ (ppm) ($CDCl_3$), 2.8 (m, 1H, oxiranyl methylene), 3.25 (m, 1H, oxiranylmethylene), 4.01 (m, 1H, oxiranyl methine), 7.65 (s, 1H, aromatic), 8.25 (m, 3H, aromatic). Compound **XVI** gave NMR δ (ppm) ($CDCl_3$), 2.8 (m, 1H, oxiranyl methylene), 3.2 (m, 1H, oxiranyl methylene), 3.99 (m, 1H, oxiranyl methine), 7.4 (d, 2H, aromatic), and 8.24 (d, 2H, aromatic).

Preparation of enzyme and enzyme assay

3α-Hydroxysteroid dehydrogenase was purified to electrophoretic homogeneity from rat liver cytosol.[18] Stock enzyme solutions contained 3.86 mg/ml in 20 mM potassium phosphate buffer, pH 7.0, containing 20% glycerol, 1 mM 2-mercaptoethanol, and 1 mM EDTA. The final specific activity was 2.0 μmol androsterone oxidized/min/mg protein under standard assay conditions. Enzyme assays were conducted in 1.0 ml systems containing 75 μM androsterone, 2.3 mM NAD^+, 100 mM potassium phosphate, pH 7.0, and 4% acetonitrile as co-solvent. Reactions were initiated by the addition of enzyme, and the change in pyridine nucleotide absorbance at 340 nm was followed over time. There was no observable absorbance change in the absence of enzyme.

Inhibition and inactivation experiments

The effect of reversible inhibitors was studied under initial velocity conditions. For the determination of IC_{50} values, increasing concentrations of inhibitor were added to the standard enzyme assay. 3α-Hydroxysteroid dehydrogenase is known to have an ordered kinetic mechanism, with pyridine nucleotide binding first and leaving last.[19] Thus, IC_{50} values are measuring inhibition through formation of an $E \cdot NAD^+ \cdot I$ ternary dead-end complex. Unless otherwise stated, derived K_i values are presumed to represent the dissociation constant for I from the $E \cdot NAD^+ \cdot I$ complex.

For inactivation studies, enzyme was first dialyzed against 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA. The dialysis was essential for the removal of 2-mercaptoethanol, which could act as a scavenging agent for the various alkylators. Inactivation was followed in 200-μl systems containing 5 to 10 μM enzyme and an excess of inactivator. Inactivator concentrations were varied over the range of 0 to 5 mM, depending on the inactivator, and at time periods (0 to 1,000 seconds), aliquots were removed and diluted at least 100-fold into the standard enzyme assay to determine the amount of enzyme activity remaining. Semilogarithmic plots of the percentage of enzyme activity remaining versus time gave estimates of the first-order rate constants for enzyme inactivation. Data were then transformed by the method of Kitz and Wilson[20] to yield K_i (dissociation constant for the binary enzyme-inactivator complex) and k_{+2} (the limiting rate constant for enzyme inactivation).

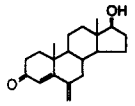
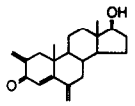
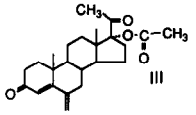
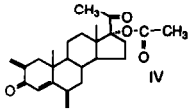
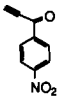
Results

Inhibition with steroid Michael acceptors

Several hydroxysteroid dehydrogenases (17β-, 3α, 20β-, and 3β-HSD) can oxidize either allylic or acetylenic alcohols to the corresponding α,β-unsaturated ketones, which then inactivate the target enzyme by Michael addition.[8–11] In exploring this route of inactivation for 3α-HSD, we choose to first synthesize Michael acceptors (compounds **I** through **IV**) that have the potential to be enzyme-generated products and evaluate these compounds as inactivators. By approaching the problem in this manner, we avoided the demanding task of synthesizing the corresponding axial 3α-hydroxysteroids containing the appropriate allylic groups. From the structures of compounds **I** through **IV**, it is also apparent that we choose to introduce exocyclic olefins. Unlike Δ^4-enes, exocyclic olefins are unhindered at the site of nucleophilic attack and, unlike 5,10-secosteroids, their introduction into the steroid does not disturb the A/B ring junction.[10]

