BBA 66531

# INHIBITION OF THE 3-KETOSTEROID $\Delta^5 \rightarrow \Delta^4$-ISOMERASE OF *PSEUDOMONAS TESTOSTERONI* BY SOME BROMO-3-KETOSTEROID DERIVATIVES*

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(Received September 27th, 1971)

## SUMMARY

Several 2α-, 4α-, and 6α-bromo-3-ketoandrostane substrate analogs of the steroid Δ-isomerase (3-ketosteroid $\Delta^4 \rightarrow \Delta^5$-isomerase, EC 5.3.3.1) of *Pseudomonas testosteroni* have been studied as inhibitors. Each was found to be a good competitive inhibitor and 2α-bromodihydrotestosterone ($K_i$, 8.2 μM) and 2α,4α-dibromodihydrotestosterone acetate ($K_i$, 6.7 μM) are among the best yet discovered for this enzyme. None of the bromosteroids studied was an irreversible inhibitor of the enzyme. The relative effectiveness as competitive inhibitors of the bromo-3-ketoandrostanes examined was correlated with their ease of enolization and with the stabilities of the corresponding enols or enolate anions. The data obtained are consistent with the presence of a basic group situated above the β-face of Ring A in the enzyme–steroid complex and with an open and readily accessible active site which is tolerant of wide structural variations in the region of the Ring A binding loci.

## INTRODUCTION

The steroid Δ-isomerase (3-ketosteroid $\Delta^5 \rightarrow \Delta^4$-isomerase, EC 5.3.3.1) of *Pseudomonas testosteroni* is one of the most intensively studied of the enzymes of steroid metabolism, particularly with respect to its mechanism of action[1,2]. The stereochemistry of the enzymic isomerization has been shown to involve direct intramolecular 4β to 6β proton transfer, usually *via* a rate-determining enolization step[3] and the available data[1–3] suggest that both a basic and an acidic group are involved in the process. A considerable body of evidence has been accumulated indicating histidine and tyrosine residues to be present at or close to the active site[1,2] and these amino acids thus present themselves as logical candidates for the roles of the catalytic

* To be regarded as Steroids and Steroidases, Part XII. For Part XI see J. B. JONES AND J. D. LEMAN, *Can. J. Chem.*, 49 (1971) 2420.

The following trivial names have been used: testosterone, 17β-hydroxyandrost-4-en-3-one; dihydratestosterone, 17β-hydroxy-5α-androstan-3-one.

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functions. However, no direct evidence of their involvement in the catalysis has yet been obtained.

Two isomerization mechanisms, one proposing a combined acid–base catalysis by histidine alone[1] and the other suggesting the involvement of both tyrosine and histidine residues[4,5], have been put forward. The data available did not permit a choice to be made between the two alternatives and accordingly, we decided to investigate the application of substrate analogues incorporating alkylating functions capable of reacting irreversibly with the active site groups in an attempt to provide more direct evidence regarding their nature and orientation. 3-Ketobromosteroids appeared to be attractive candidates for effecting the types of active site modifications envisaged and the recent success achieved in this regard with 6β-bromotestosterone acetate by BÜKI *et al.*[6] prompts us to report the results of our analogous studies on the related Compounds 1–5 possessing 2α-, 4α- and 6α-oriented bromo substituents. Although no alkylation of the enzyme was observed with any of Compounds 1–5, each was found to be a good competitive inhibitor. The 2α-bromo derivatives 1 and 2 were particularly effective in this regard and the data obtained provide further support for the general validity of the mechanistic proposals outlined above and for the location of a basic catalytic group above the β-face of the A-ring of the steroid in the *ES* complex.

## MATERIALS AND METHODS

### *Steroids*

In the preparative work the criteria of purity applied, the methods and analytical instrumentation used, specifications of the chromatographic supplies, solvents, *etc.* were as described previously[4]. Mass spectra were recorded on a Bell and Howell 21-490 spectrometer.

Testosterone acetate was obtained by acetylation of testosterone with acetic anhydride in pyridine. Dihydrotestosterone[6], 17β-acetoxy-5α-androstan-3-one[7], and androst-5-ene-3,17-dione[4] were prepared according to the literature procedures.

