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Irreversible inhibition of Δ^5-3-oxosteroid isomerase by 2-substituted progesterones

Trevor M. PENNING
Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, U.S.A.

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2α-Cyanoprogesterone (I) and 2-hydroxymethyleneprogesterone (II) were synthesized and screened as irreversible active-site-directed inhibitors of the Δ^5-3-oxosteroid isomerase (EC 5.3.3.1) from *Pseudomonas testosteroni*. Both compounds were found to inhibit the purified bacterial enzyme in a time-dependent manner. In either case the inactivated enzyme could be dialysed without return of activity, indicating that a stable covalent bond had formed between the inhibitor and the enzyme. Inactivation mediated by compounds (I) and (II) followed pseudo-first-order kinetics, and at higher inhibitor concentrations saturation was observed. The competitive inhibitor 17β-oestradiol offered protection against the inactivation mediated by both compounds, and initial-rate studies indicated that compounds (I) and (II) can also act as competitive inhibitors yielding K_i values identical with those generated during inactivation experiments. 2α-Cyanoprogesterone (I) and 2-hydroxymethyleneprogesterone (II) thus appear to be active-site-directed. To compare the reactivity of these 2-substituted progesterones with other irreversible inhibitors of the isomerase, 3β-spiro-oxiranyl-5α-pregnan-20β-ol (III) was synthesized as the C_{21} analogue of 3β-spiro-oxiranyl-5α-androstan-17β-ol, which is a potent inactivator of the isomerase [Pollack, Kayser & Bevins (1979) *Biochem. Biophys. Res. Commun.* **91**, 783–790]. Comparison of the bimolecular rate constants for inactivation (k_{+3}/K_i) mediated by compounds (I)–(III) indicated the following order of reactivity: (III) > (II) > (I). 2-Mercaptoethanol offers complete protection against the inactivation of the isomerase mediated by 2α-cyanoprogesterone (I). Under the conditions of inactivation compound (I) appears to be completely stable, and no evidence could be obtained for enolate ion formation in the presence or absence of enzyme. It is suggested that cyanoprogesterone inactivates the isomerase after direct nucleophilic attack at the electropositive 2-position, and that tautomerization plays no role in the inactivation event. By contrast, 2-mercaptoethanol offers no protection against the inactivation mediated by 2-hydroxymethyleneprogesterone, and under the conditions of inactivation this compound appears to exist in the semi-enolized form.

The final step in progesterone synthesis in mammalian tissues is catalysed by a C_{21}-Δ^5-3-oxosteroid isomerase which converts pregn-5-ene-3,20-dione (a Δ^5-3-oxosteroid) into progesterone (a Δ^4-3-oxosteroid). Our long-term goal is to synthesize selective irreversible inhibitors of the ovarian and placental isomerase, since these may have the potential to act as antiprogesterones. These mammalian enzymes have proven difficult to study, since they are microsomal and require radiochemical assays for detection. A reasonable model system on which to screen potential inhibitors is the Δ^5-3-oxosteroid isomerase from *Pseudomonas testosteroni*. This bacterial enzyme with the aid of acid/base catalyses isomerizes Δ^5-3-oxosteroids into Δ^4-3-oxosteroids via the *cis*-diaxial transfer of the 4β-proton to the 6β-position (Malhotra &

Abbreviations used: 2α-cyanoprogesterone, 2α-cyanopregn-4-ene-3,20-dione; 2-hydroxymethyleneprogesterone, 2-hydroxymethylenepregn-4-ene-3,20-dione; 3β-spiro-oxiranylpregnane, 3β-spiro-oxiranyl-5α-pregnan-20β-ol; 3α-spiro-oxiranylpregnane, 3α-spiro-oxiranyl-5α-pregnan-20β-ol.

Ringold, 1965; Talalay & Benson, 1972). Although there is some disagreement concerning the percentage of proton transfer that is intramolecular, there is a fairly good concensus that the catalytic mechanism for the bacterial and mammalian enzymes is quite similar (Murota *et al.*, 1971; Batzold *et al.*, 1976; Akhtar *et al.*, 1980; Viger *et al.*, 1981).

Interest in developing irreversible inhibitors of the Δ^5-3-oxosteroid isomerases was originated by Goldman (1968), who reported that 2α-cyano-17β-hydroxy-4,4,17-trimethylandrost-5-en-3-one and 17β-hydroxy-2-hydroxymethylene-androstan-3-one appeared to be very-tight-binding inhibitors of both the bacterial and the adrenal isomerase. It was suggested that these compounds interacted with these enzymes in a stoichiometric manner. Since these earlier observations, a number of effective irreversible inhibitors of the bacterial isomerase have been reported. These include the suicide substrates, the 5,10-seco-3-oxosteroids synthesized by Batzold & Robinson (1975, 1976), the 3β-spiro-oxiranylandrostane derivatives synthesized by Pollack *et al.* (1979) and the aromatase inhibitor 10β-(1-oxoprop-2-ynyl)oestr-4-ene-3,17-dione (Penning *et al.*, 1981*a*).