Steroids containing an exocyclic olefin at C-6 were found to be quite potent reversible inhibitors of the

Table 1 Inhibition of 3α-hydroxysteroid dehydrogenase by steroidal and nonsteroidal Michael acceptors

COMPOUND		ENZYME INHIBITION				
		REVERSIBLE		IRREVERSIBLE		
		IC_{50}	K_i	K_i	k_{+2}	$t_{1/2}$
	I	1.0 μM	0.44 μM[a]	-	-	-
	II	10.0 μM	4.4 μM[a]	-	-	-
	III	0.115 μM	0.05 μM[a]	-	-	-
	IV	10.0 μM	4.4 μM[a]	-	-	-
	VII	3.0 μM	4.0 μM[b]	Stoichiometric Inactivator		<1s
	VIII	6.0 μM	8.0 μM[b]	Stoichiometric Inactivator		<1s
	X	91 μM	N.D.	17 μM		<1s

[a] K_i values for the steroids were computed using the relationship $IC_{50} = (1 + [S]/K_m)K_i$.
[b] K_i values for nonsteroids were obtained from Dixon analysis.[22] Nonsteroids are competitive inhibitors versus NAD^+.[23]

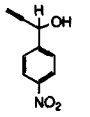
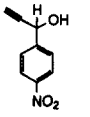
enzyme, yielding IC_{50} values of 0.10 to 10 μM (Table 1). However, despite their affinity for the E · NAD^+ complex, these steroids were unable to produce enzyme inactivation. These data suggest that either these compounds do not come into contact with a reactive nucleophile or that they are not reactive enough as alkylating agents. Recent studies show that 3α-HSD contains at least three reactive cysteines at its active site.[21] Therefore, the issue would appear to be low reactivity of the Michael acceptor at this site. The reactivity of an α,β-unsaturated ketone could be increased by introducing a powerful electron-withdrawing group either adjacent to the ketone or by appropriate substitution of the olefin or acetylene. It is difficult to examine this issue in the steroid series. Introduction of an electron-withdrawing group either adjacent or geminal to the ketone at C-3 is not easy without opening the A ring. Further, although 6-cyano or 6-bromo Δ^4-3-ketosteroids are time-dependent inactivators of 3α-HSD (Ricigliano and Penning, unpublished observations), these data could be explained by either the activation of the Michael acceptor or by direct displacement of cyanide or bromine by an incoming nucleophilic amino acid attacking C-6 of the steroid.

Inactivation with nonsteroidal Michael acceptors

To overcome the limitations imposed by steroid chemistry, we initiated a search for nonsteroidal substrates with a high k_{cat} for 3α-HSD that could be appropriately modified to yield Michael acceptors. In this regard, we reported earlier the modification of 4-nitroacetophenone, an aromatic ketone substrate for 3α-HSD, to yield 1-(4-nitrophenyl)-2-propen-1-one (**VII**) and 1-(4-nitrophenyl)-2-propyn-1-one (**VIII**). These vinyl and acetylenic ketones were found to be stoichiometric inactivators of 3α-HSD that rapidly inactivate the enzyme with halflives of less than 10 s[14] (Table 1). We hypothesized that the efficiency of inactivation was related to an increase in the electropositivity of the enzyme-generated Michael acceptors, and that this was achieved by the presence of the electron-withdrawing *p*-nitrophenyl group at C-1. Comparable studies with 1-phenyl-2-propen-1-one **X**, however, reveal that this compound is also an excellent inactivator, yielding a K_i = 17 μM and a halflife of 1 second at saturation (Table 1). These data imply that the presence of the electrophilic phenyl group is sufficient for effective enzyme inactivation.

