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INHIBITION OF β-HYDROXYSTEROID DEHYDROGENASE

I. STRUCTURAL CHARACTERISTICS OF SOME STEROIDAL INHIBITORS*

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SUMMARY

1. β-Hydroxysteroid dehydrogenase (3 (or 17)-β-hydroxysteroid:NAD(P) oxidoreductase, EC 1.1.1.51) was prepared from *Pseudomonas testosteroni*. This enzyme obeyed first-order kinetics with androst-4-ene-3,17-dione as substrate between $1.0 \cdot 10^{-4}$ M and $2.5 \cdot 10^{-6}$ M.

2. The most effective inhibitors, with the concentration at which they inhibited 50%, were: 2-hydroxymethylene-17α-methylandrostan-17β-ol-3-one ($9 \cdot 10^{-7}$ M), 2α-cyano-4,4,17α-trimethylandrost-5-en-17β-ol-3-one ($8 \cdot 10^{-7}$ M), 17β-hydroxy-4,4,17α-trimethylandrost-5-enol[3,2-c]pyrazole ($8 \cdot 10^{-7}$ M), and 4,4-dimethyl-17β-hydroxyandrost-5-eno[3,2-c]pyrazole ($8 \cdot 10^{-7}$ M). Testosterone inhibited 50% at $2 \cdot 10^{-5}$ M.

3. A study of 42 derivatives of the androstane nucleus showed that the degree of inhibition depended upon the nature of the substituents on positions 2, 3, 4 and 17 of the steroid nucleus. Spacial presentation of these groups to the enzyme by the arrangement of double bonds or by their absence also affected inhibition.

INTRODUCTION

Our laboratory reported earlier that pretreatment of rats with estradiol-17β enhanced testicular 3β-hydroxysteroid dehydrogenase (3β-ol dehydrogenase[2]) activity with androst-5-ene-3β,17β-diol as substrate while causing testicular atrophy by blockade of pituitary gonadotrophin output. Estradiol-17β was 50 times more effective than diethylstilbestrol in stimulating this enzyme in the testes of treated animals[3]. The dehydrogenase which acts on isomeric α-steroids in rat liver, 3α-hydroxysteroid dehydrogenase (EC 1.1.1.50), has been studied by KOIDE[4] and KOIDE *et al.*[5].

β-Hydroxysteroid dehydrogenase (3(or 17)-β-hydroxysteroid:NAD(P) oxidoreductase, EC 1.1.1.51), discussed in this communication, was isolated from testosterone-induced cultures of *Pseudomonas testosteroni*[6] and purified 50- to 150-fold compared v th cell-free extracts[7]. This bacterial enzyme interconverts steroidal alcohols and ketones on positions 3 and 17 and, to a lesser extent, on position 16 of

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the steroid nucleus[7]. In animal tissues, 17β-hydroxysteroid dehydrogenases with somewhat different properties have been isolated from guinea pig liver[8], from beef liver[9] and from placenta[10,11]. The 3β-hydroxysteroid dehydrogenases are present in tissues which synthesize steroid hormones, but are lacking in those that do not[12]. A comparison of the nature of the various steroidal dehydrogenases has been made by TALALAY[13]. Besides functioning as dehydrogenases, rat-liver 3α-hydroxysteroid dehydrogenase[5] and 17β-hydroxysteroid dehydrogenase from placenta[14] also serve as transhydrogenases between NAD and NADP. The bacterial enzyme employed in this study is stereospecific for the β-configuration of the steroidal alcohol and is specific in its requirement for NAD as the dehydrogenase cofactor[15]. The substrate spectrum and kinetics with testosterone as substrate have been well established by TALALAY *et al.*[15–17].

With the availability to this laboratory of steroidal pyrazoles[18,19] and isoxazoles[20,21], which have modified endocrinological activities, we were prompted to evaluate these compounds as inhibitors of the bacterial β-hydroxysteroid dehydrogenase. A summary of these studies is presented in this communication.

