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MODIFIED ACETYLENIC STEROIDS AS POTENT MECHANISM-BASED INHIBITORS OF CYTOCHROME P-450$_{scc}$

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Summary—Synthesized 20-(4-tetrahydropyranyl-1-butynyloxy)-5-pregnen-3α,20β-diol [steroid I] and 20-(3-tetrahydropyranyl-1-propargyloxy)-5-pregnen-3α,20β-diol [steroid III] have been found to inactivate purified adrenocortical cytochrome P-450$_{scc}$. When incubated with the enzyme under turnover conditions, steroid I inactivated cytochrome P-450$_{scc}$ by about 85% in 40 min. This is in contrast to the free triol analog, steroid II which inactivated the enzyme by only 45% within the same incubation period. A comparison of steroid III with its free triol analog, steroid IV, also showed that the diol is a more effective inactivator of the enzyme than the triol. The partition ratio was calculated by two different methods. Each of the steroids I–IV bound to the enzyme with spectrophotometric dissociation constant (K_s) in the micromolar range, producing Type II low spin spectra changes during titration of the enzyme. In addition, it was found that the binding of each of the compounds to the enzyme occurred without inactivation of the enzyme and that the inactivation under turnover condition, is not as a result of conversion to the denatured P-420 species. This demonstrated that steroids I and III could correctly be designated as mechanism-based (suicide) inhibitors. The kinetic studies demonstrated that steroids with the tetrahydropyranyl substituent are more potent inhibitors of cytochrome P-450$_{scc}$ as shown by an initial turnover rate of 0.06 min^{-1}, an inactivation rate constant of 0.05 min^{-1}, and a partition ratio of about 1.0 for steroid I. Based on our finding, possible mechanisms of inactivation of cytochrome P-450$_{scc}$ by these acetylenic steroids are propsoed.

INTRODUCTION

Cholesterol is the obligatory precursor for all steroid hormones. The metabolism of cholesterol in steroid hormone biosynthesis takes place within the inner membrane of mitochondria, in the cortex of mammalian adrenal glands [1] and other steroid hormone biosynthetic tissues. The first step in these processes is the enzymatic side chain cleavage of cholesterol to form pregnenolone and 4-methylpentanal. This rate-limiting step in the biosynthesis of steroid hormones, is catalyzed by cytochrome P-450$_{scc}$ [2], the terminal protein in the sequential electron transport system involving the NADPH, adrenodoxin reductase (AR), and adrenodoxin (ADX).

Although much is known about the properties of cytochrome P-450$_{scc}$, including the intermediate metabolites of its reaction [3–7], the mechanism of hydroxylation of the substrate and the nature of activation of molecular oxygen at the active site are yet to be properly defined. One way to obtain more information on the mechanism of the hydroxylation and topography of the active site of the enzyme, is by the employment of mechanism-based inhibitors. Such use of mechanism-based inhibitor of any cytochrome P-450 was first demonstrated on the hepatic cytochrome P-450 system [8]. In those experiments and others that soon followed [9–13], the incubation of rat liver microsomes with both steroid and non-steroid compounds containing terminal acetylenic and olefinic functional groups, inactivated the enzyme with concurrent formation and accumulation of a green pigment in the liver. Ortiz de Montellano *et al.*[14–16] later demonstrated that there was a covalent reaction between the substrate and the heme group of the hepatic microsomal cytochrome P-450, and postulated the formation of an oxirene intermediate as a possible mechanism for the hydroxylation of those inhibitors.

The first report of regulation of cytochrome P-450$_{scc}$ was a clinical use of the enzyme's inhibition involving an *in vivo* regulation of adrenal steroidogenesis with aminoglutethimide [17]. Since then, there have been various attempts to regulate cytochrome P-450$_{scc}$ activity both by suppression of its biosynthesis [18] as well as by use of ligand modifying substrate analogs involving amino, p-tolyl, thio, and silicon containing steroids [19–22]. A mechanism-based inhi-

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bition of purified cytochrome $P\text{-}450_{scc}$ by acetylenic compounds was first reported by Nagahisa *et al.*[23]. In the study, various acetylenic steroids were incubated with purified adrenocortical cytochrome $P\text{-}450_{scc}$, resulting in a mechanism-based inactivation of the enzyme. In our own studies, we have made use of modified acetylenic steroids I–IV (Fig. 1). Two of the steroids (I and III) were modified with tetrahydropyranyl group at the terminal end of the side chain. The other two, steroids II and IV, were free hydroxy (triol) analogs. Here we report that these steroids are potent mechanism-based inhibitors of cytochrome $P\text{-}450_{scc}$.

