Inactivation of Δ^5 -3-oxo steroid isomerase with active-site-directed acetylenic steroids

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Several steroid analogues containing conjugated acetylenic ketone groups as part of a seco-ring structure or as substituents on the intact steroid system are irreversible inhibitors of Δ^5 -3-oxo steroid isomerase (EC 5.3.3.1) from *Pseudomonas testosteronei*. Thus 10 β -(1-oxoprop-2-ynyl)estr-4-ene-3,17-dione (I), 5,10-seco-estr-4-yne-3,10,17-trione (II), 17 β -hydroxy-5,10-seco-estr-4-yne-3,10-dione (III) and 17 β -(1-oxoprop-2-ynyl)androst-4-en-3-one (IV) irreversibly inactivate isomerase in a time-dependent manner. In all cases saturation kinetics are observed. Protection against inactivation is afforded by the powerful competitive inhibitor 19-nortestosterone. The inhibition constants (K_i) for 19-nortestosterone obtained from such experiments are in good agreement with those determined from conventional competitive-inhibition studies of enzyme activity. These compounds thus appear to be active-site directed. In every case the inactivated enzyme could be dialysed without return of activity, indicating that a stable covalent bond probably had formed between the steroid and enzyme. Compound (I) is a very potent inhibitor of isomerase [$K_i = 66.0 \mu\text{M}$ and $k_{+2} = 12.5 \times 10^{-3} \text{ s}^{-1}$ (where K_i is the dissociation constant of the reversible enzyme–inhibitor complex and k_{+2} is the rate constant for the inactivation reaction of the enzyme–inhibitor complex)] giving half-lives of inactivation of 30–45 s at saturation. It is argued that the basic-amino-acid residue that abstracts the intramolecularly transferred 4 β -proton in the reaction mechanism could form a Michael-addition product with compound (I). In contrast, although compound (IV) has a lower inhibition constant ($K_i = 14.5 \mu\text{M}$), it is a relatively poor alkylating agent ($k_{+2} = 0.13 \times 10^{-3} \text{ s}^{-1}$). If the conjugated acetylenic ketone groups are replaced by α -hydroxyacetylene groups, the resultant analogues of steroids (I)–(IV) are reversible competitive inhibitors with K_i values in the range 27–350 μM . The enzyme binds steroids in the C₁₉ series with functionalized acetylenic substituents at C-17 in preference to steroids in the C₁₈ series bearing similar groups in the ring structure or as C-10 substituents. In the 5,10-seco-steroid series the presence of hydroxy groups at both C-3 and C-17 is deleterious to binding by the enzyme.

Δ^5 -3-Oxo steroid isomerase (EC 5.3.3.1) from *Pseudomonas testosteronei* is to our knowledge the only homogeneous and crystalline enzyme of steroid metabolism (Kawahara & Talalay, 1960; Kawahara *et al.*, 1962), which is of known amino-acid sequence (Benson *et al.*, 1971) and is under study by X-ray crystallography (Westbrook, 1976; Westbrook *et al.*, 1976). The enzyme catalyses the conversion of Δ^5 -3-oxo steroids to Δ^4 -3-oxo steroids by a mechanism believed to involve combined acid-base catalysis in which the base effects the intramolecular transfer of the 4 β -proton to the 6 β -position as shown in Scheme 1 (Malhotra & Ringold, 1965; Talalay &

Benson, 1972). Several attempts have been made to label the active site of the enzyme by affinity reagents (Büki *et al.*, 1971; Batzold & Robinson, 1975; Ogez *et al.*, 1977; Pollack *et al.*, 1979). The most elegant approach to this problem is that described by Robinson and his colleagues, who have synthesized two acetylenic seco-steroids, namely 5,10-seco-estr-4-yne-3,10,17-trione (X) and 19-nor-5,10-secopregn-5-yne-3,10,20-trione (XI) (Batzold & Robinson, 1976). These compounds inactivate isomerase irreversibly in a time-dependent manner (Batzold & Robinson, 1975). They are converted at the active site by the normal catalytic

Scheme 1. Mechanistic pathway for enzyme-catalysed isomerization of Δ^3 -3-oxo steroids to Δ^4 -3-oxo steroids. The enzymic isomerization is promoted by acid (AH) and base (B) catalysis at the active site. The base (B) abstracts the 4β -proton (H^*), which is transferred intramolecularly to the 6β -position. The $\Delta^{3,5}$ -dienol is a postulated intermediate.

Scheme 2. Nucleophilic addition to an $\alpha\beta$ -acetylenic ketone

A nucleophile (X) can alkylate the β -carbon of an $\alpha\beta$ -acetylenic ketone. Nucleophilic attack is facilitated by enolization.

mechanism of isomerase into the corresponding highly reactive conjugated allenic ketones (Covey & Robinson, 1976), which are thus in a unique position to alkylate a reactive nucleophile, presumably the base involved in catalysis.