MATERIALS

Lyophilized cells of testosterone-induced *P. testosteroni*, NAD and $NADH_2$ which assayed 98 and 88%, respectively, were obtained from the Sigma Chemical Company. Testosterone and 17α-methyltestosterone were obtained from the Vitamerican Oil Corporation. Androst-4-ene-3,17-dione and levigated alumina powder, AB grade, were products of Mann Research Laboratories and Buehler Ltd., respectively. The alumina powder was washed once with water and dried as suggested by HAYAISHI AND STANIER[22], otherwise all reagents were used without further purification. Synthetic steroids were obtained from Dr. R. O. CLINTON and his associates of this Institute. Details on their synthesis and purification have been published elsewhere[18–21].

METHODS

β-Hydroxysteroid dehydrogenase was prepared from *P. testosteroni* essentially by the method of MARCUS AND TALALAY[7], with the additional acetone fractionation step which they suggested[23]. The bacterial cells were disrupted with alumina in 0.05 M Tris buffer (pH 9.0) which was made 0.001 M with respect to EDTA. $(NH_4)_2SO_4$ fractionations were carried out with a solution saturated at 4° containing 2 g of EDTA per l at a final pH of 8.0. The β-enzyme was purified through the second $(NH_4)_2SO_4$ precipitation (Step 3), employing the above modifications, and used in this state of purity for all determinations. Protein was analyzed by the method of LOWRY *et al.*[24]. Specific activities of four separate preparations of purified β-hydroxysteroid dehydrogenase ranged from 9000 to 12 500 units per mg of protein when testosterone and NAD were used as substrate and cofactor, respectively, at pH 9.5 (ref. 7). The average ratio of β- to α-enzyme activities was 3.5.

The assay system used for determining the effect of steroids on reaction rate contained 100 μmoles of Sørensen's phosphate buffer (pH 5.5), 0.05 μmole of androst-4-ene-3,17-dione dissolved in methanol, variable amounts of steroid test com-

pound dissolved in methanol, and 0.1 μmole of $NADH_2$ in a total volume of 2.95 ml. The reaction was initiated by adding 50 μl of enzyme (250 β units) to the above reaction mixture in a 1.0 cm Beckman silica cuvette. Readings of absorbancy were taken 30 sec after the addition of enzyme and every 10 sec thereafter for a total of 100 sec. Initial reaction rates were determined from the straight line portions of absorbancy *vs.* time curves. One activity unit was defined as the absorbancy change at 340 mμ of 0.001/min per ml of enzyme under the above conditions.

RESULTS

Kinetics of the reaction with androst-4-ene-3,17-dione

MARCUS AND TALALAY[16] studied the velocity of testosterone oxidation to androst-4-ene-3,17-dione as a function of substrate concentration. They found that at testosterone concentrations higher than $6.0 \cdot 10^{-6}$ M, substrate inhibition was superimposed upon the first-order reaction rate. They attributed this behavior to the formation of an inactive bimolecular complex, *i.e.*, two molecules of substrate per enzyme reactive site. We found that first-order kinetics was obeyed between $1.0 \cdot 10^{-4}$ M and $2.5 \cdot 10^{-6}$ M androstenedione in its reduction to testosterone in the back reaction. Since we were interested in studying testosterone formation, the absence of substrate inhibition within the useful range of reaction-rate determinations with androstenedione as substrate greatly simplified the kinetic evaluation of inhibition by selected steroids.