Our studies in mammalian tissues indicate that separate enzymes may exist for the isomerization of androst-5-ene-3,17-dione and pregn-5-ene-3,20-dione (Penning & Covey, 1982). Thus C_{21} analogues of several of the inhibitors described above may have the potential to prevent progesterone synthesis in endocrine tissues.

With this background in mind, I have commenced our programme by synthesizing 2α-cyanoprogesterone (I) and 2-hydroxymethyleneprogesterone (II) (see Fig. 1), and have compared their ability with that of 3β-spiro-oxiranyl-5α-pregnan-20β-ol (III) to inactivate the purified bacterial isomerase irreversibly.

Materials and methods

Materials

Pregnenolone, dehydroepiandrosterone and 20β-hydroxy-5α-pregnan-3-one were purchased from Steraloids (Wilton, NH, U.S.A.). Spectroscopic-quality acetonitrile was obtained from Burdick and Jackson, Muskegon, MI, U.S.A., and crystalline bovine serum albumin was purchased from Armour Pharmaceuticals, Kankakee, IL, U.S.A. Solutions of albumin were neutralized with NaOH.

The purification of crystalline isomerase and the determination of its specific activity and molarity have been described elsewhere (Benson *et al.*, 1971, 1974, 1975).

Steroid synthesis

Synthesis of androst-5-ene-3,17-dione, 2-hydroxymethyleneprogesterone (II) *and 2α-cyanoprogesterone* (*I*). Androst-5-ene-3,17-dione was synthesized from dehydroepiandrosterone (Kawahara, 1962); 2-hydroxymethyleneprogesterone (II) and 2α-cyanoprogesterone (I) were synthesized from pregnenolone acetate in five and six steps respectively (Gut, 1956; Kissman *et al.*, 1962).

Synthesis of 3α-spiro-oxiranyl-5α-pregnan-20β-ol (*IV*). A mixture of a 50.2% suspension of NaH in oil (6 mmol, 150 mg) plus 6.26 mmol of trimethyloxo-

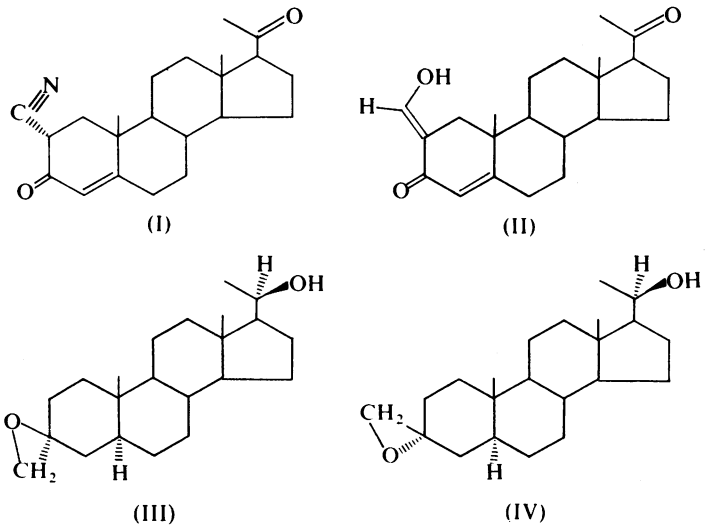


Fig. 1. *Active-site-directed irreversible inhibitors of bacterial* Δ^5*-3-oxosteroid isomerase*

sulphonium iodide (1.37g) and 30ml of dimethyl sulphoxide (redistilled over CaH_2) was stirred under N_2 at room temperature until no more gas was evolved. Solid 20β-hydroxy-5α-pregnan-3-one (100mg) was added, and the solution was stirred for 16h at room temperature and then for 2h at 50°C. The solution was then poured into 100ml of water, and the white precipitate was recrystallized from acetonitrile, with an overall yield of 40%, m.p. 184–186°C. I.r. 3600cm^{-1} (broad OH band) and absence of (C=O) stretch at 1710cm^{-1}; n.m.r. δ (p.p.m.) [(^{2}H)chloroform] 0.76 (3H, s, C-18-CH_3), 0.86 (3H, s, C-19-CH_3), 1.14 (3H, d, J = 6.24Hz, C-21-CH_3) 2.64 (2H, s, 3β-CH_2) and 4.04 (1H, multiplet, OH).