### *2α-Bromo-17β-hydroxy-5α-androstan-3-one (1)*

Trimethylphenyl ammonium perbromide (0.65 g, 1.72 mmoles, purchased from City Chemical Corp., N.Y.C.) was added slowly at 20° with stirring to a solution of 17β-hydroxy-5α-androstan-3-one (0.5 g, 1.72 mmoles) in dry tetrahydrofuran (5 ml). Stirring was continued until no further precipitate formed (15 min) and the mixture was then poured into 5% aqueous $NaHCO_3$ (200 ml) and extracted with diethyl ether (100 ml). The diethyl ether solution was washed with water (100 ml), dried ($MgSO_4$) and evaporated and the solid obtained was recrystallized from acetone–*n*-hexane or acetonitrile to give 2α-bromo-17β-hydroxy-5α-androstan-3-one (1) as needles (0.3 g), m.p. 186–187° (ref. 9, m.p. 180–181°).

### *2α,4α-Dibromo-17β-acetoxy-5α-androstan-3-one (2)*

Bromine (0.42 ml, 7.6 mmoles) in acetic acid (10 ml) was added dropwise with stirring at 20° to 17β-hydroxy-5α-androstan-3-one (1 g, 3.45 mmole) in acetic acid (100 ml). The resulting solution was stirred for a further 24 h and was then poured into water (1 l). The precipitated solid was filtered, washed with water, and dried to

give 2α,4α-dibromo-17β-acetoxy-5α-androstan-3-one (2) in quantitative yield (1.7 g). Repeated recrystallization from aqueous acetone and finally from cyclohexane yielded a pure sample, m.p. 202–204°, infrared (chloroform) 1751 and 1724 cm $^{-1}$; PMR ([$^2$H]chloroform) $\delta$ 4.60 (1,d, $J$ = 12 Hz, C-4β H) and 4.76 p.p.m. (1,d of d, $J$ = 6 and 13 Hz, C-2β H). *Analysis.* Calcd. for $C_{21}H_{30}O_3Br_2$ C, 51.44; H, 6.17; Br, 32.60. Found: C, 51.68; H, 6.30; Br, 32.55%. Mol. wt. (mass spectrum) 490.

*4α-Bromo-17β-acetoxy-5α-androstan-3-one (3)*

2α,4α-Dibromo-17β-acetoxy-5α-androstan-3-one (Compound 2, 6.1 g, 2.04 mmoles) in deoxygenated chloroform (10 ml) and acetic acid (24 ml) was treated under a $N_2$ atmosphere with anhydrous chromous acetate (2.36 g, 13.3 mmoles, prepared *in situ* by the method of WILLIAMSON AND JOHNSON[10]). The reaction mixture was stirred for 10 min at 20° after which time air was bubbled into the suspension and diethyl ether (100 ml) was then added. The resulting mixture was washed with water (150 ml) followed by 5% aqueous $NaHCO_3$ (3 times 100 ml) and finally with saturated aqueous NaCl (100 ml). Evaporation of the dried ($MgSO_4$) organic solution yielded an amorphous pink solid which was purified by thin-layer chromatography on silica gel with diethyl ether–benzene (1:1, v/v) development. Recrystallization first from aqueous acetone and then from *n*-hexane gave 4α-bromo-17β-acetoxy-5α-androstan-3-one (Compound 3, 0.22 g); m.p. 196–198° (ref. 11, m.p. 199–200°).

*2α-Bromo-17β-hydroxyandrost-4-en-3-one (4)*

Testosterone (1.15 g, 4 mmoles) was converted to its 2-ethoxalyl derivative (1.22 g) by treatment with ethyl oxalate and sodium hydride in benzene according to the procedure of YASUDA[12]. Subsequently, 2-ethoxalyl testosterone (0.7 g, 1.8 mmoles) was dissolved in methanol (15 ml) containing anhydrous potassium acetate (0.35 g, 3.6 mmoles) and a solution of bromine (0.2 ml) in carbon tetrachloride (6 ml) was added dropwise with stirring until the solution retained a permanent pale yellow coloration[13]. The reaction mixture was then concentrated to half volume, water (0.5 ml) added, and the resulting precipitate allowed to coagulate. Filtration yielded 2α-bromo-17β-hydroxyandrost-4-en-3-one (Compound 4, 0.42 g) which was purified by thin-layer chromatography on silica gel using diethyl ether–benzene (1:1, v/v) development followed by recrystallization from acetone–*n*-hexane to give plates (0.2 g). m.p. 131–132° (ref. 12, m.p. 127–128°).