Several C₁₈ and C₁₉ steroids bearing either oxopropynyl or hydroxypropynyl groups as part of the ring structure or as substituents on the steroid skeleton have been synthesized (Covey, 1979; Covey *et al.*, 1979b). Steroids in the C₁₉ series bearing a hydroxypropynyl group at C-17 and steroids in the C₁₈ series containing a hydroxypropynyl group at C-10 are of particular interest since these compounds presumably could be oxidized by 20β -hydroxy steroid dehydrogenase or by steroid aromatase respectively to the corresponding conjugated acetylene system. The enzyme-generated conjugated acetylenic ketone would then be in a position to be a very selective inhibitor of either of these enzymes, whereas the precursor hydroxy-

propynyl steroids would fall into the general category of steroid suicide substrates. An extension of this work would be to examine the ability of these oxopropynyl steroids to act as general affinity labelling reagents for steroid-transforming enzymes, since the conjugated acetylenic ketones should undergo facile attack by a nucleophile at the active site to form a Michael addition product (Scheme 2).

The present paper describes a series of oxopropynyl steroids that act as active-site-directed irreversible inhibitors of Δ^3 -3-oxo steroid isomerase from *P. testosteronei* and a series of hydroxypropynyl steroids that act as competitive inhibitors of the same enzyme. This is of particular interest, since it is possible that the nucleophile available for alkylation by several oxopropynyl steroids is the base involved in catalysis. The results reported in the present paper emphasize the importance of extending these studies to mammalian steroid isomerases, and the possibility that both the hydroxy- and

oxopropynyl compounds may modify steroid metabolism.

Materials and methods

Materials

Spectroscopic-quality acetonitrile was obtained from Burdick and Jackson, Muskegon, MI, U.S.A., and crystalline bovine serum albumin from Armour Pharmaceuticals, Chicago, IL, U.S.A. Solutions of albumin were neutralized with NaOH.

Crystalline isomerase (sp. activity 52000 μmol of androst-5-ene-3,17-dione isomerized/min per mg) was prepared and assayed by our standard procedures (Wang *et al.*, 1963; Jarabak *et al.*, 1969; Benson *et al.*, 1974). Specific activities were based on protein concentrations determined from an extinction coefficient of $A_{1\text{cm}}^{0.1\%}$ at 280 nm of 0.336 (Benson *et al.*, 1975). The molarities of enzyme solutions were expressed in terms of the subunit molecular weight of 13394 (Benson *et al.*, 1971).

Androst-5-ene-3,17-dione and pregn-5-ene-3,20-dione were synthesized from dehydroepiandrosterone and pregnenolone respectively (Kawahara, 1962). 19-Nortestosterone was purified by crystallization and high-vacuum sublimation. 10 β -(1-Oxoprop-2-ynyl)oestr-4-ene-3,17-dione (I), 17 β -(1-oxoprop-2-ynyl)androst-4-en-3-one (IV), 10 β -[(1R)-1-hydroxyprop-2-ynyl]oestr-4-ene-3,17-dione (V), 10 β -[(1S)-1-hydroxyprop-2-ynyl]oestr-4-ene-3,17-dione (VI), 17 β -[(1R)-1-hydroxyprop-2-ynyl]androst-4-en-3-one (VII) and 17 β -[(1S)-1-hydroxyprop-2-ynyl]androst-4-en-3-one (VIII) were prepared as described previously (Covey, 1979; Covey *et al.*, 1979b). 5,10-Seco-oestr-4-yne-3,10,17-trione (II), 17 β -hydroxy-5,10-seco-oestr-4-yne-3,10-dione (III) and 3 β ,17 β -dihydroxy-5,10-seco-oestr-4-yn-10-one (IX) were also synthesized (D. F. Covey & V. D. Parikh, unpublished work). The syntheses of 5,10-seco-oestr-5-yne-3,10,17-trione (X) and 19-nor-5,10-seco-pregn-5-yne-3,10,20-trione (XI) have been reported (Batzold & Robinson, 1976) and samples of these compounds were gifts from C. H. Robinson. When appropriate, the identity, purity and concentration of steroid solutions were checked by u.v. spectroscopy.