Synthesis of 3β-spiro-oxiranyl-5α-pregnan-20β-ol (III). A 50.2% suspension of NaH in oil (12mmol, 300mg) was washed in redistilled dry tetrahydrofuran. Dimethyl sulphoxide (9ml, redistilled over CaH_2) was added, and the sodium salt was prepared by heating at 70°C under N_2. The volume was then increased by addition of tetrahydrofuran (9ml), and the mixture was cooled to −5°C. Trimethylsulphonium iodide (12mmol) was added rapidly to the slurry to form the dimethylsulphonium ylide, and was followed by the addition of 20β-hydroxy-5α-pregnan-3-one (100mg) dissolved in 3.0ml of tetrahydrofuran. The reaction was allowed to proceed for 1–2h on ice, and then at room temperature overnight under N_2. The reaction mixture was poured into 150ml of water containing 2.5ml of acetic acid; the product was extracted with chloroform and dried over anhydrous $MgSO_4$, and solvent removed *in vacuo*. Analysis of the product by t.l.c. indicated the presence of one major product contaminated with some less-polar material (presumably the 20β-methyl ether). This contaminant was removed by chromatography on neutral alumina [with benzene/ethyl acetate (4:1, v/v)], and the desired material was recrystallized from diethyl ether in an overall yield of 60%, m.p. 176–178°C. I.r. 3600cm^{-1} (broad OH band) and absence of (C=O) strength at 1710cm^{-1}; n.m.r. indicated the presence of both 3α- (axial) and 3β- (equatorial) oxirane protons in the ratio of 2:1, δ (p.p.m.) [(^{2}H)chloroform] 2.56 (s, 3α-CH_2) and 2.62 (s, 3β-CH_2); other chemical shifts were 0.73 (3H, s, C-18-CH_3), 0.82 and 0.84 (two singlets in the ratio of 2:1, C-19-CH_3 from both isomers), 1.12 (d, J = 6.24Hz, C-21-CH_3) and 3.94p.p.m. (1H, multiplet, OH). Attempts to separate the 2:1 mixture of the 3β- and 3α-spiro-oxiranes by chromatography on neutral alumina in a variety of solvent systems was unsuccessful.

Enzyme studies

Isomerase activity was measured in 1.0ml systems containing 0.85ml of water, 0.100ml of 1.0M-potassium phosphate buffer pH7.0, 10μl of neutralized 1% (w/v) bovine serum albumin and 59.5μM-androst-5-ene-3,17-dione in 40μl of acetonitrile. In every assay, the final concentration of organic solvent was maintained at 4% (v/v). Reactions were initiated by the addition of enzyme, and the amount of product formed was monitored at 248nm by measuring the formation of the Δ^4-3-ketone; ε = 16300M^{-1}·cm^{-1}.

Inactivation experiments were conducted as described by Penning *et al.* (1981*a*). Essentially stoichiometric amounts of inhibitor and isomerase were incubated together and samples were removed with time and diluted 1 in 10^3 with 1% bovine serum albumin before assay. Thus the final concentration of inhibitor within the assays was always negligible. Kinetic constants for inactivation were calculated by using the method of Kitz & Wilson (1962). Competitive-inhibition experiments were conducted as previously described (Penning *et al.*, 1981*a*), and K_i values were computed from the secondary plots obtained from Dixon plots (Siegel, 1975). In every instance lines were drawn after linear-regression analysis by using the procedure FITLINE, which is available to users of the PROPHET computer.

Results and discussion

Irreversible inhibition of Δ^5-3-oxosteroid isomerase by 2-substituted progesterones

2α-Cyanoprogesterone (I) and 2-hydroxymethyleneprogesterone (II) were synthesized and evaluated as irreversible inhibitors of the Δ^5-3-oxosteroid isomerase from *Pseudomonas*. Both compounds produced a time-dependent loss of enzyme activity that was a function of inhibitor concentration (Figs. 2*a* and 2*b*). Double-reciprocal plots of the pseudo-first-order rate constants for inactivation versus inhibitor concentration (Fig. 3) gave K_i values (dissociation constants for the reversible enzyme–inhibitor complex), k_{+3} values (limiting rate constant for inactivation) and τ_{50} values (the time by which 50% of the enzyme activity is lost under saturation conditions). Values for 2α-cyanoprogesterone were as follows: K_i = 105μM, $k_{+3} = 0.13 \times 10^{-3}$s^{-1} and τ = 88min. For 2-hydroxymethyleneprogesterone the corresponding values were: K_i = 125μM, $k_{+3} = 0.3 \times 10^{-3}$s^{-1} and τ = 38min. When isomerase inactivated with compound (I) or (II) was extensively dialysed against 5mM-potassium phosphate buffer, pH7.0, there was no restoration of enzyme activity, suggesting that the inactivation event is accompanied by stable covalent-bond formation. Under these dialysis conditions, untreated enzyme retains full activity.