*6α-Bromo-17β-acetoxyandrost-4-en-3-one (5)*

This compound, needles from *n*-hexane–diethyl ether, m.p. 138–141°, was obtained from testosterone acetate in 37% overall yield by the method of DEAN AND CHRISTIANSEN[14], who quote m.p. 147–148°.

*Inhibition studies*

The standard spectrophotometric enzyme assay procedure developed by TALALAY[1] was used as described previously[4]. All kinetic runs were carried out in 0.03 M phosphate buffer of pH 7 containing 1.6% of methanol using 3 times crystallized steroid $\Delta$-isomerase of specific activity 64 300 units/mg protein, which had been diluted (by a factor of up to $10^6$) with 1% bovine serum albumin of pH 7. Androst-5-ene-3,17-dione was used as the standard substrate.

*Evaluation of Compounds 1–5 as irreversible inhibitors*

The bromosteroids 1–5 were each incubated with approx. 1 pM isomerase at 25° in 1.6% aqueous methanolic 0.03 M phosphate buffer solution of pH 7 and the reaction solutions were assayed periodically up to 48 h. A very large (approx. $10^6$ molar) excess of each inhibitor was used. Comparisons of the rates of disappearance of enzyme activity from the above mixtures with those of the corresponding reference solutions from which the inhibitors were omitted gave no indication of any irreversible inhibition by any of Compounds 1–5.

*Evaluation of Compounds 1–5 as competitive inhibitors*

The competitive inhibition studies were carried out using 1 pM enzyme solutions and with substrate concentrations in the range 7.3–43.8 $\mu$M and inhibitor concentrations varying between 0 and 60 $\mu$M. The upper limit of inhibitor concentration for each of the compounds studied was determined by the solubility in the final 1.6% methanolic reaction solvent. For each $K_i$ determination measurements were taken for three or four different substrate and inhibitor concentrations and all runs were carried out at least in duplicate and were reproducible to within $< 3\%$. The reference cell in the ultraviolet spectrophotometer contained all components except the substrate. The initial isomerization velocities were calculated from the absorbance changes during the first 3–4 min only and the inhibition constants were obtained by the method of DIXON[15]. All the kinetic data were subjected to least-squares regression analysis and correlation coefficients of approx. 0.99 were obtained. Representative Dixon plots obtained for the inhibitors 1–5 are reproduced in Fig. 1. The $K_i$ values of testosterone, testosterone acetate, dihydrotestosterone, and 17$\beta$-acetoxy-5$\alpha$-androstan-3-one, which were required for reference purposes, were determined in the same way and the complete inhibition constant data are summarized in Table I.

