Mechanism of Inactivation of 3-Oxosteroid Δ^5-Isomerase by 17β-Oxiranes†

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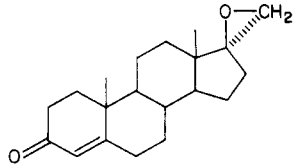
ABSTRACT: The affinity label (17*S*)-spiro[estra-1,3,5(10),6,8-pentaene-17,2′-oxiran]-3-ol (**5**β) inactivates 3-oxosteroid Δ^5-isomerase from *Pseudomonas testosteroni* by formation of a covalent bond between Asp-38 of the enzyme and the steroid. High-performance liquid chromatography (HPLC) analysis of tryptic digests of inactivated enzyme shows that two isomeric steroid-containing peptides are formed in a ratio of 9:1 at pH 7 (TPS_1 and TPS_2). Hydrolysis of each of these peptides produces a different steroid: TPS_1 releases 17α-(hydroxymethyl)estra-1,3,5(10),6,8-pentaene-3,17β-diol (S_1) whereas TPS_2 yields 17β-(hydroxymethyl)estra-1,3,5(10),6,8-pentaene-3,17α-diol (S_2). Inactivation of the enzyme by (17*S*)-spiro[estra-1,3,5(10),6,8-pentaene-17,2′-oxiran-^{18}O]-3-ol, followed by mass spectral analysis of the diacetate of the steroid released upon hydrolysis of the enzyme–inhibitor bond, reveals that TPS_1 is formed by attack of Asp-38 at the methylene carbon of the oxirane. In contrast, TPS_2 is produced by Asp-38 attack at the tertiary carbon. These results imply that inactivation occurs through concurrent S_N1 and S_N2 reactions of Asp-38 with the protonated inhibitor and that Asp-38 is located on the α face of the steroid when it is bound to the active site in the correct manner to react for both the S_N1 and S_N2 processes.

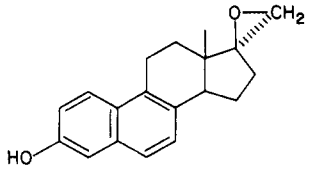
The chemical mechanism by which the 3-oxosteroid Δ^5-isomerase (EC 5.3.3.1) from *Pseudomonas testosteroni* promotes the conversion of $\Delta^{5(6)}$- and $\Delta^{5(10)}$-3-oxosteroids to their respective Δ^4 isomers has been the focus of much research in recent years (Batzold et al., 1976). Several investigators have used active site directed irreversible inhibitors as probes to determine the nature of the enzyme active site. Among these are 6β-bromotestosterone acetate (Büki et al., 1971), acetylenic and allenic 5,10-secosteroids (Batzold & Robinson, 1975; Covey & Robinson, 1976; Penning et al., 1981a,b, 1982; Penning & Talalay, 1981), and the photoaffinity label 3-oxo-4-estren-17β-yl acetate (Martyr & Benisek, 1973, 1975; Ogez et al., 1977).

In an effort to determine the mechanism of action of the isomerase and the topographical composition of the enzyme active site, we have studied the irreversible active site directed inactivation by 3β-oxiranyl steroids (Pollack et al., 1979; Bevins et al., 1984) and 17β-oxiranyl steroids (Bevins et al., 1980; Kayser et al., 1983). For both series of oxiranes, complete enzyme inactivation occurs with the covalent attachment of 1 mol of steroid inhibitor/mol of enzyme monomer through an ester bond (Kayser et al., 1983; Bevins et al., 1984).

Inactivation of the enzyme by either 3β- or 17β-oxirane, followed by tryptic digestion of the modified enzyme, produces two isomeric peptide–steroid adducts, with identical amino acid composition and sequence (residues 14–45). For enzyme inactivated with the 17β-oxirane (17*S*)-spiro-[4-androsten-17,2′-oxiran]-3-one (**4**β), each of these adducts was found to



4β



5β

have a steroid molecule bound to Asp-38 (Kayser et al., 1983). In this paper, we report the identity of the steroids released by base hydrolysis of each of the two enzyme–steroid adducts from enzyme inhibited with (17*S*)-spiro[estra-1,3,5(10),6,8-pentaene-17,2′-oxiran]-3-ol (**5**β) and the fate of labeled oxygen from ^{18}O-labeled oxirane.