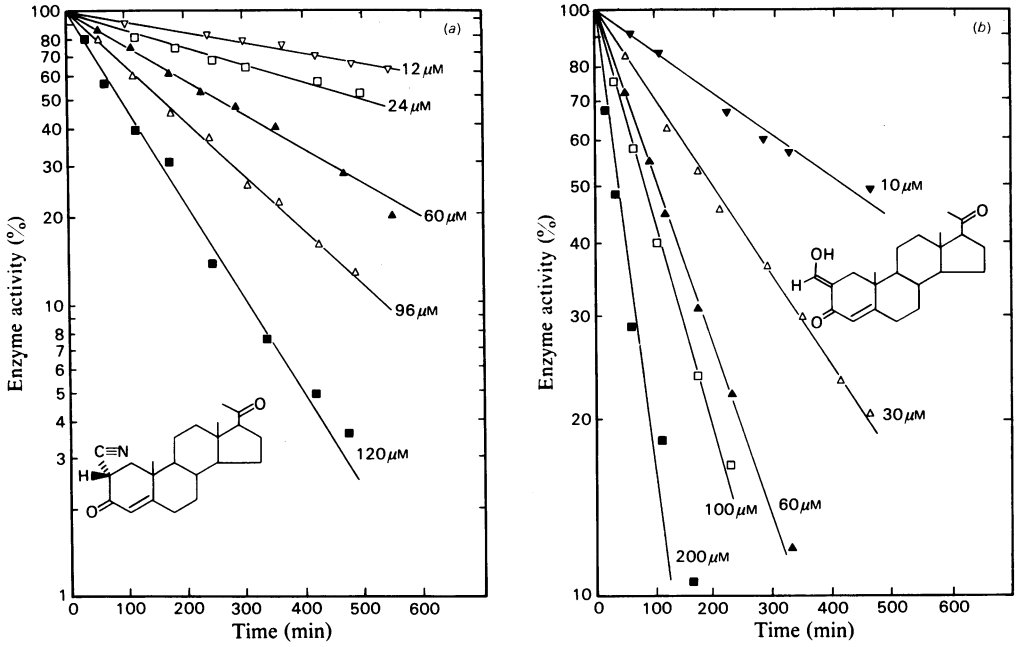


Fig. 2. *Irreversible inhibition of Δ^5-3-oxosteroid isomerase by 2α-cyanoprogesterone (a) and 2-hydroxymethyleneprogesterone (b)* Isomerase (1.0 μM) was incubated with various concentrations of 2α-cyanoprogesterone or 2-hydroxymethyleneprogesterone, as indicated, in 0.5 ml of 5 mM-potassium phosphate buffer, pH 7.0, containing 4% (v/v) acetonitrile at 25°C. Portions were removed at the times indicated, diluted with 1% bovine serum albumin and assayed for enzyme activity. A semi-logarithmic plot of percentage of enzyme activity remaining with respect to time was constructed, as shown for each inhibitor concentration.

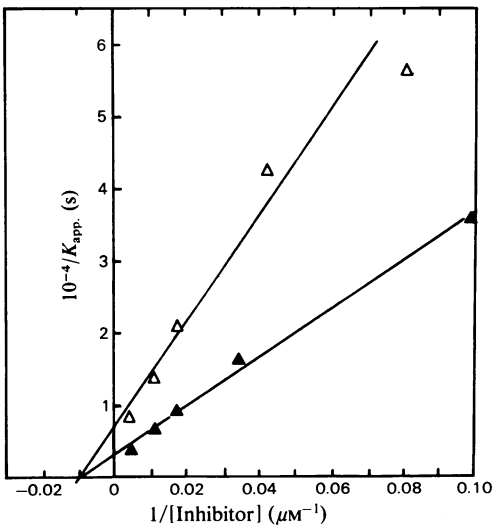


Fig. 3. *Kitz–Wilson plots for the inactivation of Δ^5-3-oxosteroid isomerase mediated by 2α-cyanoprogesterone (△) and 2-hydroxymethyleneprogesterone (▲)* Pseudo-first-order rate constants from the inactivation data shown in Fig. 2 were plotted versus the reciprocal of the inhibitor concentration. The intercept on the ordinate yields $1/k_{+3}$, and the intercept on the negative abscissa yields $-1/K_i$.