The effect of double-bond placement on inhibition

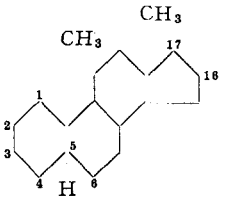
Testosterone (Compound 1, Table I) is the product of the reaction and it also inhibited the initial reaction rate at a concentration nearly equal to that of the substrate, androstenedione. 17α-Methyltestosterone (Compound 2) also inhibited the reaction, but at a slightly higher concentration. The introduction of an additional double bond at C-6 to form a 4,6-diene did not affect the degree of inhibition (*cf.* Compound 3, androsta-4,6-diene-17β-ol-3-one and Compound 4, 17α-methylandrosta-4,6-diene-17β-ol-3-one). Substitution of an α-ethyl (Compound 5, 17α-ethylandrosta-4,6-diene-17β-ol-3-one) or an α-vinyl (Compound 6, 17α-vinylandrosta-4,6-diene-17β-ol-3-one) group at position 17 caused an almost 10-fold increase in inhibitory activity. However, changing the position of the additional double bond to C-1 to form a 1,4-diene and substitution of an α-ethinyl group at position 17 as in Compound 7, 17α-ethinylandrosta-1,4-diene-17β-ol-3-one, resulted in a 100-fold decrease in inhibition compared with the above 4,6-dienes.

The effect of steroids lacking the 3- and 17-keto substituents

Compounds 8 through 15 of Table I lack keto groups at position 3 and 17; so they cannot serve as substrates for steroid dehydrogenase with NADH as the cofactor. They are also inert as inhibitors in the system. Therefore, they must not be bound to the enzyme at a place vital to androstenedione reduction because, of the eight compounds in Table I with the 3β-hydroxy substituent, none interfered with

TABLE I

INHIBITION OF β-HYDROXYSTEROID DEHYDROGENASE BY CERTAIN ANDROSTANE DERIVATIVES



Androstane nucleus

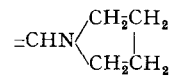
Compound No.	Substituent on position: 1	2	3	4	5	6	16	17	Degree of inhibition*
1	—	—	keto	△	—	—	—	β-OH	18 μM
2	—	—	keto	△	—	—	—	β-OH,α-CH_3	27 μM
3	—	—	keto	△	—	△	—	β-OH	16 μM
4	—	—	keto	△	—	△	—	β-OH,α-CH_3	25 μM
5	—	—	keto	△	—	△	—	β-OH,α-C_2H_5	2.0 μM
6	—	—	keto	△	—	△	—	β-OH,α-CH=CH_2	3.5 μM
7	△	—	keto	△	—	—	—	β-OH,α-C≡CH	100 μM
8	—	—	β-OH	—	△	—	—	β-OH,α-C_2H_5	None
9	—	—	β-OH	—	△	—	—	β-OH,α-CH=CH_2	None
10	—	—	β-OH	—	△	—	—	β-OH,α-CH_2CH=CH_2	None
11	—	—	β-OH	—	△	—	—	β-OH,α-C≡CCH_2OH	Stimulation
12	—	—	β-OCH_3	—	△	—	—	β-OH,α-C≡CH	Stimulation
13	—	—	β-OH	—	△	—	—	β-OCOCH_3,α-CH_3	None

* The concentrations refer to the amounts necessary to inhibit the enzyme 50%. Stimulation refers to an increased reaction rate in the presence of test steroid compared with that for the control reaction.

TABLE I (continued)

INHIBITION OF β-HYDROXYSTERIOD DEHYDROGENASE BY CERTAIN ANDROSTANE DERIVATIVES

Androstane nucleus

Compound No.	Substituent on position: 1	2	3	4	5	6	16	17	Degree of inhibition*
14	—	—	β-OH	—	△	—	—	β-$OCOC_2H_5$,α-CH_3	None
15	—	—	β-Cl	—	△	—	—	β-OH	None
16	—	—	β-OH	—	△	—	$-CH_2N(CH_3)_2$	keto	Stimulation
17	—	—	β-OH	—	△	—		keto	None
18	—	CH_3CO-	keto	—	—	—	—	β-$OCOCH_3$	Stimulation
19	—	△, CH_3CO-	CH_3OCO-	—	—	—	—	β-$OCOCH_3$	Stimulation
20	—	HOCH=	keto	—	—	—	—	β-OH,α-CH_3	0.9 μM
21	—	HOCH=	keto	△	—	—	—	β-OH	Stimulation
22	—	HOCH=	keto	△	—	—	—	β-OH,α-CH_3	None
23	—	HOCH=	keto	△	—	—	HOCH=	keto	None
24	—	$(CH_3)_2CHOCH=$	keto	△	—	—	—	β-OH,α-CH_3	None
25	—	—	keto	$(CH_3)_2$	△	—	—	β-OH,α-CH_3	None
26	—	α-C≡N	keto	$(CH_3)_2$	△	—	—	β-OH,α-CH_3	0.78 μM
27	—	$-C\equiv N$	keto	△	—	—	—	β-OH,α-CH_3	18 μM