Inactivation experiments

The inactivation of isomerase was studied at 25°C in systems of 0.25–1.0 ml final volume containing 1.0 mM-potassium phosphate buffer, pH 7.0, 2.0 μM enzyme, and various concentrations of steroids in acetonitrile (4 or 8%, v/v). Incubations were started by the addition of the steroid solution. Portions (1 μl) were removed at suitable time intervals, diluted 1000-fold with 1% bovine serum albumin and assayed for enzyme activity (Wang *et al.*, 1963). When a semi-logarithmic plot of the

percentage of activity remaining with respect to time declined in a linear manner, indicating pseudo-first-order inactivation, the results were analysed further by the method of Kitz & Wilson (1962). In this method, it is assumed that $[I] \gg [E]$, that EI (the reversible enzyme-inhibitor complex) is at all times in equilibrium with enzyme and inhibitor, and that many reversible enzyme-inhibitor complexes form before one molecule of enzyme is inactivated (i.e. $k_{+2} \ll k_{-1}$):

where E^* is the inactivated enzyme, k_{-1}/k_{+1} is K_1 (the dissociation constant of the enzyme-inhibitor complex) and k_{+2} becomes the rate constant for the inactivation reaction within the enzyme-inhibitor complex. It has been demonstrated that this condition of quasi-equilibrium holds when the value of the apparent second-order rate constant for the inactivation of the enzyme is much smaller than k_{+1} (Brocklehurst, 1979). In our experiments the values of k_{+2}/K_1 are well below $1 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$, whereas k_{+1} is commonly at least $10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ for substrates.

By taking the reciprocals of the apparent pseudo-first-order rate constants of inactivation ($1/k_{\text{app}}$) and plotting them against the reciprocal of the inhibitor concentration ($1/[I]$) for a series of values of $[I]$, a linear relationship is obtained from which both K_1 and k_{+2} can be determined (Kitz & Wilson, 1962), since:

$$\frac{1}{k_{\text{app}}} = \frac{1}{k_{+2}} + \frac{K_1}{k_{+2}[I]} \quad (1)$$

This relationship has the same form as the Lineweaver-Burk transformation of the Michaelis-Menten equation (Lineweaver & Burk, 1934):

$$\frac{1}{v} = \frac{1}{V_{\text{max}}} + \frac{K_m}{V_{\text{max}}[S]} \quad (2)$$

Incubations of isomerase in the presence of acetonitrile and buffer under the above conditions resulted in no significant loss of enzyme activity over the period of duration of the experiments. In nearly all instances, the steroids were stable in 1 mM-potassium phosphate buffer at pH 7.0. However, compounds (X) and (XI) are only moderately stable under such conditions, and are transformed via the conjugated allenic ketones to the corresponding 3,5-diketones (Covey *et al.*, 1979a; T. M. Penning, D. F. Covey & P. Talalay, unpublished work). The rates of formation of these non-enzymic products are sufficiently low to introduce only minor errors in the measurements of the rates of inactivation.

Protection by 19-nortestosterone

Protection of isomerase against irreversible inactivation by compounds (I–IV) and (X) was afforded

by 19-nortestosterone, a powerful competitive inhibitor of the enzyme (Wang *et al.*, 1963). This phenomenon was studied under conditions described above with a constant amount of irreversible inhibitor and various concentrations of 19-nortestosterone. The incubations were initiated by the addition of the enzyme. Portions (1 μ l) were removed at specified time intervals, diluted 1000-fold with 1% bovine serum albumin and assayed for enzyme activity. The final concentration of 19-nortestosterone in the assay system had a negligible effect on the velocity. A semi-logarithmic plot of the percentage of enzyme activity remaining with respect to time was constructed for each 19-nortestosterone concentration. Pseudo-first-order rate constants of inactivation ($k_{app.}$) were obtained and a secondary plot of $1/k_{app.}$ versus 19-nortestosterone concentration ($[I']$) was made. This plot, which is analogous to the Dixon plot ($1/v$ against $[I]$; Dixon, 1953) for reversible inhibitors, permits the calculation of K_i values for 19-nortestosterone (designated here as K_i'), on the assumption of competitive inhibition. The slope of the Dixon plot is given by $K_m/(V_{max} \cdot K_i[S])$, whereas the slope of the plot of $1/k_{app.}$ with respect to $[I']$ is $K_i/(k_{+2}K_i'[I])$, as may be seen by comparison of eqns. (1) and (2).

Competitive inhibition studies

Initial velocities of isomerization of either of the natural substrates pregn-5-ene-3,20-dione or androst-5-ene-3,17-dione were measured in a system containing 2.7 ml of water, 0.1 ml of 1 M-potassium phosphate buffer, pH 7.0, the steroid inhibitor in 200 μ l of acetonitrile and 10 μ l of 1% bovine serum albumin. The reaction was followed by measuring the formation of the Δ^4 -3-oxo steroid ($\lambda_{max.} = 248$ nm; $\epsilon = 16300 \text{ M}^{-1} \cdot \text{cm}^{-1}$). Non-enzymic rates were measured over 5 min and subtracted from the enzymic rate. The non-enzymic rate was found to be less than 4% of that of the enzyme-catalysed reaction. In all cases the reaction was started by addition of enzyme.