The powerful competitive inhibitor of the bacterial isomerase 17β-oestradiol ($K_i = 10\,\mu M$; Batzold *et al.*, 1976), at concentrations of 20–40 μM, protects the enzyme from inactivation by either the 2α-cyanoprogesterone (I) or the 2-hydroxymethyleneprogesterone (II). In addition, initial-rate studies with compounds (I) and (II) gave Dixon plots (Fig. 4) that yielded K_i values for competitive inhibition identical with those generated from Kitz–Wilson plots of inactivation data. Taken together, these data indicate that compounds (I) and (II) are active-site-directed irreversible inhibitors of the bacterial isomerase.

It is of interest to compare these findings with those reported by Goldman (1968), in which 2α-cyano-17β-hydroxy-4,4,17-trimethylandrost-5-en-3-one and 17β-hydroxy-2-hydroxymethyleneandrostan-3-one were used to inhibit the bacterial isomerase. Initial-rate studies with these compounds indicated that they were very potent competitive inhibitors of the isomerase, yielding K_i values of 0.40 and 1.5 μM respectively. Failure to restore enzyme activity after dialysis indicated that it was very difficult to remove these tight-binding inhibitors from the enzyme, and as a consequence these compounds were described as stoichiometric inhibitors. In these earlier studies, no time-course

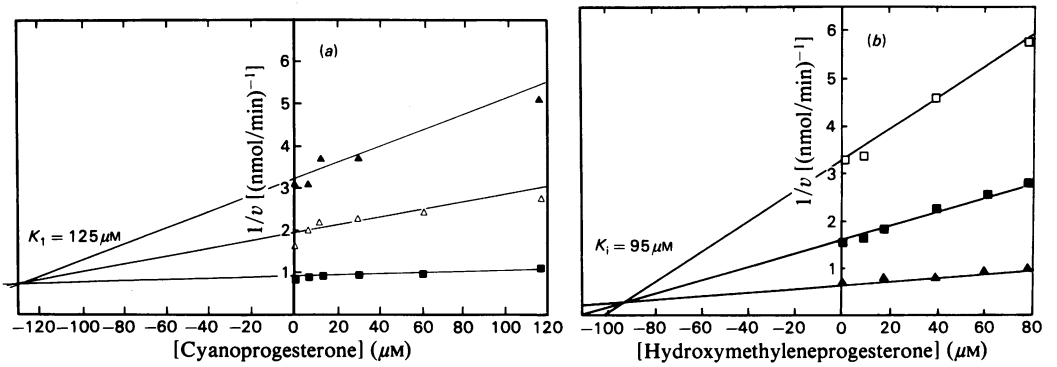


Fig. 4. *Competitive inhibition of the initial rate of Δ^5-3-oxosteroid isomerization by 2α-cyanoprogesterone (a) and 2-hydroxymethyleneprogesterone (b)*

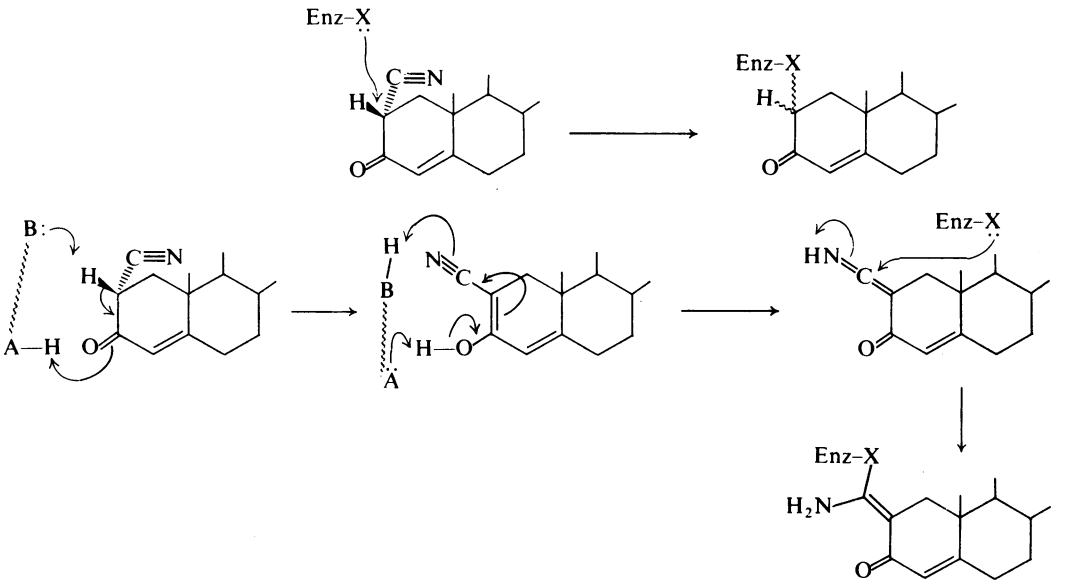
Initial rates of isomerization at fixed concentrations of androst-5-ene-3,17-dione (9.9, 19.8 and 99.6 μM) were measured in the presence of various concentrations of 2α-cyanoprogesterone (5.8–116.0 μM), or at fixed concentrations of androst-5-ene-3,17-dione (9.9, 19.8 and 49.8 μM) in the presence of various concentrations of 2-hydroxymethyleneprogesterone (19.6–78.5 μM). Dixon plots were constructed as shown.