EXPERIMENTAL

Materials

Lyophilized antipregnenolone and [7-^{3}H]pregnenolone were obtained from Radioassay Systems Laboratories while pregnenolone and cholesterol were from Sterloids. Proparyl alcohol, 3-butyn-1-ol and dihydropyran were products of Aldrich Chemical Company. The thin-layer chromatograph (TLC) was run on either Uniplate from Analtech Inc., or Redi-Coat H from Supelco Inc. Tetrahydrofuran used in the syntheses was dried and distilled from the purple solution of sodium and benzophenone.

Isolation and purification of enzyme

Cytochrome $P\text{-}450_{scc}$ was purified from bovine adrenal cortex as described by Seybert *et al.*[24], a modified procedure of Suhara *et al.*[25]. The enzyme was concentrated in an Amicon ultrafiltration unit with a PM 30 membrane, and was stored at 4°C until it was needed. The purified enzyme exhibited only the 450 nm absorption when it was reduced by sodium dithionite and then treated with carbon monoxide. The apparent molecular weight from a single band on the SDS–PAGE was 53,000 daltons. The concentration of cytochrome $P\text{-}450_{scc}$ so purified was calculated from the CO-dithionite difference spectra using a millimolar absorption coefficient of 91 $cm^{-1} \cdot mM^{-1}$.

Adrenodoxin reductase (AR) and adrenodoxin (ADX) were also purified from bovine adrenal cortex by a combination of affinity, ion exchange and gel filtration chromatography after the technique described by Lambeth and Kamin[26]. The concentration of AR was estimated based on the flavin absorption at 450 nm using an absorption coefficient of 10.9 $cm^{-1} \cdot mM^{-1}$. ADX concentration was calculated using an absorption coefficient of 11.3 $cm^{-1} \cdot mM^{-1}$ at 414 nm.

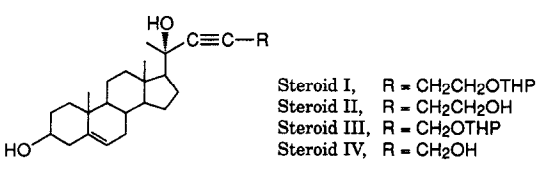


Fig. 1. Chemical structure of modified acetylenic steroids. THP = tetrahydropyranyl moiety.

Synthesis of modified acetylenic steroids

Propargyl alcohol and 3-butyn-1-ol were reacted with dihydropyran according to the method of Jones and Mann[27] to produce 2-propargyloxytetrahydropyran and 3-butynyloxytetrahydropyran respectively. The product was distilled under reduced pressure. The ethylmagnesium bromide was synthesized as in the method of Petrow and Stuart-Webb[28]. This Grignard reagent was used within 1 h of its synthesis in an exchange reaction with either 3-butyloxytetrahydro-pyran or 2-propargyloxytetrahydropyran. The product of the exchange reaction was then coupled with pregnenolone. The Grignard reagent and pregnenolone were in the molar ratio of 10:1. After refluxing for 1 h and stirring at room temperature for another 48–60 h, the reaction mixture was hydrolyzed. The organic phase was washed with water and dilute solution of $NaHCO_3$, and then dried over anhydrous Na_2SO_4. At the removal of the solvent, the oil left was triturated with hexane and the solid was recrystallized in successions from acetone, methanol, and finally with hexane. To obtain the corresponding triols, each tetrahydropyranyloxy derivative was hydrolyzed by a molar equivalent amount of concentrated HCl in THF in a salt-ice bath until complete hydrolysis as monitored by TLC. The solvent was removed *in vacuo* and the film left was dissolved in methanol. Upon removal of the methanol, a white crystalline solid obtained was recystallised from methylene chloride and then from 20% benzene in hexane.