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Materials and Methods

Acetonitrile and water used in the high-performance liquid chromatography (HPLC) analysis were HPLC grade (Baker); all other solvents and reagents were reagent grade (Baker). Water enriched in ^{18}O (98.3 atom %) was obtained from Prochem. *Pseudomonas testosteroni* (dried cells) was purchased from Sigma. Trypsin was obtained from Worthington. General procedures concerning isomerase purification and inhibition as well as the trypsin digestion, HPLC analyses, and amino acid analysis have been described (Pollack et al., 1979; Bevins et al., 1980; Kayser et al., 1981). The purified enzyme used in this work had a specific activity of 47 000 units/mg. (17*S*)-Spiro[estra-1,3,5(10),6,8-pentaene-17,2′-oxiran]-3-ol (**5**β) and (17*R*)-spiro[estra-1,3,5(10),6,8-pentaene-17,2′-oxiran]-3-ol (**5**α) were synthesized as previously described (Bevins et al., 1980).

17α-(Hydroxymethyl)estra-1,3,5(10),6,8-pentaene-3,17β-diol (S_1). A solution of dioxane/1 N KOH (1:9) was prepared and thoroughly purged with argon gas. (17*S*)-Spiro[estra-1,3,5(10),6,8-pentaene-17,2′-oxiran]-3-ol (63 mg, 0.22 mmol) was dissolved in 77 mL of the dioxane/KOH solution, and the solution was again purged with argon. The reaction flask was sealed and left stirring for 113 h at 37 °C. The reaction solution was cooled in an ice bath, and concentrated phosphoric acid (14.7 M) was added dropwise with vigorous stirring to lower the pH to 7. The dioxane was then removed on a rotary evaporator at room temperature. The precipitated material was collected by gravity filtration and allowed to air-dry. The solid was taken up in acetone and filtered, and the solvent was evaporated to yield a white solid (70 mg, 104%). Thin-layer chromatography (TLC) (ethyl acetate, silica) showed one spot, R_f 0.42.

17α-(Acetoxymethyl)-3-acetoxyestra-1,3,5(10),6,8-pentaen-17β-ol was prepared by treating S_1 (70 mg, 0.23 mmol) with acetic anhydride (0.5 mL, 5.3 mmol, redistilled from

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NaOAc) and pyridine (2 mL) at room temperature for 17 h under nitrogen. The reaction solution was then poured into ice-cold potassium phosphate buffer (50 mL, 0.013 M, pH 7). The precipitate that immediately formed was collected by gravity filtration, washed with water, and air-dried. The diacetate (60 mg, 71%) was recrystallized from ether: mp 161–162 °C; NMR ($CDCl_3$) δ 0.87 (3 H, s, 18-CH_3), 2.15 (3 H, s, $-CH_2O_2CCH_3$), 2.35 (3 H, s, $-O_2CCH_3$), 4.24 (2 H, d, J = 3.8 Hz, $-CH_2OAc$), and 7.16–8.02 (5 H, m, aromatic); mass spectrum, m/e 382 (M^+), 364 (M – H_2O), 349 (M – H_2O – CH_3). Anal. ($C_{22}H_{26}O_5$) C, H.

17β-(Hydroxymethyl)estra-1,3,5(10),6,8-pentaene-3,17α-diol (S_2) was prepared as above in 83% yield from (17*R*)-spiro[estra-1,3,5(10),6,8-pentaene-17,2′-oxiran]-3-ol and showed only one spot on TLC (ethyl acetate, silica).

17β-(Acetoxymethyl)-3-acetoxyestra-1,3,5(10),6,8-pentaen-17α-ol was obtained by acetylating S_2 in 97% yield: mp 180–181.5 °C; NMR ($CDCl_3$) δ 0.70 (3 H, s, 18-CH_3), 2.15 (3 H, s, $-CH_2O_2CCH_3$), 2.35 (3 H, s, $-O_2CCH_3$), 4.30 (2 H, d, J = 1.3 Hz, $-CH_2OAc$), and 7.17–8.04 (5 H, m, aromatic); mass spectrum, m/e 382 (M^+) 364 (M – H_2O), 349 (M – H_2O – CH_3). Anal. ($C_{22}H_{26}O_5$) C, H.