experiments were undertaken. The work described in the present paper suggests that 2-hydroxymethylene- and 2-cyano-substituted 3-oxosteroids may represent a true class of irreversible inhibitors for the Δ^5-3-oxosteroid isomerase. From this discussion, it should be apparent that the K_i values for 2α-cyanoprogesterone and 2-hydroxymethyleneprogesterone are several orders of magnitude greater than those reported by Goldman (1968) for the 2α-cyano-17β-hydroxy-4,4,17-trimethylandrost-5-en-3-one and 17β-hydroxy-2-hydroxymethylene-androstan-3-one, and suggest that the enzyme prefers to accommodate a bulky 4,4-dimethyl-substituted steroid than a Δ^4-3-ketone.

In order to compare the reactivity of 2α-cyanoprogesterone (I) and 2-hydroxymethyleneprogesterone (II) with other inhibitors of the bacterial isomerase, I have synthesized pure 3α-spiro-oxiranyl-5α-pregnan-20β-ol (IV) and a 2:1 mixture of the 3β-spiro-oxiranylpregnane (III) + 3α-spiro-oxiranylpregnane (IV). These pregnane derivatives are related to the 3β-spiro-oxiranyl-5α-androstan-17β-ol synthesized by Pollack *et al.* (1979). This latter compound is among the most potent irreversible inhibitors of the bacterial enzyme, whereas the opposite stereoisomer, the 3α-spiro-oxiranyl-5α-androstan-17β-ol, was unable to cause enzyme inactivation. In my studies, the pure 3-α-spiro-oxiranylpregnane (IV) did not inactivate the oxosteroid isomerase, whereas the 2:1 mixture of the 3α- and 3β-isomers produced a rapid time-dependent inactivation. The effect would thus seem to be due to the 3β-isomer. Double-reciprocal plots (not shown) of the inactivation data generated by the mixture gave a K_i value of 29.0 μM, a k_{+3} of 1.47×10^{-3} s^{-1} and a τ value of 7.8 min. These kinetic constants reflect the properties of the 3β-isomer and are believed to be unaffected by the contaminating 3α-isomer. Thus initial-rate studies indicate that the 3β-isomer is a good competitive inhibitor of androst-5-ene-3,17-dione isomerization ($K_i = 29\mu M$), whereas the 3α-isomer has no effect on these initial velocities and appears to be essentially unbound over its range of solubility. Such findings indicate that 3β-spiro-oxiranyl-5α-pregnan-20β-ol (III) is an active-site-directed irreversible inhibitor of the bacterial enzyme, whereas the 3α-isomer appears to be excluded from the active site. Comparison of the bimolecular rate constants k_{+3}/K_i generated during the inactivation of the isomerase with compounds (I)–(III) indicated the following order of reactivity: 3β-spiro-oxiranyl-5α-pregnane-3,20-dione (III) > 2-hydroxymethyleneprogesterone (II) > 2α-cyanoprogesterone (I).

Mechanism of inactivation mediated by 2-substituted progesterones

2α-Cyanoprogesterone can inactivate the steroid isomerase by two plausible mechanisms (Scheme 1). In the first mechanism, there could be direct nucleophilic attack at the electropositive 2-position, with subsequent release of cyanide. In the second mechanism, the base at the active site could extract the 2β-proton, and enolization via the 3-ketone would permit tautomerism to generate a keteneimine that could exist in conjugation with the ketone at C-3. The resulting enzyme-generated Michael acceptor would be prone to nucleophilic attack. This latter mechanism was postulated on the basis of a number of observations. First, when the purified bacterial isomerase binds competitive



Scheme 1. *Inactivation of bacterial Δ^5-3-oxosteroid isomerase by 2α-cyanoprogesterone*