Spectrophotometric measurements

Prior to running an experiment, the cytochrome $P\text{-}450_{scc}$ was desalted with the appropriate working buffer by filtration through a column of Sephadex G-25. The desalted enzyme was used the same day. For the titration experiments, 1 ml of the desalted enzyme solution was placed in both the sample and reference cuvettes. The sample enzyme was titrated with aliquots of a 5 mM stock solution of modified steroid in ethanol. Equal volume of ethanol was added to the reference cuvette. After each addition, the spectrum was scanned repeatedly from 500 to 350 nm until an equilibrium was established. The difference between the absorbance at the peak and that at the trough of the spectra, ΔA, was measured. Each set of data was then subjected to a non-linear least squares fit [29] to obtain the spectrophotometric dissociation constant, K_s, for the enzyme–steroid complex. To monitor the Soret absorbance change during catalytic turnover, 1 μM freshly desalted enzyme was added to 0.25 μM AR, 5 μM ADX and 10 μM of modified steroid in a cuvette. After the absolute spectrum between 500 and 350 nm had reached an equilibrium, the NADPH-generating system was added to initiate the turnover reaction. The

spectrum was scanned every 5 min for 150 min after which time the CO-dithionite difference spectrum was obtained to determine the concentration of the native enzyme species remaining in solution. To determine the partition ratio for the compounds by measurement of loss of Soret absorbance, aliquots of the steroids in ethanol were added to 1 μM of enzyme under turnover condition. The addition of NADPH-generating system which initiated the turnover reaction converted the oxidized heme iron into the reduced form and produced the maximum absorbance. The reduction of this maximum absorbance at the addition of each aliquot of the steroid was monitored. The percent of initial absorbance was plotted against the concentration of the steroid added. To obtain the rate of decrease in the concentration of cytochrome P-450$_{scc}$ remaining at various time intervals, an experiment was set up as above. At specific time intervals, 1 ml of the reaction solution was withdrawn and a CO-dithionite difference spectrum was obtained.

Kinetic assay

The measurement of the amount of pregnenolone formed during catalytic reaction was as described by Lambeth *et al.*[30] using radioimmunoassay (RIA). All enzyme incubations were carried out in a Fisher Scientific shaking water bath at 37°C in 35 mM phosphate buffer containing 0.1 mM DTT, pH 7.40. All reaction mixtures were preincubated at that temperature for a predetermined time prior to initiation of the reaction. To assay for the amount of pregnenolone produced as a result of catalytic turnover, a calculated volume of hexane was withdrawn from each vial based on the reported turnover of the enzyme [26] and from our own result. The hexane was evaporated and the radioimmunoassay was performed according to the method of Abraham[32]. Radioactivity was counted for a preset value of 20,000 counts.

RESULTS

The purity of the synthesized steroids were estimated from the TLC and elemental analysis. All the steroids showed elemental analyses within 0.3% of the calculated values. Each of the steroids showed a single spot on TLC plates, made visible by iodine vapor and then with 50% H_2SO_4 and charred on a hot-plate, were purple. These were distinguishable from that of pregnenolone which exhibited an orange color with the same treatment.

Cytochrome P-450$_{scc}$ purified and stored in 20% glycerol buffer at 4°C, was isolated as mixture of high spin and low spin forms. The CO-dithionite difference spectrum obtained to determine the quality and concentration of the enzyme, showed that little or no cytochrome P-420 species was present. The SDS–PAGE showed a single band corresponding to an apparent molecular weight of 53,000 Da.