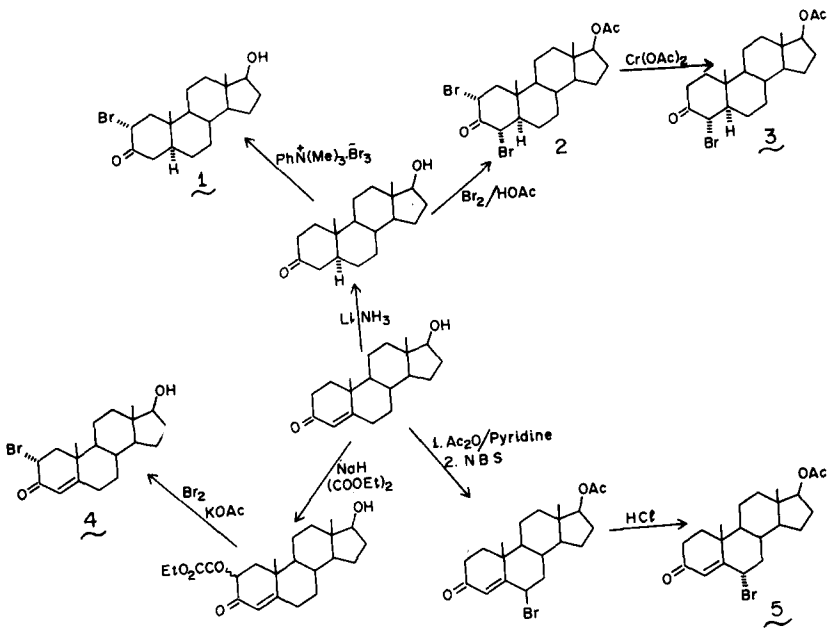
TABLE I

INHIBITION CONSTANTS OF THE BROMOANDROSTANES 1–5 AND OF THEIR UNSUBSTITUTED PARENT COMPOUNDS

The inhibition constants were determined by the method of DIXON[14] and all the kinetic data were subjected to least-squares regression analysis. Representative Dixon plots for the inhibitors 1–5 are reproduced in Fig. 1. All kinetic runs were carried out at least in duplicate and were reproducible to $< \pm 3\%$.

| *Inhibitor* | $K_i$ ($\mu M$) |
|---|---|
| Dihydrotestosterone | 25.7 |
| 17-$\beta$-Acetoxy-5$\alpha$-androstan-3-one | 25.2 |
| 2$\alpha$-Bromodihydrotestosterone (1) | 8.2 |
| 2$\alpha$,4$\alpha$-Dibromo-17$\beta$-acetoxy-5$\alpha$-androstan-3-one (2) | 6.7 |
| 4$\alpha$-Bromo-17$\beta$-acetoxy-5$\alpha$-androstan-3-one (3) | 22.0 |
| Testosterone | 26.0* |
| Testosterone acetate | 30.4 |
| 2$\alpha$-Bromotestosterone (4) | 31.6 |
| 6$\alpha$-Bromotestosterone acetate (5) | 39.7 |

* H. J. RINGOLD (personal communication) recorded a value of 30 $\mu$M.




Scheme 1.

## RESULTS

Of the literature methods available for the preparations of the bromoandrostanes 1–5 only that leading to 6α-bromotestosterone acetate (5) was used without modification. The remaining Compounds 1–4 were obtained by the hybrid routes outlined in Scheme 1. For some of the C-17β-hydroxy compounds investigated purification problems were encountered; these were overcome by protection of the C-17 function as the acetate, as in Compounds 2, 3 and 5.

None of the above bromosteroids turned out to be an irreversible inhibitor of the isomerase as had been hoped. Although an immediate decrease in specific activity was noted when a large excess of each potential inhibitor was incubated at 25° with enzyme at pH 7 this was attributable in each case to competitive inhibition. While the enzymic activity continued to decrease during 2 days, the loss of activity was not significant in comparison with the rate of denaturation of the enzyme in the absence of inhibitors. In fact, the better competitive inhibitors, such as 2α-bromodihydrotestosterone (1), afforded substantial protection against denaturation. In the presence of the latter compound, the inactivation rate was reduced to one tenth of that of the control enzyme solution. That each of Compounds 1–5 was in fact acting as a competitive inhibitor was confirmed by the method of DIXON[15]. The plots obtained using androst-5-ene-3,17-dione as the substrate are shown in Fig. 1 and the inhibition constants calculated from the intercepts are summarized in Table I. Also included in Table I for comparison purposes are the $K_i$ values of dihydrotestosterone, 17β-acetoxy-5α-androstan-3-one, testosterone and testosterone acetate, each of which was likewise determined by the Dixon procedure.