Equilenin-17-^{18}O was prepared according to a modification of the procedure of Lawson et al. (1969). Equilenin (100 mg) was dissolved in 1.35 mL of dioxane. To this solution was added 270 μL of $H_2{}^{18}O$ followed by the addition of 1.35 mL of 0.02 N HCl in dioxane in a glove bag under an atmosphere of nitrogen. The reaction flask was stoppered and then incubated in oil bath at 75 °C for 24 h. The reaction was cooled and poured into 20 mL of ice-cold 0.03 M phosphate buffer (pH 7). The steroid was extracted with methylene chloride (4 times 6 mL), washed with water, and dried to give 90 mg of product. The sample was found to contain about 5% residual ^{16}O. Mass spectrum showed m/e 268 (M^+) and 253 (M – CH_3).

(17S)-Spiro[estra-1,3,5(10),6,8-pentaene-17,2′-oxiran-^{18}O]-3-ol (*^{18}O-labeled 5β*) was prepared from equilenin-17-^{18}O by the procedure used for **5β** (Bevins et al., 1980), purified by column chromatography on silica, and recrystallized from methanol. The sample was found to contain about 25% ^{16}O by mass spectrometry: mass spectrum, m/e 282 (M^+), 249 (M – $CH_2{}^{18}OH$).

17α-(Hydroxymethyl)estra-1,3,5(10),6,8-pentaene-3,17β-diol-17β-^{18}O was prepared by hydrolysis of ^{18}O-labeled **5β**. A total of 6 mg (21 μM) of the oxirane dissolved in 100 μL of dioxane was added to a stirring mixture of 700 μL of 0.5 N NaOH (dioxane/water, 1:1). The solution was purged with argon, stoppered, and incubated at 37 °C. After 113 h, the reaction was cooled, neutralized with phosphroric acid, and extracted with ethyl acetate (3 times 1.5 mL). The extracts were combined and washed with water. Solvent was removed under nitrogen.

17α-(Hydroxy-^{18}O-methyl)estra-1,3,5(10),6,8-pentaene-3,17β-diol. A solution of $Na^{18}OH$ (ca. 1.5 M) was prepared in a nitrogen glove bag with ca. 20 mg of NaOH and 350 μL of $H_2{}^{18}O$. (17*S*)-Spiro[estra-1,3,5(10),6,8-pentaene-17,2′-oxiran]-3-ol (6 mg), dissolved in 350 μL of dioxane, was added to the above solution. The reaction flask was purged with argon, stoppered, and incubated at 37 °C for 96 h. Longer reaction times (ca. 113 h) under these conditions (dioxane/water, 1:1) gave a significant amount of an unidentified byproduct. The reaction was neutralized with phosphoric acid and extracted with ethyl acetate (3 times 1.5 mL), the extract was washed with water, and the solvent was removed under nitrogen.

Acetylation of the ^{18}O-Enriched Alcohols To Produce **1** *and* **2**. Crude alcohols were acetylated directly at room temperature in 800 μL of pyridine (distilled over KOH) and 200 μL of acetic anhydride (distilled from sodium acetate) for ca. 24 h. The solution was neutralized with pH 7 sodium phosphate buffer and extracted with methylene chloride (3 times 2 mL). The extracts were combined, washed with water, and dried. The solvent was removed under nitrogen. The two diacetates were purified by HPLC (C_{18} μBondapak, 70% methanol, elution at 20 mL). Mass spectrum showed the following: **1**, m/e 384 (M^+), 366 (M – H_2O), 351 (M – H_2O – CH_3); **2**, m/e 384 (M^+), 364 (M – $H_2{}^{18}O$), 349 (M – $H_2{}^{18}O$ – CH_3).