The addition of 20-(4-tetrahydropyranyl-1-butynyloxy)-5-pregnen-3α,20β-diol; 20-(1-butynyl)-5-pregnen-3α,20β,25-triol; 20-(3-tetrahydro-pyranyl-1-propargyloxy)-5-pregnen-3α,20β-diol; or 20-(1-propynyl)-5-pregnen-3α,20β,24-triol to the purified and desalted cytochrome P-450$_{scc}$ resulted in Type II spectral shift [31]. However, unlike other substrate analogs employed (results not shown), the spectral conversion from high spin to low spin was incomplete at the end of the titration. This was especially true for the triol analogs. The absolute spectrum of the titrated enzyme showed that only about 50% of the enzyme had been converted to the low spin state even at molar ratios of ten equivalents of steroid per mole of enzyme (Fig. 2). Unlike other analogs employed,

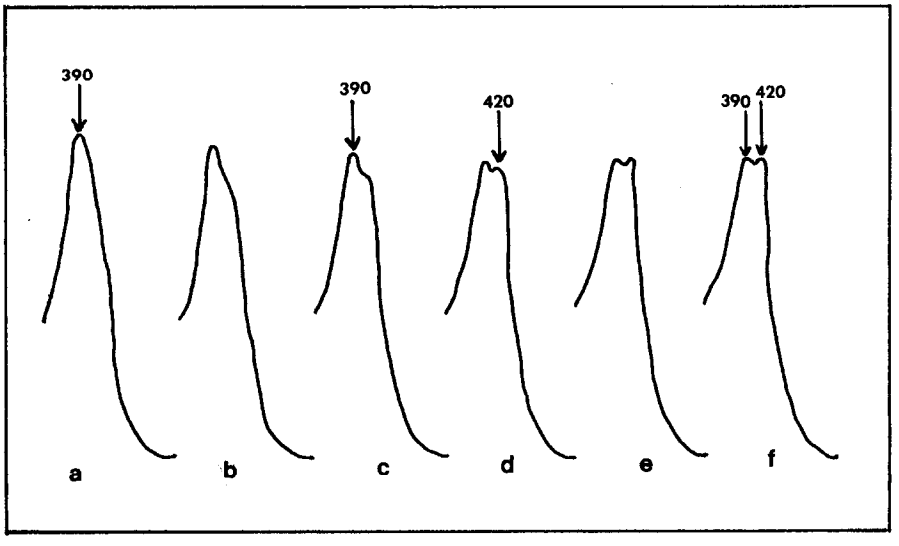


Fig. 2. Absolute spectra of cytochrome P-450$_{scc}$ during titration with steroid IV. **a** represents a high spin absolute spectrum before the addition of any steroid. **b–f** represent the spectra after the addition of aliquots of the steroid to the enzyme and the gradual conversion of the high spin form.