The *p*-nitro group has a marked effect on the efficiency of oxidation of the parent alcohols. Thus, the k_{cat} is two orders of magnitude lower for the unsubstituted 1-phenyl-2-propen-1-ol (**IX**) than that observed for 1-(4-nitrophenyl)-2-propen-1-ol (**V**) (Table 2). This

Table 2 Oxidation of nonsteroidal latent Michael acceptors by 3α-hydroxysteroid dehydrogenase

COMPOUND		TURNOVER[a]			
		Km (mM)	Vmax (μmol/min/mg)	Vmax/Km (μmol/min/mg) mM	kcat (min^{-1})
	V	2.0 ± 0.9[b]	0.58 ± 0.14[b]	2.9	19.72
	VI	0.75 ± 0.05[b]	0.29 ± 0.01[b]	0.38	9.85
	IX	2.16 ± 0.36	0.0053 ± 0.0006	0.0024	0.18

[a] The kinetic constants were derived using mixtures of the racemic alcohols. We have previously shown that 3α-HSD will oxidize only one of the stereoisomers in the mixture[14]; the configuration of the isomer oxidized has not been determined. The ability of the unoxidized isomer to act as a competitive inhibitor of the reaction has not been evaluated.
[b] Data from Ricigliano and Penning.[14]

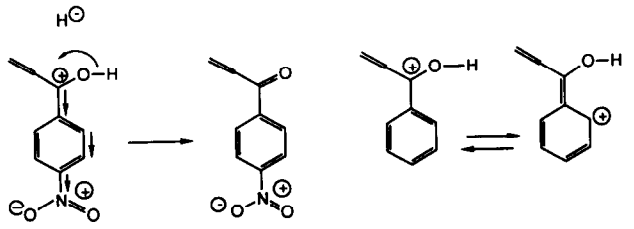


Figure 2 Influence of the *p*-nitrophenyl ring on proton abstraction. The *p*-nitrophenyl ring destabilizes the intermediate carbonium ion facilitating proton abstraction, while the phenyl ring stabilizes the intermediate carbonium ion as a phenonium ion.

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 2).

An alternative explanation could involve electrostatic repulsion of the *p*-nitro group to facilitate desorption of the product from the active site. However, this is unlikely since analysis of the kinetic mechanism shows that the rate-limiting step is the dissociation of the pyridine nucleotide and not the dissociation of the steroidal product.[19]

In summary, our 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 with a more powerful electron-withdrawing group (*p*-nitrophenyl ring) since this will destabilize the intermediate carbonium ion, which in turn facilitates abstraction of the hydroxylic proton. Clearly, these findings could only have been obtained using nonsteroidal active and latent Michael acceptors.

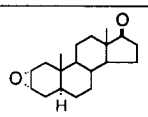
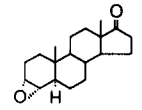
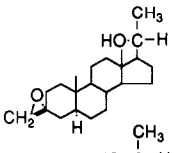
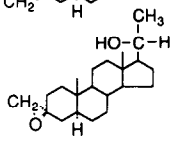
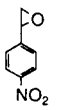
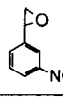
Inhibition studies with steroidal epoxides

By substituting the acceptor carbonyl at C-3 of the steroid with an oxiranyl oxygen, it was hypothesized that this would act as a carbonyl mimetic; polarization of the oxiranyl oxygen by the acid at the active site would increase the strain on the epoxide ring and enhance nucleophilic attack, leading to enzyme inactivation. To achieve this goal, a selection of steroid epoxides at and around the reactive center at C-3 were synthesized, including the 3α- and 3β-spiroepoxides (compounds **XI** through **XIV**).