Identification of Steroids Released by Base Hydrolysis of TPS_1 and TPS_2. Isomerase was inhibited with **5β** until the residual activity was constant. Tryptic digestion and isolation of the modified peptides were performed as before (Kayser et al., 1983). The solvent was removed by a gentle stream of nitrogen, and ca. 0.2 nmol of each peptide (TPS_1 and TPS_2) was incubated in NaOH (ca. 0.5 N, pH 12.5) at 37 °C for 2 h. The solution was neutralized with 1 N HCl, and the steroids were extracted with ethyl acetate (3 times 1.5 mL). The ethyl acetate was dried and removed under a slow stream of nitrogen to give S_1 and S_2. These compounds were found to coelute with authentic samples on HPLC [C_{18} μBondapak, acetonitrile/water (42:58 v/v), with 0.1% H_3PO_4 in the water, retention time 18.8 mL (S_1) and 20.3 mL (S_2)].

Inactivation with ^{18}O-Labeled **5β**. Isomerase (22.5 μM) was inhibited with ^{18}O-labeled **5β** (20 μM) as above (total volume 32 mL). TPS_1 was collected by HPLC; reinjection of a portion of the collected sample showed no contamination by TPS_2. Hydrolysis of TPS_1 gave a sample of S_1 that was acetylated and analyzed by mass spectrometry (Bevins et al., 1984).

Results

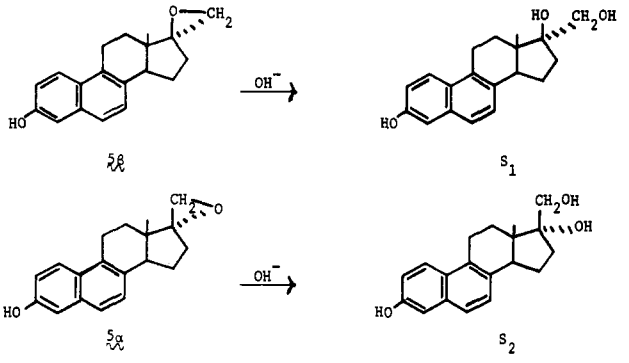
Enzyme inhibited with **5β** was denatured and digested with trypsin. HPLC analysis of the tryptic digests showed two steroid-containing peptides (TPS_1 and TPS_2) in a 9:1 ratio that were separated and isolated as before (Kayser et al., 1983). The peptide–steroid bond is base labile; hydrolysis of TPS_1 yields only steroid S_1, whereas hydrolysis of TPS_2 produces S_2. These two steroids were collected by HPLC.

Rates of hydrolysis of both TPS_1 and TPS_2 were determined at pH 12 (28% acetonitrile, v/v, 0.036 M sodium phosphate, 25 °C) by HPLC analysis of reaction aliquots. The loss of TPS_1 with time is consistent with a first-order process having a rate constant of 5.5×10^{-4} s^{-1}. Identical rate constants were obtained from an analysis of the concomitant increase in peptide TP (residues 14–45) and hydrolyzed steroid S_1. Similar experiments with TPS_2 gave a rate constant for hydrolysis of 2.0×10^{-4} s^{-1}.

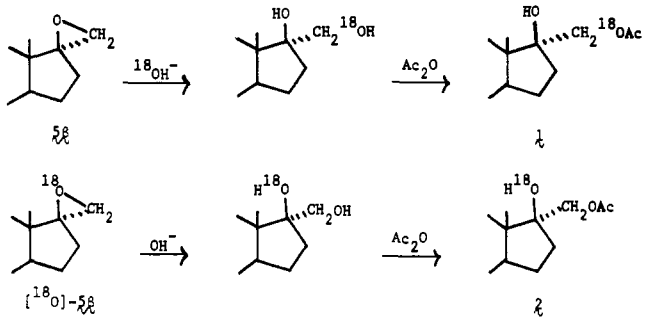
The steroids released from TPS_1 and TPS_2 were identified as S_1 and S_2, respectively, by coelution on HPLC with authentic samples prepared from base-catalyzed hydrolysis of the corresponding 17β- and 17α-oxiranes (Scheme I). The two steroids (S_1 and S_2) are well resolved by HPLC with retention times of 18.8 and 20.3 mL, respectively.