In every experiment the K_m and $V_{max.}$ values for the natural substrates were determined over the concentration range 0–25 μ M for pregn-5-ene-3,20-dione and 0–250 μ M for androst-5-ene-3,17-dione (the upper concentrations approach the limits of solubility). The effect of a range of inhibitor concentrations was examined for each of three to five substrate concentrations. For C_{19} -steroids bearing a 1-hydroxyprop-2-ynyl substituent at C-17 the concentration of inhibitor was varied from 0 to 25 μ M. For steroids in the C_{18} series bearing an α -hydroxyacetylene group either in the ring structure or as a substituent, concentrations of inhibitor between 0 and 200 μ M were used. Dixon plots were then constructed for at least three substrate con-

centrations and the slope of each line was used to construct a secondary plot (i.e. slope of Dixon plot against $1/[S]$) from which the K_i values were computed. In all cases the lines were drawn by linear regression analysis.

Dialysis experiments

Inactivated isomerase obtained from the various experiments (containing less than 2% of the initial activity) was diluted to 1.0 ml and dialysed for 24 h against three changes of 1 litre of 1 mM-potassium phosphate, pH 7.0. At the end of the dialysis a portion of the enzyme was diluted 1000-fold with 1% bovine serum albumin and assayed for enzyme activity under standard conditions. Isomerase that was incubated in the absence of steroid can be dialysed under identical conditions without loss of enzyme activity.

Results

The following compounds were found to be active-site-directed irreversible inhibitors of Δ^5 -3-oxo steroid isomerase from *P. testosteronei*: 10 β -(1-oxoprop-2-ynyl)oestr-4-ene-3,17-dione (I), 5,10-seco-oestr-4-yne-3,10,17-trione (II), 17 β -hydroxy-5,10-seco-oestr-4-yne-3,10-dione (III) and 17 β -(1-oxoprop-2-ynyl)androst-4-en-3-one (IV). In addition the following compounds were found to be competitive inhibitors: 10 β -[(1R)-1-hydroxyprop-2-ynyl]oestr-4-ene-3,17-dione (V), 10 β -[(1S)-1-hydroxyprop-2-ynyl]oestr-4-ene-3,17-dione (VI), 17 β -[(1R)-1-hydroxyprop-2-ynyl]androst-4-en-3-one (VII), 17 β -[(1S)-1-hydroxyprop-2-ynyl]androst-4-en-3-one (VIII) and 3 β ,17 β -dihydroxy-5,10-seco-oestr-4-yn-10-one (IX).

10 β -(1-Oxoprop-2-ynyl)oestr-4-ene-3,17-dione (I)

Compound (I) was found to be an extremely potent irreversible inhibitor of isomerase, whereas compounds (V) and (VI) did not inactivate the enzyme (Fig. 1). This is not surprising since the acetylene group is not activated for nucleophilic attack in the absence of the carbonyl group. The half-life of 2.0 μ M-isomerase in the presence of 5–100 μ M-compound (I) ranged from 12 min to 45 s (Figs. 2a and 2b). The inactivation event displayed saturation kinetics. Kitz–Wilson plots of the pseudo-first-order inactivation data gave a K_i of 66.0 μ M and a k_{+2} of $12.5 \times 10^{-3} \text{ s}^{-1}$ (Figs. 2a and 2b and Table 1). The term k_{+2}/K_i describes the efficiency of the inhibitor and in this case was $188 \text{ M}^{-1} \cdot \text{s}^{-1}$. Thus compound (I) appears to be three to four times more effective as an irreversible inhibitor than the corresponding enzyme-generated inhibitor derived from the acetylenic suicide substrate in the C_{18} series (compound X), for which $k_{+2}/K_i = 50.9 \text{ M}^{-1} \cdot \text{s}^{-1}$ as described by Robinson and his colleagues (Batzold

& Robinson, 1975; Covey & Robinson, 1976). The powerful competitive inhibitor 19-nortestosterone retards the rate of inactivation by compound (I). In the presence of a constant concentration of I ($50\text{ }\mu\text{M}$) and increasing concentrations of 19-nortestosterone ($0\text{--}100\text{ }\mu\text{M}$), the half-times of inactivation increased

Fig. 1. Inactivation of Δ^5 -3-oxo steroid isomerase with 10β -(1-oxoprop-2-ynyl)estr-4-ene-3,17-dione (I). Isomerase ($2.0\text{ }\mu\text{M}$) was incubated with either $100\text{ }\mu\text{M}$ -compound (I) (x), $200\text{ }\mu\text{M}$ - 10β -(1R)-1-hydroxyprop-2-ynyl]estr-4-ene-3,17-dione (V) (■) or $200\text{ }\mu\text{M}$ - 10β -(1S)-1-hydroxyprop-2-ynyl]estr-4-ene-3,17-dione (VI) (Δ), in 1 ml of 1 mM-potassium phosphate buffer, pH 7.0, containing 4% (v/v) acetonitrile at 25°C . Portions were removed at specified times and assayed for enzyme activity.