Examination of these compounds as reversible enzyme inhibitors failed to yield IC_{50} values even at the limit of their solubility (100 μM), implying that these compounds are unable to bind to the E · NAD^+ complex. Moreover, no enzyme inactivation was observed with any of these compounds in the presence and absence of pyridine nucleotide (Table 3). The inability of the steroidal epoxides to bind to the enzyme may imply that an oxiranyl ring at or near C-3 is too bulky a functionality to have near the catalytic center. In addition, it could be argued that an oxiranyl oxygen is a poor substitute for the carbonyl group, since the sp^2 carbon at C-3 on the steroid was replaced by an sp^3 hybridized carbon.

Since Michael acceptors based on the nitrophenyl ring system were superior inactivators than their steroidal counterparts, nitrophenyl oxides were synthesized based on 3- and 4-nitrostyrene (**XV** and **XVI**). These compounds acted as poor reversible inhibitors, yielding IC_{50} values for the E · NAD^+ complex of 250 to 300 μM. However, 4-nitrostyrene oxide produced rapid time-dependent inactivation of 3α-HSD (K_i = 1.0 mM for

Table 3 Inhibition of 3α-hydroxysteroid dehydrogenase by steroidal and nonsteroidal epoxides

COMPOUND		ENZYME INHIBITION				
		REVERSIBLE		IRREVERSIBLE		
		IC_{50}	K_i	K_i	k_{+2}	$t_{1/2}$
	XI	>>100 μM[a]	N.D.	-	-	-
	XII	>>100 μM[a]	N.D.	-	-	-
	XIII	>>100 μM[a]	N.D.	-	-	-
	XIV	>>100 μM[a]	N.D.	-	-	-
	XV[b]	250 μM	85 μM[c]	1 mM	$8.3 \times 10^{-3} s^{-1}$	83s
	XVI[b]	300 μM	133 μM[c]	>>20 mM	Curved Rates	

[a] IC_{50} values for steroidal epoxides were far in excess of 100 μM, which is their limit of solubility in the assay.
[b] The chirality of compounds **XV** and **XVI** is unknown.
[c] K_i values for the reversible inhibition observed with the nonsteroidal epoxides were obtained by Dixon analysis.[22] The nonsteroidal epoxides were competitive inhibitors against androsterone.

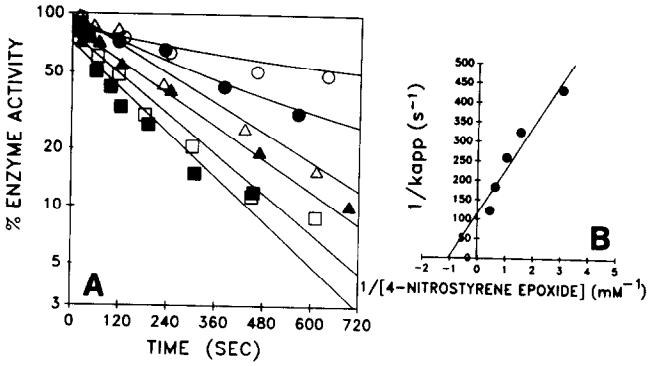


Figure 3 Time-dependent inactivation of 3α-HSD by 4-nitrostyrene oxide. Semilogarithmic plots of the percentage of enzyme activity remaining versus time are shown for varying concentrations of 4-nitrostyrene oxide; 165 μM (○), 330 μM (●), 660 μM (△), 986 μM (▲), 1.64 mM (□), and 2.32 mM (■). (A) Transformation of the data according to the method of Kitz and Wilson[20] produced the secondary plot. (B) In these experiments, the concentration of the enzyme was 5.3 μM and inactivation studies were performed as described in Experimental.