Oxirane **5β** was synthesized enriched with 75% ^{18}O in the oxirane oxygen, and the isomerase was inhibited with ^{18}O-labeled **5β**. TPS_1 (^{18}O labeled) was isolated by HPLC and hydrolyzed, and the steroid released was acetylated. The acetylated ^{18}O-labeled steroid was analyzed by mass spectrometry to determine the location of the labeled oxygen. Authentic samples with ^{18}O in the primary acetate (**1**) and ^{18}O in the tertiary alcohol (**2**) were synthesized and used for comparison (Scheme II). For authentic **1** and **2**, molecular ions at m/e 384 were observed along with peaks due to M –

Scheme I



Scheme II

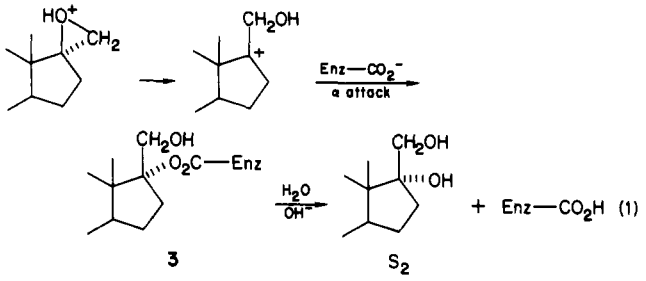


H_2O (*m/e* 366 for **1** and 364 for **2**) and M − H_2O − CH_3 (*m/e* 351 for **1** and 349 for **2**). Compound **2** showed 25% of ^{16}O, as expected, since the oxirane precursor was only 75% enriched in ^{18}O. The mass spectrum of the acetylated steroid of S_1 isolated from TPS_1 (^{18}O labeled) showed peaks at *m/e* 384, 364, and 349, establishing that the ^{18}O was in the tertiary alcohol (**2**). Although a peak corresponding to *m/e* 351 was detected at ca. 12% the intensity of *m/e* 349, we conclude that there was no contamination of this sample by isomer **1**. This conclusion is based upon the observation that **2** also shows *m/e* 322, whereas **1** gives *m/e* 324. No trace of *m/e* 324 was found in the spectrum of acetylated S_1 despite the fact that the *m/e* 322 peak is more intense than the *m/e* 349 peak.

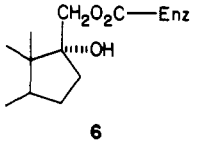
Discussion

We have previously shown that the 17β-oxiranes **4**β and **5**β rapidly inactivate the 3-oxosteroid Δ^5-isomerase (Bevins et al., 1980). Tryptic digestion of the modified enzyme yields two covalent enzyme–steroid adducts, which we have labeled TPS_1 and TPS_2, in the ratio of 1:1 for **4**β and 9:1 for **5**β (Kayser et al., 1983). For enzyme modified with **4**β, each of these adducts was found to involve Asp-38 as the nucleophilic amino acid residue. The release of different steroids upon hydrolysis of TPS_1 and TPS_2 derived from **4**β confirms the conclusion that the cause of the occurrence of two steroid-containing fragments is isomerism about the steroid end of the Asp–steroid bond. Similar experiments with **5**β-modified enzyme showed that Asp-38 is also the nucleophile in TPS_1, but the low yield of TPS_2 made it impossible to determine the nucleophilic amino acid. Nevertheless, the similarity of results, coupled with the fact that TPS_1 and TPS_2 release different steroids for both **5**β- and **4**β-modified enzymes, argues strongly that Asp-38 is the nucleophile for TPS_2 derived from **5**β as well.