In an attempt to provide estimates of the facility with which the inhibitors 1–5

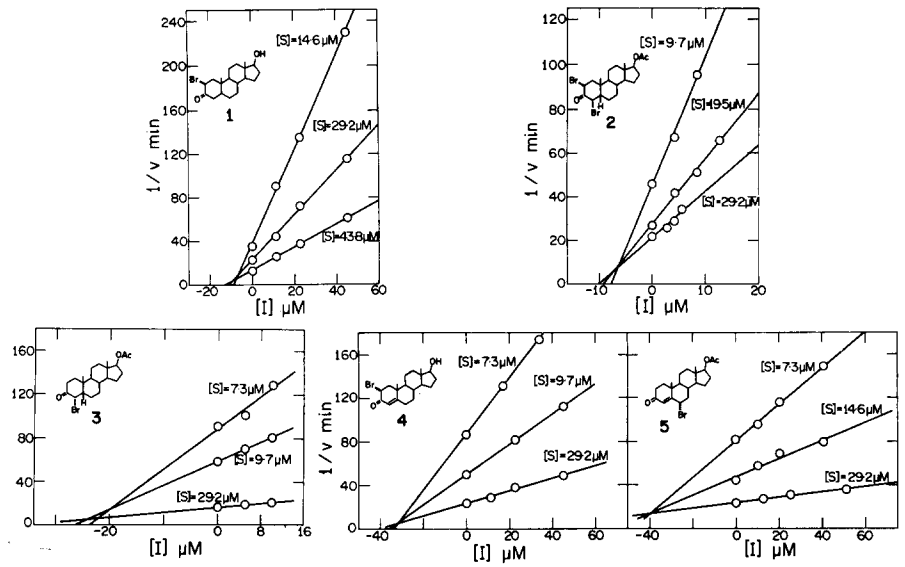



Fig. 1. Representative Dixon plots for the inhibitors 1–5. Androst-5-ene-3,17-dione was the substrate used.

would be expected to alkylate active site histidine or tyrosine residues, the reactions of the bromosteroids with the model nucleophiles imidazole and phenol were surveyed under a variety of conditions. These included the use of $KHCO_3$, $K_2CO_3$ and KOH as basic catalysts, and methanol, dimethyl sulfoxide, acetone, acetonitrile, dioxane, *N*-methylpyrrolidone, and dimethylformamide as solvents. However, although displacement of bromide was relatively facile in many cases, no steroidal imidazolyl or phenoxy derivatives were detected in any reaction mixture. The reactions undergone by 2α-bromodihydrotestosterone (2) are illustrative of the general situations encountered. On treatment with imidazole and $KHCO_3$ in methanol the only isolable products were those resulting from overall attack by the solvent *viz.* 2β- and 4β-methoxydihydrotestosterone. In contrast, during the attempts to effect alkylation of phenol using KOH as the basic catalyst, significant proportions of 2α-hydroxydihydrotestosterone were formed. In aprotic solvents complex mixtures were formed in which olefinic products, resulting from elimination rather than the desired substitution reactions, predominated.

## DISCUSSION

In order to account for the stereospecific 4β→6β diaxial proton transfer and the extremely high ($16.7 \cdot 10^6$ for androst-5-ene-3,17-dione) molecular activity[1] of the enzyme it seemed reasonable to assume that in the *ES* complex the basic catalytic group would occupy a fairly central location between the C-4 and C-6 positions above the β-face of the steroid substrate. Accordingly, the substrate analogues considered first were those in which the reactive functions would be oriented such that alkylation of a β-face histidine or other basic group residue would be favored. Bromo-steroids of the types represented by Structures 1–5 appeared to meet the desired criteria since

for each compound the activated ($\alpha$-haloketo or allylic) C-2, C-4, or C-6 bromo substituent would be advantageously aligned for $S_N2$ displacement by a nucleophile positioned above the $\beta$-face of the C-4 region of Ring A in the *ES* or *EI* complex. Furthermore, each of Compounds 1–5 retained the C-3 carbonyl group considered to be important both mechanistically and with respect to binding and orientation of the $\Delta^5$-3-keto substrates in the *ES* complexes.

It was realized that nucleophilic displacements of the types desired are generally much less readily effected on the steroid nucleus than is the case for analogous acyclic compounds[16]. Nevertheless, examples of reactions of the types desired are known[18–20] although in other cases the stereochemistry of the products is often complicated by rearrangement and/or epimerization prior to or following substitution of the leaving group. However, it was felt that binding of Compounds 1–5 at the active site of the isomerase would markedly reduce the incidence of the above types of undesired reactions. Furthermore, we were hopeful that favorable orientation and proximity factors in the *EI* complex would compensate to a large degree for the decreased susceptibility of the bromo substituent towards nucleophilic displacement.