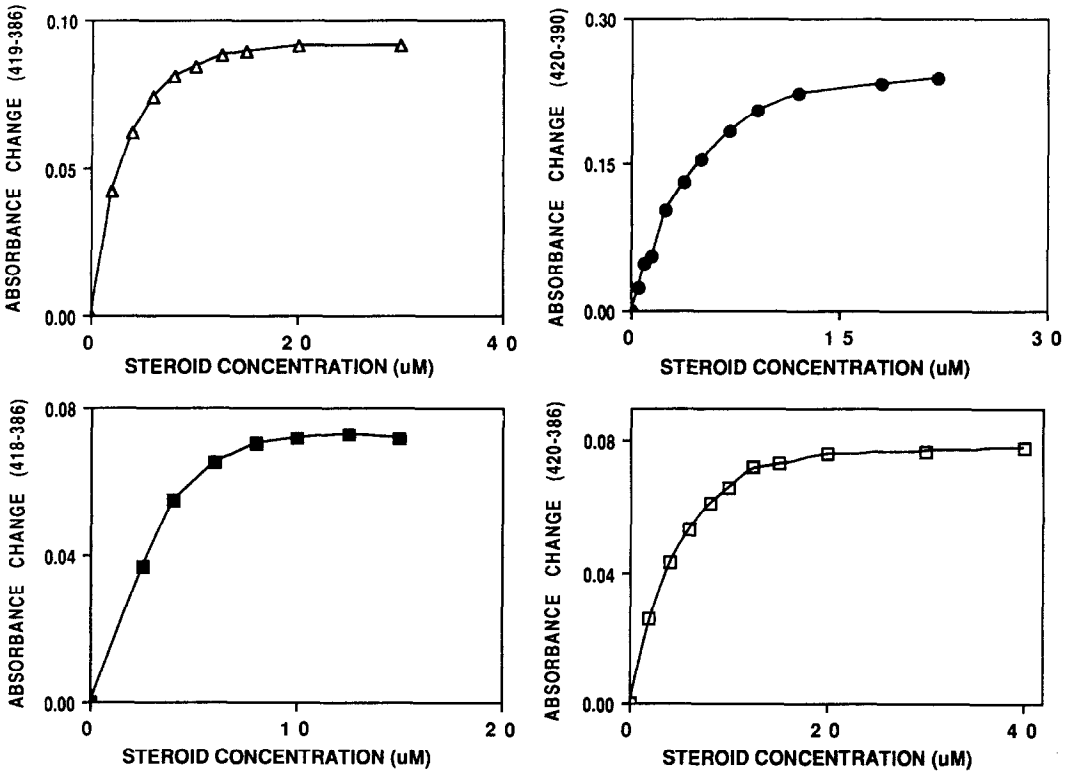


Fig. 3. Titration of the purified cytochrome P-450$_{scc}$ with steroids I (△), II (●), III (■), and IV (□). One milliliter of the enzyme solution in the sample cuvette was titrated with aliquots of an ethanol solution of each steroid. An equal amount of ethanol was added to the reference cuvette. After the addition of each aliquot, the spectrum was scanned from 500 350 nm until the attainment of equilibrium. A set of absorbance change (ΔA) values and their corresponding steroid concentrations were subjected to a non-linear least squares fit [29]. The solid line represents the theoretical curve through the data points.

these four steroids bind more loosely to the enzyme as evident by slow rate of equilibration and a 1–4 molar stoichiometry. The change in absorbance between the peak and the trough was plotted against corresponding concentration of the steroid added, to produce a titration curve for each steroid (Fig. 3). From the non-linear least squares fit [24], the spectrophotometric dissociation constant (K_s) for each steroid was obtained (Table 1). The change in absorbance of the enzyme during catalytic processing of steroid I was plotted against the ratio of the concentrations of the steroid and enzyme to produce a sharp break in the curve (Fig. 4) corresponding to a partition coefficient of about 2.0.

With cholesterol as substrate, the cytochrome P-450$_{scc}$ used in this study gave a turnover number of 3.1 min^{-1}. The rate of reaction was linear over 10-min period. When each of steroids I–IV was incubated with the enzyme under turnover conditions, each steroid was metabolized to varying degree ranging from an initial turnover rate of 0.06 min^{-1} for steroid I to 0.80 min^{-1} for steroid II (Table 2). In addition to low metabolism, steroid I produced the most remarkable inactivation of cytochrome P-450$_{scc}$. Under catalytic conditions, the tetrahydropyranyl-containing steroid I reduce the activity of the enzyme by 85% over a 40-min preincubation period, with an inactivation rate constant of 0.050 min^{-1} (Fig. 5, Table 3).

Table 1. Spectrophotometric dissociation constants (K_s) of the synthesized steroids

Steroid	K_s (μM)
I	2.37
II	1.50
III	1.30
IV	3.00

Titration procedures and treatment of data were as described for Fig. 3.

DISCUSSION

In these studies, all the steroid analogs were designed as 20-hydroxy derivatives. One reason for this is the relative ease of synthesis. The other is to promote enhanced binding capacity to the enzyme as shown by the dissociation constant of 20-hydroxycholesterol [19, 32]. Each of the steroids used bind to the enzyme with a comparable dissociation constant as does 20-hydroxycholesterol. However, these steroids have higher dissociation constants when compared with other steroids used by us (data not shown) or those reported by other groups [23, 33]. The decrease in the affinity of these steroids can probably be attributed to the increase in the