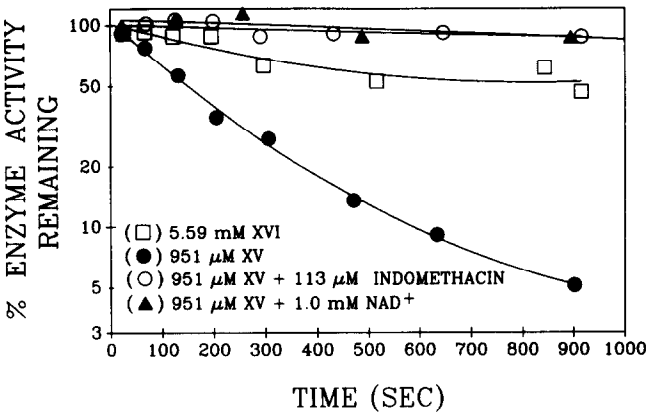


Figure 4 Inactivation of 3α-HSD by 3- and 4-nitrostyrene oxides and protection with indomethacin and NAD^+. Semilogarithmic plots of the percentage of enzyme activity remaining versus time are shown for 5.59 mM 3-nitrostyrene oxide (□), 951 μM 4-nitrostyrene oxide (●), 951 μM 4-nitrostyrene oxide plus 113 μM indomethacin (▲), and 951 μM 4-nitrostyrene oxide plus 1.0 mM NAD^+ (○). Indomethacin and NAD^+ also afford protection against inactivation by 3-nitrostyrene oxide (data not shown). In all these experiments, the concentration of the enzyme was 5.3 μM. For incubations containing indomethacin, the amount of enzyme activity remaining was corrected, after measuring the inhibition of the initial velocity due to the presence of low micromolar concentrations of indomethacin in the enzyme assay. These corrections were made from the appropriate dose-response curves.

E · I; halflife at saturation of 83 seconds; Figure 3 and Table 3). (The differences in K_i values for reversible and irreversible inhibition are anticipated since they are dissociation constants for ternary and binary complexes, respectively.) In contrast, 3-nitrostyrene oxide was less efficient as an enzyme inactivator since high concentrations (20 mM) were required to achieve greater than 80% loss of enzyme activity, and the curvilinear rates obtained precluded accurate determination of the kinetic constants for the inactivation event. These data imply that the reactivity of the epoxide improves considerably if an electron-withdrawing group (*p*-nitrophenyl rather than an *m*-nitrophenyl or phenyl) is placed on a ring carbon. One could argue that all that has been achieved is the activation of a nonspecific enzyme alkylating agent. However, inactivation of the 3α-HSD by either 3- or 4-nitrostyrene oxide is slowed significantly by either pyridine nucleotide or by compounds that compete for the androsterone-binding site (e.g., indomethacin[18]; Figure 4).

Moreover, using 4-nitrostyrene oxide as a reversible enzyme inhibitor, initial velocity studies reveal that this compound acts as a competitive inhibitor against androsterone and as an uncompetitive inhibitor against NAD^+. These data are consistent with the ordered kinetic mechanism for 3α-HSD, in which pyridine nucleotide binds first and leaves last.[19] Thus, the uncompetitive inhibition pattern observed with 4-nitrostyrene oxide and varying NAD^+ is due to the formation of an E · NAD^+ · I ternary complex instead of the E · NAD^+ · Androsterone complex.

In summary, our findings indicate that the reactivity of epoxide inactivators can be increased by the introduction of an appropriate electron-withdrawing group at C-1. This effect can be produced by the introduction of a *p*-nitrophenyl but not by an *m*-nitrophenyl ring.

The proposed mechanism of enzyme inactivation mediated by 4-nitrostyrene oxide is shown in Figure 5.

Discussion

This paper shows that Michael acceptors and epoxides based on simple aromatic systems may provide important leads to the development of nonsteroidal mechanism-based inactivators of HSDs. Previous work in this field initially focused on the incorporation of latent Michael acceptors in the steroid nucleus and side chain. In several instances, the synthesis of these steroids was difficult.[10,11] In developing mechanism-based inactivators for human placenta 17β-HSD, in which the acetylenic alcohol was on the side chain, the rate of oxidation