* The concentrations refer to the amounts necessary to inhibit the enzyme 50%. Stimulation refers to an increased reaction rate in the presence of test steroid compared with that for the control reaction.

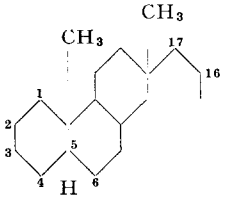
androstenedione reduction. Altering the substituents on the 17α-position did not seem to increase binding of these 3β-hydroxyandrost-5-ene derivatives to the enzyme. A change in the position of the double bond from position 4 to position 5 may itself affect binding, so that a blanket statement cannot be made concerning the 3α-hydroxy substituent. Undoubtedly, attachment of steroids to the enzyme surface involves multiple sites, of which positions 3 and 17 have been shown to be involved with respect to substrate reactivity[16].

The effect of a substituent adjacent to position 3 or 17

MARCUS AND TALALAY[16] demonstrated that a substituent at position 16 of estradiol-17β interfered with utilization of the latter as a substrate, but not with the ability of the 16,17-disubstituted derivatives to act as inhibitors of the NAD-catalyzed reaction. In the present study, 2-hydroxymethylene-17α-methylandrostan-17β-ol-3-one, 2α-cyano-4,4,17α-trimethylandrost-5-en-17β-ol-3-one and 2-cyano-17α-methylandrost-4-en-17β-ol-3-one (Compounds 20, 26, and 27 of Table I, respectively)

TABLE II

THE EFFECT OF BRIDGED RING STRUCTURES AT POSITIONS 3 AND 17 OF THE STEROID NUCLEUS ON β-HYDROXYSTEROID DEHYDROGENASE ACTIVITY



Androstane nucleus

Compound No.	Substituent on position: 2, 3	4	5	6	16, 17	17	Degree of inhibition*
1	[3,2-d]Aminothiazole	—	—	—	—	β-OH	140 μM
2	3β-OH	—	△	—	[16,17-c]Pyrazole	—	Stimulation
3	[3,2-c]Pyrazole	△	—	—	—	β-OH,α-CH_3	4.8 μM
4	[3,2-c]Pyrazole	△	—	△	—	β-OH	4.3 μM
5	[3,2-c]Pyrazole	△	—	△	—	β-OH,α-CH_3	1.5 μM
6	[3,2-c]Pyrazole	$(CH_3)_2$	—	—	—	β-OH,α-CH_3	10 μM
7	[3,2-c]Pyrazole	$(CH_3)_2$	△	—	—	β-OH	0.83 μM
8	[3,2-c]Pyrazole	$(CH_3)_2$	△	—	—	β-OH,α-CH_3	0.83 μM
9	[3,2-c]Isoxazole	—	—	—	—	β-OH,α-CH_3	26 μM
10	[2,3-d]Isoxazole	—	—	—	—	β-OH,α-CH_3	None
11	[2,3-d]Isoxazole	△	—	—	—	β-OH,α-CH_3	45 μM
12	[2,3-d]Isoxazole	△	—	—	—	β-$OCOC_2H_5$	None
13	[2,3-d]Isoxazole	△	—	△	—	β-OH,α-CH_3	28 μM
14	[2,3-d]Isoxazole	△	—	△	—	β-$OCOCH_3$	Stimulation
15	[2,3-d]Isoxazole	$(CH_3)_2$	△	—	—	β-OH,α-CH_3	8.5 μM