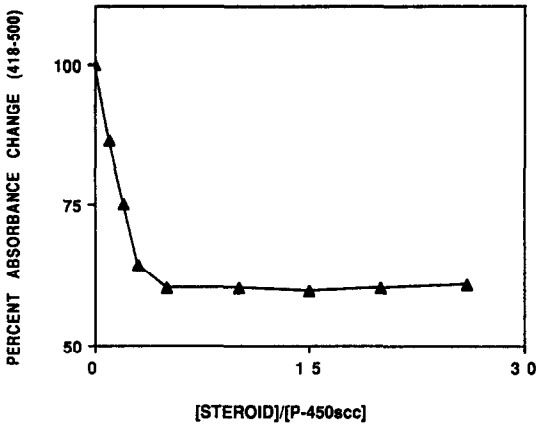


Fig. 4. Decrease in the absolute Soret absorbance of cytochrome P-450$_{scc}$ during turnover of steroid I. The reaction medium contained 1 μM cytochrome P-450$_{scc}$, 0.5 μM AR, 20 μM ADX and the NADPH-generating system. The reaction was initiated by the addition of aliquots of a stock solution of the steroid. At periodic intervals, a sample was withdrawn, reduced with sodium dithionite and ligated to carbon monoxide, and the difference spectra were obtained. The change in the Soret absorbance before the addition of the first aliquot of the steroid was designated as 100% and subsequent absorbance values were compared to it.

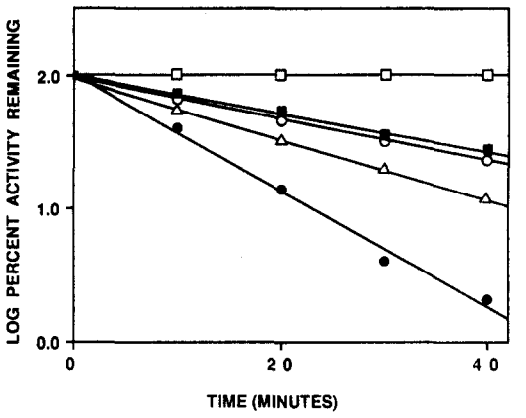


Fig. 5. First-order plot of the kinetics of inactivation of purified cytochrome P-450$_{scc}$ by steroid inhibitors I (●), II (*), III (△), and IV (■). (⊡) represents the incubation of the enzyme for the same amount of time but without the addition of any steroid. The enzyme was incubated with each steroid under turnover conditions at 37°C in 35 mM phosphate buffer containing 0.1 mM DTT. The reaction was initiated by the addition of the NADPH-generating system. At time intervals, 100 μl aliquots were withdrawn and were filtered through a minicolumn of Bio Gel P-6DG. One hundred microliters of the filtered enzyme was injected into a tube containing the substrate and NADPH-generating system to initiate the cholesterol catabolism.

hydrophilicity of their side chains as oxygen-containing functional groups. Because the side chain of cholesterol is hydrophobic, it can be speculated that the cytochrome P-450$_{scc}$ active site pocket that accommodates the side chains shows complementary hydrophobicity. Binding of the polar oxygen moieties in this pocket may therefore lead to unfavorable thermodynamic interaction. This is evident in the observation that steroid IV which contains a hydroxy group and is presumably more hydrophilic than the tetrahydropyranyl-containing steroid III, has slightly lower affinity for the enzyme. These dissociation constants nonetheless, are considerably lower than the 85 μM value reported by Jefcoate[34] for cholesterol itself. The side chain hydroxy group is also responsible for the incomplete conversion of the high spin to low spin state. This is in agreement with the observation of Jefcoate[34] and Mason *et al.*[18], that 25-hydroxycholesterol produced only a partial high spin response of cholesterol-free cytochrome P-450$_{scc}$.

Our findings in this study showed that each of the steroids I–IV exhibited the typical characteristics of mechanism-based inhibition when incubated with cytochrome P-450$_{scc}$ under turnover conditions. Steroid I proved to be the most potent of the suicide inhibitors with a progressive loss in both the cleavage activity of cholesterol as well as Soret absorbance. Nagahisa *et al.*[20, 23] reported that when 20-hydroxy steroids designed as suicide inhibitors were incubated with cytochrome P-450$_{scc}$ under catalytic conditions, there was a concomitant decrease in the low spin Soret absorbance of the enzyme as a result of mechanism-based enzyme inactivation. Based on the original calculations of Tatsunami *et al.*[37] on the kinetics of suicide substrates, Nagahisa *et al.*[20, 23] showed that such decrease in the low spin Soret absorbance is related to the partition ratio of inactivation. Unlike that reported by the Nagahisa group, incubation of each of the steroids I–IV did not produce such a dramatic decrease in Soret absorbance. Steroid I with the largest decrease, produced only 35% compared to the 50% decrease reported in the initial Soret absorbance with an acetylenic steroid and near 100% with a silicon-containing steroid. Notwithstanding the small decrease, when the data for steroid I were plotted, a partition ratio of 2.0 was obtained. This number is smaller than the value of 6 reported for the most