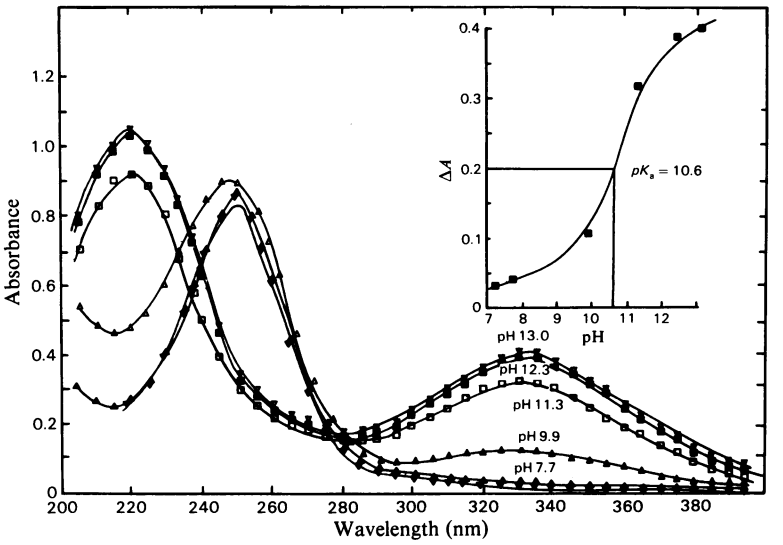


Fig. 5. *Effect of base and 2-mercaptoethanol on the u.v.-absorption spectrum of 2α-cyanoprogesterone*
The u.v.-absorption spectrum of 70 μM-cyanoprogesterone in 5 mM-potassium phosphate buffer containing 4% (v/v) acetonitrile was taken at the pH values shown (pH was adjusted with 0.1 M-NaOH). The effect of 20 mM-2-mercaptoethanol on the u.v.-absorption spectrum measured at pH 7.0 is also indicated (▼).

inhibitors such as 19-nortestosterone, red-shifts in the u.v.-absorption spectrum of the enzyme–steroid complex occur and suggest that enolization via extraction of the 2β-proton can take place (Kawahara *et al.*, 1962). Secondly, the α-cyano-ketone group of 2α-cyanoprogesterone is analogous to the βγ-acetylenic ketone present in 5,10-seco-oestr-5-yne-3,10,17-trione, which is a suicide substrate for the bacterial isomerase. In this analogy, the 4β-proton is abstracted by the base at the active site. Enolization of the carbanion via the 3-ketone yields the highly reactive allenic ketone, which then inactivates the enzyme (Batzold & Robinson, 1975; Covey & Robinson, 1976; Pen-

ning *et al.*, 1981*b*). I believed that the formation of a keteneimine in conjugation with the 3-ketone could be formally analogous to the formation of the allenic ketone. Thirdly, Alston (1980) proposed that the α-cyanoketone (2α-cyano-17β-hydroxy-4,14,17α-trimethylandrost-5-en-3-one) tested by Goldman (1968) may inactivate the 3β-hydroxysteroid dehydrogenase/Δ^5-3-oxosteroid isomerase by keteneimine formation.

The experimental findings given below help to discriminate between the two mechanisms. First, in potassium phosphate buffer, pH 7.0, 2α-cyanoprogesterone is completely stable and the u.v.-absorption spectrum shows a single peak of strong absorbance ($\lambda_{max.}$ 248nm; $\varepsilon = 13500 M^{-1} \cdot cm^{-1}$; Fig. 5). Upon titration in base, the enolate ion can be generated, as shown by the appearance of a new peak ($\lambda_{max.}$ 335nm; $\varepsilon = 5500 M^{-1} \cdot cm^{-1}$). These titrations yield a pK_a for the enol of 10.6, suggesting that strong base will generate the enolate. However, no evidence for the formation of a keteneimine could be obtained. Secondly, the bacterial isomerase is inactivated by 2α-cyanoprogesterone over a considerable period of time, the τ_{50} value being 88min. If the isomerase were generating its own alkylating agent (e.g. keteneimine), it would have time to diffuse into solution, and yet u.v. spectrometry indicates that the spectrum of 2α-cyanoprogesterone is unaffected by the inactivation event. Thirdly, when the enzyme is incubated with 2α-cyanoprogesterone in the presence of 20mM-2-mercaptoethanol at pH 7.0, complete protection against inactivation is observed (Fig. 6). Under these conditions, there is no change in the u.v.-absorption spectrum of the steroid (Fig. 5). Thus, in these model nucleophilic addition reactions, the conjugation of the Δ^4-3-ketone of 2α-cyanoprogesterone is not extended, and indicates that the thiol reagent scavenges via direct displacement of the 2α-cyano group. (This concentration of 2-mercaptoethanol has no effect on untreated enzyme and will not restore activity to dialysed enzyme that has been inactivated by more than 99% by 2α-cyanoprogesterone.) Together, these data suggest that the mechanism of inactivation is via direct nucleophilic displacement of the 2α-cyano group and not by enzyme-generated keteneimine formation.