* The concentrations refer to the amounts necessary to inhibit the enzyme 50%. Stimulation refers to an increased reaction rate in the presence of test steroid compared with that for the control reaction.

showed that 2,3-disubstituted steroids were strongly inhibitory. 2-Hydroxymethylene- or 2-cyano-3-ketones were among the most effective inhibitors of steroid dehydrogenase. Certain criteria had to be met for strong binding to occur, however. In the case of 2-hydroxymethylene derivatives, ring A had to be saturated. With 2-cyano derivatives, the 4,4-dimethyl group enhanced inhibition 20-fold. That the in-inhibition was due to the α-cyano group was attested by the ineffectiveness of 4,4,17α-trimethylandrost-5-en-17β-ol-3-one (Compound 25, Table I).

The effect of ring-bridging at positions 3 or 17

Bridging C-2 and C-3 with aminothiazole, pyrazole or with isoxazole produced an inhibitory effect on β-hydroxysteroid dehydrogenase (see Table II). The one 16,17-pyrazole tested, 3β-hydroxyandrost-5-eno[16,17-c]pyrazole, had a 3β-hydroxy substituent, which did not inhibit the enzyme well according to results given in Table I. All of the [3,2-c]pyrazoles listed in Table II inhibited steroid dehydrogenase. The presence of one or two double bonds or 17α-methyl in addition to the 17β-hydroxy substituent did not seem to affect inhibitory activity. Among the 4,4-dimethyl pyrazoles, the absence of unsaturation at position 5 imposed strain on the ring system[19] and decreased inhibitory ability.

The isoxazoles, generally, were weak inhibitors of steroid dehydrogenase. Inhibition by the [3,2-c]conjugated isoxazole (Compound 9, Table II) may be contrasted with the inactivity of the [2,3-d]conjugated isoxazole (Compound 10, Table II) in the androstane series. Among the androstene[2,3-d]isoxazoles, acylation of the 17β-hydroxy substituent nullified inhibition. The most active isoxazole, 17β-hydroxy-4,4,17α-trimethylandrost-5-eno[2,3-d]isoxazole (Compound 15, Table II), was the 4,4-dimethyl derivative. The presence of the 4,4-dimethyl group increased inhibitory ability among the pyrazoles also.

DISCUSSION

An androstane or androstene derivative must meet at least three structural criteria for maximal inhibition of β-hydroxysteroid dehydrogenase. The first of these, among the simpler steroids, is the presence of a free keto group at position 3 with or without an additional electronegative group at position 2. 2-Hydroxymethylene-3-keto- or 2-cyano-3-ketosteroids are effective inhibitors. Therefore, the presence of certain substituents adjacent to the reactive 3-position does not prevent inhibition of the enzyme. This inhibitory property is also confirmed in the case of 4,4-dimethyl derivatives. Bridging ring A carbons 2 and 3 by the electronegative aminothiazole, pyrazole or isoxazole rings also yields effective inhibitors without the necessity of the 3-keto group. The 2-hydroxymethylene-3-keto combination exists as a hydrogen-bonded ring system which may have properties analogous to the above bridged ring-A compounds.

Since the 4,4-dimethyl steroids require the androst-5-ene nucleus for maximal inhibitory ability, the degree of unsaturation and the position of the double bond comprise the second criterion. A saturated A ring is required for inhibition by the 2-hydroxymethylene-3-keto combination, while unsaturation at positions 4 or 5 is necessary for the isoxazole derivative to be effective. These observations are not sur-

prising, since the position of a double bond or the lack of one changes the spacial presentation of reactive groups to the enzyme.

The third criterion for inhibition is the type of substituent at position 17, which is another location on the steroid undergoing oxidation–reduction. All of the effective inhibitors listed in this study possess a free 17β-hydroxyl group. Esterification of this group cancels the inhibitory effect. The presence of a variety of small substituents at position 17 orientated α to the hydroxyl group either do not prevent the steroid from exerting its inhibitory effect or enhance inhibition depending upon the nature of the 17α-substituent.

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