From the knowledge that the steroids released upon hydrolysis of TPS_1 and TPS_2 are S_1 and S_2, respectively, it is possible to deduce the structures of the two covalent inhibitor complexes that are formed on inactivation with **5**β. The identification of S_2 as a 17α-hydroxy-17β-(hydroxymethyl) steroid suggests that the structure for TPS_2 is best represented by **3**. This adduct could result from an S_N1 reaction of Asp-38 with the protonated oxirane, if Asp-38 is located on the α side of the steroid nucleus (eq 1). The inability of unprotonated

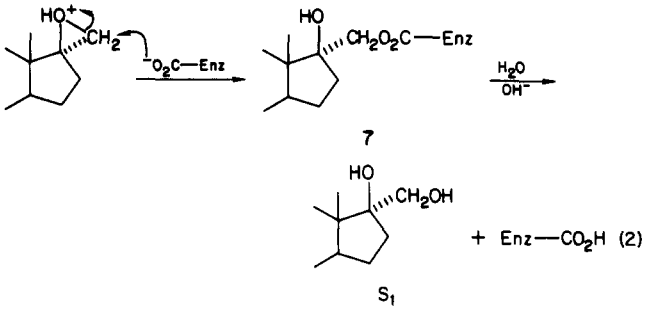


epoxides to readily undergo nucleophilic attack in the absence of electrophilic assistance (Buchanan & Sable, 1972) argues that protonation of the oxygen is required for this reaction. The other ester (**6**) which would hydrolyze to S_2 may be ruled

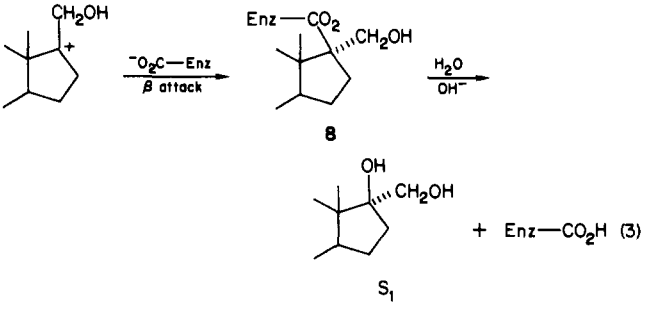


out since no reasonable mechanism is available for its formation.

On the other hand, there are two conceivable structures for TPS_1 that would hydrolyze to produce S_1. Bimolecular displacement by Asp-38 at the methylene carbon (eq 2) would



product **7**, whereas collapse of the tertiary carbonium ion of eq 1 with Asp-38 from the β face of the steroid would give **8** (eq 3). Each of these structures would hydrolyze to form S_1.



It is possible to assign structure **7** to TPS_1 on the basis of the ^{18}O-labeling experiments. If **8** were the correct structure, inhibition with ^{18}O-labeled oxirane followed by hydrolysis and acetylation would produce **1** with the ^{18}O in the primary acetate. If **7** were correct, the labeled oxygen would be in the tertiary hydroxyl group producing **2**. The mass spectral results show conclusively that **2** is produced rather than **1** when these reactions are carried out with ^{18}O-labeled oxirane, demonstrating that **7** is indeed the correct structure.

It is clear from the above labeling experiments that inactivation of 3-oxosteroid Δ^5-isomerase by **5**β occurs by attack of Asp-38 from the α side of the steroid at either the methylene carbon to give **7** or the tertiary carbon to give **3** in a ratio of 9:1. Similar results have previously been obtained for the inactivation of the isomerase by 3β-oxiranes (Bevins et al., 1984). For the 3β-oxiranes, attack from the α side at both the primary and tertiary carbons occurs in about equimolar amounts.

Chemical precedent for nucleophilic attack at both primary and tertiary sites may be found in the corresponding nonenzymic reactions of epoxides. Although nucleophilic attack on unprotonated epoxides generally occurs at the least substituted carbon, attack on protonated epoxides usually leads to a mixture of products, probably through a borderline bimolecular mechanism in which substantial positive charge develops at the carbon being attacked (Biggs et al., 1971). The mixture of products results from a competition between steric factors that favor reaction at the less substituted carbon and electronic factors that favor attack at the more highly substituted site. Although we have interpreted the reaction of Asp-38 with **5**β in terms of competing S_N1 and S_N2 reactions, a borderline mechanism is equally consistent with our results.