In view of these considerations it was initially a disappointment to discover that none of the substrate analogues 1–5 was capable of effecting alkylation of the isomerase. That their failure to act as irreversible inhibitors was not ascribable to their inability to bind well or orient correctly at the active site was provided by the indication that they were acting as competitive inhibitors of the enzyme. This conclusion, based on the reduction in isomerization rates and protection afforded against denaturation in the presence of each compound (particularly with Compounds 1 and 2), was confirmed by a detailed study of this aspect using the Dixon procedure[15].

The plots obtained (Fig. 1) showed clearly that each of the bromosteroids examined was a competitive inhibitor of the enzyme and application of the graphical analysis of SMITH *et al.*[21] established that each inhibition involved one bromosteroid molecule per active site. The inhibition constants are recorded in Table I. That the minor variation in the C-17 substituent of Compounds 1–5 between hydroxyl and acetate did not exert an important influence on the inhibition process was established by determining the $K_i$'s of the pairs of inhibitors, dihydrotestosterone and 17$\beta$-acetoxy-5$\alpha$-androstan-3-one, and testosterone and testosterone acetate. As the values recorded in Table I show, the differences are not significant. It is of interest to note that this observation is in direct contrast to the effect of substituting acetate for hydroxyl at C-17$\beta$ of inhibitors of the 3(and 17)$\beta$-hydroxysteroid dehydrogenase from the same microorganism. However, in this regard it should be noted that competitive inhibitors of the isomerase often turn out to be noncompetitive inhibitors of the dehydrogenase[22–24].

The $K_i$ values of Compounds 1–5 show them to be among the most effective of the competitive inhibitors which have been surveyed to date. The fact that the inhibition constants of the bromosteroids are either of the same order of magnitude, or lower than, those of the unsubstituted parent inhibitors provides clear evidence that a bulky bromide substituent at C-2, -4, or -6 does not interfere sterically with the active site binding process. These data, taken in conjunction with the previous data on the efficacy of some other 3-keto C-2, -4, and -6 substituted competitive inhibitors[23] indicate that in the region of the Ring A binding loci, the topology of the active site is fairly open and that in general, its accessibility to substrates or inhibitors

is not affected by Ring A substituents, other than those at C-10. This concept of a readily accessible active site tolerant of wide structural variations and permitting unhindered approach of the substrate, is in accord with the extremely high molecular activity of the enzyme. Furthermore, it is significant that the 5$\alpha$-androstane derivatives, in which the A-rings are in non-planar chair conformations, are as good or better inhibitors than the analogous $\Delta^4$-compounds even though the latter approximate more closely the near-planar transition-state of an *ES* complex[25].

Although each of Compounds 1–5 is a good inhibitor, the 2$\alpha$-bromo derivatives of the 5$\alpha$-androstane series (Compounds 1 and 2) are much more effective than the other bromo-compounds or either of the parent androstanes dihydrotestosterone and 17$\beta$-acetoxy-5$\alpha$-androstan-3-one. In contrast, for the bromosteroids 3–5 the $K_i$ values are very similar to those of their non-brominated parent compounds.