7-fold. Since the competitive inhibitor under steady-state conditions binds a constant fraction of enzyme as a reversible E-I complex, less enzyme is available for an affinity-labelling reagent that binds to the same site as 19-nortestosterone. It may be inferred that compound (I) is an active-site-directed inhibitor. A plot of the reciprocal of the first-order rate constant for inactivation with respect to 19-nortestosterone concentration gave a K_i value for 19-nortestosterone of $6.0\text{ }\mu\text{M}$. This is comparable with the value of $12.8\text{ }\mu\text{M}$ determined for 19-nortestosterone from direct competition studies in which the initial velocity of isomerization of a constant concentration of androst-5-ene-3,17-dione was measured in the presence of various concentrations of 19-nortestosterone (Table 1).

Incubation of $4.0\text{ }\mu\text{M}$ -isomerase with $5.0\text{ }\mu\text{M}$ -compound (I) leads eventually to 90% inactivation of the enzyme, suggesting that 1 molecule of inhibitor is incorporated per subunit of isomerase. Incubation of $4.0\text{ }\mu\text{M}$ -isomerase with excess compound (I) ($>10\text{ }\mu\text{M}$) results in retention of less than 0.5% of the initial activity. If such preparations are left for 3 days at 25°C , no recovery of enzyme activity is observed. Finally, the inactivated enzyme does not regain activity on dialysis. These findings suggest that compound (I) can cause a total inactivation of enzyme activity and that a stable covalent linkage is formed.

5,10-Seco-estr-4-yne-3,10,17-trione (II) and 17β -hydroxy-5,10-seco-estr-4-yne-3,10-dione (III)

Compounds (II) and (III) were found to be good irreversible inhibitors of Δ^5 -3-oxo steroid isomerase,

Fig. 2. Kinetics of inactivation of Δ^5 -3-oxo steroid isomerase with 10β -(1-oxoprop-2-ynyl)estr-4-ene-3,17-dione (I). Isomerase ($2.0\text{ }\mu\text{M}$) was incubated with variable concentrations of compound (I) as indicated, in 1 ml of 1 mM-potassium phosphate buffer, pH 7.0, containing 4% (v/v) acetonitrile at 25°C . Portions were removed at various times and assayed for enzyme activity. A semi-logarithmic plot of the percentage of enzyme activity remaining with respect to time was constructed as shown for each inhibitor concentration (a). The resulting Kitz-Wilson plot (Kitz & Wilson, 1962) from which the K_i and k_{+2} values were determined is shown (b).

Table 1. *Inactivation of Δ^5 -3-oxo steroid isomerase with active-site-directed acetylenic steroids*

Δ^5 -3-Oxo steroid isomerase ($2.0\ \mu\text{M}$) was incubated in 1 mM-potassium phosphate buffer, pH 7.0, in the presence of compound (I) (5 – $100\ \mu\text{M}$), compound (II) (10 – $400\ \mu\text{M}$), compound (III) (50 – $400\ \mu\text{M}$), compound (IV) (2.5 – $20\ \mu\text{M}$), compound (X) (20 – $400\ \mu\text{M}$) or compound (XI) (10 – $400\ \mu\text{M}$). The inactivation results were analysed by Kitz–Wilson plots from which K_i and k_{+2} values were determined. Isomerase ($2.0\ \mu\text{M}$) was also incubated in the presence of a constant amount of compound (I) ($5.0\ \mu\text{M}$), compound (II) ($200\ \mu\text{M}$) or compound (X) ($50\ \mu\text{M}$) and the concentration of 19-nortestosterone was varied (0 – $200\ \mu\text{M}$). From these protection experiments the K_i values for 19-nortestosterone were determined (see the Materials and methods section). Experimental conditions A and B refer to the presence of either 4 or 8% by volume of acetonitrile respectively in the incubations. The acetonitrile concentration was increased to overcome the low solubility of some of the compounds. The K_i values for 19-nortestosterone were also determined by competitive-inhibition studies performed in a 3 ml reaction system containing 33 mM-potassium phosphate buffer, $10\ \mu\text{l}$ of 1% bovine serum albumin and either 4 or 8% by volume of acetonitrile containing androst-5-ene-3,17-dione as substrate.

Experimental conditions	Inhibitor		Kinetic constants for inactivation			K_i values for 19-nortestosterone obtained from:	
	No.	Structure	K_i (μM)	k_{+2} (s^{-1})	k_{+2}/K_i ($\text{M}^{-1}\cdot\text{s}^{-1}$)	Protection studies (μM)	Competitive-inhibition studies (μM)
A	(I)		66.0	12.5×10^{-3}	188	6.0	12.8
A	(X)		52.0	2.65×10^{-3}	50.9		
B	(II)		100.0	3.02×10^{-3}	30.2	8.0	25.0
B	(III)		166.0	0.77×10^{-3}	4.6		
B	(X)		66.0	2.30×10^{-3}	34.8	22.0	
B	(XI)		32.0	4.10×10^{-3}	128.0		
B	(IV)		14.5	0.13×10^{-3}	9.0	15.0