Conclusive proof of this mechanism would require detection of the cyanide released during the inactivation event. This is beyond the scope of the present study, since it would require introduction of a [^{14}C]cyano group into progesterone, which is not a trivial undertaking. Another concern is that, if it is assumed that the stoichiometry of inactivation is one molecule of cyanoprogesterone incorporated per subunit of enzyme, the radiolabelled cyanoprogesterone would have to be of sufficiently high specific radioactivity so that detection of a small amount of cyanide would be possible.

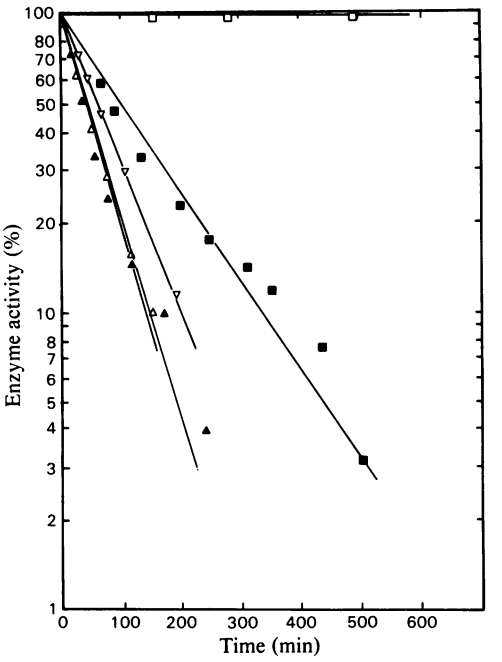


Fig. 6. *Effect of 2-mercaptoethanol on the inactivation of Δ^5-3-oxosteroid isomerase mediated by 2α-cyanoprogesterone and 2-hydroxymethyleneprogesterone*

The inactivation of Δ^5-3-oxosteroid isomerase by 2α-cyanoprogesterone (■) and 2-hydroxymethyleneprogesterone (▲) was monitored, as described in the legend to Fig. 2. The effect of 2-mercaptoethanol on the rates of inactivation was also determined: □, 100μM-2α-cyanoprogesterone plus 20mM-2-mercaptoethanol; △, 100μM-2-hydroxymethyleneprogesterone plus 20mM-2-mercaptoethanol; ▽, 100μM-2-hydroxymethyleneprogesterone plus 50mM-2-mercaptoethanol.

2α-Hydroxymethyleneprogesterone, when incubated in potassium phosphate buffer, pH 7.0, shows two absorbance peaks in the u.v. region that correspond to the Δ^4-3-ketone ($\lambda_{max.}$ 248nm; $\varepsilon = 13500 M^{-1} \cdot cm^{-1}$) and the semi-enolized form of the keto-aldehyde ($\lambda_{max.}$ 355nm; $\varepsilon = 6500 M^{-1} \cdot cm^{-1}$). Acid/base titration of this compound yields a pK_a of 6.0 for the keto-aldehyde. Thus, during the inactivation event, the enzyme is incubated with the semi-enolized form. With either tautomer, it is difficult to envisage how alkylation via nucleophilic attack can take place. This is borne out by scavenging experiments with 2-mercaptoethanol. Thus, even when concentrations as high as 50mM-2-mercaptoethanol are used, it is difficult to protect the isomerase from inactivation by the 2-

hydroxymethyleneprogesterone (Fig. 6). It should be recalled that, as this compound is a tautomer of a dicarbonyl, a possible mechanism for inactivation is that it could react with the guanidino group of arginine. Although further experimentation will be required to establish this point, dialdehydes, e.g. malondialdehyde (Vallee & Riordan, 1969), can act as arginine-modifying reagents. It is of interest that butane-2,3-dione (a diketone arginine-modifying reagent) will inactivate the purified isomerase in borate buffer at pH8.0 and that protection can be afforded by the powerful competitive inhibitors 19-nortestosterone and 17β-oestradiol (results not shown).

The present paper describes the irreversible inhibition of the bacterial steroid isomerase by 2-substituted progesterones and presents some data alluding to their mechanism of inactivation. These steroids may have the potential to inhibit the C_{21}-Δ^5-3-oxosteroid isomerase of mammalian endocrine tissues, and thereby prevent progesterone biosynthesis.

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