The most satisfactory rationalization for the differences in $K_i$ between the inhibitors of Table I was provided by considering the consequences of their relative ease of enolization within the *EI* complex and the stabilities of the enols or enolate anions so produced. Evidence for enolization within 19-nortestosterone–isomerase complexes has been presented by WANG *et al.*[26] and the possible significance of this with respect to other 3-ketosteroid inhibitors has been noted[24,27]. In base-catalyzed enolization of six-membered ring ketones, removal of an axial proton occurs preferentially[28]. Thus for all the 5$\alpha$-androstane derivatives of Table I, binding at the active site in the manner envisaged for the $\Delta^5$-3-ketosteroid substrates would facilitate enolization since the basic group assumed to be situated above Ring A would be favorably oriented for abstraction of the axial 2$\beta$ (or 4$\beta$) hydrogens. From the literature data on the direction of enolization[17] and the pattern of bromination of steroid-3-ketones[29] it can be predicted that for all the 5$\alpha$-androstan-3-one inhibitors studied enolization in the $\Delta^2$-direction will be much preferred over $\Delta^3$. Thus in the *EI* complexes any enolized inhibitor would be expected to be bound as the $\Delta^2$-isomer. The fact that 2$\alpha$-bromodihydrotestosterone (1) is a much better inhibitor than its parent compound dihydrotestosterone would then be attributable to the increased facility of enolization of Compound 1 resulting from the greater acidity of the 2$\beta$-proton. The marginal superiorities of inhibitors such as 2$\alpha$,4$\alpha$-dibromo-17$\beta$-acetoxy-5$\alpha$-androstan-3-one (2) and 4$\alpha$-bromo-17$\beta$-acetoxy-5$\alpha$-androstan-3-one (3) over Compound 1 and 17$\beta$-acetoxy-5$\alpha$-androstan-3-one, respectively, are also accommodated by this analysis since the inductive effect of a C-4 bromide substituent should be reflected in somewhat increased acidities of the 2$\beta$-axial protons of Compounds 2 and 3. For the Table I inhibitors in which a $\Delta^4$-double bond is present, the $K_i$ range (26.0–39.7 $\mu$M) is not very great and no self-consistent pattern can be drawn from considerations of enolizations, relative acidities of protons etc.

Although more concrete evidence is obviously required before the above hypothesis can be fully substantiated it is considered that the facility with which bromo-3-ketoandrostanes such as Compound 1 would be expected to enolize may also account for their ineffectiveness as irreversible inhibitors since enolization by an active site base would preclude $S_N2$ displacements of the type desired. The products obtained in the model alkylation studies with imidazole and phenol under basic conditions are also compatible with this view.

Initially it was felt that brominated 3-ketoestranes would be better alkylating substrate analogues than the androstanes 1–5 since comparisons of estrane and

androstane inhibitors show that the 19-methyl group does interfere with binding at the active site[1,26,27]. Unfortunately, the bromoestranes corresponding to Compounds 1–5 are all thermodynamically unstable[30] and none of the preparative routes surveyed afforded any compounds retaining the desired bromide configuration. However, with the steric effect of the C-19 methyl group removed, such derivatives would be expected to enolize even more readily than their androstane counterparts[31] and following the recognition of the importance of this factor, synthetic approaches to estrane inhibitors were discontinued.

At this stage in our investigation, BÜKI *et al.*[6] showed that 6$\beta$-bromotestosterone acetate acted as an active-site directed irreversible inhibitor upon prolonged incubation with the enzyme*. It is of interest to contrast the data on the inhibitors of Table I with those reported[5] for 6$\beta$-bromotestosterone acetate. In short term experiments the latter compound was also found to be a competitive inhibitor of the enzyme. However, its relatively high $K_i$ value of 57 $\mu$M suggests that steric interaction of the large 6$\beta$-axial** bromide substituent with the active site is more serious than any involved in the *EI* complexes of Compounds 1–5 and the normal orientation envisaged for a good inhibitor at the active site is obviously sufficiently changed for displacement of bromide by the basic catalytic group to become possible. None of the deviations from linearity observed in the Dixon plots for 6$\beta$-bromotestosterone acetate inhibition at high $[I]:[S]$ ratios (considered indicative of a possible irreversible inhibition reaction), were observed for any of Compounds 1–5 even when much higher $[I]:[S]$ ratios were employed.

ACKNOWLEDGMENTS

Financial support by the National Research Council of Canada, and the award (to S.S.) of an NRCC Science Scholarship, are gratefully acknowledged.

We also express our thanks to Dr. P. Talalay for a generous gift of pure enzyme.

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* Ironically, although this compound was an intermediate in our route to the 6$\alpha$-epimer 5 (Scheme 1), we concluded that it was not worthwhile considering as an active site alkylating agent since the bromo-substituent was $\beta$-oriented!

** Owing to the severe 1:3 C-19–C-6 Br interactions to be expected in 6$\beta$-bromotestosterone acetate, the orientation of the bromo group will not be truly axial.

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