Table 2. Rates of initial turnover of the synthesized steroids by cytochrome P-450$_{scc}$

Steroid	Initial turnover rate (min^{-1})
Cholesterol	3.10
I	0.06
II	0.80
III	0.27
IV	0.11

Conditions of reactions were as described for Fig. 4.

Table 3. Rate constants for the inactivation of purified cytochrome P-450$_{scc}$ by steroid inactivators

Steroid	Inactivation rate constant (min^{-1})
Cholesterol	0.00
I	0.050
II	0.018
III	0.021
IV	0.016

The reaction conditions were as in Fig. 5.

potent acetylenic steroid by the Nagahisa group under similar experimental condition. In control experiments, the incubation of cholesterol with cytochrome P-450$_{scc}$ under the same condition as above exhibited a similar decrease in the low Soret absorbance. This decrease was only partially reversible in contrast to the complete reversal reported by Nagahisa and coworkers [23]. Our results thus suggest that the decrease in the Soret absorbance may not be sufficiently reliable measure of the extent of mechanism-based inactivation of cytochrome P-450$_{scc}$. The observed decreases may be connected to a number of factors, among which are binding of substrate, molecular oxygen or intermediates, change in the steady-state redox level of the enzyme, and loss of the prosthetic group, factors which have been reported in cytochrome P-450 from other sources [36, 37]. We suggest that for the use of this concept to be valid, the result should be coupled with other corroborating experimental data from which the partition ratio (k_{cat}/k_{inact}) is calculated. The result of such calculation gave a partition ratio of 1.2 for steroid I. From the change in Soret absorbance during catalytic processing of the same steroid I was also obtained a partial coefficient of 2.0. It is only interesting that in our studies, the two partition ratio values calculated from such different sets of experimental data are in so much agreement. This, however, may not always be so and should not be taken for granted.

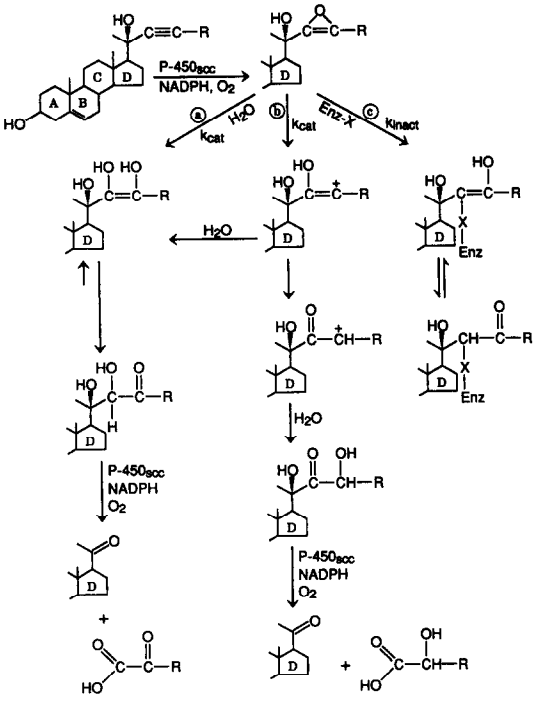
Of the four steroids utilized in this investigation, steroids I and III are the most effective suicide inhibitors of cytochrome P-450$_{scc}$. These two steroids were prepared as intermediates in the synthesis of steroids II and IV respectively. Both steroids have low turnover number with steroid III being metabolized four times as fast as steroid I. For steroid I, enzyme activity decreased with a first order rate constant of 0.050 min^{-1} ($t_{1/2}$ = 13.8 min) to a level of 15% of the initial activity. This value is the same as the concentration of the 450 nm absorbing species remaining when steroid I was incubated with cytochrome P-450$_{scc}$ and the time-course of loss of enzyme was monitored by CO-dithionite difference spectroscopy (data not shown). These data also point to the apparent invalidity of using the change in the Soret absorbance alone as a direct measure of inactivation of cytochrome P-450$_{scc}$ by an inhibitor. As stated earlier, there was an 85% loss in the specific content of the P-450 form although there was only a 35% loss in the Soret absorbance of the enzyme on incubation with steroid I.