Fig. 3. Inactivation of Δ^5 -3-oxo steroid isomerase with 5,10-seco-oestr-4-yne-3,10,17-trione (II) and 17 β -hydroxy-5,10-seco-oestr-4-yne-3,10-dione (III)

Isomerase (2.0 μ M) was incubated with either 200 μ M-compound (II) (x), 200 μ M-compound (III) (O), 200 μ M-compound (II) plus 200 μ M-19-nortestosterone (Δ), or 200 μ M-3 β ,17 β -dihydroxy-5,10-seco-oestr-4-yn-10-one (IX) (□), in 0.5 ml of 1 mM-potassium phosphate buffer, pH 7.0, containing 8% (v/v) acetonitrile at 25°C. Portions were removed at various time intervals and their enzyme activity was determined.

Fig. 4. Inactivation of Δ^5 -3-oxo steroid isomerase with 17 β -(1-oxoprop-2-ynyl)androst-4-en-3-one (IV)

Isomerase (2.0 μ M) was incubated with either 20 μ M-compound (IV) (x), 20 μ M-compound (IV) plus 100 μ M-19-nortestosterone (●), 20 μ M-17 β -(1S)-1-hydroxyprop-2-ynyl]androst-4-en-3-one (VII) (■), or 20 μ M-17 β -(1R)-1-hydroxyprop-2-ynyl]androst-4-en-3-one (VIII) (Δ), in 0.25 ml of 1 mM-potassium phosphate buffer, pH 7.0, containing 8% (v/v) acetonitrile at 25°C. Portions were removed at various times and assayed for enzyme activity.

whereas the related 3 β ,17 β -dihydroxy-5,10-seco-oestr-4-yne-10-one (IX) was without effect (Fig. 3). The half-lives with compound (II) (10–400 μ M) ranged from 40 to 5 min, whereas those for compound (III) (50–400 μ M) ranged from 100 to 18 min. The fact that compound (III) is a poorer inhibitor than compound (II) is borne out by the higher K_i and lower k_{+2} values (Table 1). Indeed the k_{+2}/K_i ratio indicates that compound (II) is six to seven times more effective as an inhibitor than compound (III). The inactivation produced by (II) shows saturation kinetics and is retarded by 19-nortestosterone (K_i from competitive inhibition studies was 25.0 μ M), suggesting that the process is active-site directed. When incubations are run overnight with 200 μ M-compound (II) or -compound (III) less than 2% of the initial enzyme activity remains, and the enzyme does not regain activity after prolonged dialysis.

5,10-Seco-oestr-5-yne-3,10,17-trione (X) and 19-nor-5,10-secopregn-5-yne-3,10,20-trione (XI) are suicide substrates of Δ^5 -3-oxo steroid isomerase (Batzold & Robinson, 1975; Covey & Robinson, 1976). Table 1 reports K_i and k_{+2} values obtained from Kitz–Wilson plots for the pseudo-first-order inactivations by these compounds. The values shown are measured under the conditions described in Table 1, and are comparable with those previously

reported (Batzold & Robinson, 1975). Covey & Robinson (1976) noted that the rates of isomerization of suicide substrates [compounds (X) and (XI)] to enzyme-generated conjugated allenic ketones, the actual inactivators of isomerase, were very fast relative to the rates of inactivation seen with the allenic ketones. The similarity of the k_{+2}/K_i ratios of 3.02 and 3.48 for compounds (II) and (X) respectively suggests that compound (II) and the conjugated allenic ketones derived from compound (X) are equally effective as affinity labels of isomerase.

17 β -(1-Oxoprop-2-ynyl)androst-4-en-3-one (IV)

Compound (IV) was found to be a relatively poor irreversible inhibitor of Δ^5 -3-oxo steroid isomerase from *P. testosteronei* and, as expected, the corresponding hydroxy compounds were not irreversible inhibitors (Fig. 4). Indeed the K_i and k_{+2} values obtained from Kitz–Wilson plots of pseudo-first-order inactivation data show compound (IV) to be bound extremely tightly ($K_i = 14.5 \mu$ M) but very slow in alkylating ($k_{+2} = 0.13 \times 10^{-3} \text{ s}^{-1}$), yielding a k_{+2}/K_i ratio of $0.90 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Table 1). Protection can be afforded against inactivation with 19-nortestosterone ($K_i = 15 \mu$ M), suggesting that the process is active-site directed. The covalent bond that forms is stable to dialysis.

Competitive-inhibition studies

Compounds (V–IX) were found to be competitive inhibitors in initial-rate studies in which either androst-5-ene-3,17-dione or pregn-5-ene-3,20-dione was used as substrate. K_i values obtained for these compounds (Table 2) were found to be the same with either substrate (endorsing the view that C_{19} -

and C_{21} -steroids are bound at the same steroid-binding site). Fig. 5 shows a typical family of Dixon plots for such competitive-inhibition studies. The inset shows a secondary plot of the slope of the Dixon plots with respect to $1/[S]$. The slope of this secondary plot yields the K_i .