In our initial work we postulated, on the basis of spectral evidence, that the ability of 17β-oxiranes to inhibit the isomerase was due to "backward binding" of these steroids at the active site (Bevins et al., 1980). The 17β-oxirane group could then react with the same group on the enzyme that reacts with the 3β-oxiranes. Although we do not know, as yet, the identity of the amino acid that is modified by the 3β-oxiranes, photoaffinity labeling experiments that have shown Asp-38 to be in the vicinity of the A ring in at least one binding mode support our theory (Ogez et al., 1977).

We suggested that the two possible modes of steroid binding to the isomerase differ by 180° rotation about an axis perpendicular to the plane of the steroid nucleus (Bevins et al., 1980). Our finding that Asp-38 is located on the α face of the D ring is consistent with this hypothesis since we have shown that the enzymic carboxylate modified in the case of a 3β-oxirane is also located on the α side of the A ring (Bevins et al., 1984). In addition, it is suggestive evidence that Asp-38 is the group modified by the 3β-oxiranes.

Backward binding has also been suggested to account for the dual activity of 3α,20β-hydroxysteroid dehydrogenase (Sweet & Samant, 1980; Strickler et al., 1980). These authors have postulated that the two modes of binding, one for 3α activity and the other for 20β activity, are related through a 180° rotation parallel to the steroid ring system rather than a 180° rotation perpendicular to the steroid plane as we observe for the isomerase.

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REFERENCES

Batzold, F. H., & Robinson, C. H. (1975) *J. Am. Chem. Soc. 97*, 2576–2578.

Batzold, F. H., Benson, A. M., Covey, D. F., Robinson, C. H., & Talalay, P. (1976) *Adv. Enzyme Regul. 14*, 243–267.

Bevins, C. L., Kayser, R. H., Pollack, R. M., Ekiko, D. B., & Sadoff, S. (1980) *Biochem. Biophys. Res. Commun. 95*, 1131–1137.

Bevins, C. L., Bantia, S., Pollack, R. M., Bounds, P. L., & Kayser, R. H. (1984) *J. Am. Chem. Soc. 106*, 4957–4962.

Biggs, J., Chapman, N. B., & Wray, V. (1971) *J. Chem. Soc. B*, 71–74.

Buchanan, J. G., & Sable, H. Z. (1972) in *Selective Organic Transformations* (Thyagarajan, B. S., Ed.) Vol. 2, pp 1–93, Wiley-Interscience, New York.

Büki, K. G., Robinson, C. H., & Talalay, P. (1971) *Biochim. Biophys. Acta 242*, 268–277.

Covey, D. F., & Robinson, C. H. (1976) *J. Am. Chem. Soc. 98*, 5038–5040.

Kayser, R. H., Bounds, P. L., Bevins, C. L., & Pollack, R. M. (1983) *J. Biol. Chem. 258*, 909–915.

Lawson, A. M., Leemans, F. A. J. M., & McCloskey, J. A. (1969) *Steroids 14*, 603–615.

Martyr, R. J., & Benisek, W. F. (1973) *Biochemistry 12*, 2172–2178.

Martyr, R. J., & Benisek, W. F. (1975) *J. Biol. Chem. 250*, 1218–1222.

Ogez, J. R., Tivol, W. F., & Benisek, W. F. (1977) *J. Biol. Chem. 252*, 6151–6155.

Penning, T. M., & Talalay, P. (1981) *J. Biol. Chem. 256*, 6851–6858.

Penning, T. M., Covey, D. F., & Talalay, P. (1981a) *Biochem. J. 193*, 217–227.

Penning, T. M., Covey, D. F., & Talalay, P. (1981b) *J. Biol. Chem. 256*, 6842–6850.

Penning, T. M., Heller, D. N., Balasubramanian, T. M., Fenselau, C. C., & Talalay, P. (1982) *J. Biol. Chem. 257*, 12589–12593.

Pollack, R. M., Kayser, R. H., & Bevins, C. L. (1979) *Biochem. Biophys. Res. Commun. 91*, 783–790.

Strickler, R. C., Covey, D. F., & Tobias, B. (1980) *Biochemistry 19*, 4950–4954.

Sweet, F., & Samant, B. R. (1980) *Biochemistry 19*, 978–986.