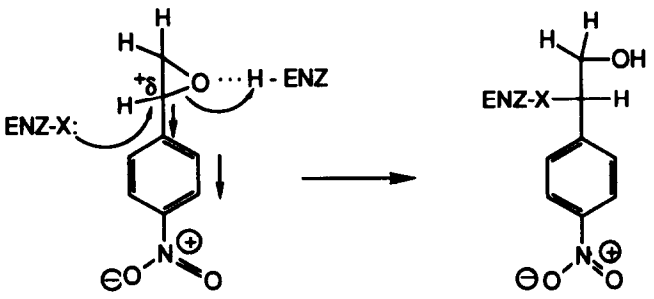


Figure 5 Mechanism of inactivation of 3α-HSD mediated by 4-nitrostyrene oxide. ENZ-X, enzyme nucleophile; ENZ-H, enzyme proton donor.

was low, while the rate of inactivation mediated by the active Michael acceptor was fast.[8] By placing a methylene group at C-16 in estradiol, the resultant allylic alcohol was found to be a good substrate, while the active Michael acceptor had low affinity and low reactivity for 17β-HSD.[24]

Covey and colleagues[25] have been concerned about these issues and have begun to systematically alter the reactivity of latent and active Michael acceptors by influencing the nature of the electron-withdrawing group at the β-carbon. Studies of the inactivation of 3α/20β-HSD from *Streptomyces* sp with side chain ethoxyacetylenic alcohols show that the electron-donating ethoxy group disturbs the equilibrium between alcohol and ketone and slows the rate of oxidation, but, on the other hand, extends the conjugation observed in the α,β-unsaturated ketone to make a more reactive electrophile. This in turn may enhance the rate of inactivation. Studies with this compound have not been reported for placental 17β-HSD. In contrast, we have altered the electronic environment of latent and active Michael acceptors based on nonsteroids. By placing an electron-withdrawing group adjacent to the alcohol of the latent Michael acceptors, we have facilitated the rate of oxidation. The reactivity of the active Michael acceptor remains essentially unchanged (*p*-nitrophenyl versus phenyl) and is exceedingly fast. It should be emphasized that these effects have been achieved by introducing an electron-withdrawing rather than an electron-donating group on the opposing side of the ketone.

In studies of 17β-HSD, Covey and co-workers[26] have attempted to improve the rate of turnover of the latent Michael acceptor and increase the reactivity of the active Michael acceptor by synthesizing partial steroids that lack a D ring. The resultant acetylenic secoestradiols were poor substrates and unimpressive as inactivators. However, it is interesting to note this trend toward developing nonsteroid mechanism-based inactivators for 17β-HSD.

Our approach has been to start with nonsteroid simple aromatic systems as leads. In the case of the *p*-nitrophenyl Michael acceptors and epoxides, it is clear that rates of inactivation can be extraordinarily fast, too fast to measure accurately in some instances. In addition, rates of oxidation of *p*-nitrophenyl allylic and acetylenic alcohols are excellent. Because of the simplicity of these molecules, one has to be concerned about their selectivity for the target enzyme and whether they just randomly alkylate the surface of the protein. Studies indicate that these may not be important concerns. Thus, in previous work, we were able to demonstrate that 1-(4-nitrophenyl)-2-propen-1-ol and 1-(4-nitrophenyl)-2-propyn-1-ol were selective in inactivating only 3α-HSD dehydrogenase from rat liver.[14] Thus, when these compounds were screened against a library of hydroxysteroid dehydrogenases as well as aliphatic alcohol dehydrogenases, they were ineffective as inactivators. Studies also suggest that the *p*-nitrophenyl Michael acceptors alkylate the pyridine nucleotide-binding site.[23] In contrast, the studies reported here show that the *p*-nitrophenyl oxides compete for and alkylate the steroid-binding site. To our knowledge, these compounds represent the first epoxide inactivators for an HSD.

Acknowledgments

This study was supported by grants from the National Institutes of Health (GM-33464 and CA-01335). T.M.P. is a recipient of a Research Career Development Award from the National Cancer Institute. A U.S. Patent application, serial no. 187,832, is pending for compounds **V** through **X**.

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