In these studies 10β -(1*R*)-1-hydroxyprop-2-

Table 2. *Competitive inhibition of Δ^5 -3-oxo steroid isomerase by acetylenic steroid analogues*

The values for V_{max} are approx. 50% of those observed under standard assay conditions [33 mM-potassium phosphate of pH 7.0, 1.67% (v/v) methanol and 33 μ g of bovine serum albumin/ml]. The values reported were obtained in the presence of 6.7% (v/v) acetonitrile. The nature and concentration of organic solvent profoundly affect the kinetic parameters of the reaction (Jones & Wigfield, 1968; Falcoz-Kelly *et al.*, 1968; Weintraub *et al.*, 1977). Abbreviation used: N.D., not determined.

Inhibitor		Kinetic constants for competitive inhibition with:					
		Androst-5-ene-3,17-dione			Pregn-5-ene-3,20-dione		
		K_m (μ M)	V_{max} (μ mol/min per μ mol of subunit)	K_i (μ M)	K_m (μ M)	V_{max} (μ mol/min per μ mol of subunit)	K_i (μ M)
no.	Structure						
(V)		211	1.54×10^6	81.0	12.5	0.6×10^6	71.0
(VI)		234	1.65×10^6	165.0	14.5	0.7×10^6	140.0
(VII)		297	2.49×10^6	27.0	8.0	0.63×10^6	33.0
(VIII)		250	2.24×10^6	27.0	6.9	0.588×10^6	25.0
(IX)		217	1.58×10^6	346.0	N.D.	N.D.	N.D.
		241 ± 15.3 (mean $\pm$ S.E.M.; $n = 5$)	$(1.80 \pm 0.20) \times 10^6$ (mean $\pm$ S.E.M.; $n = 5$)		10.5 ± 1.79 (mean $\pm$ S.E.M.; $n = 4$)	$(0.63 \pm 0.27) \times 10^6$ (mean $\pm$ S.E.M.; $n = 4$)

Fig. 5. Competitive inhibition of initial rate of isomerization of androst-5-ene-3,17-dione by 17β -[(1S)-1-hydroxyprop-2-ynyl]androst-4-en-3-one (VII)

Initial rates of isomerization of fixed concentrations of androst-5-ene-3,17-dione (26.4, 52.8, 105.6 and 158.3 μM) were measured in the presence of variable concentrations of compound (VII) (0–20 μM). Dixon plots were then constructed as shown. The inset shows a secondary plot of the slope of the Dixon plots with respect to $1/[S]$, from which the K_i of the competitive inhibitor was calculated.

ynyl]oestr-4-ene-3,17-dione (V) was found consistently to be a better inhibitor than 10β -[(1S)-1-hydroxyprop-2-ynyl]oestr-4-ene-3,17-dione (VI). For example, K_i values of 81 μM and 165 μM respectively were obtained with androst-5-ene-3,17-dione as substrate and K_i values of 71 and 140 μM respectively with pregn-5-ene-3,20-dione as substrate.

Compounds (VII) and (VIII) appear to be equipotent as reversible inhibitors. In addition they bind fairly tightly to the enzyme with K_i values between 25.0 and 33.0 μM respectively (Table 2), as is normally the case with C_{21} -steroids. This suggests that the enzyme has no preference as to whether the polar group at C-20 is in the α - or β -configuration. Compound (IX) was found to be a very poor competitive inhibitor of isomerase ($K_i = 346 \mu\text{M}$), indicating that when the acetylenic group occupies position-4 in the seco-steroid a $3\beta,17\beta$ -diol is not well tolerated.

Discussion

After the pioneering studies of Bloch (1971), suicide substrates have been designed for use both in novel methods of affinity labelling and in selective

approaches to drug design (Seiler *et al.*, 1978). Many of the enzyme inactivators in this category are acetylenic compounds that are converted by their target enzymes into Michael acceptor systems. This activation may give rise to conjugated allenic ketones as described for compounds (X) and (XI) (Batzold & Robinson, 1975; Covey & Robinson, 1976), or to an allene conjugated with a Schiff's base, as in the case of the inactivation of pyridoxal phosphate-linked enzymes by propargylglycine (Abeles & Walsh, 1973). Alternatively, the acetylene may be activated by direct conjugated with a Schiff's base, as in the case of the inactivation of γ -aminobutyric acid transaminase by 4-aminohex-5-ynoic acid (Metcalf *et al.*, 1978). However, little attention appears to have been accorded to the possibility of creating affinity-labelling reagents by introducing conjugated acetylenic ketone groupings into substrate analogues. The present paper describes four steroid analogues that contain such reactive groups and are active-site-directed irreversible inhibitors of Δ^5 -3-oxo steroid isomerase. We suggest that introduction of this functionality into substrates for enzymes requiring a base for their reaction mechanism could lead to new types of effective affinity-labelling reagents.