We have shown that steroids I–IV were metabolized by cytochrome P-450$_{scc}$ as demonstrated by the time-dependent production of pregnenolone. The time-course for inactivation of the enzyme shows that all four steroids are inactivators of the enzyme as well. Unlike the case of inactivation of liver microsomal P-450 by acetylenic and olefinic compounds [8–16, 38–41], no formation of any green pigment was observed when steroids I–IV were incubated with cytochrome P-450$_{scc}$ under catalytic conditions. The green pigments observed in the liver microsomal system have been credited to the N-alkylation of a pyrrole nitrogen of the protoporphyrin. The lack of green pigment formation in our experiments is in agreement with the findings of the Nagahisa group, and points to the probability of the alkylation of an amino acid side chain residue within the vicinity of the active site. And the fact that DTT did not prevent the inactivation of the enzyme implies that the modification that led to inhibition of enzyme activity was a covalent one.

It has been proposed that since the inactivation of cytochrome P-450$_{scc}$ occurs only in the presence of molecular oxygen in addition to other cofactors, the intermediate, as in the case of liver microsomal cytothcome P-450 [42], might be an oxirene [23]. It is of interest to note that all of the steroids used were metabolized despite the multiple bonds between C22 and C23. The absence of hydrogen on C22 of these steroids suggests that the mechanism of cleavage of these inactivators cannot be entirely the same as it is for cholesterol.

It may be asked why steroid I is the most potent suicide inhibitor of cytochrome P-450$_{scc}$. One possible explanation is that the tetrahydro-pyranyl oxygen in the ring directly interacts with an amino acid residue at or near the active site of the enzyme. The interaction might have promoted a proper orientation to the oxirene intermediate moiety to bring the desired covalent modification, leading to enzyme inactivation as a result. Steroid III was not as effective as steroid I probably because the length of its side chain did not allow the tetrahydropytranyl group to adequately interact with the hypothetical amino acid in the same manner and to the same extent as it did in steroid I. The importance of the tetrahydropyranyl group could be further investigated by replacing it with a phenyl or cyclohexyl substituent. Such variation may answer questions as to whether the THP group effect is a result of the bulkiness of the unit or if indeed the oxygens of this moiety are critical to efficacy of these compounds.

In view of the ability of cytochrome P-450$_{scc}$ to convert the acetylenic steroids to pregnenolone, we propose a mechanism (Scheme 1) in which the oxirene intermediate is opened up to an enediol (pathway **a**), a vinyl cation (pathway **b**), or an enol by covalent reaction with an amino acid side chain residue on the enzyme (pathway **c**). The α-hydroxy ketone tautomers formed in pathways **a** and **b** can undergo further reaction with the enzyme to produce pregnenolone. The side chain cleavage products formed however are different from the normally formed 4-methylpentanal when cholesterol is used as the substrate. Isolation of these cleavage products could therefore be used to demonstrate the validity of the proposed mechanisms. The partitioning of the proposed oxirene between pathways **a**/**b** and pathway



Scheme 1. Proposed mechanism of catalytic processing of acetylenic steroids by cytochrome P-450$_{scc}$.

c, should be the determinant as to the extent of inactivation of the enzyme and the value of partition coefficient.

Several suicide inhibitors of cytochrome P-450$_{scc}$ have been designed and characterized, and the likely contribution of tetrahydropyranyl substituent in the inactivation of the enzyme have been presented. The employment of this type of mechanism-based inactivators may be useful in the understanding of the mechanism of steroid hydroxylation and of cholesterol side chain cleavage by cytochrome P-450$_{scc}$, and the topography of the active site of the enzyme.

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