Of the compounds described in the present paper, compound (I) appears to be especially interesting, since it is one of the most potent irreversible inhibitors of Δ^5 -3-oxo steroid isomerase described. The presence of the bulky 10β -(1-oxoprop-2-ynyl) group was considered to be unfavourable for binding to the active site of the enzyme because of steric hindrance, since 19-nor-steroids (e.g. 19-nortestosterone, 17β -oestradiol) are far more potent as reversible inhibitors of the enzyme than steroids carrying a 10-methyl substituent (Wang *et al.*, 1963; Weintraub *et al.*, 1977). This conclusion may require revision in the light of the finding that 10β -(1-oxoprop-2-ynyl) groups and 10-oxo substituents are associated with relatively low K_i values, in comparison with those obtained for 19-methyl steroids. Thus, the chemical nature rather than the presence of a substituent at C-10 may be a far more important factor in controlling the binding of the steroid analogue. It has been suggested on the basis of crystallographic studies of isomerase substrates and suicide inhibitors (Carrell *et al.*, 1978) that the intramolecular transfer of the 4β -proton to the 6β -position that occurs during the isomerization reaction may be mediated by a basic residue that is symmetrically located with respect to the 4β -proton and the 19-methyl group. The potent inactivation of isomerase by compound (I) suggests that the space occupied by the 10β -(1-oxoprop-2-ynyl) Michael acceptor system may be located close to this important nucleophile that abstracts the 4β -proton (Scheme 3).

Scheme 3. Mechanistic pathway for enzyme-catalysed isomerization of Δ^3 -3-oxo steroids to Δ^4 -3-oxo steroids based on the X-ray crystallographic structure for androst-5-ene-3,17-dione

The isomerization reaction is facilitated by an acid (AH) and a base (B) at the active site. The base (B) abstracts the 4β -proton. The crystallographic structure of androst-5-ene-3,17-dione clearly indicates that this base must be positioned above the plane of the A-ring of the steroid and is symmetrically located with respect to both the 4β -proton and the 19-methyl group. The planar $\Delta^{3,5}$ -dienol is an intermediate and the 4β -proton is subsequently transferred intramolecularly to the 6β -position to give the Δ^4 -3-oxo steroid. A structure for the 10β -(1-oxoprop-2-ynyl)androst-4-ene-3,17-dione is shown (lower right) in which the α,β -acetylenic ketone substituent is in close proximity to the base (B). This could explain the inhibitory potency of this compound.

Another surprising finding is that compound (II) (containing the 17-oxo group) is about six to seven times more potent as an alkylating agent than compound (III) (containing the 17β -hydroxy group). In contrast, there is little difference in the K_1 values for androst-4-ene-3,17-dione ($200\mu\text{M}$) and testosterone ($150\mu\text{M}$) (Weintraub *et al.*, 1977). We conclude that the changes in the conformation of the A/B ring system, resulting from removal of the 5,10 bond and the introduction of an acetylenic linkage,

may affect the spatial position of the D ring and place the 17β -hydroxy group of compound (III) in an unfavourable (hydrophobic) environment. That the conformation of the A ring can affect the topographic position of the D ring and influence binding affinities is also suggested by the studies of Weintraub *et al.* (1977), who have shown that oestra-1,3,5-triene derivatives (with planar A rings) carrying 17α -acetylenic substituents are bound very efficiently to the enzyme, whereas the analogous

androstene derivatives with 17 α -acetylenic groups are not well tolerated. The interactions near position-17 of the steroid are further emphasized by the findings that steroids bearing a hydroxypropynyl group at C-17 are good reversible inhibitors of the isomerase, irrespective of the configuration of the hydroxy group at C-20 (K_i values of 25–33 μ M) for compounds (VII) and (VIII). This behaviour would appear to be in marked contrast with the already described large differences in the inhibitory potency of 17 α -hydroxyandrost-4-en-3-one, which is not bound, versus 17 β -hydroxyandrost-4-en-3-one (testosterone), which has a K_i value of 150 μ M (Weintraub *et al.*, 1977). These values were determined in the presence of 10% (w/v) methanol as co-solvent, whereas the kinetic parameters reported in Table 2 were measured in 6.7% (w/v) acetonitrile. Despite the profound effects of organic solvents on kinetic parameters of the isomerization reaction (Jones & Wigfield, 1968; Falcoz-Kelly *et al.*, 1968; Weintraub *et al.*, 1977) we believe these comparisons to be sound. The hydroxy groups of 17 β -hydroxypropynyl substituents could rotate so that they lie outside the influence of any unfavourable interactions with the enzyme surface.

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